Effect of Polymer Types and Architectures on the Properties and Activities of Polymer-Protein Conjugates

Benjaporn Treetharnmathurot

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Author	Miss Benjaporn Treetharnmathurot
Major Program	Pharmaceutical Sciences

Major Advisor :

.....

(Assoc. Prof. Dr. Ruedeekorn Wiwattanapatapee)

Co-advisors :

.....

(Assist. Prof. Dr. Chitchamai Ovatlarnporn)

.....

(Assist. Prof. Dr. Juraithip Wungsintaweekul)

.....

(Prof. Dr. Ruth Duncan)

Examining Committee :

.....Chairperson

(Assist. Prof. Dr. Bhutorn Canyuk)

.....

(Dr. Luelak Lomlim)

.....

(Dr. Rachaneekorn Jevprasesphant)

.....

(Assoc. Prof. Dr. Ruedeekorn Wiwattanapatapee)

.....

(Assist. Prof. Dr. Chitchamai Ovatlarnporn)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Pharmaceutical Sciences

.....

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

ชื่อวิทยานิพนธ์	ผลของชนิดและลักษณะโครงสร้างของพอลิเมอร์ต่อคุณสมบัติและการออกฤทธิ์ของพอลิเมอร์-โปรตีนคอนจุเกต
ผู้เขียน	นางสาวเบญจพร ตรีธารมธุรส
สาขาวิชา	เภสัชศาสตร์
ปีการศึกษา	2551

บทคัดย่อ

การสังเคราะห์ conjugate ทำได้โดยการเชื่อมต่อ trypsin ซึ่งใช้เป็นแบบจำลองโปรตีนกับพอลิเมอร์ที่มีลักษณะโครงสร้างแตกต่างกัน 5 ชนิด ได้แก่ monomethoxy polyethylene glycol (mPEG; น้ำหนักโมเลกุล 1,100, 2,000 และ 5,000 g/mol) β-cyclodextrin (β-CD; น้ำหนักโมเลกุล 1,135 g/mol) polyamidoamine (PAMAM) dendrimer (รุ่นที่ 3 น้ำหนักโมเลกุล 6,941 g/mol) dextrin (dextrin I; น้ำหนักโมเลกุล 8,100 g/mol และ dextrin II; น้ำหนักโมเลกุล 61,000 g/mol) และ semitelechelic poly[*N*-(2-hydroxypropyl)methacrylamide] (ST-HPMA; น้ำหนักโมเลกุล 10,100 g/mol) จุดมุ่งหมายของงานวิจัยนี้คือการศึกษาผลของชนิดและลักษณะโครงสร้างของพอลิเมอร์ต่อการออกฤทธิ์, การสลายตัว และความคงตัวต่ออุณหภูมิของ polymer-protein conjugates การออกฤทธิ์ของ trypsin และ polymer-trypsin conjugates ประเมินโดยใช้ *N*-benzoyl-*L*-arginine *p*-nitroanilide (BAPNA) เป็น substrate เมื่อเปรียบเทียบการออกฤทธิ์ ค่าจลนศาสตร์ต่างๆ (ได้แก่ K_M, V_{max} และ K_{cat}) ความคงตัวต่ออุณหภูมิ (30-70 ºC) และอัตราการสลายตัวที่อุณหภูมิ 40 °C ของ trypsin และ polymer-trypsin conjugates แล้ว พบว่าการออกฤทธิ์ของ trypsin และ polymer-trypsin conjugates ทุกตัวมีค่าลดลงหลังจากการเชื่อมต่อกับพอลิเมอร์

การสังเคราะห์ activated PEG โดยใช้ตัวเชื่อมที่ต่างกัน 3 แบบ โดยการนำ PEG (1,100, 2,000 and 5,000 g/mol) มาทำปฏิกิริยากับ succinic anhydride (SA) cyanuric chloride (CC) หรือ tosyl chloride (TC) จากการศึกษาผลของน้ำหนักโมเลกุลและตัวเชื่อม พบว่า คอนจุเกตที่ประกอบด้วยพอลิเมอร์ที่มีน้ำหนักโมเลกุลสูงกว่าจะมีความคงตัวมากกว่าคอนจุเกตที่ประกอบด้วยพอลิเมอร์ที่มีน้ำหนักโมเลกุลต่ำกว่า และ trypsin-CC-mPEG conjugate มีความคงตัวมากที่สุดเมื่อเปรียบเทียบกับตัวเชื่อมแบบอื่นๆ ซึ่งใช้ PEG ที่มีน้ำหนักโมเลกุลเท่ากัน

พอลิเมอร์ต่างๆ ซึ่งถูกดัดแปลงโดยอาศัยปฏิกิริยา succinoylation เพื่อให้มีหมู่ –COOH ซึ่งสามารถเชื่อมต่อกับหมู่ $–NH_2$ ของ trypsin ได้ด้วยพันธะ amide จากการเปรียบเทียบพบว่า dextrin II conjugate มีความคงตัวมากกว่า free trypsin และ polymer-trypsin conjugates ชนิดอื่นๆ เมื่อทดลองที่อุณหภูมิ 30-70 ºC อีกทั้งยังช่วยป้องกันการสลายตัวที่อุณหภูมิ 40 °C อีกด้วย เมื่อเปรียบเทียบค่าครึ่งชีวิต ($t_{1/2}$) ของ polymer-trypsin conjugates เหล่านี้ พบว่าสามารถเรียงลำดับจากมากไปน้อย ได้ดังนี้ dextrin II-trypsin > β-CD-trypsin > trypsin-SA-mPEG 5000 > dextrin I-trypsin > ST-HPMA-trypsin > native trypsin > PAMAM-trypsin conjugates

นอกจากนี้ ยังมีการสังเคราะห์คอนจุเกตที่มีฤทธิ์ต้านมะเร็ง โดยใช้ ribonuclease A (RNase A) เชื่อมต่อกับ dextrin I, dextrin II and ST-HPMA-COOH จากการศึกษาความเป็นพิษต่อเซลล์ B16F10 โดยใช้ MTT assay พบว่า RNase A ไม่เป็นพิษต่อเซลล์ B16F10 เมื่อความเข้มข้นของ RNase A น้อยกว่า 1 มิลลิกรัมต่อมิลลิลิตร ขณะที่ความเป็นพิษต่อเซลล์ B16F10 ของคอนจุเกตเรียงลำดับจากมากไปน้อย เป็นดังนี้ ST-HPMA conjugates > dextrin I conjugates ≥ dextrin II conjugates อย่างไรก็ตาม เมื่อพิจารณาทั้งด้านความคงตัวและความเป็นพิษต่อเซลล์แล้ว พบว่า dextrin II เป็นพอลิเมอร์ที่น่าสนใจ และสามารถนำไปพัฒนาเป็น bioresponsive polymer-protein conjugate ที่มีฤทธิ์ต้านมะเร็งได้ต่อไป

การศึกษาทั้งหมดนี้แสดงให้เห็นว่า ลักษณะโครงสร้างของพอลิเมอร์ น้ำหนักโมเลกุล และตัวเชื่อมที่ใช้จะมีผลต่อการออกฤทธิ์ อัตราการสลายตัว และความคงตัวต่ออุณหภูมิของโปรตีน ปัจจัยที่สำคัญต่างๆ เหล่านี้ สามารถใช้เป็นแนวทางเบื้องต้นในการเลือกใช้พอลิเมอร์ให้มีความเหมาะสมต่อการสังเคราะห์คอนจุเกตที่มีฤทธิ์ในการรักษาเพื่อการพัฒนาเป็นสูตรตำรับต่อไป

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ABSTRACT

Model conjugates were synthesized by coupling a model protein, trypsin to five polymers with different architectures including monomethoxypolyethylene glycol (mPEG; Mw 1,100, 2,000 and 5,000 g/mol), β-cyclodextrin (β-CD; Mw 1,135 g/mol), polyamidoamine (PAMAM) dendrimer (generation 3, Mw 6,941 g/mol), dextrin (dextrin I; Mw 8,100 g/mol and dextrin II; Mw 61,000 g/mol) and semi-telechelic poly[*N*-(2-hydroxypropyl)methacrylamide] (ST-HPMA; Mw 10,100 g/mol). The enzyme activity and thermal stability of native trypsin and the polymer-trypsin conjugates were compared using *N*-benzoyl-*L*-arginine *p*-nitroanilide (BAPNA) as a substrate. The kinetic parameters (K_M, V_{max} and K_{cat}) were also calculated. The effect of conjugate chemistry on trypsin thermal stability was investigated at temperature between 30-70 ºC and the autolysis rate was examined at 40 °C. It has been found that the activity of trypsin of all conjugates reduced after polymer conjugation.

The mPEGs (Mw 1,100, 2,000 and 5,000 g/mol) were prepared using succinic anhydride (SA), cyanuric chloride (CC) or tosyl chloride (TC) and used to synthesize a library of trypsin conjugates. It was found that PEG-trypsin conjugates containing higher molecular weight of mPEG (5,000 g/mol) were more stable against temperature than free trypsin and other lower Mw of PEG, and the conjugate containing CC-mPEG had the best thermal stability than other type of linker when using the same Mw of PEG.

Using the succinoylated polymers prepared by succinoylation produced –COOH groups for coupling with $–NH_2$ of trypsin with amide bonds. The dextrin II-trypsin conjugate showed the most thermal stability than the other conjugates and native trypsin at all temperatures

between 30-70 °C. It exhibited improving in the autolysis assays at 40 °C and also displayed the greatest half-life. The half-life ($t_{1/2}$) ratio of conjugates showed as follow: dextrin II-trypsin > β-CD-trypsin > trypsin-SA-mPEG 5000 > dextrin I-trypsin > ST-HPMA-trypsin > native trypsin > PAMAM-trypsin conjugates.

Moreover, anti-cancer conjugates were also synthesized using ribonuclease A (RNase A) conjugated to dextrin I, dextrin II and ST-HPMA-COOH for development of therapeutic conjugates as anti-cancer conjugate. The preliminary cytotoxic studies of free RNase A and polymer-RNase A conjugates were performed using MTT assay in B16F10 melanoma cell. RNase A alone was not cytotoxic (less than 1 mg/mL) whereas the cytotoxic of conjugates to B16F10 melanoma cells showed as follow: ST-HPMA conjugates > dextrin I conjugates $\geq$ dextrin II conjugates. However, following thermal stability and cytotoxicity studies, dextrin II is interesting and has the potential to use as bioresponsive polymer-protein conjugates for further development of anti-cancer conjugates.

Overall the observations made in these studies revealed that polymer architectures, their molecular weight and linking chemistry affect the activity of protein, the rate of protein autolysis and thermal stability. These important factors can be used as a guidance for suitable selectivity of optimal polymer/chemistry for synthesis of a lead polymer therapeutic suitable for later formulation development.

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ABBREVIATIONS AND SYMBOLS

°C	degree Celsius
5-ASA	5-aminosalicylic acid
5-FU	5-fluorouracil
β-CD	β-cyclodextrin
μg	microgram
μL	microliter
μM	micromolar
μmol	micromole
AANHS	acetic acid *N*-hydroxysuccinimide ethyl ester
Abs	absorbance
AcOH	acetic acid
ALL	acute lymphoblastic leukemia
AP	ammonium persulfate
ARG	arginine
ASP	aspartic acid
ATCC	american type culture collection
B16F10	murine melanoma cell line
BAEE	N_{α}-benzoyl- *L* -arginine-ethyl-ester
BAPNA	N_{α}-Benzoyl-*L*-arginine-*p*-nitroanilide
BSA	bovine serum albumin
BS-RNase	bovine seminal ribonuclease
BuOH	butanol
^{13}C	Carbon-13
CB	Coomassie blue
CC	cyanuric chloride
CC-PEG	cyanurate PEG

CD	cyclodextrin
CDBN	butylenediamine cyclodextrin
CDEN	ethylenediamine cyclodextrin
$CDNH_2$	monoamine cyclodextrin
CDPN	propylenediamine cyclodextrin
CMC	carboxymethylcellulose
Contd	continued
CV-1	african green monkey fibroblasts
CYS	cysteine
D_2O	deuterated water
Da	dalton
DCC	*N, N*-dicyclohexylcarbodiimide
DCU	dicyclohexylurea
ddH_2O	double distilled water
DMAP	4-dimethylaminopyridine
DMF	*N,N′*-dimethylformamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EC_{50}	effective concentration to achieve 50 % maximal response
EDC	*N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
EPR	enhanced permeability and retention effect
Equiv.	equivalent
EU	european Union
Fab′	antibody fragment
FBS	foetal bovine serum
FDA	food and drug administration
FMOC	9-fluorenylmethoxycarbonyl

FPLC	fast protein liquid chromatography
FT-IR	fourier transform infrared
g	gram
G	generation
G-CSF	granulocyte colony stimulating factor
GLN	glutamine
GLU	glutamic acid
GLY	glycine
GM-CSF	granulocyte-macrophage colony stimulating factor
GPC	gel permeation chromatography
h	hour
^{1}H	hydrogen-1
HA	hyaluronic acid
HAase	hyaluronidase
HCl	hydrochloric acid
HGR	human growth hormone receptor antagonist
HIS	histidine
HPMA	*N*-(2-hydroxypropyl)methacrylamide copolymer
IC_{50}	concentration which inhibits cell growth by 50 %
ILE	isoleucine
IP	interperitoneal
IR	infrared
i.t.	intratumoral
i.v.	intravenous
K_{cat}	turnover rate
K_M	affinity constant or Michaelis constant
kDa	kilodalton
LEU	leucine

LYS	lysine
M	molar
mA	milliampere
MCF7	human breast cancer cell lines
MET	methionine
MeOH	methanol
mg	milligram
Min	minute
mL	milliliter
ML2	human myeloid leukaemia cell line
MLC	mixed lymphocyte culture
mM	millimolar
Mn	number average molecular weight
mol	mole
mmol	millimole
mPa	millipascal
mPEG	monomethoxy(polyethylene glycol)
MSH	melanocyte stimulating hormone
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-terazoliumbromide
Mw	weight average molecular weight
MWCO	molecular weight cut-off
N_2	nitrogen
NA	not applicable
NaCl	sodium chloride
NaOH	sodium hydroxide
NCS	neocarzinostatin
ND	not determined
NHS	*N*-hydroxysuccinimide

NIH	National Institues of Health
nm	nanometer
NMR	nuclear magnetic resonance
NPC	*p*-nitrophenyl chloroformate
NS	not stated
NSCLC	non-small cell lung cancer
PAMAM	polyamidoamine dendrimer
PBS	phosphate buffered saline
PDI	polydispersity index
PEG	polyethylene glycol
PEI	poly(ethyleneimine)
PGA	polyglutamic acid
pH	-log hydrogen ion concentration
PHE	phenylalanine
PHEG	poly((*N*-hydroxyethyl)-*L*-glutamine)
PHPMA	poly[*N*-(2-hydroxypropyl)methacrylamide] copolymer
PK1	HPMA copolymer-Gly-Phe-Leu-Gly-doxorubicin conjugate
PK2	HPMA copolymer-Gly-Phe-Leu-Gly-doxorubicin conjugate containing *N*-acetated galactosamine residue
pKa	acid dissociation constant
PLA_2	phospholipase A_2
PLG	poly(DL-lactide-co-glycolide)
PNA	*p*-nitroaniline
ppm	parts per million
PR	phenol red
PRO	proline
PUMPT	Polymer-masking-UnMasking-Protein Therapy
PVA	polyvinyl alcohol

RCC	renal cell carcinoma
RES	reticuloendothelial system
rhEGF	recombinant human epidermal growth factor
RI	ribonuclease inhibitor
RNA	ribonucleic acid
RNase	ribonuclease
RNase A	ribonuclease A
RPMI	rose park memorial institute
rRNA	ribosomal ribonucleic acid
RT	room temperature
Russ.	russian translation
s	second
S	substrate concentration
SA	succinic anhydride
SANS	small-angle neutron scattering
SA-PEG	succinoylated PEG
SC-PEG	succinimidyl carbonate PEG
SCID	severe combined immunodeficiency disease
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SER	serine
SMA	styrene-co-maleic anhydride
SMANCS	styrene-co-maleic anhydride-neocarzinostatin
SOD	superoxide dismutase
ST	semitelechelic
ST-HPMA	semitelechelic *N*-(2-hydroxypropyl)methacrylamide copolymer

Sulfo-NHS	*N*-hydroxysulfosuccinimide
scFv	single chain Fv antibody fragment
$t_{1/2}$	half-life
T_{50}	temperature which retains 50 % of the initial activity
TAME	tosyl-*L*-arginine methyl ester
TEMED	*N,N,N′,N′*-tetramethylethylenediamine
TC	tosyl chloride
TC-PEG	tosylated PEG
THR	threonine
TLC	thin layer chromatography
TNBS	2,4,6-trinitrobenzenesulfonate
TNF	tumor necrosis factor
TPC	2, 4, 5-trichlorophenylchloroformate
Tris-HCl	tris[hydroxymethyl]aminomethane hydrochloride
TRP	tryptophan
TYR	tyrosine
UKF-NB-3	cell derived from evans stage 4 NB
UV	ultraviolet
UV-vis	ultraviolet-visible
v	velocity
V	volt
VAL	valine
V_{max}	maximum velocity
v/v	volume by volume
w/w	weight by weight
wt	weight

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

More peptide and protein drugs are in development than ever before and many have become important therapeutic agents. The use of peptides and proteins as human therapeutics has expanded in recent years due to: (1) the discovery of novel peptides and proteins, (2) a better understanding of their mechanism of action *in vivo*, (3) improvements in expression or synthesis of proteins and peptides that closely resemble fully human proteins and peptides, and (4) improvements in formulation or molecule-altering technologies that have the ability to deliver polypeptides *in vivo* with improved pharmacokinetic and pharmacodynamic properties (Walsh, 2000). However, these proteins and peptides still have several limitations that restrict broad application. These disadvantages include proteolytic cleavage, short circulation half-life, rapid kidney clearance, low solubility *in vivo* and their propensity to generate neutralizing antibodies (immunogenicity). In addition, most polypeptides drugs must be delivered by either subcutaneously or intravenously injection (Harris and Chess, 2003). Their inherent drawbacks of short circulation half-life and rapid kidney clearance of protein drugs, mean they are inconvenient for patient use.

The field of drug delivery is rapid expanding and its potential has already been proved by the development of many products to the market. Several strategies have been used to improve clinical properties of polypeptide drugs. For examples: manipulation of amino acid sequence to reduce immunogenicity and proteolytic degradation, fusion or conjugation to immunoglobulins or serum protein (e.g. albumin) to increase half-life as biodegradable polymeric matrices, incorporation into drug-delivery vehicles (e.g. liposomes for protection and slow release), and the last but not least conjugation to natural or synthetic polymers to protect protein

from degradation and improve its pharmacokinetics and pharmacodynamics (Roberts et al, 2002; Harris and Chess, 2003).

Among above approaches, polymer conjugation is one of particularly interesting and has already shown itself to be an important technique in pharmaceutical chemistry for the delivery peptide and protein drugs. Polymer conjugation has been widely exploited to improve the therapeutic properties of peptides, enzymes, proteins, small molecules and oligonucleotides that available in many marketed products (Duncan, 2003). Covalent conjugation of a polymer to a protein provides the possibility to improve protein stability and prolong circulating half-life of protein (by increasing their hydrodynamic volume and decreasing their elimination rate). Furthermore, increasing the number of polymer chains per protein can prevent the approach of proteolytic enzymes, antibodies or antigen processing cells due to steric hindrance (Pasut and Veronese, 2007). Over the last few decades, many polymers have been used for protein conjugation. The most popular have been monofunctional polymer such as monomethoxy (polyethylene glycol) (mPEG) and poly-functional polymer such as dextran. The following sections provide examples of protein conjugates synthesized using with polyethylene glycol (PEG), dextran and other polymers recently being used for polymer conjugation.

The aim of the present study was to define the physico-chemical features that are important in the design of novel polymer-protein conjugation using PEG, β-cyclodextrin (β-CD), polyamidoamine (PAMAM) dendrimer, dextrin and a semi-telechelic *N*-(2-hydroxypropyl) methacrylamide (ST-HPMA) homopolymer. The effect of the polymer used, its shape and molecular weight and the linking chemistry on the activity and thermal stability of polymer-protein conjugates were studied. Since, formulation development of protein drug, the active ingredient must be stable during formulation and manufacture. Therefore, thermal stability of protein drug in formulation is one of a major factor that associate to its biological activity.

This introduction reviews the challenges of polymer conjugation in protein delivery and in particular the development in the field of polymer-protein conjugation. The specific areas of PEGylation and protein drugs used as anti-tumor agents are also discussed. In

these studies, PEG, β-CD, PAMAM dendrimer, dextrin and ST-HPMA homopolymer were used as model polymers. The enzymes, trypsin and ribonuclease A were chosen as model proteins.

1.2 Review of literatures

1.2.1 Polymer conjugation and polymer therapeutics

Since the pioneering studies of Ringdorf (Ringdorf, 1975) in polymer conjugation has become of increasing interest as a tool for delivery of low molecular weight drugs and more complex compounds such as proteins, peptides and oligonucleotides. Both natural and synthetic polymers have been used for conjugation. Many different synthetic procedures have been used for polymer conjugation and all were characterized by high specificity and mild reaction condition. This field is now known as "bioconjugation chemistry". Bioconjugation is a very versatile procedure as it can be tailor-made using polymers with the wanted molecular weight, shape, structure and with the functional groups needed for coupling at the positions in the protein chain (Veronese and Morpurgo, 1999).

The term "Polymer Therapeutics" (Figure 1.1) was introduced by Duncan et al. (1996) to describe the classes of new pharmaceutical products that include polymeric drugs, polymer-drug conjugates, polymer-protein conjugates, polymer-DNA complexes (polyplexes) and polymeric micelles. These systems use nano-sized water soluble polymers as delivery systems that are more like new chemical entities than conventional drug delivery systems or formulations. These systems simply entrap, solubilize or control drug release without resorting to chemical conjugation. Conceptually, polymer therapeutics share many features with other macromolecular drugs (proteins, antibodies, oligonucleotides) and macromolecular prodrugs including immunoconjugates (Duncan, 2003).

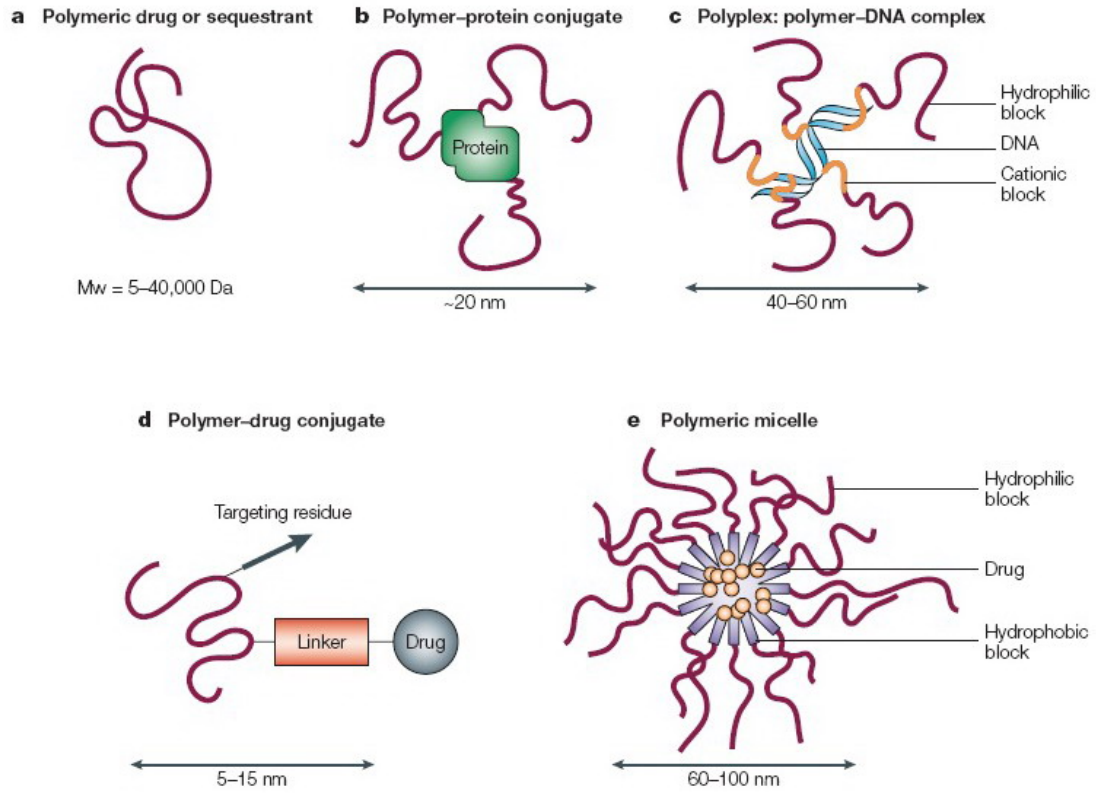


Figure 1.1 Schematic representation of polymer therapeutics. The nano-sized and multicomponent natures of these structures are displayed (from Duncan, 2003).

1.2.2 Polymer-protein conjugates

Many peptides and proteins possess specific biological activities that mark them as potential protein therapeutics. However, the clinical use of protein and peptides as parenteral therapeutic agents still prevents a number of physicochemical and biological challenges (Table 1.1). Polymer-protein conjugation is just one approach that has been used as its advantages are also shown in Table 1.1. Polymer-protein conjugates are now widely employed for applications in medicine, biotechnology and nanotechnology. The field of polymer-protein conjugation began with the first pioneering work (in the area of pharmaceutics) was carried out by Torchillin et al. (1982). They conjugated the polysaccharide dextran to the enzyme streptokinase. In parallel, Davis, Abuchowski and co-workers in 1977 were also investigating polymer-protein conjugation

for drug development and they were the first described a method for the covalent attachment of mPEG and named this method as “PEGylation” (Abuchowski et al., 1977b). They termed “PEGylation” and showed that it was also able to alter the pharmacokinetic and pharmacodynamic properties of protein (Abuchowski and Davis, 1979). Since their studies, a number of alternative polymers have been explored for polymer conjugation but PEG has emerged as the most popular candidate for protein modification. PEGylated proteins form the majority of the protein conjugates on the market (Table 1.2) and there are many others already in advanced clinical investigation (Table 1.3). Such polymer conjugates have been developed for the treatment of several severe diseases, demonstrating that the potential of this technique is not restricted to a narrow therapeutic area (Pasut and Veronese, 2007).

Table 1.1 Challenges for the delivery of proteins and peptides as therapeutic agents and advantages of polymer-protein conjugates (adapted from Torchilin et al, 2003; Pasut et al, 2004).

Challenges of peptide and protein delivery	**Advantages of polymer-protein conjugates**
Physicochemical	Physicochemical
- Poor solubility	- Improve solubility
- Susceptibility to pH	- More stable against pH
- Susceptibility to temperature	- More stable against temperature
Biological	Biological
- Rapid Reticuloendothelial system clearance	- Decreased renal filtration
- Rapid renal elimination	- Protection against enzyme degradation
- Proteolytic degradation	- Lowered immunogenicity
- Immunogenicity	- Selective accumulation in tumors via the enhanced permeability and retention effect
- Premature metabolism	
- Sensitivity on repeated administration	

Table 1.2 Polymer-protein conjugates currently on the market

Polymer-protein conjugate	Drug name (Company)	Indication	Year to market	Reference
Dextran-streptokinase	Streptodekase	Cardiovascular and ophthalmologic	1982	Torchilin et al., 1982
PEG- adenosine deaminase	Adagen™ (Enzon)	SCID syndrome	1990	Levy et al., 1988
SMANCS	Zinostatin, Stimalamer (Yamanouchi Pharmaceutical)	Hepatocellular carcinoma	1993 (Japan)	Maeda and Konno, 1997
PEG- L-asparaginase	Oncaspar® (Enzon)	Acute lymphoblastic leukaemia	1994	Graham, 2003
PEG-interferon alpha-2b	PEG-Intron® (Schering Plough/ Enzon)	Hepatitis C	2000	Wang et al., 2002
PEG-G-CSF	Pegfilgrastim, Neulasta® (Amgen)	Prevention of neutropaenia associated with cancer chemotherapy	2002	Kinstler et al., 2002
PEG-HGR	Pegvisomant, Somavert® (Pfizer)	Acromegaly	2002	Parkinson et al., 2003
PEG-interferon alpha-2a	Pegasys® (Roche/Nektar)	Hepatitis C	2002	Bailon et al., 2001 Reddy et al., 2002

Contd.

Table 1.2 Polymer-protein conjugates currently on the market (Contd.)

Polymer-protein conjugate	Drug name (Company)	Indication	Year to market	Reference
Branch PEG-anti-VEGF aptamer	Pegaptanib, Macugen™ (EyeTech Pharmaceuticals, Pfizer)	Age-related macular degeneration	2004	Gragoudas et al., 2004
PEG-anti-TNF Fab	CDP870 (Certolizumab pegol) Cimzia® (UCB)	Crohn's disease	2008	FDA, 2008

EU = European Union; G-CSF = Granulocyte colony stimulating factor; HGR = Human growth hormone receptor antagonist; PEG = polyethylene glycol; SCID = Severe combined immunodeficiency; SMANCS = styrene maleic anhydride; TNF = tumour necrosis factor; NS = not stated

Table 1.3 Polymer-protein conjugates currently in clinical trials

Polymer-protein conjugate	Drug name / Company	Indication	Status	Reference
A PEGyated diFab antibody	CDP791 (UCB-ImClone System)	Non-Small Cell Lung Cancer (NSCLC) Targets VEGF receptor-2	Phase II	Ton et al., 2007
PEG-Anti TNF Fab	CDP870 (Certolizumab pegol)	Chronic plaque psoriasis	Phase II	NIH, 2008a
		Rheumatoid arthritis	Phase III	NIH, 2009a
PEG-Arginine deiminase	ADI-PEG	Melanoma, Neoplasm Metastatic	Phase I	NIH, 2006a
	ADI-PEG 20	Hepatocellular carcinoma	Phase II	NIH, 2006b
PEG-Glucocerebrosidase	Lysodase	Gaucher's disease	Phase I	NIH, 2008b
PEG-haemoglobin	Ro 50-3821	Anaemia, Lung cancer	Phase II	NIH, 2009b
PEG-interferon alpha-2a	NS*	Non-melanomatous skin cancer	Phase I/II	NIH, 2009c
		Chronic hepatitis B and C	Phase III	NIH, 2007a
PEG-interferon alpha-2b	PEG Intron®	Clinical evaluation on solid tumor, multiple sclerosis and HIV/AIDS	Phase II	Bukowski et al., 2002 Wang et al., 2002

Contd.

Table 1.3 Polymer-protein conjugates currently in clinical trials (Contd.)

Polymer-protein conjugate	Drug name / Company	Indication	Status	Reference
PEG-interferon alpha-2b	PEG Intron®	Gastrointestinal carcinoid tumour	Phase II	NIH, 2009d
		Chronic myeloid leukaemia		NIH, 2007b
		Brain and CNS tumours		NIH, 2009e
		Renal cell carcinoma (RCC)	Phase III	Odaimi et al., 2004
		Advanced stage IV melanoma		Krown et al., 2004
PEG-sTNF-a-R1	pegsunercept	Rheumatoid Arthritis	Phase II	NIH, 2009f
PEG-uricase	pegloticase	Gout	Phase II	NIH, 2009g

NS* = not stated

Polymer-protein conjugates represent a tripartite structure composed of; (1) a water-soluble polymer bearing a functional group(s) that will be used in conjugation. (2) A covalent linker and (3) a protein. The polymer used can either contain inherent functionality or it must first be chemically activated in order to incorporate a suitable functional group for protein conjugation. Conjugation can be either random or at specific site on the protein.

Functional stability often limits the practical application of enzymes in both medicine and biotechnology processes. Therefore, protein modification by macromolecular substances constitutes a useful tool for improving the stability of these biomolecules. In general, the effectiveness of polymer-protein conjugation depends on selection the appropriate conditions based on: (i) the type, size and structure of the protein; (ii) the structure and size of the modifying polymer; and (iii) the type and conditions for the chemical reaction involved in the modification process (Venkatesh and Sundaram, 1998).

1.2.3 Polymers used for conjugation

Several polymers have been studied as candidates for protein drug delivery. In general, an ideal polymer for parenteral use should be biodegradable or have a sufficiently low molecular weight that it can be eliminated from the body thus avoiding progressive accumulation. Homogeneity of the polymer-protein conjugate is also important so the polymer used should have low polydispersity and it should be preferably have only one reactive group to avoid undesired cross-linking. The polymer used should be able to extend body residence time either to prolong the action of conjugate or to allow distribution and accumulation in the desired body compartments. Although, there is a long list of available polymers, unfortunately not one combines all of these properties (Pasut and Veronese, 2007).

Polymers usually selected to synthesize macromolecular conjugates can be categorized according to: (a) their chemical nature: vinylic or acrylic polymer, polysaccharides, poly(amino acids), (b) the backbone stability: biodegradable or stable polymers, (c) the origin: natural or synthetic polymers, and (d) the molecular weight: oligomers, polymers (Soyez et al., 1996). Examples of some of the most popular polymers used are illustrated in Table 1.4.

Table 1.4 Examples of some of the most popular polymers

Polymer source	Example
Synthetic polymers	■ PEG ■ HPMA homopolymers ■ HPMA copolymers ■ Poly(ethyleneimine) (PEI) ■ Poly(styrene-co-maleic anhydride) (SMA) ■ Polyvinyl alcohol (PVA) ■ Polyamidoamine (PAMAM) dendrimer
Natural polymers	■ Dextran ■ Dextrin ■ Pullulan ■ Mannan ■ Chitosans ■ Hyaluronic acid (HA)
Pseudo synthetic polymers	■ Polyglutamic acid (PGA) ■ Poly(*L*-lysine) ■ Poly(aspartamides) ■ Poly(malic acid) ■ Poly((*N*-hydroxyethyl)-*L*-glutamine) (PHEG).

Even though, several polymers have been utilized for protein modification, only three polymers have been successfully brought to market for protein modification. The two non-PEGylated polymer-protein conjugates including dextran-streptokinase (Streptodekase®) and SMANCS (Zinostatin stimalamer®) and PEGylated proteins, currently and widely used as therapeutics are reviewed in the following section.

1.2.3.1 Non-PEGylated polymer-protein conjugates

Polysaccharides have been widely studied in drug delivery. Their pharmacokinetics was influenced by their molecular weight, charge, chemical modification and degree of polydispersity and/or branching. They were used for the delivery system of small drugs and for the preparation of protein conjugate (Mehvar, 2003). A large number of polysaccharides have been successfully used to modify enzymes (Table 1.5). However, dextran (α, 1-6 polyglucose) is a poly-functional natural polymer that has been the most extensively used in this class. Dextran was initially approved as plasma expander (Brocchini and Duncan, 1999) and as mentioned above. Dextran was conjugated to streptokinase to give the first polymer-protein product (Streptodekase®) that was marketed in Russia. It was commercialized for the treatment of cardiovascular and opthalmological pathologies cause by thrombosis (Torchilin et al., 1982. *Russ*.). Since, both polysaccharides and proteins are poly-functional molecules. Their coupling, using periodate oxidation yielding aldehyde groups to react with protein can cause both undesired cross-linking product and a complex or heterogeneous conjugate. Mono-functional polymers, such as PEG were thus first used to avoid such problem (Veronese and Morpurgo, 1999).

Poly(styrene-co-maleic anhydride) (SMA) is a poly-functional hydrophobic synthetic polymer obtained from styrene and maleic anhydride. The anti-tumor protein neocarzinostatin (NCS), which exhibited cytotoxicity against mammalian cells and Gram-positive bacteria, was covalently bound to SMA to form the conjugate "Styrene-co-maleic anhydride-neocarzinostatin" (SMANCS, Zinostatin stimalamer®). It was approved only in Japan and brought to market in 1993 for treatment of primary liver cancer (hepatocellular carcinoma). Due to the increase in hydrophobicity of SMANCS, it can be solubilized in lipid media (Lipiodol; poppy seed oil) and after intrahepatic-artery administration, using the femoral artery to access the hepatic artery, the Lipiodol allow visualization of tumors killing. This conjugate increases plasma half-life, and tumor-targeting has shown excellent quality in patients with primary liver cancer (Maeda and Konno, 1997; Maeda, 2001).

Table 1.5 Polysaccharide-protein conjugates

Polymer	Derivatization	Protein	Reference
Carboxymethylcellulose	Periodate oxidation	α-amylase	Villalonga et al., 1999
	Polyaldehyde	Trypsin	Villalonga et al., 2000
	Modified with β-cyclodextrin	Trypsin	Villalonga et al., 2003
Chitosan	Acetamide	Invertase	Gomez et al., 2000
	Periodate oxidation	Cellulase	Darias and Villalonga, 2001
α- , β-, and γ-cyclodextrin	Monoamine	Trypsin	Fernandez et al., 2002
β-cyclodextrin	Monoamine ($CDNH_2$)	Trypsin	Fernandez et al., 2003
	Ethylenediamine (CDEN)		
	Propylenediamine (CDPN)		
	Butylenediamine (CDBN)		
	O-carboxymethyl	α-Chymotrypsin	Fernandez et al., 2004b
	Dicarboxylic acid	Trypsin	Fernandez et al., 2004a
	Carboxylic acid	Oxytocin	Bertolla et al., 2008

Contd.

Table 1.5 Polysaccharide-protein conjugates (Contd.)

Polymer	Derivatization	Protein	Reference
Dextran	Cyanogen bromide	Trypsin	Marshall and Rabinowitz, 1976
		Catalase	Marshall and Humphreys, 1977
		α-amylase	Donlan, 1988
	Methylation or acetylation	α-amylase	Srivastava, 1991
	Aminated	Catalase	Valdivia et al., 2006
Dextrin	Succinoylation	Trypsin	Duncan et al., 2008
		rhEGF†	Hardwicke et al., 2008
		Phospholipase A_2	Ferguson and Duncan, 2009
Oxidized sucrose polymer	Aldehyde	Trypsin	Venkatesh and Sundaram, 1998
Pectin	Ethylenediamine	Invertase	Gomez and Villalonga, 2000
Sodium alginate	Periodate oxidation	α-amylase	Gomez et al., 2001

†rhEGF = Recombinant human epidermal growth factor

1.2.3.2 PEGylated proteins

PEGylation is now a well recognized tool that can improve the properties of protein and peptide drugs. PEG (Figure 1.2a) is formed from repeating units of ethylene glycol and the polymer can have linear or branched chain of different molecular mass that is terminated with hydroxyl groups. PEG is synthesized by anionic ring polymerization of ethylene oxide using anhydrous alkanols such as methanol or derivatives including methoxyethoxy ethanol as initiator to yield diol PEG or monoalkyl capped PEG such as mPEG, respectively. As mPEG (Figure 1.2b) is monofunctional, it is most useful for protein modification due to it does not give rise to cross-linking (Greenwald, 2003; Harris and Chess, 2003). The presence of trace amounts of water during polymerization resulted in a number of diol PEG therefore commercially available mPEG can typically contain some diol PEG as an impurity. This diol PEG content is dependant on the molecular weight (% diol PEG content is lower for low Mw PEG) due to polymerization at both ends of the polymer. The amount of diol PEG can exceed 1-10% and this can significantly increase the heterogeneity of the conjugates synthesized by cross-linking which can also result in protein inactivation (Veronese et al., 2001).

(a) PEG **(b) mPEG**

Figure 1.2 Structures of (a) polyethylene glycol (PEG) and (b) monomethoxy polyethylene glycol (mPEG)

PEG is a non-toxic, inert, amphiphilic polymer. It can be synthesized in a variety of molecular weights. In comparison to other polymers, PEG has a relatively narrow polydispersity (Mw/Mn) in the range of 1.01 (for < 5,000 g/mol) to 1.1 (for > 50,000 g/mol). This polymer is soluble in both water and organic solvents, non-toxic, biocompatible and lacks immunogenicity. The FDA has approved PEG for using as a vehicle or base in foods, cosmetics and pharmaceuticals including injectable, topical, rectal and nasal formulations. PEG of

molecular weight < 30,000 g/mol is eliminated from the body via the kidney, and PEG > 20,000 g/mol is additionally excreted in the faeces (Greenwald, 2003; Harris and Chess, 2003). PEG shows a high flexibility of the main chain in aqueous solution due to a high level of hydration with 2-3 water molecules tightly associated with each ethylene glycol subunit. The apparent molecular weight of PEG is 5-10 times larger than a soluble protein of similar molecular weight. All these factors contribute to PEG is ability to reduce immunogenicity and antigenicity, and prevent degradation of covalently bound proteins by enzymes (Roberts et al., 2002).

As has been previously studied, PEG has been successfully used for conjugation of many therapeutic proteins (shown in Table 1.2). The first FDA approved conjugate, PEG-adenosine deaminase (Adagen™) came to market in 1990 and was approved for the treatment of severe combined immunodeficiency disease (SCID). It was successfully commercialized by Enzon, Inc. In this case, multiple chains of PEG (5,000 g/mol) were covalently bound to one molecule of bovine adenosine deaminase (40,000 g/mol) using amide bond. Previously, SCID patients were transfused with red blood cells as they contain adenosine deaminase. However, repeated blood transfusion runs the risk of iron overload and transfusion-associated viral infection. PEG-adenosine deaminase had an increased plasma half-life and it was less immunogenic and more effective than previous therapies involving blood transfusion or injection of the free enzyme (Hershfield et al., 1987; Harris and Chess, 2003).

PEG-*L*-asparaginase (Oncaspar®) was the second FDA approved product to come to market (Enzon, Inc.) in 1994. In this case, *L*-asparaginase (135,000 g/mol) was conjugated to multiple strands of PEG (5,000 g/mol) via an amide bond and the product became an effective drug for the treatment of acute lymphoblastic leukemia (ALL). Leukemic cells are unable to synthesize asparagines, and need an extend source of this amino acid in order to grow. When administered, *L*-asparaginase derived from *E. coli* can hydrolyze asparagine present in the blood and this inhibits leukaemic cells growth. However, administration of free *L*-asparaginase can caused hypersensitivity reactions and the production of neutralizing antibodies that shorten its half-life. Synthesis of the PEG-*L*-asparaginase conjugate reduces allergic reaction (to less than 8%) and increases the circulating half-life of protein. The latter reduces the frequency of

administration needed and decreased the requirement for daily doses to an injection once every 2 weeks (Park et al., 1981; Greenwald et al., 2003).

PEGylated interferon-α-2b (PEG-Intron®) was introduced to market in 2000 and it is used for the treatment of hepatitis C. PEG-Intron® contains a single strand of linear PEG (12,000 g/mol) linked to one molecule of interferon (19,000 g/mol). The PEG used is first activated as the succinimidyl carbonate derivative (SC-PEG) and this is then reacted with target amino acids in the protein to give an amide bond. The resulting product is a mixture of positional isomers (Wang et al., 2002). The circulating half-life of PEG-Intron-α-2b was shown to be 8 times higher than that of native interferon-α-2b. This allows treatment by a weekly subcutaneous injection of PEG-Intron® (Greenwald et al., 2003). Another conjugate developed to treat hepatitis C is PEGylated interferon-α-2a, PEGASYS®. It was commercialized in 2002 in this case using a branched PEG (40,000 g/mol) conjugated to interferon-α-2a. Native interferon-α-2a is given by injection every other day, but its short circulating half-life leads to pulsed blood concentration levels. When high molecular weight branched PEG was conjugated to Interferon -α-2a, a longer circulating half-life conjugation gave a great improvement in the pharmacokinetic profiles of the protein was achieved. Administration of the conjugate also resulted in prolonged absorption from subcutaneous injection site (Reddy et al 2002) and once weekly injection led to near constant blood concentrations above the therapeutic level over the one week period (Harris and Chess, 2003).

PEGylation of granulocyte-colony stimulating factor (G-CSF), Neulasta® or PEG-filgrastim, gave a product that was approved by U.S. FDA for the market in 2002. It is used to prevent neutropaenia associated with cancer chemotherapy. A single PEG chain (monofunctional mPEG aldehyde, 20,000 g/mol) was covalently bound to the *N*-terminal amino group of the methionyl residue of G-CSF (19,000 g/mol). Conjugation reduces renal clearance, improves efficacy and gives better patient's compliance (Kinstler et al., 2002; Greenwald et al., 2003).

Other PEG conjugates have been developed for clinical use e.g. human growth hormone receptor antagonist (HGR) (Parkinson et al., 2003). Recently, a PEGylated anti-TNF

antibody fragment (CDP870, Certolizumab pegol or Cimzia®) came to market as a new treatment for Crohn's disease (FDA, 2008). It is also being developed (Clinical trial phase III) as a treatment for rheumatoid arthritis (Sandborn et al., 2005; NIH, 2009a). In each case the conjugated protein has an extended circulating half-life.

1.2.4 Polymer-protein conjugation chemistry

An extensive variety of conjugation chemistries have been used to prepare PEGylated proteins. The PEG must first be functionalization and then protein conjugation can be accomplished by binding to a hydroxyl, an amino or a carboxyl group present in the protein (Harris and Chess, 2003).

In order to avoid protein cross-linking during polymer conjugation, a semi-telechelic polymer (one with a single reactive group at one terminal end) is required. As mentioned above, in the early studies, the protein was bound to multiple PEG chains. However, today a 1:1 molar ratio of polymer : protein is often preferred (Chapman et al., 1999) as it allows the synthesis of better defined products. Linear mPEG has been widely used as the one terminal functional group prevents cross-linking. Most frequently a molecular weights of 5,000 g/mol has been used (Levy et al., 1988). The mPEG terminal hydroxyl group must first be activated before protein conjugation. The method used for activation is important as it determines the type of covalent bond that will be formed between the polymer and protein and consequently effect the biological properties of the conjugate. Furthermore, the linker used must be chosen carefully to make certain that the linking chemistry will not generate toxicity or immunogenicity (Harris and Chess, 2003). There are a number of different amino-acid residues to which PEG may be conjugated. These include: arginine (ARG), aspartic acid (ASP), cysteine (CYS), glutamic acid (GLU), histidine (HIS), lysine (LYS), serine (SER), threonine (THR), and tyrosine (TYR), which their *N*- and *C*-terminal groups could be conjugated (Roberts et al., 2002).

The most useful protein functionalities for specific chemical modification are the natural amino acids side chains of cysteine (CYS), lysine (LYS), tyrosine (TYR) and glutamine (GLN) together with the α-*N* terminus (α-*N*) of the peptide backbone. The thiol side chain in

CYS is a mild nucleophile and this fact has been extensively utilized for the specific chemical modification of protein. CYS can be modified selectively, rapidly and in a quantitative fashion under appropriate conditions. The reactions used for CYS modification include alkylation and disulfide formation. In both cases, the overall charge of the protein is maintained, which is an additional advantage of reacting with CYS (Thordarson et al., 2006). Site-specific conjugation has been achieved using thiol reactive PEGs such as PEG-maleimide, PEG-vinyl sulfone, PEG-iodoacetamide and PEG-orthopyridyl disulfide (Sato et al., 2002). These specifically conjugate to CYS or thiol residues present in the protein to form thioether linkages. However, it should be noted that the conjugation of thiol reactive PEG may be reversed in a thiol-reducing environment.

In the absence of a free CYS in a native protein, one or more CYS residues would be inserted into the protein sequence by genetic engineering. This approach can be used to minimize loss in bioactivity, prepare well defined conjugates and to decrease immunogenicity. However, with this approach it is possible to increase incorrect disulfide formation and protein dimerization (Roberts et al., 2002).

1.2.5 Polymers used for conjugation in this study

For this study, five polymers were used. They were chosen as they are different in both structure and molecular weight. The polymers include PEG, β-CD, PAMAM dendrimer, dextrin and ST-HPMA homopolymer.

1.2.5.1 PEG

As described above (section 1.2.3.2), PEG has been widely used for conjugation with many kinds of protein such as cytokine, enzyme and hormone. Bioconjugated therapeutics are summarized in Table 1.6. A library of PEG-trypsin conjugates synthesized using PEGs of different molecular weight (Mw 1,100, 2,000 and 5,000 g/mol) and different linking chemistries are described and also discussed in Chapter 3.

Table 1.6 Examples of studies on the PEG-protein conjugates

Protein	Type of study	Reference
Cytokine		
Erythropoietin	*In vivo* studies on efficacy and therapeutic effects	Malik et al., 2000
GM-CSF	*In vivo* studies on efficacy and immunogenicity	Malik et al., 1992
Interleukin (-6, -2 and -15)	*In vivo* studies on immunogenicity	Gonzalez-Garcia et al., 1996
Enzyme		
Adenosine phosphorylase	Alternative enzyme therapy for adenosine deaminase deficiency	Brewerton et al., 2003
Alkaline phosphatase	Effect of coupling chemistry on activity	Yoshinga and Harris, 1989
Catalase	*In vivo* studies on immunogenicity	Abuchowski et al., 1977a
	In vitro studies on pharmacological properties	Beckman et al., 1988
Chymotrypsin	Activity studies	Chiu et al., 1993
	Characterization of binding sites	Schering et al., 2004
Elastase	Enzymatic activity studies	Koide and Kobayashi, 1983
Galactosidase (α and β)	Assessment of activity and properties	Beecher et al., 1990; Matsuo et al., 1997
Glutaminase	Anticancer agent	Harzmann et al., 2004
Gulonolactone oxidase	Catalytic activity studies	Hadley and Sato, 1989

Contd.

Table 1.6 Examples of studies on the PEG-protein conjugates (Contd.)

Protein	Type of study	Reference
Enzyme (Contd.)		
Lysozyme	Identification of PEGylation site	Veronese et al., 2001a
Peroxidase	Effect of PEG size on modification	Fortier and Laliberte, 1993
Ribonuclease	Antibody recognition, substrate accessibility and conformational stability studies	Caliceti et al., 1990
	Aspermatogenic and anti-tumor activity studies	Matousek et al., 2002
	Biological and anti-tumor activity studies	Matousek et al., 2004
Superoxide dismutase	Kinetic studies	Argese et al., 1993
	In vitro studies on pharmacological properties	Beckman et al., 1988
Tissue plasminogen activator	Characterization of functional activity in different species	Berger and Pizzo, 1988
Trypsin	Activity studies	Abuchowski and Davis, 1979; Gaertner and Puigserver, 1992; Zhang et al., 1999; Murphy and O'Fagain, 1996
Urokinase	Activity studies	Sakuragawa et al., 1986 Garman and Kalindjian, 1987

Contd.

Table 1.6 Examples of studies on the PEG-protein conjugates (Contd.)

Protein	Type of study	Reference
Hormone		
Glucagon	Efficacy and pharmacokinetics	Lee et al., 2004
Human growth hormone	*In vivo* studies on physical and biological properties	Clark et al., 1996
Insulin	Microencapsulation, administration route	Reviewed in Hinds and Kim, 2002
Melanin	Biotechnological application	Ishii et al., 1995
Other		
Albumin	*In vivo* studies on immunogenicity	Abuchowski et al., 1977a
Avidin	Structure activity relationship study	Caliceti et al., 2002
Biphalin	*In vivo* studies on analgesia	Huber et al., 2003
Desmopressin	*In vivo* studies on efficacy	Veronese, 2004
Fab′	Half-life and retention of activity	Chapman et al., 1999
Haemoglobin	Effect of PEG size on properties	Manjula et al., 2003
Ovalbumin	Melanoma	Jackson et al., 1987

GM-CSF = Granulocyte-macrophage colony stimulating factor; Fab′ = antibody fragment

1.2.5.2 β-Cyclodextrin

Cyclodextrins (CDs) are a family of cyclic non-reducing α-(1,4)-linked-D-glucopyranose. They composed of 6 (cyclohexaamylose), 7 (cycloheptaamylose) or 8 (cyclooctaamylose) glucose units in the chair conformation namely α-, β- and γ-CDs, respectively (Szejtli, 1998). CDs are potential candidates for drug development because they can be used either for complexation or as functional carrier materials in pharmaceutical formulations. β-CD, the most common natural CD contains 21 hydroxyl groups, 7 groups are primary and the rest are secondary hydroxyls (Figure 1.3). All of these hydroxyl groups are available as starting points for structural modifications. Various functional groups have been introduced into the macrocyclic ring (Table 1.7). Many CDs derivatives have been extensively used to improve drug properties such as solubility, dissolution rate, stability or bioavailability. They were used to modify drug release in oral preparation for either immediate-release, prolonged-release, delayed-release or site-specific release such as colonic delivery (Hirayama and Uekama, 1999).

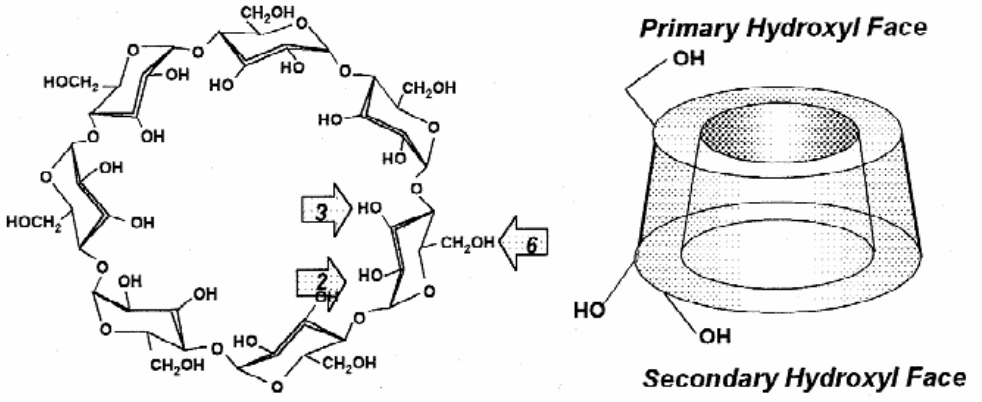


Figure 1.3 Structure of β-CD shows hydroxyls located on the edge of the β-CD ring (from Hirayama and Uekama, 1999).

CDs can be chemically modified to introduce a variety of functional groups including amines, thiols, aldehydes etc. These modified oligosaccharides can be bound to polymers, peptides and also surfaces of nanoparticles as previously summarized in Table 1.5. For

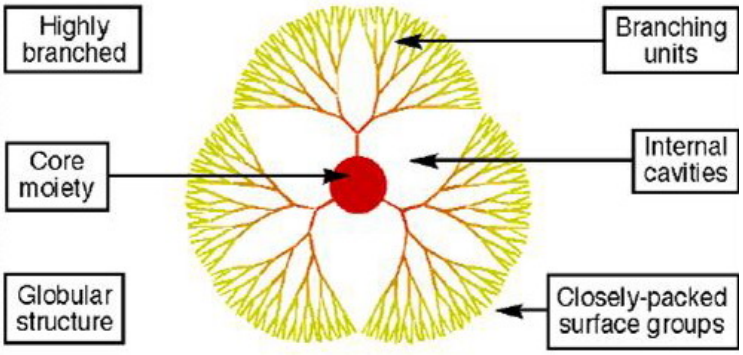
example, the monoamine derivatives of α- , β-, and γ-CD has been used to modify bovine trypsin. The esterolytic activity and the affinity of trypsin for substrate were improved by the attachment of the CD residues (Fernandez et al., 2002). Trypsin and chymotrypsin conjugates were also prepared with several β-CDs including monosubstituted amino-β-CDs (Fernandez et al., 2003) or dicarboxyl-β-CDs (Fernandez et al., 2004b; Fernandez et al., 2004a). β-CD was chosen for these studies because it can be conjugated to protein amino acid residues under mild conditions and it has a potential to enhance stability of proteins (as described above).

Table 1.7 Pharmaceutical useful of β-CD derivatives obtained by substitution of the -OH groups located on the edge of the β-CD ring (from Hirayama and Uekama, 1999).

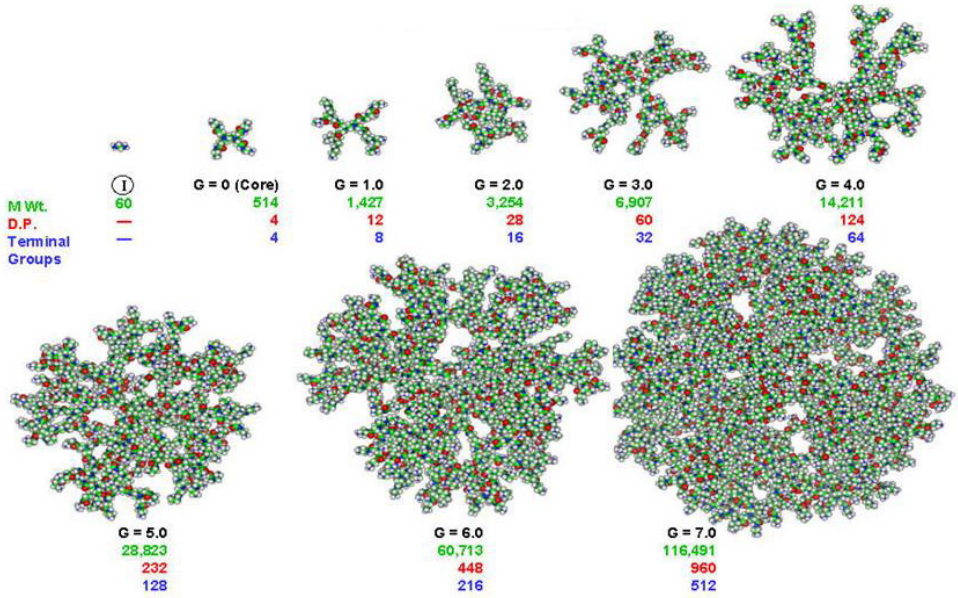
Derivative	**Position of substituent**	**Substituent**
Hydrophilic derivative		
Methylated β-CD	2, 6-; 2, 3, 6-	-O-CH_3
Hydroxylate β-CD	Random	-O-CH_2-CH(OH)-CH_3
Branch β-CD	6-	-Glucosyl; -maltosyl
Hydrophobic derivatives		
Ethylated β-CD	2, 6-; 2, 3, 6-	-O-C_2H_5
Peracylated β-CD	2, 3, 6-	-O-CO$(CH_2)_n$-CH_3
Ionizable derivatives		
Carboxyalkyl β-CD	Random	-O-$(CH_2)_n$-COONa
Carboxymethyl β-CD	2, 6-; 3-	-O-CH_2COONa
Ethyl β-CD	2, 6-; 3-	-O-C_2H_5
Sulfates	Random	-O-SO_3Na
Alkylsulfones	Random	-O-$(CH_2)_n$- SO_3Na

1.2.5.3 Polyamidoamine (PAMAM) dendrimer

Dendrimers are branched macromolecules that are synthesized using a series of reactions radiating out from an inner core (Figure 1.4). Dendrimers can be synthesized starting from the central core and working out toward the periphery, divergent synthesis (Tomalia et al., 1985) or using a top-down approach, starting from the outermost residues providing wedges that are eventually bound to a core, convergent synthesis (Hawker and Frechet, 1990) (Figure 1.5).



(a)



(b)

Figure 1.4 Scheme of (a) a dendrimer molecule and (b) three-dimension structure of dendrimer in each generation (from http://www.eng.buffalo.edu/Courses/ce435/Dendrimers/Dendrimers.html).

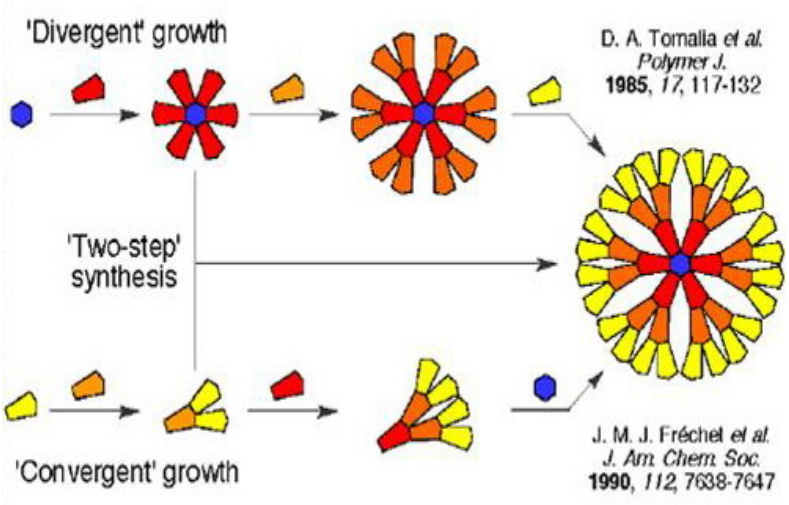


Figure 1.5 Dendrimer synthesis approaches: divergent synthesis and convergent synthesis (from http://www.eng.buffalo.edu/Courses/ce435/Dendrimers/Dendrimers. html).

Dendrimers possess a well-defined structure that allows precise control of size, shape and terminal group functionally (Tomalia et al, 1985). They can theoretically be synthesized to be monodisperse, and they are hyperbranched synthetic macromolecules with a large number of surface groups. The ideal dendrimer carrier for development as a parenteral polymer therapeutic should exhibit high aqueous solubility and high drug-loading capacity, biodegradability, low toxicity, favorable retention and distribution characteristics, specificity for the target disease, and have appropriate bioavailability. Dendrimers potentially have a number of pharmaceutical applications such as enhancing the solubility of poorly soluble drugs, enhancing the delivery of DNA and oligonucleotides and as novel carriers for drug delivery (Wolinsky and Grinstaff, 2008).

Dendrimers have been investigated as drug carriers using two strategies. First, dendrimer-drug conjugates (Figure 1.6a), where a drug is covalently conjugated to form a macromolecular drug conjugate. Alternatively, drug-encapsulation (Figure 1.6b), dendrimers have been proposed as containers which physically entrap drug molecules inside the dendritic structure. a drug is non-covalently encapsulated in the interior of the dendrimer (Wolinsky and Grinstaff, 2008). This section reviews dendrimer conjugation, particularly conjugation of PAMAM dendrimers with macromolecules as this is most relevant to this study.

The dendrimer terminal functional groups determine their solubility, and physical and chemical interaction in the surrounding environment (Zeng and Zimmerman, 1997). Many families of dendrimers have been synthesized using a wide variety of core molecules and building monomers. The PAMAM dendrimer (Figure 1.7), is a commercially available framework, containing ethylenediamine core and branch units are constructed from both methylacrylate and ethylenediamine (Tomalia et al., 1985).

PAMAM dendrimers have been widely used to prepare drug conjugates, generally with the drug covalently attached to the peripheral groups of the dendrimer (Table 1.8). Multiple drug molecules can be attached to each dendrimer molecule and the release of these therapeutic molecules is partially controlled by the nature of the linkages (Wolinsky and Grinstaff, 2008). PAMAM conjugates have been proposed to increase the solubility of Naproxen, the poorly water-soluble drug (Najlah et al., 2006). Naproxen was conjugated to zero generation (G0-NH_2) PAMAM dendrimer directly, by an amide bond or by ester bond using *L*-lactic acid or diethylene glycol as a linker. It was found that all of the prodrugs were more hydrophilic than parent drug. Conjugates formed by amide linkage were not suitable for the development of prodrug for oral delivery due to their high chemical and enzymatic stability. On the other hand, it was suggested the conjugates formed by lactic acid ester linkage displayed potential as controlled release systems or prodrugs for drug targeting. For site-specific colonic drug delivery, Wiwattanapatapee and coworker (2003) conjugated 5-aminosalicylic acid (5-ASA) to third generation (G3-NH_2) PAMAM dendrimer using either *p*-aminobenzoic acid or *p*-aminohippuric acid as a spacer. The amount of drug released over 24 h when incubated was between 45% and 57% for these conjugates compared with 80% in 6 h from the commercial prodrug, sulfasalazine.

PAMAM dendrimers have potential for many different uses ranging from gene delivery to magnetic resonance imaging the development of vaccines, antiviral and antibacterial agents, and especially as anticancer therapeutics (Gillies and Frechet, 2005). Duncan and Malik (1996) reported the first PAMAM dendrimer anticancer conjugate. Cisplatin (Duncan and Malik, 1996; Malik et al., 1999) and paclitaxel (Khandare et al., 2006) conjugates have been described.

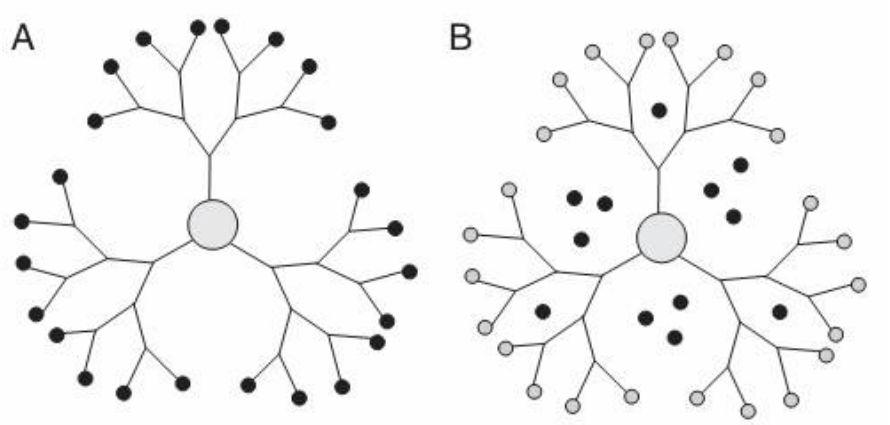


Figure 1.6 Strategies for dendrimer drug delivery. Panel (A) dendrimer-drug conjugates and panel (B) drug-encapsulation. Black dots represent drug molecules (from Klajnert et al., 2003).

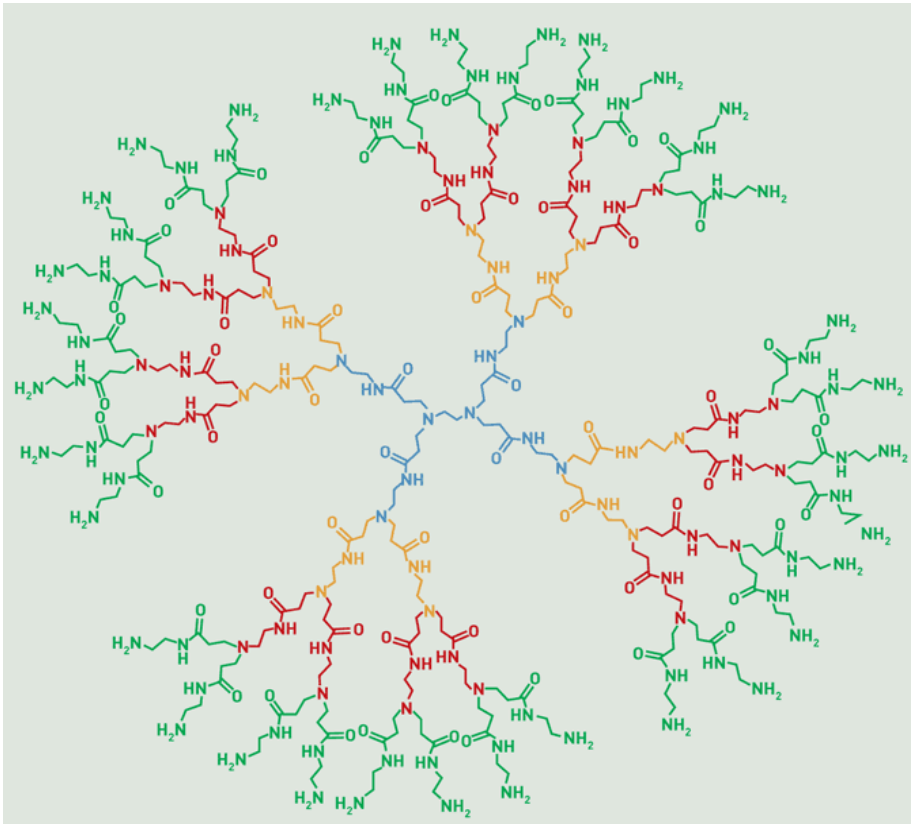


Figure 1.7 Structure of PAMAM dendrimer (G3-NH_2). The core is shown in blue and each successive generation is shown in a different color.
(from http://pubs.acs.org/cen/coverstory/83/8324dendrimers.html)

The anticancer agent, cisplatin has been conjugated to a G3.5-NH_2 PAMAM dendrimer giving a highly water-soluble dendrimer-platinate which released platinum slowly *in vitro*. The dendrimer-platinate conjugate was between 3- and 15- fold less toxic than cisplatin and its selective accumulation in solid tumor tissue by the enhanced permeability and retention (EPR) effect was appreciably higher (Malik et al., 1999).

Paclitaxel, a poorly soluble anticancer drug, was covalently conjugated with PEG or G4-OH PAMAM dendrimer to compare the anticancer activity of the drug delivery by a linear or dendritic carrier. The potential enhancement of drug solubility and cytotoxicity were determined. Both PEG and PAMAM dendrimer increased the aqueous solubility of paclitaxel (0.3 μg/mL) dramatically to 2.5 mg/mL and 3.2 mg/mL, respectively. The conjugates were evaluated using A2780 human ovarian carcinoma cells. PEG-paclitaxel conjugates reduced the efficacy of the drug 2.5-fold, but PAMAM-paclitaxel conjugates showed the IC_{50} decreased or cytotoxic increased by 10-fold compared with free drug. The results demonstrated that the availability of the drug was dramatically influenced by the architecture of its polymer conjugates (Khandare et al., 2006).

There are relatively few studies describing PAMAM dendrimer conjugated to a drug and a targeting moiety e.g. folic acid, monoclonal antibodies or peptides. These targeting residues have been used to increase tumor specific delivery (Wolinsky and Grinstaff, 2008). G4-NH_2 PAMAM dendrimer conjugation to porphyrin has also been used for gene therapy using to facilitate transfection irradiation (under non-toxic conditions) (Shieh et al., 2008).

PAMAM-protein conjugate has been rarely studied. It is interesting to study the effect of the globular structure of PAMAM dendrimer on activity and stability of the conjugated protein. Therefore PAMAM-trypsin conjugate is synthesized and its biological properties is determined and discussed further in Chapter 4.

Table 1.8 Examples of reported PAMAM-drug conjugates

Drug	Generation-Type	Type of study	Reference
5-fluorouracil	G4-NH_2	Slow release and reduce the toxicity	Zhuo et al., 1999
	G5-NH_2		
5-ASA	G3-NH_2	Colon-specific drug carriers	Wiwattanapatapee et al., 2003
Biotin	Acetylated G5	Targeting cancer cells	Yang et al., 2009
Cisplatin	G3.5-NH_2	Cancer therapy	Duncan and Malik, 1996
			Malik et al., 1999
Doxorubicin	Bow-tie dendrimer	*In vivo* studies on colon carcinomas	Lee et al., 2006
Ibuprofen	G4-OH	Preparation, cellular transport and activity studies	Kolhe et al., 2006
Methotrexate	Folate-PAMAM	Drug carriers with tumor cell specificity	Kono et al., 1999
Methotrexate	G2.5-COOH	Activity studies on cell line	Gurdag et al., 2006
	G3-NH_2		
Methylprednisolone	G4-OH	Synthesis, cellular transport and activity studies	Khandare et al., 2005
Naproxen	G0-NH_2	Stability and bioavailability studies	Najlah et al., 2006
Paclitaxel	G4-OH	Anticancer drug	Khandare et al., 2006

Contd.

Table 1.8 Examples of reported PAMAM-drug conjugates (Contd.)

Drug	Generation-Type	Type of study	Reference
Penicillin V	G2.5-NH_2	*In vitro* release study	Yang and Lopina, 2003
	G3-NH_2		
Porphyrin	G4-NH_2	Phototriggered gene transfection	Shieh et al., 2008
Propanolol	G3-NH_2	Effects on solubility, efflux transporters and bioavailability	D'Emanuele et al., 2004
Venlafaxine	G2.5-NH_2	Extended release	Yang and Lopina, 2005

G = generation; 5-ASA = 5-aminosalicylic acid

1.2.5.4 Dextrin

Dextrin (Figure 1.8a) is a natural α-1,4 poly(glucose) polymer obtained by enzymatic hydrolysis of corn or potato starch. It displays minimal branching containing few (< 5%) α-1,6 linkages. Dextrin is non-toxic and non-immunogenic and it has recently been used to develop novel biodegradable protein and peptide conjugates (Duncan et al., 2008; Hardwicke et al., 2008). α-Amylase randomly hydrolyzes the α-1,4-D-glucosidic linkages of dextrin to yield disaccharide degradation products such as maltose (α-1,4) and isomaltose (α-1,6) (Kost and Goldbart, 1997).

The similar structure of dextrin, pullulan, dextran and amylase were shown in Figure 1.3. Pullulan (Figure 1.8b) is a linear polysaccharide with maltotriosyl repeating units joined by α-1,6 linkages. Alternatively, the structural formula of pullulan may be presented as a regular sequence of panoses bonded by α-1,4 linkages. However, a small proportion of α-1,3 linkages are also present in the structure (Shingel, 2004). Dextran (Figure 1.8c) contains only α-1,6 linkages while amylose (Figure 1.8d) contains only α-1,4 linkages in the structure.

Dextrins have been used clinically for many years as a dietary supplement (Maltodextrin, Caloreen®) in cases of renal and hepatic failure (Alsop, 1994). They were FDA approved for routine parenteral administration as dialysis solution (Icodextrin™) (Mistry and Gokal, 1994). It is also used as a formulation solution for peritoneal administration of 5-fluorouracil (5-FU) (Kerr et al., 1996), and to prevent postoperative adhesions (Adept®) (Verco et al., 2000). Dextrin-2-sulfate was also studied clinically as an intraperitoneal (IP) treatment for HIV AIDS (Thornton et al., 1999). Dextrin modified by succinoylation (Hreczuk-Hirst et al., 2001b) was shown to be reproducible and was used to introduce the reactive groups necessary for covalent drug conjugation e.g. anticancer drug, doxorubicin (Hreczuk-Hirst et al., 2001a) and antifungal agent, amphotericin B (German et al., 2000). Duncan and colleagues studied (2008) to investigate dextrin as a polymer for generation of polymer therapeutics. The degree of dextrin succinoylation can be used to tailor the rate of amylase degradation (Hreczuk-Hirst et al., 2001a).

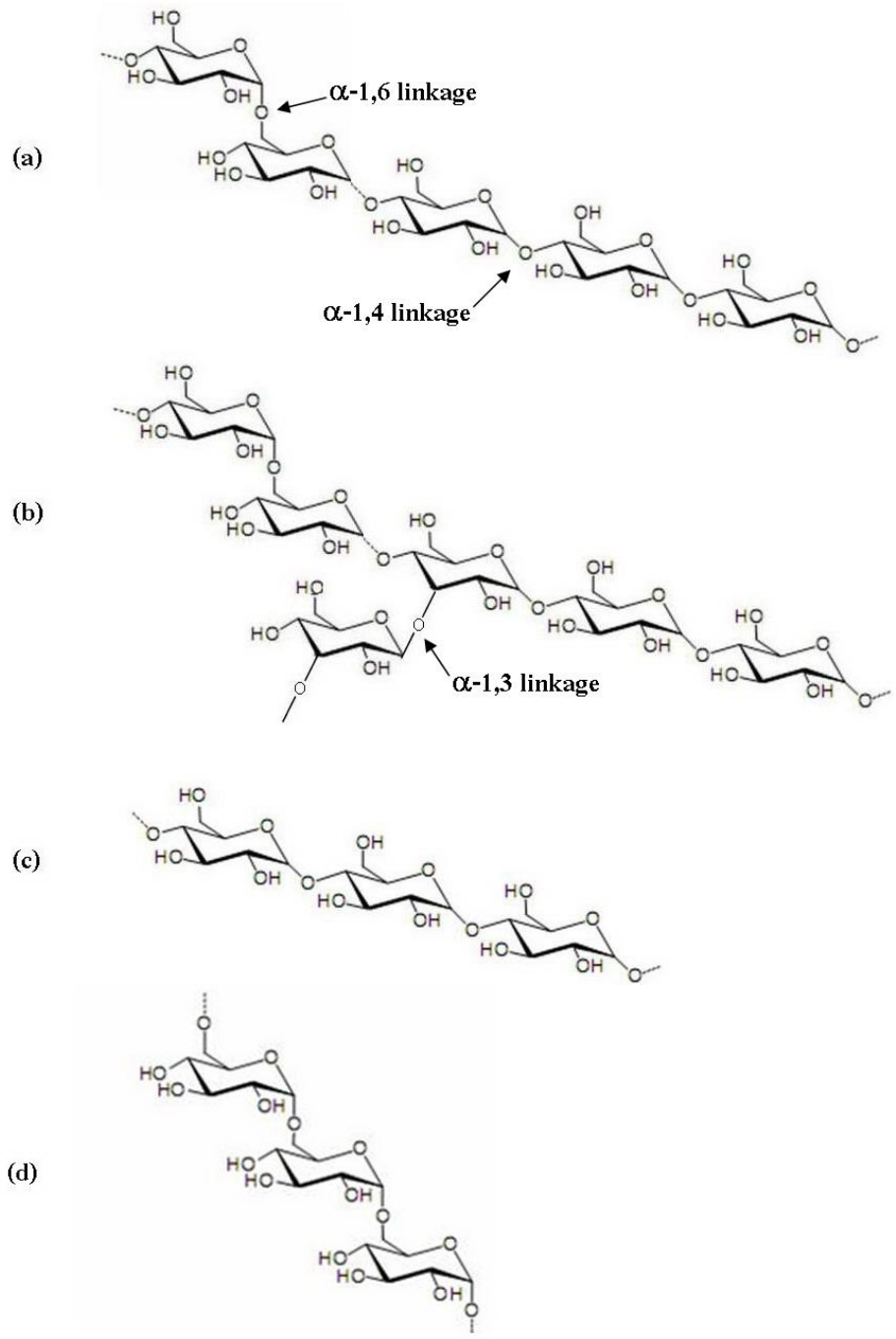


Figure 1.8 Structure formula of the repeating unit of (a) dextrin [α-1,4 and (< 5%) α-1,6 linkages] in comparison with (b) pullulan [α-1,4, α-1,6 and α-1,3 linkages], (c) dextran [α-1,6 linkages] and (d) amylose [α-1,4 linkages] (adapted from Shingel, 2004).

A number of dextrin-protein conjugates have been investigated (Chapter 1, Table 1.5). Recently, it was used as a model polymer to generate novel, bioresponsive protein conjugates for use in the context of a new concept called **P**olymer-masking-**U**n**M**asking-**P**rotein **T**herapy (PUMPT) (Duncan et al., 2008). In this approach, a biodegradable polymer is used to transiently mask a protein during transit (thus stabilizing/inactivating the protein), whilst subsequently allowing triggered polymer degradation, protein unmasking and the restoration of protein bioactivity in a controlled manner. In the context of PUMPT, succinoylated dextrin has been used to prepare bioresponsive (α-amylase triggered degradation) conjugates of recombinant human epidermal growth factor (rhEGF), a polymer therapeutic designed to promote wound healing (Hardwicke et al., 2008), and phospholipase A_2 (PLA_2), an anticancer conjugate (Ferguson and Duncan, 2009).

1.2.5.5 *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer

Semitelechelic (ST) polymers are linear macromolecules containing a functional group at one end of the polymer chain. This functional group can be used for conjugating the polymer chain to other molecular species or surface without causing cross-linking (Lu et al., 1998). HPMA copolymers have been widely used for drug conjugation and in particular, doxorubicin conjugates were the first anticancer polymer therapeutics to be tested clinically (Duncan, 2003; Duncan, 2006). HPMA is not charged, non-toxic, non-immunogenic, does not bind to blood protein and has shown good biocompatible clinically. HPMA copolymers of molecular weight < 40kDa are readily excreted via renal elimination and HPMA copolymers of molecular weight above renal threshold remain in circulation for long period and can be retain in the body by the reticuloendothelial system (RES) and skin (Satchi-Fainaro et al., 2002). Conjugation of HPMA copolymer with proteins resulted in decreased immunogenicity and resistance to proteolytic degradation.

The HPMA copolymer chain has less hydrated radius than PEG at the same molecular weight (Kamei and Kopecek, 1995), and GPC indicated that ST-HPMA copolymer

chain is smaller and more tightly bound conformations than PEG in water. This suggested that larger ST-HPMA copolymer chains would be needed to match effect of PEG. The attachment of such multifunctional HPMA copolymer most probably results in multiple points of attachment to the protein, and can bring the disadvantage of cross-linking. More recently, the synthesis of ST-HPMA copolymers has been used to permit one-point attachment of HPMA to surfaces of proteins (Lu, et al., 1998).

In the context of protein modification, ST-HPMA with different functional end groups (carboxyl and amino groups) were successfully synthesized by chain transfer free-radical polymerization and linked to the model protein, α-chymotrypsin (Lu et al., 1998; Oupicky and Ulbrich, 1999). These studies showed that the type of bond between the enzyme and the polymer affected activity of the conjugates. It is known that the carboxyl group of an aspartic acid is important in the active site of chymotrypsin and this was consistent with the observed decrease in the activity of the carboxyl group-modified chymotrypsin conjugates. In contrast to the activity of modified chymotrypsins which was attached to amino group of the polymer was higher than that of the native enzyme.

In early studies, HPMA copolymers and ST-HPMA-COOH (Figure 1.9) were used for the modification of proteins e.g. acetylcholinesterase (Laane et al., 2003), bovine seminal ribonuclease (BS-RNase) (Ulbrich et al., 1997 and 2000; Oupicky and Ulbrich, 1999; Soucek et al., 2001; Pouckova et al., 2004), insulin (Chytry et al., 1996), superoxide dismutase (Sure et al., 2002), and ribonuclease A (RNase A) (Soucek et al., 2002; Pouckova et al., 2004). Studies with these HPMA copolymer- and ST-HPMA-protein conjugates are summarized in Table 1.9. Therefore, ST-HPMA-COOH was a polymer of interest chosen for the modification of the model proteins both trypsin and RNase A in this study.

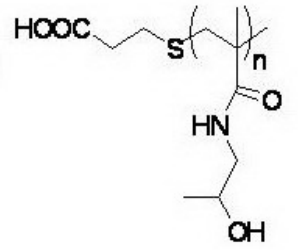


Figure 1.9 Structure of ST-HPMA containing carboxylic group (ST-HPMA-COOH)

Table 1.9 Examples of studies on the HPMA-protein conjugates

Polymer	Protein	Type of study	Reference
HPMA copolymer	Acetylcholinesterase	*In vivo* studies on prolong circulation times	Laane et al., 2003
	Antibody	Biological properties	Rihova and Kopecek, 1985
		Studies for tumor selective prodrug activation	Satchi-Fainaro et al., 2002
	Bovine seminal ribonuclease	Cancer therapy	Ulbrich et al., 1997
		In vivo studies on inhibition of solid tumor growth	Soucek et al., 2001
	Chymotrypsin	Covalent attachment	Laane et al., 1981
	Insulin	Effects on smooth muscle cell proliferation	Chytry et al., 1996
	Peptide vaccine	*In vitro* studies to improve oral vaccination	Morgan et al., 1996
	Superoxide dismutase	Synthesis and physicochemical studies	Sure et al., 2002
ST-HPMA	Bovine seminal ribonuclease	Activity studies	Oupicky et al., 1999
		Preliminary biological evaluation studies	Ulbrich et al., 2000
		In vivo studies on therapeutic effect	Pouckova et al., 2004
	Chymotrypsin	Activity studies on different functional end group of ST-HPMA	Lu et al., 1998
		Activity studies	Oupicky et al., 1999
	Ribonuclease A	*In vitro* and *in vivo* studies on anti-tumor effects	Soucek et al., 2002
		In vivo studies on therapeutic effect	Pouckova et al., 2004

1.2.6 Model proteins for conjugation in this study

To study the effect of the aforementioned polymer/and conjugation chemistry on protein activity and stability, it was necessary to choose model protein. Two proteins, trypsin and ribonuclease A (RNase A) were chosen. They have been widely studied before as polymer conjugates being recognized as good models for the studies on protein chemistry and enzymology. Examples of previously described polymer-trypsin and polymer-ribonuclease conjugates are listed in Tables 1.12 and 1.13, respectively. These proteins are briefly described below.

1.2.6.1 Trypsin

Trypsin have been isolated and characterized from many species e.g. bovine and porcine. Moreover, bovine and porcine trypsins have been used in a variety of medical and scientific applications (Johnson et al., 2002). This enzyme is a pancreatic serine protease, and it is produced in a pro- form, inactive trypsinogen. Trypsinogen transforms to trypsin by a single peptide bond (LYS-ILE) cleavage near the *N*-terminus depending on species as shown in Table 1.10. The amino acid compositions of bovine and porcine trypsin are summarized in Figure 1.10, and the enzyme consists of a single polypeptide chain of 223 amino acid residues.

There are three commonly occurring forms of trypsin: α-, β- and ε-trypsin. Commercially available trypsins are combination of the active α or β-trypsin and the inactive ε-trypsin (ExPASy, 2008). Extensive characterization information including a well defined crystal structure and active site information is available for trypsin. The structure of native trypsin has a catalytic triad which is typical of serine proteases such as HIS-57, SER-195 and ASP-102 (Figure 1.11). The aspartate residue (Asp 189) located in the catalytic pocket (S1) of trypsins is responsible for attracting and stabilizing positively-charged lysine and/or arginine, and it is thus responsible for the specificity of the enzyme.

Table 1.10 *N*-terminal sequences of various trypsinogen (from Walsh, 1970)

Species	*N*-terminal sequence
Bovine	Val-Asp-Asp-Asp-Asp-Lys-
Porcine	Phe-Pro-Thr-Asp-Asp-Asp-Asp-Lys-

(a) Amino acid sequence of bovine trypsin

```
         10         20         30         40         50         60
FIFLALLGAA VAFPVDDDDK IVGGYTCGAN TVPYQVSLNS GYHFCGGSLI NSQWVVSAAH

         70         80         90        100        110        120
CYKSGIQVRL GEDNINVVEG NEQFISASKS IVHPSYNSNT LNNDIMLIKL KSAASLNSRV

        130        140        150        160        170        180
ASISLPTSCA SAGTQCLISG WGNTKSSGTS YPDVLKCLKA PILSDSSCKS AYPGQITSNM

        190        200        210        220        230        240
FCAGYLEGGK DSCQGDSGGP VVCSGKLQGI VSWGSGCAQK NKPGVYTKVC NYVSWIKQTI

ASN
```

(b) Amino acid sequence of porcine trypsin

```
         10         20         30         40         50         60
FPTDDDDKIV GGYTCAANSI PYQVSLNSGS HFCGGSLINS QWVVSAAHCY KSRIQVRLGE

         70         80         90        100        110        120
HNIDVLEGNE QFINAAKIIT HPNFNGNTLD NDIMLIKLSS PATLNSRVAT VSLPRSCAAA

        130        140        150        160        170        180
GTECLISGWG NTKSSGSSYP SLLQCLKAPV LSDSSCKSSY PGQITGNMIC VGFLEGGKDS

        190        200        210        220        230
CQGDSGGPVV CNGQLQGIVS WGYGCAQKNK PGVYTKVCNY VNWIQQTIAA N
```

Figure 1.10 Amino acid sequence of (a) bovine trypsin and (b) porcine trypsin. These are the length of the partial sequence of the unprocessed precursor. The highlighted fonts represent activation peptide (from UniProtKB/Swiss-Prot, 2008).

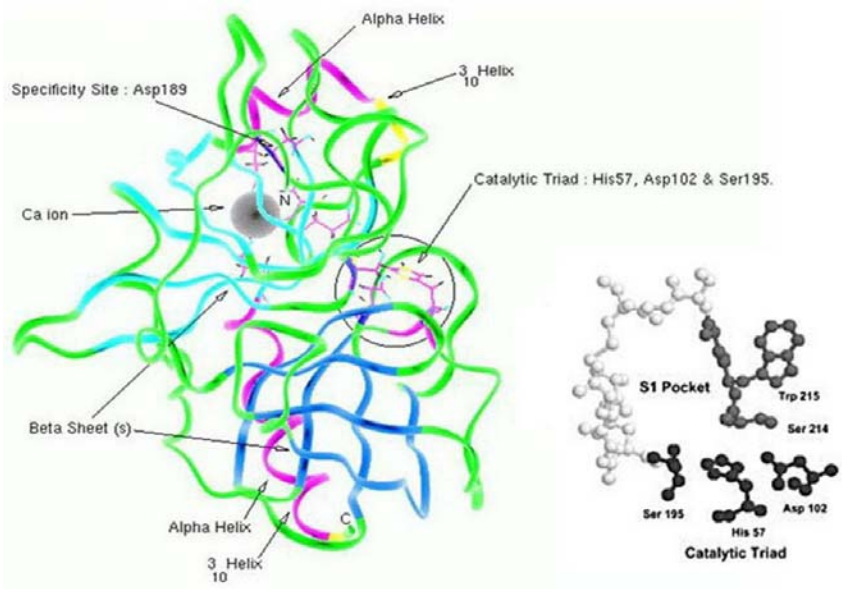


Figure 1.11 Three-dimension structure of bovine trypsin and its catalytic triads (adapted from http://www.soc.nii.ac.jp/jbiochem/jb/131-2/2fbaiff1.html).

Trypsin displays a narrow substrate specificity and it hydrolyzes peptide bonds (*L*-lysyl and *L*-argininyl bonds of polypeptides) at the carboxylic side of lysine and arginine. However, when proline is at the carboxylic side of lysine or arginine the bond is almost completely resistant to cleavage by trypsin. Cleavage may also be considerably reduced when acidic residues are present on either side of a potentially susceptible bond (Wilkinson, 1986).

The choice of substrate and the experimental conditions used are known to affect the kinetic parameters determined for trypsin (Johnson et al., 2002). Many types of synthetic substrates have been used for trypsin and there are listed in Table 1.11. Trypsin displays optimum activity at pH 7-9 (Hooper, 2002), and this range of pH has been used in all kinetics studies (Table 1.12). As mentioned above, the lysines present in proteins are often used for conjugation to carboxylic groups of polymer. Therefore, the number and location of lysine residues might be used as an indicator of potential polymer conjugation sites. In trypsin, there are no lysines in the catalytic triad, so the probability of direct conjugation to the active site, and thus irreversible reduction of trypsin activity is likely to be low. Murphy and O'Fagain (1996) determined the number of lysines present in bovine trypsin using a 2,4,6-trinitrobenzenesulfonate (TNBS) assay.

They found that only 8 of 14 lysines were modified by mPEG conjugate when using mPEGs derivatized by acetic acid *N*-hydroxysuccinimide ester. When mPEGs were activated by *p*-nitrophenyl chloroformate and then conjugated to bovine trypsin, it was found that only 11 of the 14 lysines available were modified (Zhang et al., 1999). This indicated that not all of the lysines present were accessible for conjugation. Furthermore, the different reaction conditions may result in a different degree of substitution and the positioned lysine that was involved in conjugation.

Trypsin was chosen as a model protein in the present study as it has already been conjugated to several polymers (summarized in Table 1.13) including PEG (Abuchowski and Davis, 1979; Gaertner and Puigserver, 1992; Zhang et al., 1999), carboxymethylcellulose (Villalonga et al., 2000; 2003), β-CD (Fernandez et al., 2002; 2003; 2004a; 2004b) and dextrin (Duncan et al., 2008).

Table 1.11 Some synthetic substrates for trypsin (from Walsh, 1970)

Type	Substrate
Amides	Benzoyl-*L*-arginine *p*-nitroanilide (BAPNA)
	L-Lysyl-*p*-nitroanilide
	Benzoyl-*L*-argininamide
	N-α-Benzyloxycarbonyl-*L*-arginine *p*-toluidide
Esters	Benzoyl-*L*-arginine ethyl ester (BAEE)
	Tosyl-*L*-arginine methyl ester (TAME)
	α-*N*-Acetyl S-(β-aminoethyl)cysteine ethyl ester
	α-*N*-Tosylyl S-(β-aminoethyl)cysteine ethyl ester
	α-*N*-Acetyl lysine ethyl ester
	Ethyl *p*-guanidinophenylacetate
Natural substrates	*N*-acetyl-L-tyrosine ethyl ester
	N-Acetyl-glycine ethyl ester
	N-α-Benzoyl-L-citrulline methyl ester
	N-Benzoyl-*L*-heptyline methyl ester

Table 1.12 Kinetic constants for some synthetic substrates for trypsin and their conditions

Trypsin	Substrate	Concentration (mM)	Buffer	pH	Temp. (°C)	K_M (mM)	K_{cat} (S^{-1})	Reference
Bovine	BAPNA	NS*	100 mM Tris-HCl, 20 mM $CaCl_2$	8.15	15	0.94	0.6	Erlanger et al., 1961
		NS*	50 mM Tris-HCl, 20 mM $CaCl_2$	8.2	25	0.80	2.5	Gaertner and Puigserver, 1992
	BAEE	NS*	67 mM Tris-HCl	8.0	25	0.0387	12.4	Fernandez et al., 2003
Porcine	BAPNA	0.1-1.5	100 mM Tris-HCl, 10 mM $CaCl_2$	8.0	25	1.72	1.78	Johnson et al., 2002
				8.0	30	1.51	1.99	
				8.0	35	2.07	2.89	

NS* = not stated

Table 1.13 Enzyme activity and kinetic parameters of polymer-trypsin conjugates

Polymer	Substrate	Enzyme activity			Reference
		Units	Free	Conjugated	
Oxidized sucrose polymer	BAPNA	T_{50} (°C)	54 ± 1.0	64 ± 3.0	Venkatesh and Sundaram, 1998
Carboxymethylcellulose	Milk casein	Specific activity (%)	100	42	Villalonga et al., 2000
	BAEE	Specific activity (%)	100	62	Villalonga et al., 2000
		K_m (μM)	39.4 x 10^{-6}	17.8 x 10^{-6}	Villalonga et al., 2000
β-cyclodextrin modified Carboxymethylcellulose	Milk casein	Proteolytic activity (%)	100	95	Villalonga et al., 2003
	BAEE	Esterolytic activity (%)	100	110	Villalonga et al., 2003
		K_m (μM)	35.5	23.0	Villalonga et al., 2003
Cyclodextrin, monoamine derivatives	BAEE	Specific activity (U/mg)	36	50 (140%) (αCDNH$_2$) 52 (145%) (βCDNH$_2$) 58 (160%) (γCDNH$_2$)	Fernandez et al., 2002
	BAEE	K_m (μM)	35.5	20.0 (αCDNH$_2$) 23.8 (βCDNH$_2$) 24.3 (γCDNH$_2$)	Fernandez et al., 2002

Contd.

Table 1.13 Enzyme activity and kinetic parameters of polymer-trypsin conjugates (Contd.)

Polymer	Substrate	Enzyme activity			Reference
		Units	Free	Conjugated	
Amino β-cyclodextrin derivative	BAEE	Esterolytic activity (U/mg)	36	34 ($CDNH_2$) 35 (CDEN) 31 (CDPN) 44 (CDBN)	Fernandez et al., 2003
	BAEE	K_m (μM)	38.7	26.6 ($CDNH_2$) 24.6 (CDEN) 44.8 (CDPN) 22.6 (CDBN)	Fernandez et al., 2003
	BAEE	K_{cat} (s^{-1})	12.4	12.7 ($CDNH_2$) 13.0 (CDEN) 12.2 (CDPN) 13.9 (CDBN)	Fernandez et al., 2003

Contd.

Table 1.13 Enzyme activity and kinetic parameters of polymer-trypsin conjugates (Contd.)

Polymer	Substrate	Enzyme activity			Reference
		Units	Free	Conjugated	
β-cyclodextrin containing dicarboxylic acid	BAEE	Specific activity (U/mg)	36	70.0 (adipic acid) 51.3 (pimelic acid) 57.8 (dodecanodioic acid)	Fernandez et al., 2004b
	BAEE	K_m (μM)	38.7	29.6 (adipic acid) 28.6 (pimelic acid) 31.8 (dodecanodioic acid)	Fernandez et al., 2004b
	BAEE	K_{cat} (s^{-1})	12.4	24.1 (adipic acid) 17.7 (pimelic acid) 19.9 (dodecanodioic acid)	Fernandez et al., 2004b
Dextran	TAME	Esterolytic activity (%)	100	53	Marshall and Rabinowitz, 1976
	Milk casein	Caseinolytic activity (%)	100	7	Marshall and Rabinowitz, 1976

Contd.

Table 1.13 Enzyme activity and kinetic parameters of polymer-trypsin conjugates (Contd.)

Polymer	Substrate	Enzyme activity			Reference
		Units	Free	Conjugated	
Dextrin	BAPNA	Trypsin activity (%)	100	51 (7,700 g/mol) 34 (47,200 g/mol)	Duncan et al., 2008
	BAPNA	K_{cat} (s^{-1})	2.52-2.93	2.59 ± 0.66 (7,700 g/mol) 1.25 ± 0.38 (47,200 g/mol)	Duncan et al., 2008
PEG (5,000 g/mol)	BAEE	Esterolytic activity (μmol min^{-1} $nmol^{-1}$ enzyme)	1.1	1.7	Gaertner and Puigserver, 1992
	BAPNA	Amidase activity (mmol min^{-1} $nmol^{-1}$ enzyme)	58	211	Gaertner and Puigserver, 1992
		K_m (affinity) (mM)	0.80 ± 0.01	0.42 ± 0.09	Gaertner and Puigserver, 1992
		K_{cat} (s^{-1})	2.5 ± 0.2	6.5 ± 1.0	Gaertner and Puigserver, 1992
	BAPNA	Half life at 60°C (min)	23	231	Zhang et al., 1999

1.2.6.2 Ribonuclease A (RNase A)

Ribonucleases (RNases) constitute a large superfamily crossing over many species. They are best known of secretary enzymes that operate at the crossroads of transcription and translation. RNA synthesis is catalyzed by RNA polymerases. RNA degradation is catalyzed by RNA depolymerases, which are most often called "ribonucleases" (Figure 1.12) (Leland and Raines, 2001). The fact that they degrade RNA gives them potential cytotoxic properties and they have been studied as anticancer agent.

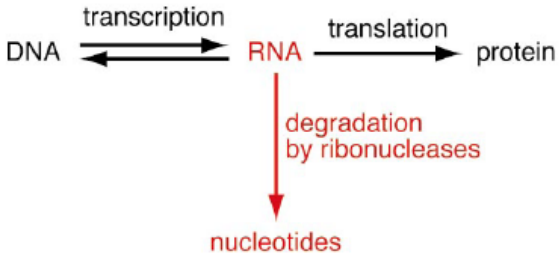


Figure 1.12 Biochemical basic for the potential cytotoxicity of ribonucleases (From Leland and Raines, 2001).

There are many sources of ribonuclease and compared their cytotoxicity e.g. bovine pancreatic (RNase A), bovine seminal (BS-RNase) and northern leopard frog (Onconase) have been intensively studied (Raines, 1998).

RNase A (EC 3.1.27.5) (Fig 1.13a), is a small monomeric protein and it is typical of mammalian extracellular RNases. The mature enzyme is secreted by exocrine cells of the bovine pancreas and it is not cytotoxic when added to mammalian cells. It has only 124 amino acid residues, including 4 disulfide bonds (Cys^{26}-Cys^{84}, Cys^{40}-Cys^{95}, Cys^{58}-Cys^{110} and Cys^{65}-Cys^{72}) and these are critical for the stability of the native enzyme. RNase A contains 19 of the 20 natural amino acids, lacking only tryptophan. This enzyme is an endonuclease which specifically cleaves at the 3′- end of pyrimidine nucleosides (Raines, 1998). The molecular formula of the native, uncharged enzyme is $C_{575}H_{909}N_{171}O_{193}S_{12}$. Its theoretical pI is 8.64, corresponds to a molecular mass of 13690 g/mol. The protein contains 10 lysines that could be available for polymer conjugation (ExPASy, 2008).

BS-RNase (Figure 1.13b) has 83% amino acid sequence identical to that of RNase A but possesses a distinct quaternary structure (Lee and Raines, 2005). BS-RNase exists as a homodimer with two subunits of 124 residues cross-linked by two disulfide bonds between Cys31 of one subunit and Cys32 of the other. It has been suggested that the greater size of BS-RNase may affect its ability to cross the lipid bilayer and reach the RNA. However, unlike RNase A, BS-RNase was found to have much greater endogenous cytotoxic activity than RNase A itself. BS-RNase has demonstrated cytotoxicity in a wide variety of *in vitro* and *in vivo* assays (Matousek et al., 2003). For a ribonuclease to be cytotoxic, it must evade ribonuclease inhibitor (RI). Whereas the BS-RNase is not monomeric cytotoxic due to RI, the dimeric protein is cytotoxic due to resistance to RI (Kim et al., 1995).

Onconase (P-30 protein) (Figure 1.13c) is a ribonuclease found in the oocytes and early embryos of *Rana pipiens*, the Northern leopard frog. The amino acid sequence of Onconase is ~30% identity with RNase A and its tertiary structure is similar to RNase A. It is cross-linked by 4 disulfide bonds, 3 of which are conserved in RNase A (Leland and Raines, 2001) and the protein has 104 residues in total with a molecular weight of 11.8 kDa. This protein expresses RNase biological activities and it is insensitive to inhibition by the RI (Sexana et al., 2002). It is more cytotoxic than BS-RNase, even though BS-RNase has greater ribonucleolytic activity than Onconase (Matousek et al., 2003). Onconase has advanced to Phase III in human clinical trials for the treatment of malignant mesothelima, an asbestos-related lung cancer (Mikulski et al., 2002; Saxena et al., 2003).

Human ribonucleases are present in extracellular fluids and correspondingly are not immunogenic (Erickson et al., 2006). Intracellularly, they have been shown to degrade transfer ribonucleic acid (tRNA) in the cytosol and consequently inhibit protein synthesis and induces apoptosis (Ardelt et al., 2003). Putative cellular routing of ribonuclease-mediated cell death consists of three steps as shown in Figure 1.14 (Leland and Raines, 2001):

(1) The ribonuclease first interacts with the cell surface binding to the target receptors on the plasma membrane.

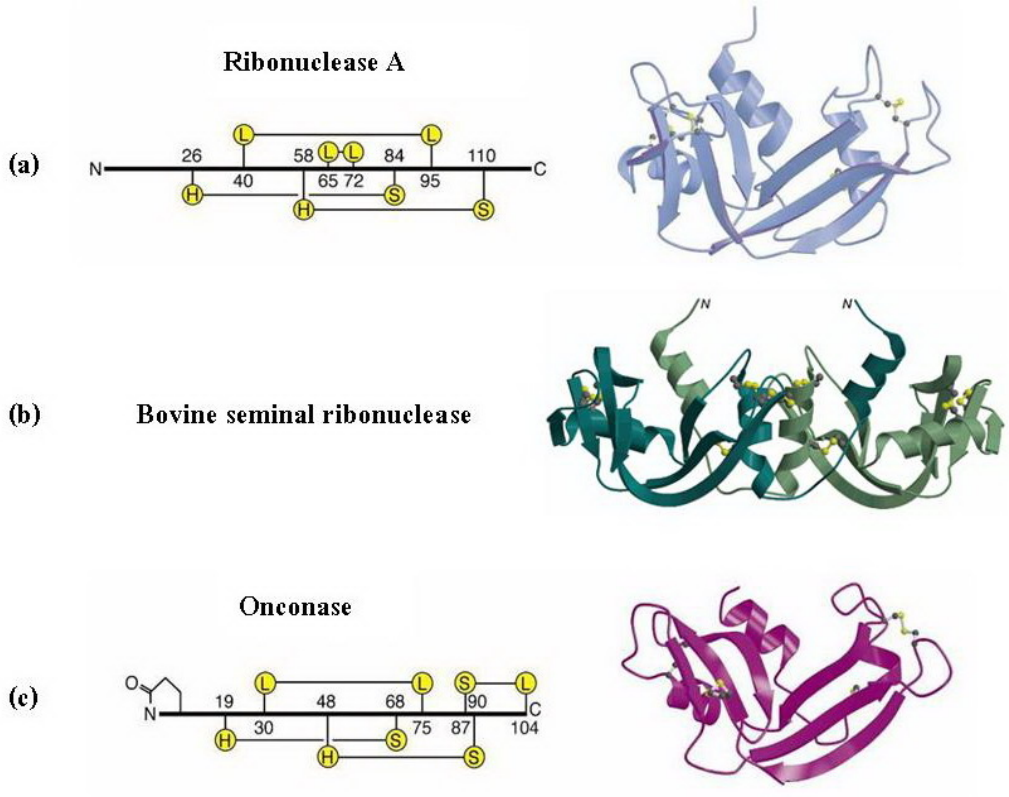


Figure 1.13 Ribbon diagrams of three dimensional structure of ribonucleases. The secondary structure context of each half-cystine is indicated by H (α-helix), S (β-sheet) or L (loop). (from Leland and Raines, 2001).

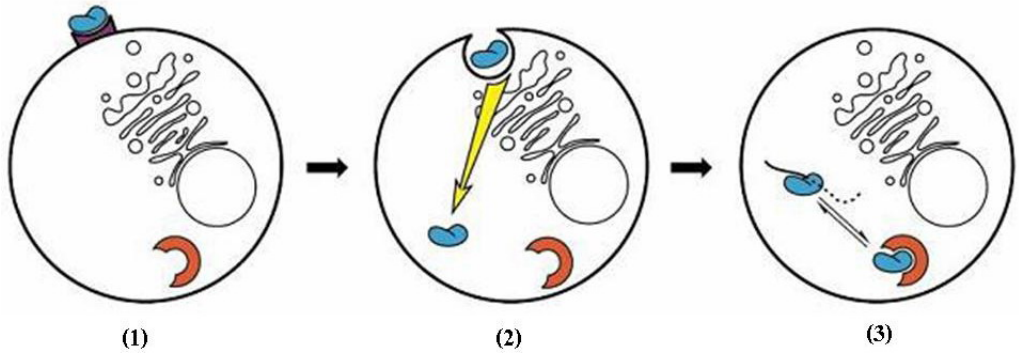


Figure 1.14 Putative cellular routing of cytotoxic ribonuclease. (1) binding receptors, (2) cytosolic internalization and (3) RNA cleavage (from Leland and Raines, 2001).

(2) It is then internalized by endocytosis and appears to cross a lipid bilayer of the endosome to reach the cytosol. The internalization pathway of ribonuclease and trafficking to the cytosol is uncertain but it must reach the cytosol to degrade cellular RNA. It has been suggested that RNase A is internalized using the clathrin- or dynamin-mediated endocytic pathway (Haigis and Raines, 2002).

(3) In the cytosol, they encounter the ribonuclease inhibitor (RI) protein, and ribonucleases that evade RI are able to catalyze cleavage of cellular RNA which leads to cell death.

Although, the specific mechanism of action of cytotoxicity of ribonuclease is not well known, it has been suggested that host cells are protected from endogenous RNases by RI. RI is a 50 kDa protein present in the cytosol of mammalian cells at high concentrations (0.01-0.1% of total cellular protein). RI requires a reducing environment (such as the cytosol) for activity and oxidation of the cysteine residues of RI causes it to lose the ability to bind to RNase A. Also RI is degraded rapidly by cellular proteases.

The key factors that affect ribonuclease as cytotoxicity are: (i) the affinity for the RIs, (ii) the form of ribonuclease (monomer or dimer), (iii) the mechanism of internalization or cellular uptake of the ribonuclease, and (iv) the mode of administration.

(i) Ribonuclease affinity for the RIs

RI binds to the active site of most of the RNase A family members with femtomolar affinities, and this binding is among the tightest of known protein-protein interactions. This high affinity has likely evolved to protect cells against rogue ribonucleases that access the cytosol. RI forms a 1:1, noncovalent complex with RNase A as shown in Figure 1.15 (Leland and Raines, 2001; Dickson et al., 2003; Erickson et al., 2006). RNase A is not cytotoxic when bound tightly by cytosolic RI (Kim et al., 1995). In contrast to RNase A, BS-RNase and onconase are toxic to mammalian cells because they could evade cytosolic RI or resistant to RI (Erickson et al., 2006).

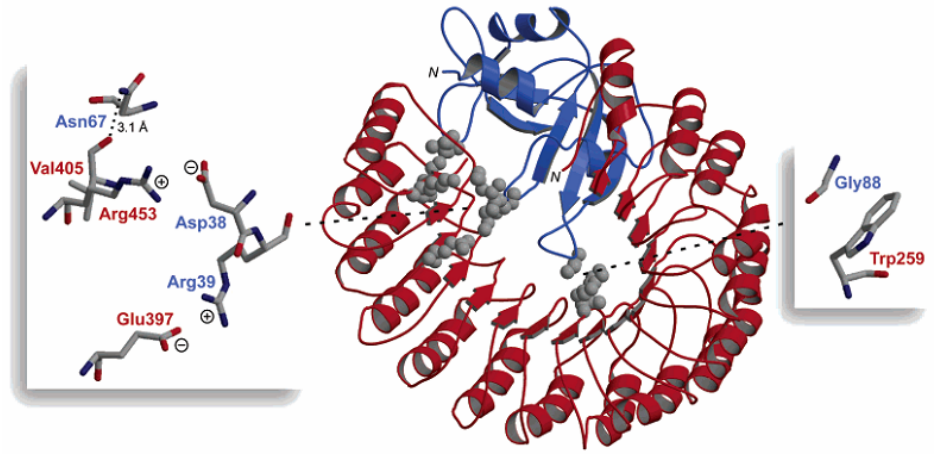


Figure 1.15 Three-dimension structure of the complex between ribonuclease inhibitor (red) and RNase A (blue) (from Lee and Raines, 2005).

(ii) Form of ribonuclease- monomer or dimer

The affinity of the ribonuclease for the RI is also affected by form (monomer or dimmer) described above. Wild-type BS-RNase is a homodimer, and its quaternary structure endows BS-RNase with resistance to inhibition by the RI. RNase A and monomeric BS-RNase, which bind tightly to RI, are not cytotoxic. However, artificially dimerized RNase A is cytotoxic, but less so than BS-RNase. A semisynthetic enzyme was constructed to understand mechanism of ribonuclease cytotoxicity, where the disulfide bonds that cross-link the monomeric subunits of dimeric BS-RNase were replaced with thioether bonds, to withstand the reducing environment of the cytosol. It was found that this semisynthetic enzyme had a cytotoxicity comparable to the wild-type BS-RNase (Kim et al., 1995).

(iii) Internalization or cellular uptake of ribonuclease

Several studies have tried to understand better the effect of intracellular location on ribonucleolytic activity and cytotoxicity. Microinjection of ribonucleases into the cytosol made them more toxic than seen when adding them to cell externally. This suggests that poor internalization can limit toxicity (Saxena et al., 1991). Moreover, the level of cationic charge of a ribonuclease can affect its internalization or cellular uptake. It has been found that increasing of the degree of cationic charge subsequently increases internalization via binding to glycolipids and

glycoproteins on the plasma membrane. Thus, the cytotoxicity of the ribonuclease is increased (Ilinska et al., 2004; Lee and Raines, 2005).

(iv) Mode of administration

The route of administration of the ribonuclease *in vivo* has also been demonstrated to affect cytotoxicity. RNase A displayed a poor *in vivo* antitumor activity profile when administered i.v., but inhibition of tumor growth was found when administered intra-tumoral (i.t.) (Daly et al., 2005). Similarly, Ulbrich et al. (2000) reported that free BS-RNase administered by i.t. administration has a significant antitumor effect while i.v. administration of free BS-RNase was totally ineffective. However, Ulbrich et al. (2000) found that it was possible to maintain significant cytotoxicity with i.v. administration of HPMA-BS-RNase conjugate. This might be expected as the conjugation of ribonuclease would prevent degradation and also potentially improve tumor targeting (Ulbrich et al., 2000).

From these studies, it was decided to focus on RNase A and efforts have been made to block the binding of RI. Conjugation of RNase A (Table 1.14) with HPMA, PEG or hyaluronidase (HA) seems to solve this important problem (Ulbrich, et al., 2000; Matousek, et al., 2002; Soucek, et al., 2002; Matousek, et al., 2003; Matousek, et al., 2004). These polymer-RNase conjugates are discussed further in Chapter 6.

1.2.7 Method for determining enzyme activity

Following the synthesis of polymer-protein (enzyme) conjugates, it is always necessary to measure the retained enzyme activity compared to that of free enzyme. In these studies, to determine the activity of conjugate and free trypsin or RNase A, a direct continuous assay was used with a substrate either BAPNA or yeast RNA respectively. In this way it was possible to determine the initial linear rate of substrate degradation.

Table 1.14 Examples of studies on the polymer-ribonuclease conjugates

Polymer	Protein	Type of study	Reference
Hyaluronidase	RNase A	Biological and anti-tumor activity studies	Matousek et al., 2004
PEG	BS-RNase	*In vivo* studies on systemic efficacy	Michaelis et al., 2002
		Aspermatogenic and anti-tumor activity studies	Matousek et al., 2002
	RNase A	Aspermatogenic and anti-tumor activity studies	Matousek et al., 2002
		Biological and anti-tumor activity studies	Matousek et al., 2004
		Adsorption of conjugates to poly(lactide-co-glycolide) surface	Daly et al., 2005
Poly(HPMA)	BS-RNase	Cancer therapy	Ulbrich et al., 1997
		Preliminary biological evaluation studies	Ulbrich et al., 2000
		In vivo studies on inhibition of solid tumor growth	Soucek et al., 2001
		In vivo studies on therapeutic effect	Pouckova et al., 2004
	RNase A	*In vitro* and *in vivo* studies on anti-tumor effects	Soucek et al., 2002
		In vivo studies on therapeutic effect	Pouckova et al., 2004
ST-HPMA	Bs-RNase	Activity studies	Oupicky et al., 1999

It was considered important to use Michaelis-Menten kinetics not only to describe enzyme activity, but to establish kinetic parameters. Interpretation of the obtained raw data can be used to determine the affinity constant (K_m), maximum velocity (V_{max}) and the turnover rate (K_{cat}) of an enzyme-substrate interaction. These parameters can then be used to make comparisons between different enzymes and different conditions, or in this case to compare the activity of free and polymer-bound enzyme either trypsin or RNase A.

Several different mathematical models have been explored as tools to calculate these enzyme kinetic parameters (Cornish-Bowden, 2004) (Figure 1.16). The models including:-

- Lineweaver-Burk plot (Double reciprocal plot)
- Hanes-Woolfe plot (Half-reciprocal plot)
- Eadie-Hofstee plot

All are based on the assumption that the Michaelis-Menten equation applies (Cornish-Bowden, 2004; Tipton, 1998): $v = (V_{max} S)/(K_m + S)$

where, v = velocity

V_{max} = maximum velocity

S = substrate concentration

K_m = affinity constant

Three factors need to be considered for this equation. Firstly, that any reverse reaction occurring is negligible. Secondly, the measurement of the reaction rate is made under the steady state conditions. Finally, the formation of the enzyme-substrate complex will not significantly affect the concentration of free substrate. These factors are validated experimentally if the initial rate is linear with time and linearly proportional to enzyme concentration, i.e. zero order kinetics (Cornish-Bowden, 2004).

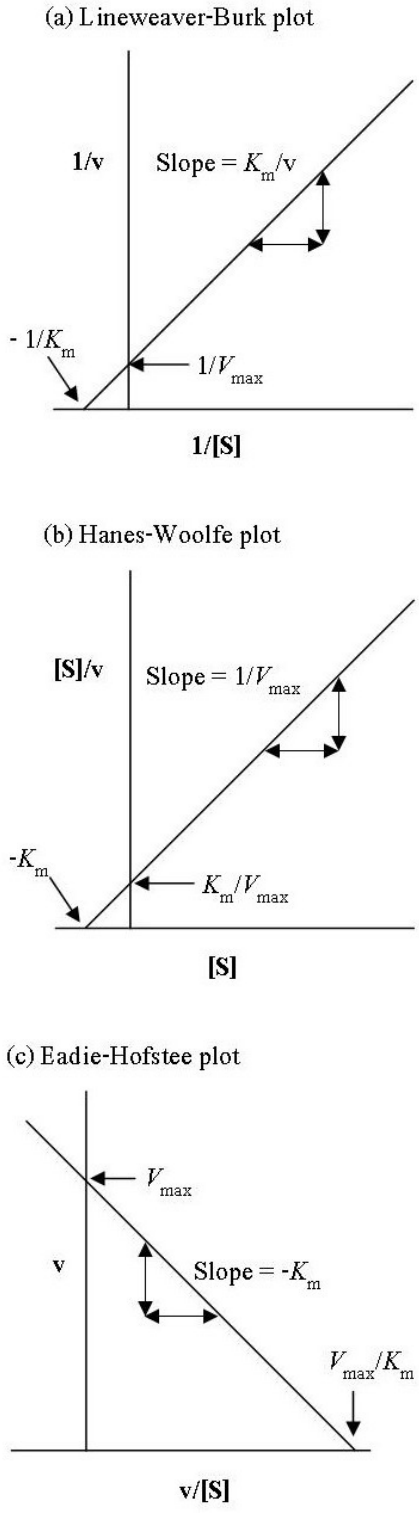


Figure 1.16 Enzyme kinetics models for data interpretation, based on the Michaelis-Menten equation.

The most frequently used plots are the Lineweaver-Burk, Hanes-Woolfe and Eadie-Hofstee plots (Samoshina and Samoshin, 2005) (Figure 1.16). Even though popular, the Lineweaver-Burk plot, has been proven to be the most statistically unreliable (Cornish-Bowden, 2004; Samoshina and Samoshin, 2005). Similarly, the Eadie-Hofstee plot has been criticised for introducing error to both axes. Despite the initial acceptance of the Lineweaver-Burke and Eadie-Hofstee plots, they rely on subjective, and sometimes inaccurate, estimations of initial velocity and they have failed to produce reliable kinetic constants.

Therefore, the Hanes-Woolfe plot was chosen for data analysis in these studies. This method of calculating kinetic parameters is not statistically ideal, but the errors shown by this type of plot can be minimized by ensuring that a suitably large range of substrate concentrations is used in the experimental design (Cornish-Bowden, 2004). The main requirements of a linear reaction rate velocity with both time, and enzyme concentration, and a correction made for blank absorbance were met to enable application of Michaelis-Menten based data analysis (Cornish-Bowden, 2004). Although K_m and V_{max} were determined, it is known that experimental conditions such as temperature, pH and the method of measurement can cause variability in the K_m values obtained (Samoshina and Samoshin, 2005). Enzyme concentration and enzyme purity can also influence V_{max} values (Cornish-Bowden, 2004; Samoshina and Samoshin, 2005), these variables were kept constant unless being specifically studied (i.e. when effect of pH and enzyme concentration were measured). However, the constant K_{cat} is not greatly affected by the same experimental conditions as K_m and V_{max}. Therefore, it was possible to compare the K_{cat} values obtained in several studies.

1.3 Objectives of this study

The primary objectives of this study were to synthesize, purify and characterize polymer-protein conjugates using trypsin as a model protein. Defined architecture and study the effect of polymer type and its molecular weight on the protein activity and thermal stability of conjugated protein in order to define the most effective polymer for protein stabilization.

Five polymers of different chemistry, shape and molecular weights were chosen including:

- *Linear polymer*: PEG (Mw 1,100, 2,000 and 5,000 g/mol)
- *Globular polymer*: β-cyclodextrin (Mw 1,135 g/mol) and PAMAM dendrimer (Mw 6,941 g/mol)
- *Branched polymer*: dextrin (Mw ~8,100 and ~61,000 g/mol) and ST-HPMA copolymer (Mw ~10,100 g/mol)

Polymer type and polymer architecture and its molecular weight affect on the activity and thermal stability of conjugated protein. These important factors are often overlooked but they can guide the early choice of optimal polymer/chemistry for synthesis of a lead polymer therapeutic suitable for later formulation development. The effect of molecular weight (1,100, 2,000 and 5,000 g/mol) of linear polymer (PEG) and different types of linker chemistry (using succinic anhydride, cyanuric chloride and tosyl chloride) on the rate of trypsin autolysis and thermal stability was determined (Chapter 3). To study the effect of polymer types, the globular structure of β-CD, a PAMAM dendrimer (Chapter 4), and dextrin and ST-HPMA (Chapter 5) were conjugated to trypsin and then the trypsin activity and thermal stability of conjugated trypsin was defined. A second objective of this study was to explore the properties of conjugates with potential therapeutic use. The enzyme, RNase A was chosen and RNase A conjugates were synthesized. The dextrin and ST-HPMA copolymer were used to synthesize polymer-RNase A conjugates (Chapter 6). These conjugates were used to determine ribonuclease A activity and cytotoxicity in B16F10 melanoma cancer cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

All chemicals used for this research were analytical grade. A detailed of the equipment, compounds, general reagents and their suppliers are itemized below.

2.1.1 Compounds and reagents

2.1.1.1 Polymers

Polyethylene glycol monomethyl ether (1,100, 2,000 and 5,000 g/mol) and β-cyclodextrin (β-CD) were purchased from Fluka (USA). Dextrin I, type 1 from corn (~8,100 g/mol) was supplied by Sigma (UK). Polyamidoamine (PAMAM) dendrimer (-OH) generation 3 (6.941 g/mol) was from Aldrich (USA). Dextrin II (~61,000 g/mol) and semi-telechelic ST-HPMA homopolymer (-COOH) (~10,100 g/mol) were from the Centre for Polymer Therapeutics, Cardiff University. ST-HPMA-COOH polymer was prepared by Lucile Dieudonné.

2.1.1.2 Proteins

The enzymes; bovine pancreatic trypsin, porcine pancreatic trypsin (type 1X-S), and the peptides; N_{α}-benzoyl- *L*-arginine-nitroanilide hydrochloride (BAPNA) were from Sigma (UK). N_{α}-Benzoyl-*L*-arginine ethyl ester hydrochloride (BAEE) and bovine serum albumin (BSA) were from Sigma (Switzerland). RNase A was supplied by Applichem (Germany).

2.1.1.3 Molecular weight standards for chromatography

Pullulan standards of molecular weight 5,900, 11,800, 22,800, 47,300, 112,000 and 788,000 g/mol were from Polymer Laboratories Ltd (UK). Dextran blue (2,000,000 g/mol) and glucose (180 g/mol) were supplied by Sigma (UK).

Protein standards of bovine lung aprotinin (6,500 g/mol), horse heart cytochrome C (12,400 g/mol), bovine erythrocytes carbonic anhydrase (29,000 g/mol), bovine serum albumin (BSA) (66,000 g/mol), and alcohol dehydrogenase (150,000 g/mol) were also supplied by Sigma (UK). Protein standards of bovine pancreas ribonuclease A (13,700 g/mol), chymotrypsinogen A (25,000 g/mol), bovine pancreas ovalbumin (43,000 g/mol), hen egg albumin (67,000 g/mol) and bovine serum blue dextran 2000 were from GE Healthcare (UK).

2.1.1.4 Chemicals for SDS-PAGE

Prestained SDS-PAGE standard of molecular weight in the range 6,919-194,239 g/mol and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were supplied by Bio-Rad (USA). Acrylamide/bis-acrylamide (40 % solution, mix ratio 37.5:1), ammonium persulfate, Brilliant Blue R-250, glycine (Electrophoresis Grade), isopropanol, sodium dodecyl sulphate (SDS), and tris[hydroxymethyl]aminomethane hydrochloride (Trizma®, Tris-HCl) were from Sigma (UK).

2.1.1.5 Chemicals for cell culture

The B16F10 murine melanoma cell line was supplied by the American type culture collection (ATCC) (USA). The cell culture media used to maintain this cell line was Rose Park Memorial Institute (RPMI) 1640 with phenol red (PR) and Glutamax. Foetal bovine serum (FBS) and trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) (0.05 % w/w trypsin, 0.53 mM EDTA) were from Invitrogen Life Technologies (UK). BOC Industrial (UK) supplied CO_2 and N_2 (medical grade, 95 % v/v) and liquid N_2. Trypan blue solution (0.4 % w/v), 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyl-2H-terazoliumbromide (MTT) and sterile dimethyl sulphoxide (DMSO) were supplied by Sigma-Aldrich (UK).

2.1.1.6 Additional Reagents

Anhydrous calcium chloride, bromothymol blue, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), sodium acetate and sodium tetraborate were from Sigma (UK). Succinic anhydride, *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS) and 4-dimethylaminopyridine (DMAP) were from Fluka (Switzerland). Cyanuric chloride and tosyl chloride were from Sigma (Germany). Coomassie brilliant blue G-250 and 2,4,6-trinitrobenzenesulfonic acid were supplied by Sigma (USA). Anala R® (England) supply di-sodium hydrogen orthophosphate dodecahydrate and sodium dihydrogen orthophosphate. *N,N′*-Dimethylformamide, anhydrous (DMF) was from Aldrich (UK). Phosphoric acid (85%) was purchased from Merck (USA). Sodium azide was supplied by USB cooperation (USA). Sodium dodecyl sulphate (SDS) was from Bio-Rad (USA). All other chemicals and solvents (glacial acetic acid, butanol, chloroform, dichloromethane, diethyl ether, hydrochloric acid, methanol, sodium chloride, sodium hydroxide, sodium sulphate, sodium sulphite and toluene) were supplied by Lab-scan Asia (Thailand).

2.1.2 Equipments

2.1.2.1 Analytical equipment

The equipment used for chemical analysis in these studies included Fourier transform infrared (FT-IR) spectrophotometer (section 2.1.2.1.1), UV spectrophotometer (section 2.1.2.1.2), gel permeation chromatography (GPC) (section 2.1.2.1.3) and fast protein liquid chromatography (FPLC) (section 2.1.2.1.4) are described below.

2.1.2.1.1 Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR analysis of the polymers was performed using either (i) an AVATAR OMNI-sampler 360 FT-IR from Nicolet Instrument Corporation (USA) with EZ OMNIC E.S.P. 5.2 software for data analysis, or (ii) a PerkinElmer Spectrum One FT-IR Spectrometer from PerkinElmer Life and Analytical Sciences (Beaconsfield, UK). Data were plotted as transmittance (%) in function of the wavenumber (cm^{-1}) and analyzed using Spectrum v5.0.2 software from PerkinElmer Life and Analytical Sciences (Beaconsfield, UK).

2.1.2.1.2 UV Spectrophotometer

UV-vis absorbance was measured using either (i) a Hewlett Packard 8452A Diode array spectrophotometer (Ontario, Canada) (ii) a Shimadzu UV 1601 Spectrometer (Kyoto, Japan), or (iii) a Cary 1G UV-Vis spectrophotometer (Varian, Australia) with Varian 1E software (Varian, Australia). A Sunrise UV-vis absorbance plate reader (Tecan, Austria) was used for analysis of samples in 96 well plates.

2.1.2.1.3 Gel permeation chromatography (GPC)

GPC analysis was carried out using a JASCO PU-980 high performance liquid chromatography consists of pump (Jasco UK Ltd, UK), with two TSK-gel columns in series ($G4000PW_{XL}$ followed by $G3000PW_{XL}$) and a guard column ($Progel\ PW_{XL}$). A Gilson 133 differential refractometer (Gilson Inc, USA) and programmable UV absorbance detector (Severn Analytical, UK) were used as dual channel detectors. PL Caliber Instrument software, version 7.0.4, from Polymer Laboratories (UK) was used for data analysis.

2.1.2.1.4 Fast protein liquid chromatography (FPLC)

FPLC was conducted using either (i) a FPLC system (Pharmacia LKB, Amersham Pharmacia Biotech, Uppsala, Sweden) with a Sephacryl S-100 HiPrep 16/60 high resolution FPLC column (Sweden), FRAC-100 Fraction Collector, LCC-500 Plus Controller, MV-7 Motor value, P-500 Pump, UV-1 Monitor, Superloop (10 mL). EYELA Toriconder TR250

recorder was obtained from Tokyo Rikakikat co., LTD (Japan), or (ii) an ÄKTA FPLC system (Amersham Pharmacia Biotech, UK) with a Superdex HR 10/30 SEC column with a UPC-900 detector. The UV absorption was performed at 280 nm. Data analysis was conducted using Unicorn 3.20 software (Amersham Pharmacia Biotech, UK).

2.1.2.2 Purification procedure

Purification of samples was carried out using either (i) dialysis tubing of cellulose membrane (molecular weight cut-off (MWCO) of 3,500 or 12,400 g/mol) purchased from Sigma-Aldrich (USA), (ii) spectra/por regenerated cellulose dialysis membrane (MWCO of 2,000 or 10,000 g/mol) purchased from BDH Merck (UK), or (iii) Vivaspin (MWCO 30,000 g/mol) centrifugal filters (Vivascience AG, Sartorius group, Germany) or (iv) Amicon Ultracel^{TM} (MWCO 10,000 g/mol) from Millipore (USA).

2.1.2.3 Freeze drying process

Samples were freeze dried using either (i) an EYELA freeze dryer FD-1 (Japan) or (ii) a Flexi Dry FD-1.540 freeze dryer (FTS systems, USA) connected to a high vacuum pump (DD75 double stage) from Javac (Australia).

2.1.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to evaluate the purity of synthesized polymer-protein conjugates. Electrophoresis were conducted using either (i) a Hoefer SE 245 Dual Gel Caster and electrophoresis power supply EPS 301 from Amersham Biosciences (USA), and a Power Pac 300 and GelAir Drying Frame from Bio-Rad (UK), or (ii) a Hoefer SE 250 Mighty Small II and EPS 301 power supply were from Amersham Biosciences (USA).

2.1.2.5 Cell culture and cell assays

A Galaxy S incubator and a Procell incubator (Wolf Laboratories and Jencons-PLS, UK) with Bioair (Bioair, Italy) and Microflow (Servicecare, UK) class II laminar flow hoods were used for all tissue culture experiment. A silver stained Neubauer haemocytometer (Marienfield, Germany) and a bright-field microscope (Inverted DM IL) from Leica (Germany) was used for cell counting. Cells were centrifuged using a Varifuge 3.0 RS, swing out rotor buckets (type 8080, rmax 21.1 cm) made by Heraeus Instruments (Germany). Cell culture consumables (Costar) were purchased from Corning Inc (USA). Pipettes, bijous and universal containers were purchased from Elkay (Ireland).

2.1.2.6 Additional equipment

Balance was from Sartorius BP210S (USA). Centrifuge was from either Kubota 5922 (Japan) or Hermle Z 323 K (Germany). Hot air oven was supplied by Memmert (Germany). Thermolyne Cimarec® 2 (USA) supplied Hot plates and stirrers. Incubator was from either Thermomixer comfort, Eppendorf (Germany) or Thermolyne Dri-bath (USA). Micropipettes (2-20 μL, 20-200 μL, 100-1000 μL) were from either Bio-Rad (USA) or Socorex (Switzerland). pH meter was supplied by Toledo 320 pH meter (Switzerland). Sharp (Thailand) supplied Refrigerators (5 °C and -20 °C). TLC-UV lamp was from UVGL-58 Handheld UV lamp (USA) and Vortex was supplied by Scientific industries (USA).

2.2 Methods

The typical reaction conditions used for the synthesis of PEG-trypsin conjugates (Chapter 3), β-CD-trypsin and PAMAM-trypsin conjugates (chapter 4), dextrin-trypsin and ST-HPMA-trypsin conjugates (Chapter 5), and dextrin-RNase A and ST-HPMA-RNase A conjugates (Chapter 6) are stated in the respective experimental chapters. The characterization techniques for analysis of specific polymer-protein conjugates are also described in the respective experimental

chapters. However, the general methods used are described here. Techniques used to purify functionalized parent polymer and the polymer-protein conjugates are described in following section (section 2.2.1). The physicochemical and biochemical methods used to characterize these conjugates are described in section 2.2.2 and 2.2.3, respectively. Cell culture methods are also described in section 2.2.4.

2.2.1 Purification and lyophilization of modified polymers and polymer-protein conjugates

The functionalized polymers or the product from the synthesis of polymer-protein conjugates were purified by following methods such as dialysis (section 2.2.1.1), FPLC fractionation (section 2.2.1.2) and Vivaspin 6 centrifugal filters (section 2.2.1.3). After purification, the product was lyophilized as described in section 2.2.1.4. However, the specific methods for polymer-protein conjugates preparation are further described in the appropriate experimental chapter.

2.2.1.1 Dialysis

Samples were dissolved in minimum amount of double distilled water (ddH_2O) and then purified by dialysis against ddH_2O (6 x 3 L) for 48 h. Dialysis membranes (MWCO 2,000, 3,500, 10,000 and 12,400 g/mol) were chosen according to the polymers or conjugate size and the size of impurity need to be removed.

2.2.1.2 FPLC fractionation

FPLC was used primarily to determine the purity (free protein) in the conjugates (described in section 2.2.3.4). This technique was also used to pool the conjugate fraction, separating them from any unconjugated protein and other impurities.

Method I: The Sephacryl S-100 HiPrep 16/60 High resolution FPLC column was equilibrated with the mobile phase (0.1 M Tris-HCl buffer; pH 8, 0.02% sodium azide and 0.1 M sodium chloride) for 2 h at a flow rate of 1 mL/min. UV absorbance (280 nm) was used to determine the retention time of the sample. The conjugate reaction mixture (2 mL) was injected onto the FPLC column using 10 mL sample loop. The FPLC was programmed to fractionate the sample into 10 mL aliquots. The conjugate fractions were collected, pooled and purified using Amicon Ultracel^{TM} (MWCO 10,000 g/mol) (section 2.2.1.3). The resulting product was then freeze dried (section 2.2.1.4). A calibration curve of Log molecular weight against elution volume was constructed using the protein standards with known molecular weight were analyzed (Figure 2.1a) and it used to estimate the molecular weight of the polymer-protein conjugates synthesized (Figure 2.1b).

Method II: The Superdex HR 10/30 FPLC column was equilibrated with the mobile phase (0.1 M phosphate buffer; pH 7.4) for 1.5-2 h at a flow rate of 0.5 mL/min and upper pressure limit of 1.8 mPa. The UV absorbance (280 nm) was determined using a UPC-900 detector to verify the retention time of the sample. The conjugate reaction mixture (1 mL) was then injected onto the FPLC column (Superdex 10/30) in 2 mL sample loop. The FPLC was however additionally programmed to fractionate the sample into 0.5 mL aliquots. A molecular weight standard calibration curve was constructed by plotting of Log molecular weight against elution volume and used to estimate the molecular weight of the polymer-protein conjugates (Figure 2.2). The conjugate fractions were collected, pooled and then purified using Vivaspin 6 (section 2.2.1.3). The resulting product was then freeze dried (section 2.2.1.4).

2.2.1.3 Vivaspin 6 centrifugal filtration

Solubilized sample was loaded (6 mL) in the top compartment of a Vivaspin centrifugal filter and centrifuged at 4000 g for 10 min. The filtrate was then discarded and the residue was topped up to 6 mL with ddH_2O. The sample was re-centrifuged and this procedure was repeated for a further two times to remove all phosphate buffered saline (PBS) salts from the solubilized sample. The residue was then lyophilized (section 2.2.1.4).

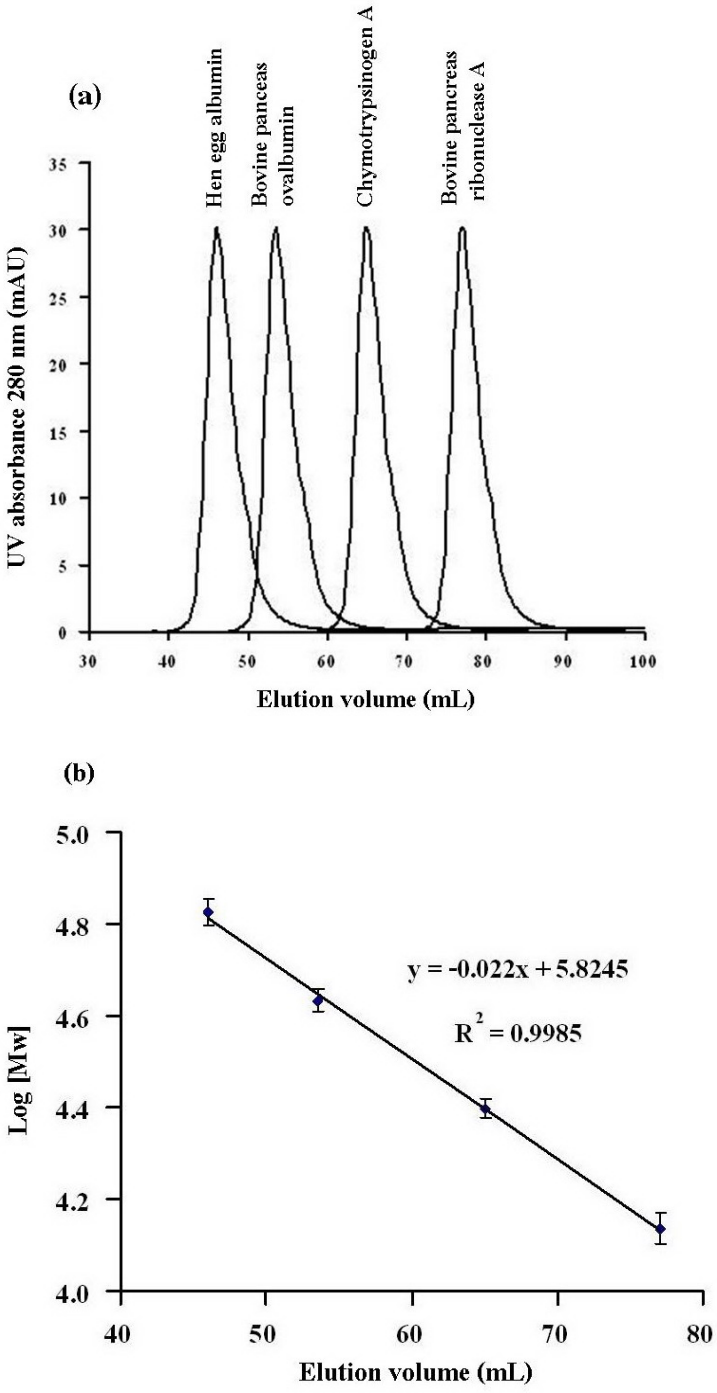


Figure 2.1 FPLC analysis using Sephacryl S-100 HiPrep 16/60 High resolution FPLC column. Panel (a) the FPLC chromatogram of protein molecular weight standards and panel (b) the calibration curve obtained by plotting Log molecular weight against elution volume to estimate molecular weight of sample.

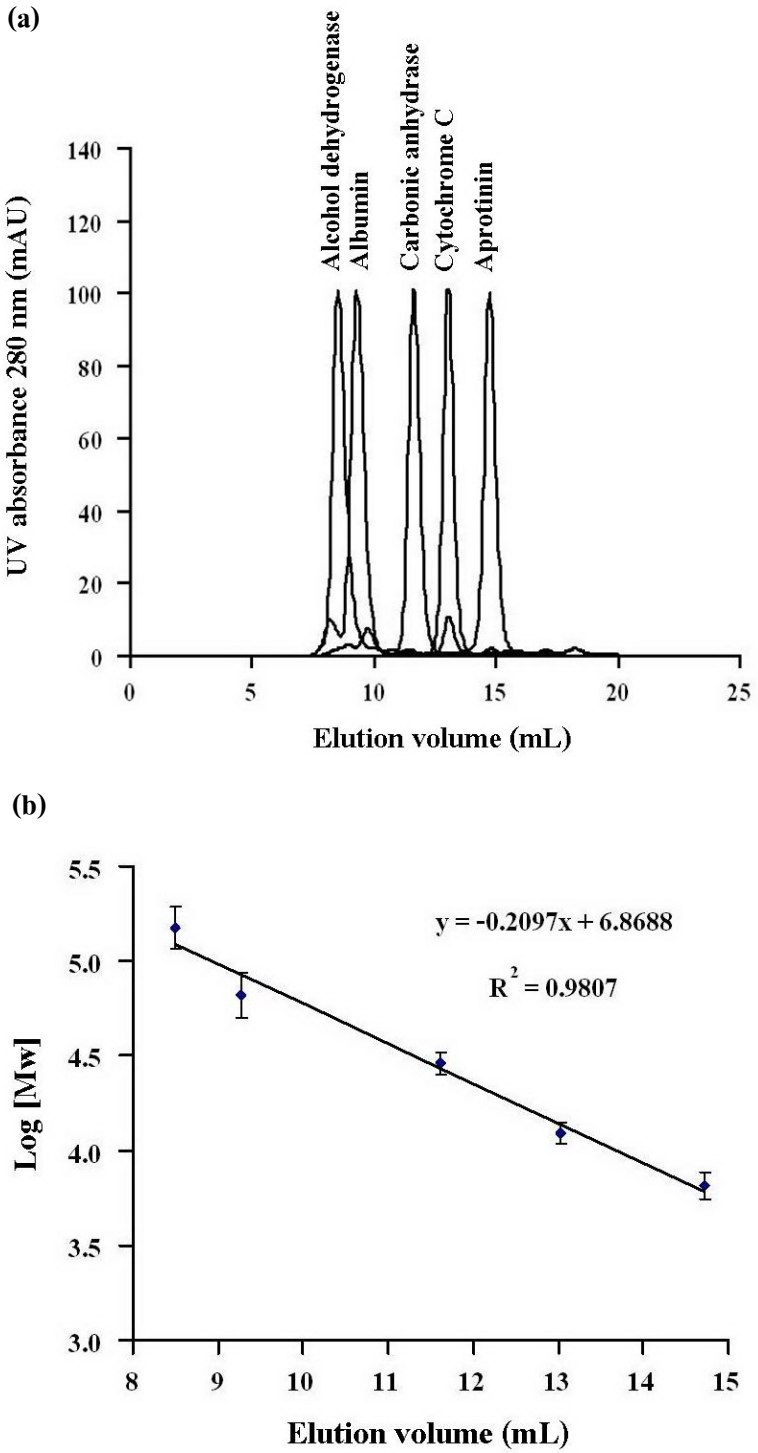


Figure 2.2 FPLC analysis using Superdex HR 10/30 FPLC column. Panel (a) the FPLC chromatogram of protein molecular weight standards and panel (b) represents the calibration curve plotted to estimate molecular weight of sample.

2.2.1.4 Lyophilization or freeze drying

Solubilized sample (up to 20 mL) was placed in a plastic tube (50 mL) which was covered with foil that was pierced for ventilation. The sample was snap frozen in liquid N_2 (5 min) and then lyophilised (24 h) to give dry the product. The obtained products were collected in refrigerator at -20 °C until needed.

2.2.2 Physicochemical characterization of modified polymers and polymer-protein conjugates

2.2.2.1 Titration of carboxyl groups in succinoylated polymers

The number of carboxylic acid groups (expressed as mol %) incorporated when polymer was reacted with succinic anhydride was quantified by titration (Hreczuk-Hirst et al., 2001b). Prior to titration, the pH meter was calibrated using standards of pH 4, 7 and 10. Succinoylated polymer (3 mg) in ddH_2O (2 mL) was titrated against NaOH (5 x 10^{-4} M), using bromothymol blue as an indicator (1 % w/v in ethanol, pH range: 6-7.6). The end point of the titration was indicated by a change in the solution colour from yellow to blue and a pH of ~7.6. Each sample was titrated three times and the mol % modification, i.e. the number of carboxylic acid groups incorporated, was calculated.

2.2.2.2 Structure determination of parent and modified polymer by FT-IR

Polymers and modified polymers included mPEG, succinoylated mPEG, cyanurate mPEG, tosylated mPEG, β-CD, succinoylated β-CD, dextrin, succinoylated dextrin, PAMAM dendrimer and succinoylated PAMAM dendrimer were analyzed by FT-IR to confirm their structural composition. Sample preparation used either (i) KBr, (ii) Neat or (iii) microscope. Samples were usually analyzed using 64 scans over the mid-infrared (400-4000 cm^{-1}) region and the background interference subtracted. The number of scans was increased from 64 to 300 when

spectra needed greater resolution. This improved the signal to noise ratio resulting in a much clearer spectra. Qualitative analysis of FT-IR spectra (% transmittance and wavenumbers (cm^{-1})) was conducted by interpretation of the peaks in the double-bond region; 2000-1500 cm^{-1} such as carbonyl characteristic peak of carboxylic or ester (Stuart, 2002).

2.2.2.3 Determination of molecular weight and polydispersity of polymers, proteins and polymer-protein conjugates using GPC

GPC was used to analyze samples in order to estimate their molecular weights and to determine their polydispersity. The GPC columns were firstly equilibrated with the mobile phase [0.1 M PBS, pH 7.4, filtered (0.2 μm) and sonicated] for 2 h at a flow rate of 1 mL/min, and the baseline zeroed against the reference cell every 30 min. A refractive index detector (sensitivity of 4) and UV absorbance detector was set at 280 nm were used to monitor sample elution.

Samples were prepared to have a concentration of 3 mg/mL in 0.1 M PBS buffer, pH 7.4, 60 μL was injected onto a G4000PW_{XL} followed by G3000 PW_{XL} column in sequence and the GPC elution run for 25 min. Gel filtration pullulan standards of known molecular weights and polydispersity were also analyzed using the same method and a standard calibration curve was generated by plotting Log molecular weight against retention time (Figure 2.3). The pullulan molecular weight standards used were listed in section 2.1.1.3. The retention time of dextran blue (2,000,000 g/mol) and glucose (180 g/mol) were also used to establish the void volume (Vo) and bed volume (Vb) of the column respectively (Meloan, 1999). The molecular weight; number average (Mn), weight average (Mw), and the polydispersity index (PDI) (Mw/Mn) of the analyzed samples were derived from the respective calibration curves using the PL calibre reanalysis software.

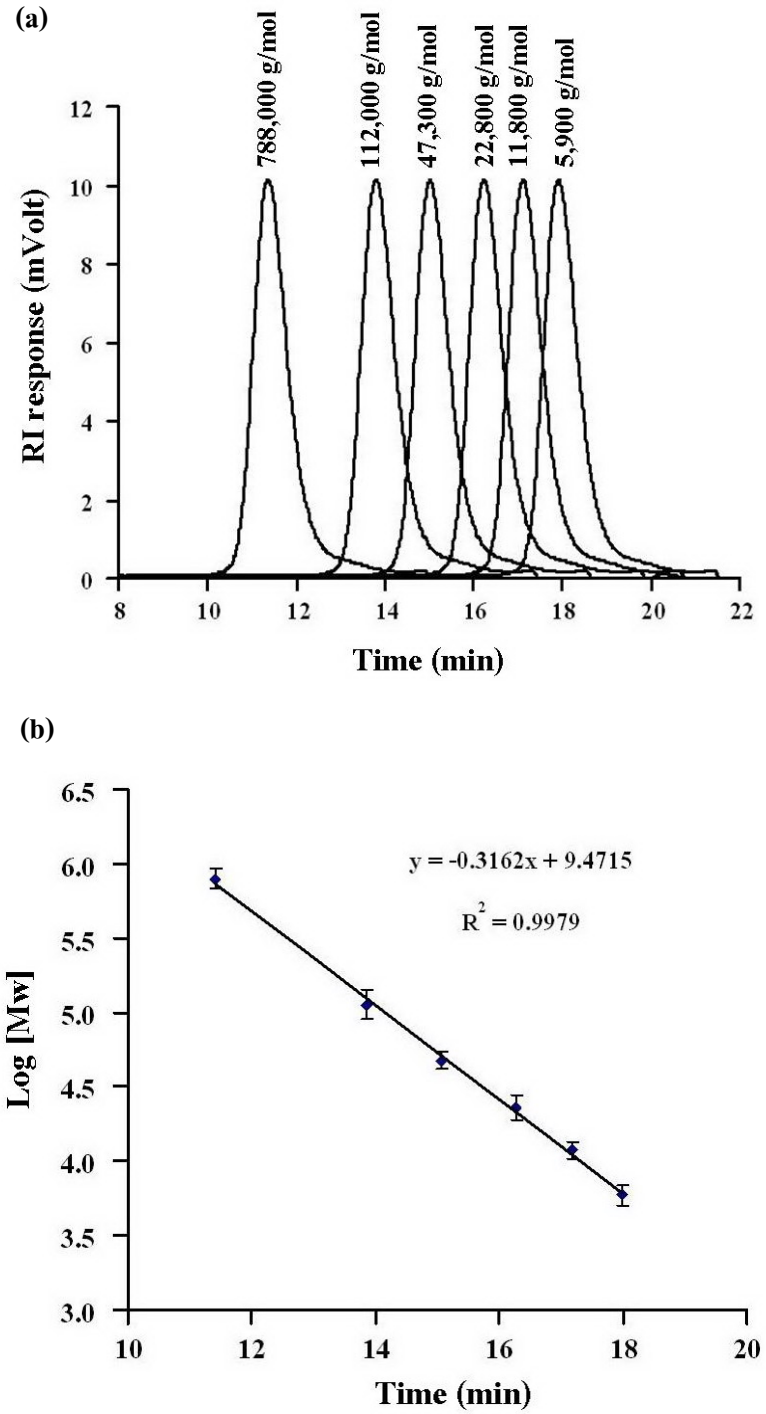


Figure 2.3 GPC analysis of pullulan molecular weight standards (a) shows the chromatogram of the pullulan standards and (b) shows the calibration curve plotted using the Polymer Laboratories software, to estimate molecular weight and polydispersity of sample.

2.2.3 Biochemical and analytical characterization of proteins and polymer-protein conjugates

A combination of methods was used to characterize free and total protein content of the polymer-protein conjugates. The total protein content of the conjugates was measured using a Bradford assay (section 2.2.3.2). In order to identify the presence of free protein in the conjugate, samples were run on SDS-PAGE (section 2.2.3.3). Analytical characterization of the conjugates was carried out using both GPC (section 2.2.2.3) and FPLC (section 2.2.3.4) to estimate their molecular weights and purity.

2.2.3.1 Quantification of the number of primary amines using TNBS assay

The extent of the protein modification was also estimated by determining the number of remaining free amino groups using the TNBS assay as described by Fields (1971). The sample (20 μL) was added to 0.1 M sodium tetraborate buffer in 0.1 M NaOH (0.5 mL) and the volume was made up to 1 ml. Then, TNBS solution (20 μL) was added and the solution was rapidly mixed. After 5 min the reaction was stopped by adding 0.1 M sodium phosphate buffer containing 1.5 M sodium sulfite (2.0 mL), and the absorbance at 420 nm was determined. A blank was prepared similarly without sample. The reaction was carried out in borate buffer at pH 9.5. At this pH, there is however a reaction with hydroxide ion to give a blank extinction. The latter reaction was stopped by lowering the pH to neutral after the amino groups have been trinitrophenylated (Figure 2.4).

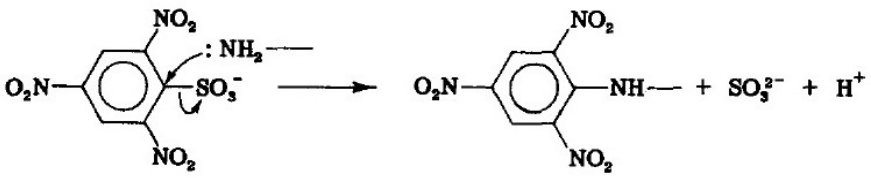


Figure 2.4 Mechanism of TNBS assay (from Fields, 1971).

2.2.3.2 Quantification of the total protein content of the polymer-protein conjugates using a Bradford assay

In all cases, the Bradford protein assay was used to determine the total protein content of the conjugates. This assay is based on the observation that the maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when protein binding occurs (Figure 2.5). Both hydrophobic and ionic interactions stabilize the anionic form of the dye causing a visible colour change (Bradford, 1976).

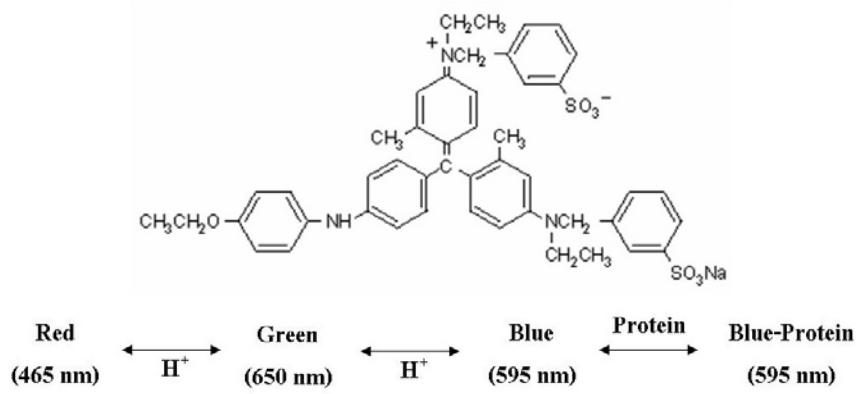


Figure 2.5 Structure of Coomassie blue G-250 dye and mechanism of Bradford assay (from Bradford, 1976).

The Bradford reagent was prepared as described below. Briefly, Coomassie Brilliant blue G-250 (100 mg) was dissolved in 95% ethanol (10 mL) and 85% phosphoric acid (25 mL), stirred thoroughly for 10 min. the mixture was adjusted to a volume of 250 mL with distilled water, filtered through Whatman filter paper No.1 and stored in a dark colored bottle at room temperature. The Bradford assays were conducted using BSA as a protein standard. 0.1 mg/mL BSA (5, 10, 20, 30 40 μL) or 1 mg/mL in ddH_2O of sample (20 μL) was added to a 1 mL cuvette and each was made up to 50 μL by adding ddH_2O. Blank was prepared similarly but, 50 μL of ddH_2O was added instead of BSA. Then, the Bradford reagent (950 μL) was added. The cuvettes were left to stand for 5 min at room temperature and then the absorbance measured at 595 nm. A calibration curve of absorbance against concentration of protein standard was

constructed and the protein concentration of unknown samples was estimated using the BSA calibration curve (Figure 2.6). This was expressed as weight percent of protein in the conjugates (%w/w).

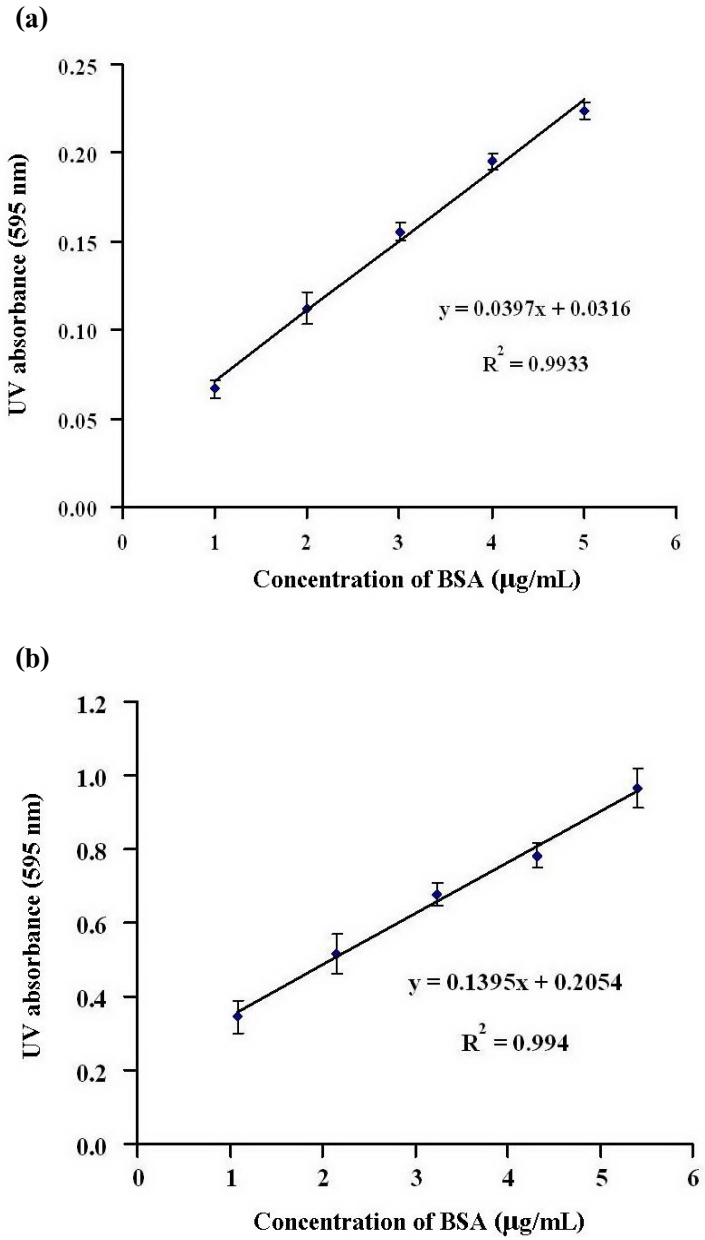


Figure 2.6 Bradford assay calibration curve using (a) a Hewlett Packard 8452A Diode array spectrophotometer (Ontario, Canada) or (b) a Sunrise UV-vis absorbance plate reader (Tecan, Austria)

2.2.3.3 Purity determination of the polymer-protein conjugates using SDS-PAGE

SDS-PAGE was used to measure free protein in the conjugates. This method was adapted from Kaufman et al., (1995) and the reagents for SDS-PAGE were prepared as described in Table 2.1. Their solution compositions are given in Table 2.2.

Table 2.1 Preparation procedure and storage conditions of reagents used in SDS-PAGE

Reagents	Composition	Storage
10% w/v SDS	Sodium dodecyl sulphate (10 g) and dd H_2O (100mL)	RT
10% Ammonium persulfate	Ammonium persulphate (20 mg) and dd H_2O (200 µL)	RT
Running gel buffer (1.5M Tris HCl, pH 8.8)	Tris base (91 g) and dd H_2O (200 mL), adjust pH to 8.8 with 2 N HCl, made up to 500 ml with ddH_2O.	4°C
Stacking gel buffer (0.5 M Tris HCl, pH 6.8)	Tris base (6 g) and dd H_2O (50 mL), adjust pH to 6.8 with 2 N HCl, made up to 100 mL with ddH_2O.	4°C
1%w/v Bromophenol blue	bromophenol blue (0.05 g) and sucrose (20 g), made up to 50 mL with ddH_2O.	-20°C
Denaturing solution (Tris HCl gel)	ddH_2O (3.8 mL); 0.5 M Tris HCl, pH 6.8 (5 mL); 10 % w/v SDS (8 mL); glycerol (4 mL), 2-mercaptoethanol (2 mL); 1% w/v bromophenol blue (0.4 mL)	RT
Running buffer (Tris HCl buffer, pH 8.3)	Tris HCl (3 g) + Glycine (14.4 g) + 10%w/v SDS (10 ml), made up to 1L with ddH_2O	4°C
Coomassie blue stain solution	1% CB solution (125 mL); methanol (500 mL) and acetic acid 100 mL, made up to 1 L with ddH_2O	RT
Strong destaining buffer	35 % methanol, 5 % acetic acid	RT
Weak destaining buffer	5 % methanol, 7 % acetic acid	RT

Table 2.2 Composition of separating and stacking gels for preparing a SDS-PAGE gel of 12.5 % cross-linkage

Components	Separating Gel pH 8.8 0.375M Tris HCl (20 mL)	Stacking Gel pH 6.8 0.125M Tris HCl (10 mL)
Stock solution	12.5%	4%
ddH_2O	8.44 mL	5.85 mL
Running buffer, Tris HCl pH 8.8 (1.5M)	5 mL	-
Stacking buffer, Tris HCl pH 6.8 (0.5M)	-	2.5 mL
10% w/v SDS	200 µL	100 µL
40% Acrylamide/bis	6.25 mL	1 mL
10% w/v Ammonium persulfate (0.05% final concentration)	100 µL	50 µL
TEMED	10 µL	5 µL

To conduct SDS-PAGE electrophoresis, first the equipment was assembled (2 glass plates, 0.75 mm spacers, locked into a casting stand) and checked for leakage by filling ethanol. The separating gel (0.375 M Tris, pH 8.8, 12.5 % cross linking, 20 mL) was prepared and transferred in between the glass plates up to a level of 4 cm from the top. Isopropanol (0.3 mL) was pipetted on top to smooth the gel surface to prevent the oxidation that would inhibit polymerization. The gel was then allowed to polymerize at RT for 30-60 min. The isopropanol was removed and a stacking gel (0.125 M Tris, pH 6.8, 10 mL) was prepared and transferred into the remaining space. Sample spacer combs were inserted into the top of the gel. The volume of the stacking gel was topped up and the gel was left to polymerize at RT for 30-45 min. The polymerized gels were removed from the casting stand and assembled in an electrophoresis tank that filled with running buffer (Tris HCl buffer, pH 8.3). Then, the conjugate samples (1 mg/mL in ddH_20) and reference samples (trypsin or RNase A) were prepared by diluting (1:1) with

denaturing solution and heated for 5 min at 100 °C. The denatured samples (conjugate or free protein) and the pre-stained protein molecular weight markers were loaded (10 μL) onto the polyacrylamide gels and the electrophoresis run at 200 V, 40 mA for 70 min until dye reached the bottom of gel. The gel was then stained for 1 h with Coomassie blue stain. The gel was cleared of non-specific staining by soaking in a strong destaining buffer for 1 h. Then, it was rehydrated using a weak destaining buffer for 30 min or until background was clear. Finally, the gel was rinsed with ddH_2O and dried between cellophane sheets to remove air bubbles, and it was clamped in a Gelair drying frame at room temperature over night.

2.2.3.4 Determination of polymer, protein and polymer-protein conjugates purity using FPLC

As previously described in section 2.2.1.2, FPLC was used to separate and collect the conjugate fractions. In addition, this technique was used as an analytical technique as to determine the presence of free protein in the conjugates and to estimate the molecular weight of the polymer-protein conjugates.

Before running the samples, the FPLC column (Superdex HR 10/30) was equilibrated with the mobile phase (0.1 M phosphate buffer, pH 7.4) for 1.5-2 h at a flow rate of 0.5 mL/min and upper pressure limit of 1.8 mPa. The samples (polymers, proteins or polymer-protein conjugates) were prepared to have a concentration of 3 mg/mL in phosphate buffer solution (PBS, pH 7.4). The solubilized sample (100 μL) was loaded onto the FPLC column in the 100 μL sample loop. The elution of sample was determined by UV absorbance (280 nm) using a UPC-900. Visual analysis of the chromatogram was carried out to determine the presence of free protein in the polymer-protein conjugate and also to identify the presence of any degradation products. A molecular weight standard calibration curve was constructed by plotting of Log molecular weights against elution volume and used to estimate the molecular weight of the polymer-protein conjugates.

2.2.4 Cell culture

2.2.4.1 Defrosting cells

Cryopreserved cells that had been stored in liquid N_2 were rapidly thawed (37 °C) and re-suspended in cell culture media (10 mL) to dilute the DMSO in the freezing medium. The composition of both the cell culture media and freezing medium for B16F10 cell culture is described in Table 2.3. The cell suspension was centrifuged (1000 g, 5 min, 20 °C) to remove the cryopreservative, and then the supernatant was removed. The cells were re-suspended in 10 mL of fresh media supplemented with 10 % FBS.

Table 2.3 B16F10 cell culture maintenance parameters

	Description
Cell line	B16F10 murine melanoma (ATCC, USA)
Passage range	10-30
Medium	90% RPMI 1640+ phenol red + Glutamax; 10 % FBS
Freezing medium	10% DMSO; 90% FBS
Seeding density (cells/mL)	1×10^4 and 4×10^4
Split ratio	1:9

2.2.4.2 Maintenance of cell line

Cells were maintained in a 75 cm^3 cell culture flask in medium (RPMI supplemented with 10 % FBS) and stored at 37 °C in a CO_2 regulated environment. The cells were subcultured twice weekly when > 70 % confluence was achieved using the following method. The cells were rinsed with 0.1 M PBS (10 mL) to remove dead cells. Then, trypsin-EDTA (1.5 mL) was added and the cells incubated (37 °C) for 2 min to detach them from the

flask. Medium (8.5 mL) was added to dilute cells and they were transferred to a universal centrifuge tube. The cell suspension was centrifuged (1000 g, 20 °C, 5 min) and then re-suspended in 10 mL complete medium. A split ratio of 1:9 of B16F10 melanoma cells was used for subculture and they were maintained and used in experiments for up to 30 passages before defrosting a new aliquot of cells.

2.2.4.3 Counting cells

Cells were prepared for subculture as described in section 2.2.4.2. They were then re-suspended in 10 mL of complete medium. Cell suspension (100 μL) was diluted 1 : 1 with 0.2 % v/v trypan blue in PBS (100 μL), mixed and left to stand (1 min) to stain dead cells blue. 10 μL of this solution was then pipetted under the cover slip on the haemocytometer and viewed using a bright-field microscope. Viable cells located in top left, top right, bottom left, bottom right and centre of 0.1 mm^3 squares of the haemocytometer were counted and cells touching outer lines of big square were ignored. Cell counting was always performed duplicate and the total cell numbers calculated using the following equation:

$$\text{Viable cells/mL} = (\text{Number of cells in each } 0.1\ mm^3 \text{ squares}/10) \times 10^4$$

$$= [(a + b + c + \ldots)/10] \times 10^4$$

2.2.4.4 Freezing cells

Aliquots of cells (10^6 cells/mL) were routinely frozen and stored in liquid N_2 to maintain a viable supply of cells of a low passage number. Firstly, cells were counted (section 2.2.4.3), re-centrifuged and re-suspended in freezing medium (10% DMSO, 90% FBS; Table 2.4). Aliquots of 1 mL were pipetted into cryogenic vials, wrapped and insulated in polystyrene and kept at –20 °C for 1 h then –80 °C for 24 h, before storing in liquid N_2 or in a –80 °C freezes.

2.2.4.5 MTT assay for characterization

Experiments were always performed during the exponential growth phase of cell cycle. The MTT assay was chosen to characterize cell growth and also used to evaluate the cytotoxicity of the polymer-RNase A conjugates (Chapter 6). This assay measures the formation of formazan crystals (blue) caused by the reduction of MTT by mitochondrial dehydrogenase (Mosmann, 1983) (Figure 2.7).

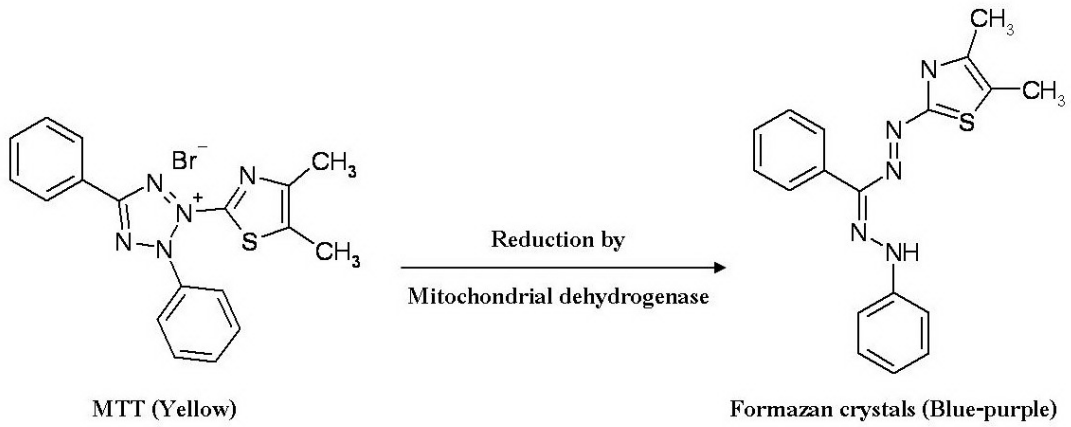


Figure 2.7 Reduction of MTT to formazan crystals by mitochondrial dehydrogenase (from Mosmann, 1983).

2.2.4.5.1 MTT assay for cell growth characterization

B16F10 cells were seeded at the desired cell density (1 x 10^4 and 4 x 10^4 cells/mL) (100 μL) in a 96 well plate and incubated at 37 °C for 24 h to enable cell adhesion. The outer wells of the 96 well plate were filled with 0.1M PBS, pH 7.4 (100 μL) to prevent evaporation of the medium and subsequent variations in growth rate. Cell were maintained at 37°C and cell numbers were then assessed by MTT assay after 24, 48, 72, 96, 120 and 144 h incubation by adding 20 μL of MTT solution (5 mg/mL in 0.1 M PBS, pH 7.4, sterile filtered) was added to a single row of the 96 well plate each day and cells incubated with MTT for 5 h at 37 °C under 5% CO_2. The medium containing MTT (120 μL) was then withdrawn and replaced

by sterile DMSO (100 μL) to dissolve the formazan crystals formed. The cells were incubated for a further 30 min at 37 °C and 5% CO_2 to completely dissolve the formazan crystals. Finally, the blue-purple color was quantified by UV-vis spectroscopy at 550 nm. The experiment were repeated three times (n = 6 in each experiment) and a typical growth curve over time was determined.

2.2.4.5.2 MTT assay for cytotoxicity determination

The cytotoxicity of samples (parent polymer, RNase A, polymer-RNase A conjugates or a medium control) was measured using the MTT assay in B16F10 melanoma cells. B16F10 cells were seeded at a density of 4 x 10^4 cells/mL in a 96 well plates. After culturing for 24 h, the incubation medium was removed and samples (100 μL) were added to the cells at the concentrations in the range 0.001–10 mg/mL RNase A equivalent (n=6). The cells were then incubated for a further 72 h at 37 ºC under 5% CO_2. MTT (20 μL) was added to each of the sample wells 5 h before the end of the 72 h incubation period. The remainder of the assay was carried out as described above. The experiment were repeat three times (n=6 in each experiment) and % cell viability was determined.

2.2.5 Statistical analysis

Samples were analyzed in a minimum of three replicates and the error calculated as either standard deviation (S.D.). Microsoft® Office Excel 2003 was used to measured statistical signification.

CHAPTER 3

PEG-TRYPSIN CONJUGATES: EFFECT OF PEG MOLECULAR WEIGHT AND LINKING CHEMISTRY ON THE BIOLOGICAL ACTIVITY AND THERMAL STABILITY

3.1 Introduction

As mentioned in the general introduction (Chapter 1, section 1.2.3.2), PEG has been most widely used for protein conjugation. Despite the large number of studies on PEG-protein modification, including the emergence of novel linking chemistry (Brocchini et al., 2006), there has been little attempt to systematically study the effect of PEG molecular weight and linking chemistry on protein properties such as thermal stability. A library of PEG-modified trypsin conjugates were synthesized using PEGs of different molecular weight (Mw 1,100, 2,000 and 5,000 g/mol) and different linking chemistries. The role of PEG in this study was acting as a modifier agent where a covalent bond is formed between the PEG molecules and protein of choice.

Bovine pancreatic trypsin was chosen as a convenient model protein. It is a pancreatic serine protease, is composed of a single polypeptide chain of 223 amino acid residues, and it displays a narrow substrate specificity hydrolyzing *L*-lysyl and *L*-argininyl bonds of polypeptides (amidase activity; Walsh, 1970 and esterase activity; Bergmeyer, 1974). The covalent attachment of *p*-nitrophenylchloroformate (NPC) activated mPEG to bovine pancreatic trypsin can be used to increase thermal stability (Gaertner and Puigserver, 1992; Zhang et al., 1999). In the present work, PEGs with different molecular weight (Mw 1,100, 2,000 and 5,000 g/mol) were functionalized by reacting with succinic anhydride (SA), cyanuric chloride (CC) or tosyl chloride (TC) which was conjugated to amino group of trypsin.

As mentioned above, amino groups were the first target of PEGylation but now conjugation to thiol, hydroxyl or amide groups is also possible by using several specific chemical

or enzymatic methods (Veronese and Pasut, 2005). The coupling reactions between amino groups of lysine in proteins and mPEG equipped with an electrophilic functional group have been used in most cases for preparation of PEG-protein conjugates (Zalipsky, 1995; Roberts et al., 2002). Such reactions usually result in conjugates composed of a globular protein in its core which are covalently linked to a numerous polymer chains. The composition of conjugates depend on (i) the number of available attachment sites (amino and sometimes other nucelophilic groups) on the starting polypeptide, (ii) excess and reactivity of the mPEG reagent, and (iii) the conditions of the modification reaction (Zalipsky, 1995).

The early PEG conjugation chemistry usually used the amino groups (both α and ε) of LYS or the *N*-terminal group of the proteins as conjugation sites (Roberts et al., 2002). Common linkages used include: (1) an *ester linkage* such as PEG-succinimidyl succinate (Carter and Meyerhoff, 1985), (2) a *carbamate linkage* such as PEG-benzotriazole carbonate, PEG-carbonylimidazole, PEG-*p*-nitrophenyl carbonate, PEG-succinimidyl carbonate and PEG-trichlorophenyl carbonate (Zalipsky, 1995), and (3) an *amide linkage* such as PEG-dichlorotriazine (Zalipsky, 1995) and PEG-tresylate (Francis et al., 1998).

Some linking chemistries used to synthesize the first generation of PEG conjugates, in particular PEG-benzotriazole carbonate, -succinimidyl carbonate, -dichlorotriazine and –tresylate, had poor selectivity. They preferentially reacted with lysine, but also displayed side reactions with hydroxyl groups of serine and tyrosine, and the imidazole side chain of histidine residues. This formed unstable sulfamate and imidazolecarbamate linkages, respectively. The product was a heterogeneous mixture of conjugates with undesired linkages that could easily degrade (Roberts et al., 2002). The fact that first generation PEGylation was a random reaction, often resulted in either steric hindrance or direct blocking of the protein active site. In this way, PEGylation could lead to a reduction in bioactivity and even the possibility of protein inactivation (Chapman, 2002).

Second generation PEGylation chemistry rapidly developed to avoid a randomly modified protein surface (mixture of isomers), unstable linkages, side reactions and lack of selectivity in substitution. mPEG-propionaldehyde, PEG-carboxylic acids and PEG-*N*-

hydroxysuccinimide ester were used for amine conjugation to give a stable secondary amine or amide linkages (Roberts et al., 2002). High molecular weight (up to 60,000 g/mol) branched PEGs were also used to enhance pharmacokinetic and pharmacodynamic properties of the protein bound (Pasut and Veronese, 2007).

In this review, these activated PEGs may be divided into three important classes (Zalipsky, 1995; Veronese, 2001). Examples of PEG derivatives were illustrated below.

3.1.1 Arylating PEGs

These derivatives contain a reactive aryl chloride residue, which is displaced by a nucelophilic amino group upon a reaction with peptides or proteins.

3.1.1.1 PEG-triazine

The cyanuric chloride method was the most widely used for activation of PEG in first generation conjugation reactions. In the pioneering work of Abuchowski et al. (1977b), PEG-proteins were prepared by modification of bovine serum albumin (BSA) with PEG-dichlorotriazine (Figure 3.1a), resulting in a conjugate with dramatically reduced immunogenicity and antigenicity. The classical approach couples one triazine ring per PEG molecule, despite the fact that cyanuryl halide derivatives are known as some of the least selective protein modifiers. Cyanuric chloride can react not only with amino groups, but also reacts with sulfhydryl (-SH) groups and this is likely responsible for loss of enzymatic activity of many proteins to which it was bound (Zalipsky, 1995). Moreover, PEG-dichlorotriazine can react with multiple nucelophilic functional groups such as LYS, SER, TYR, CYS and HIS, which results in displacement of one of the chlorides and produces a conjugate with retained charge in the form of a secondary amine linkage (Zalipsky, 1995; Roberts et al., 2002). The remaining chloride is less susceptible to react with nucelophilic residues but the reactivity is sufficient to allow cross-linking of protein molecules containing additional nucelophilic residues, so this methodology

probably destroys more biological activity in the bound protein than other methods used (Delgado et al., 1992; Roberts et al., 2002).

2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine (PEG_2-chlorotriazine, Figure 3.1b) was prepared and utilized in protein conjugation to avoid cross-linking when using PEG-dichlorotriazine. The lower reactivity of the remaining chlorine translates into a more selective modification of LYS and CYS residues without further side reaction (Roberts et al., 2002). Since this reagent is derived from trichloro-*s*-triazine it reacts by replacement of two of the most reactive chlorides with PEG-*O* residues, and leaving the least reactive third chloride (Zalipsky, 1995). The PEG_2-chlorotriazine seems to be more effective in rendering proteins non-immunogenic without compromising the biological activity as much as the PEG-dichlorotriazine. The mPEG-dichlorotriazine is easy to prepare, gives stable products with both amino and thiol groups and its chemistry is well-known (Veronese, 2001).

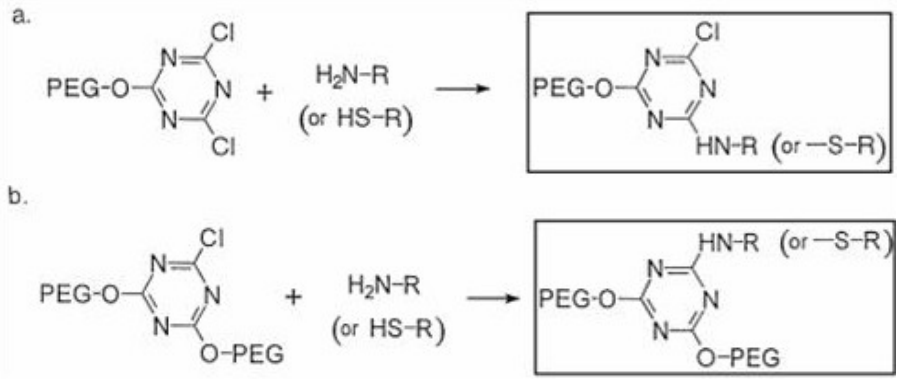


Figure 3.1 A typical reaction scheme for PEG-triazine-protein conjugation (from Veronese, 2001).

Since many of the beneficial properties of PEG-proteins (extended blood circulation time, reduced immunogenicity, etc.) are directly dependent on the PEG content of conjugates, a two-armed reagent, such as PEG_2-chlorotriazine, has an intrinsic advantage over single mPEG chain reagents (PEG-dichlorotriazine). Its ability to bind double the amount of polymer for the same number of attachment sites could be achieved (Zalipsky, 1995). For example, in order to eliminate antigenicity of BSA approximately half the amino groups (30 out

of 60 amines) must be modified with PEG-dichlorotriazine (Abuchowski et al., 1977b), while only 15 residues have to be modified with PEG_2-chlorotriazine (Matsushima et al., 1992).

3.1.2 Alkylating PEGs

Alkylating PEGs react with amino groups of protein to form secondary amine moieties. This method could maintain the positive charge of the starting amino groups. Three types of alkylating PEGs are often used in protein conjugations which are PEG-aldehyde, PEG-tresylate and epoxy PEG (Veronese and Pasut, 2005).

3.1.2.1 PEG-aldehyde reactions (Figure 3.2a)

This reaction gives a highly stable amine linkage between PEG and protein after Schiff base formation followed by cyanoborohydride reduction (Delgado et al., 1992; Veronese, 2001). This is a convenient conjugation where amino group technique positive charge is critical for retention of biological activity. However, the reaction rate for the Schiff base formation is relatively low, sometimes up to a day is necessary for complete reaction leading to inactivation of labile molecules. It has been found that the best reaction takes place with α- and ε-amino groups of protein at neutral or mild alkaline pHs, while good selectivity for the α-amino terminal of amino acid occurs at pH 5-6 (Veronese, 2001).

3.1.2.2 PEG-tresylate reactions (Figure 3.2b)

This is an alternative method used for conjugation to maintain positive charge and also to give a permanent amine linkage (Delgado et al., 1992). The chemistry used for conjugation, and the conjugation products are not unique and well defined, although more specific to amino groups than PEG-dichlorotriazine was reported (Roberts et al., 2002). The optimum reaction takes place with amine at pH 8 and at low temperature. The positive charge of amino group is maintained. In this case, the reaction rate depends upon the properties of the protein and the structure of the reaction products (Veronese, 2001).

3.1.2.3 Epoxy-PEG reactions (Figure 3.2c)

This activated PEG is rarely used because its reactivity is low, and the specificity of interaction is not certain since hydroxyl groups may also react. Reaction at high pH (pH 8.5-9.5) is preferred for amino conjugation, although binding to hydroxyl, imidazole and thiol groups also may take place at this pH (Veronese, 2001; Veronese and Pasut, 2005).

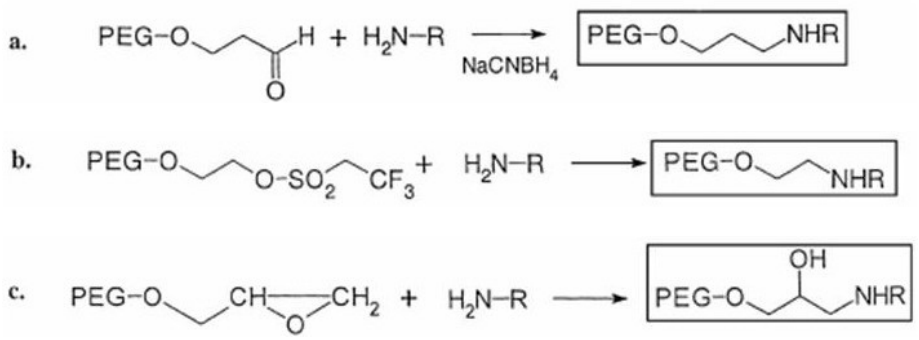


Figure 3.2 Reaction scheme of alkylating PEGs for amino group conjugation (from Veronese, 2001).

3.1.3 Acylating PEGs

These derivatives contain reactive acyl groups. Protein modification with these agents results in acylated amine-containing linkages such as amides and carbamates.

3.1.3.1 PEG-*N*-hydroxysuccinimidyl ester (Figure 3.3a)

To react PEG-*N*-hydroxysuccinimidyl ester with a protein, an anhydride such as succinic anhydride, is added followed by activation with *N*-hydroxysuccinimide (NHS) in the presence of carbodiimide (Delgado et al., 1992). This reaction is highly reactive towards amino groups. The kinetic rate of conjugation depends on the number of -CH_2 groups linked to the carboxyl group. However, it is important to note that changing the distance between the active ester and the PEG backbone by the addition of methylene units has a profound influence on the

reactivity towards amino groups and water (Veronese, 2001; Roberts et al., 2002). For example, at pH 8 and 25 °C, the succinimidyl ester of carboxymethylated PEG (PEG-O-CH_2-COOH) is extremely reactive with the half-life of hydrolysis rate of 0.75 min. This makes it was not possible to use in conjugation process (Harris et al., 1995). In contrast, the half-life of hydrolysis for the propanoic acid (PEG-O- CH_2CH_2-COOH) and the butanoic acid (PEG-O-$CH_2CH_2CH_2$-COOH) derivatives of PEG is 16.5 and 23 min, respectively (Harris et al., 1997). Reactivity of PEG active esters towards amino group and water can be decreased by introducing an α-branching moiety to the carboxylic acid (Roberts et al., 2002).

3.1.3.2 PEG-*p*-nitrophenylcarbonate or PEG-trichlorophenyl carbonate

When the hydroxyl group of PEG is activated by *p*-nitrophenylchloroformate (NPC) or 2, 4, 5-trichlorophenylchloroformate (TPC), these activated PEGs can be used for conjugation. This conjugation method requires a very high pH (e.g. 8.5-9.2) for the coupling step (Delgado et al., 1992), but the extent and rate of modification can easily be followed in the case of PEG-*p*-nitrophenylcarbonate and PEG-trichlorophenylcarbonate by monitoring the phenolate-ion leaving group by colorimetric analysis (Roberts et al., 2002). Both reagents exhibit lower reaction rate than the NHS activated PEGs. This enables exploitation of the different reactivity of the amino groups in the protein to stop the reaction to give the desired degree of modification. A carbamate derivative is obtained. Generally, this reaction methodology gives a slower reaction, and greater specific its towards certain amino groups in the protein. Furthermore, reactions in aqueous-organic solvents are feasible.

There is an interesting different between these two carbonate-activate PEGs (Veronese, 2001; Roberts et al., 2002). PEG-*p*-nitrophenylcarbonate (Figure 3.3b1) has a pale yellow color, whose intensity increases with the conjugation reaction due to the release of *p*-nitrophenol. PEG-trichlorophenylcarbonate (Figure 3.3b2) is a colorless product. The extent of amino groups' modification can be followed directly by colorimetric analysis which quantifies the residual amino groups in the reaction mixture without the need for removing excess reagent or reaction by-products (Veronese, 2001).

3.1.3.3 PEG-carbonylimidazole or PEG-benzotriazole carbonate

Using PEG-carbonylimidazole derivatives to react with amino groups, carbamate linkages can be generated between the protein and PEG (Delgado et al., 1992). PEG-oxycarbonylimidazole (Figure 3.3c1) is characterized by a slower reaction rate compared to chloroformates (Veronese, 2001) and a reaction time of up to 72 h and unphysiological conditions are needed to couple the polymer to the protein (Delgado et al., 1992). On the other hand, PEG-benzotriazole carbonate (Figure 3.3c2) was very reactive, although less so than the succinimidyl activated carboxyl PEGs (Veronese, 2001).

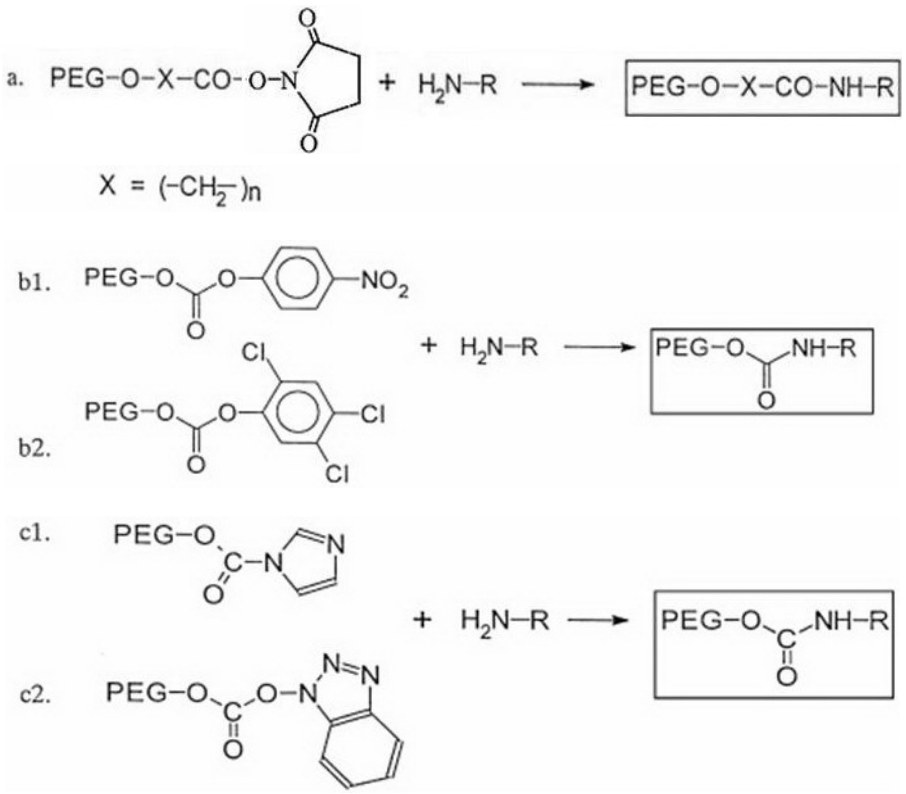
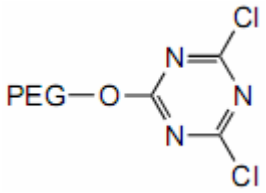
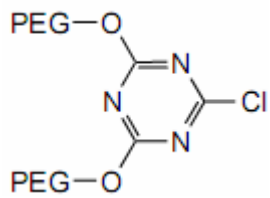
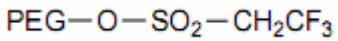


Figure 3.3 Reaction scheme of acylating PEGs for amino group conjugation (from Veronese, 2001).

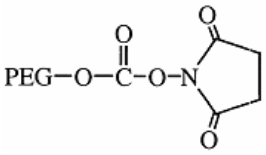
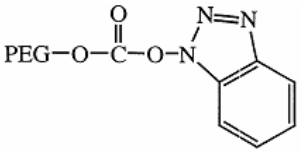
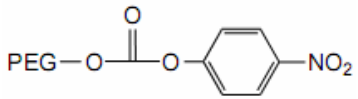
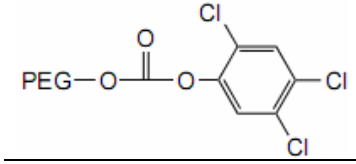
In general, PEGylation techniques have been described as early (first generation) and more recent (second generation) techniques. The properties of these methods are summarized in Table 3.1.

Table 3.1 Properties of PEG derivatives for amino conjugation and some examples of PEG-protein conjugates

PEG derivative	Properties	Linkage	Protein
First generation			
PEG-dichlorotriazine 	- Cross linking due to remaining chloride - Now they are abandoned for therapeutic application because of their toxicity	Amide	- BSA (Abuchowski et al., 1977b)
PEG_2-chlorotriazine 	- Toxicity	Amide	- BSA (Matsushima et al., 1992) - Trypsin (Gaertner and Puigserver, 1992)
PEG-tresylate 	- Non-specific conjugation can result in a degradable sulfamate linkage. -Not much used because the chemistry leads to a mixture of products.	Amide	- Albumin (Delgado et al., 1990) - Alkaline phosphatase (Yoshinga and Harris, 1989)

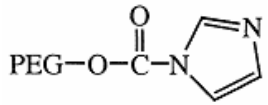
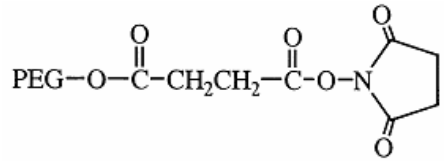
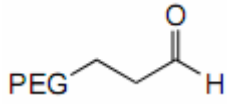
Contd.

Table 3.1 Properties of PEG derivatives for amino conjugation and some examples of PEG-protein conjugates (Contd.)

PEG derivative	Properties	Linkage	Protein
First generation (Contd.)			
PEG-succinimidyl carbonate 	- Non-specific conjugation to HIS or TYR rather than LYS, produces a hydrolytically unstable imidazolecarbamate linkage - Low reactivity	Carbamate	- SOD (Veronese et al., 1990)
PEG-benzotriazole carbonate 	- Non-specific conjugation to HIS or TYR rather than LYS, produces a hydrolytically unstable imidazolecarbamate linkage - Low reactivity	Carbamate	- α-interferon (Lee and McNemar, 1999)
PEG-*p*-nitrophenyl carbonate 	- Toxic by-products of 4-nitrophenol	Carbamate	- Trypsin (Gaertner and Puigserver, 1992; Zhang et al., 1999)
PEG-trichlorophenyl carbonate 	- Toxic by-products of 2, 4, 5-trichloro phenol	Carbamate	- SOD (Veronese et al., 1985)

Contd.

Table 3.1 Properties of PEG derivatives for amino conjugation and some examples of PEG-protein conjugates (Contd.)

PEG derivative	Properties	Linkage	Protein
First generation (Contd.)			
PEG-carbonylimidazole 	- Low reactivity	Carbamate	- SOD (Beauchamp et al., 1983)
PEG-succinimidyl succinate 	- Succinate tag left attached to the protein following degradation - The ester bond between succinic acid and PEG is susceptible to hydrolysis.	Ester	- Asparaginase (Abuchowski et al., 1984) - Adenosine deaminase (Hershfield et al., 1987) - Uricase (Tsuji et al., 1985)
Second generation			
PEG-aldehyde 	- A two step reaction; a schiff base is reduced by $NaCNBH_3$ and the coupling reaction is carried out at low pH (4.5-5). - It labels only the α-amino group.	2° amine	- G-CSF (Kinstler et al, 2002)

(adapted from Delgado et al., 1992; Zalipsky, 1995; Veronese, 2001;Roberts et al., 2002; Veronese and Pasut, 2005)

BSA = Bovine serum albumin; G-CSF = Recombinant human granulocyte colony-stimulating factor; SOD = Superoxide dismutase

3.1.4 The aim of this study

To summarize the specific technical aim of this study were:

(1) To synthesize and characterize PEG-trypsin conjugates using SA, CC or TC for activating PEG (Mw 1,100, 2,000 and 5,000 g/mol).

(2) To determine trypsin activity in the conjugates using BAPNA and BAEE as substrates for amidase and esterase activity, respectively.

(3) To calculate the kinetic parameters (K_M, V_{max} and K_{cat}) and compare them for the different PEG-trypsin conjugates.

(4) To measure the thermal stability and autolysis of the different PEG-trypsin conjugates and compare to native trypsin.

In these studies, it was hoped to identify the PEG molecular weight and the linking chemistry that would confer optimum activity, stability and formulation properties.

3.2 Materials

All chemicals used were of analytical grade. A detailed list of the equipment, compounds, general reagents and their suppliers, used in this study are listed in Chapter 2 (section 2.2). Compounds and reagents specifically used here were as follows. mPEGs of molecular weight 1100, 2000 and 5000 g/mol, succinic anhydride and DMAP were from Fluka (Switzerland). Bovine pancreatic trypsin (EC 3.4.21.4), cyanuric chloride, tosyl chloride, EDC, TNBS, BAPNA, BAEE, BSA and the Coomassie blue protein assay were all from Sigma (Germany). All other reagents and chemicals were of analytical grade.

3.3 Methods

3.3.1 Preparation of activated mPEG

3.3.1.1 Synthesis of succinoylated mPEG (SA-mPEG) (Figure 3.4a)

The mPEG (1100, 2000 or 5000 g/mol; 1 mmol) was first dried by azeotropic distillation using toluene and then dissolved in anhydrous DMF (10 mL). Succinic anhydride (4 mmol) was added, followed by DMAP (4 mmol). The mixture was stirred overnight at room temperature, the resulting polymer was then precipitated using ether, recrystallized twice from CH_2Cl_2/ether (1:40) and characterized by TLC (BuOH/AcOH/H_2O, 4:1:1) (Zalipsky et al., 1983), FT-IR and ^{1}H-NMR. The acid groups were quantified by titration against standardized NaOH solution using bromothymol blue as an indicator (Hreczuk-Hirst et al., 2001b).

3.3.1.2 Synthesis of cyanurate mPEG (CC-mPEG) (Figure 3.4b)

Cyanuric chloride (3 mmol) was dissolved in CH_2Cl_2 containing anhydrous sodium sulphate. mPEG (1,100, 2,000 or 5,000 g/mol; 1 mmol) was added and the mixture was stirred overnight at room temperature. The solution was then filtered, and ether was added slowly with stirring. The finely divided precipitate was collected on a filter and re-dissolved in CH_2Cl_2. This precipitation and filtration process was repeated several times until the ether was free of residual cyanuric chloride as determined by TLC ($CHCl_3$/MeOH, 7:1) following the method of Schiavon et al (2004). The product was characterised by FT-IR and ^{13}C-NMR.

3.3.1.3 Synthesis of tosylate mPEG (TC-mPEG) (Figure 3.4c)

Tosyl chloride (3 mmol) was first dissolved in CH_2Cl_2 containing anhydrous sodium sulphate. mPEG (1,100, 2,000 or 5,000 g/mol; 1 mmol) and then triethylamine (1 mmol) was added and the mixture was stirred overnight at room temperature. The solution was filtered, and ether was added slowly with stirring. The resulting white powder was collected on a filter and

re-dissolved in CH_2Cl_2, washed several times until the ether was free of residual tosyl chloride as determined by TLC ($CHCl_3$/MeOH, 9:1). The product was characterised by FT-IR and ^{1}H-NMR.

3.3.2 PEGylation of trypsin

3.3.2.1 Synthesis of trypsin-SA-mPEG (Figure 3.4a)

A molar ratio of trypsin: SA-mPEGs of 1:20, 1:40 and 1:60 was used. SA-mPEG was dissolved in 50 mM potassium phosphate buffer, pH 6.0 (10 mL). EDC (using the same molar ratios as SA-mPEG) was added, and the reaction mixture was stirred for 10 min. Then sulfo-NHS (using the same molar ratios as SA-mPEG) was added and the reaction mixture was stirred for a further 45 min. Trypsin was then added to the reaction mixture and the pH adjusted to pH ~ 8 by addition of NaOH dropwise. The reaction mixture was left stirring for 1 h at RT and then for 16 h at 4 °C. It was then dialyzed at 4 °C for 48 h against water using a dialysis membrane of MWCO 12,400 g/mol and the product was finally lyophilized to yield a white powder (adapted from Fernandez et al., 2003).

3.3.2.2 Synthesis of trypsin-CC-mPEG (Figure 3.4b)

CC-mPEG was reacted with trypsin solubilized in 0.1 M sodium tetraborate buffer (pH 9.5, 2 mg/mL). As above a molar ratio of trypsin: CC-mPEGs of 1:20, 1:40 and 1:60 was used. The resulting mixture was stirred at RT for 4 h, and then brought to pH 3.0, and dialyzed at 4 °C and the product was lyophilized to yield a white powder as above (Abuchowski and Davis, 1979).

3.3.2.3 Synthesis of trypsin-mPEG (Figure 3.4c)

TC-mPEG was reacted with trypsin solubilized in 0.1 M sodium tetraborate buffer (pH 9.5, 2 mg/mL) using the same molar ratios as given above. The resulting mixture was stirred at RT for 4 h, the solution then brought to pH 3.0, and dialyzed at 4 °C and the product lyophilized to yield a white powder as above (Gaertner and Puigserver, 1992).

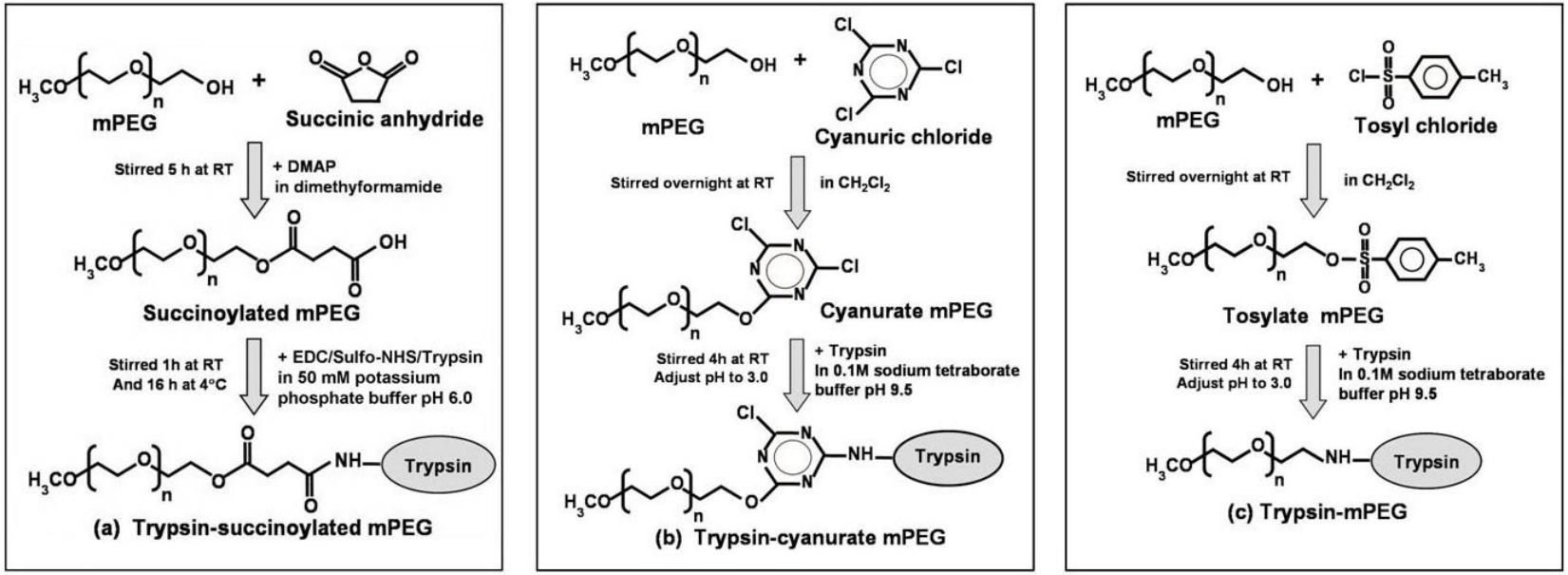


Figure 3.4 Reaction for synthesis of PEG-trypsin conjugates. Panel (a) Trypsin-succinoylated mPEG, panel (b) Trypsin-cyanurate mPEG and panel (c) Trypsin-mPEG conjugate (taken from Treetharnmathurot et al., 2008).

3.3.3 Characterization of activated mPEG and the PEG-trypsin conjugates

The general methods used to characterize both the succinoylated polymer and the polymer-trypsin conjugates are described in Chapter 2. These include dialysis (section 2.2.1.1), Amicon Ultracel^{TM} centrifugal filters (section 2.2.1.3), freeze-drying (section 2.2.1.4), titration (section 2.2.2.1) and FT-IR (section 2.2.2.2). The methods used for conjugate characterization were also previously described in Chapter 2 e.g. TNBS assay (section 2.2.3.1) and Bradford assay (section 2.2.3.2).

3.3.4 Determination of trypsin (native and conjugate) enzyme activity

3.3.4.1 Determination of trypsin amidase activity using BAPNA

Amidase activity of native and the PEGylated-trypsin conjugates was measured using BAPNA as a substrate (Murphy and O'Fagain, 1996). Briefly, 4.2 mM BAPNA (18.2 mg dissolved in 1 mL DMSO and added to 9 ml of 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$) (400 μL) and 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$ (580 μL) were added to a 1 mL cuvette. Then, 20 μL of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv. in 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$) was added and the solution was thoroughly mixed. The mixture was incubated at 30 °C for 10 min and the absorbance was read using a Shimadzu UV 1601 Spectrometer (Kyoto, Japan) at 410 nm. One unit of activity is defined as the amount of enzyme that hydrolyses 1 μmol BAPNA/min. Thus trypsin activity could be calculated for a range of substrate concentrations (0.4 – 2.0 mM) and knowing the molar extinction coefficient 8800 $M^{-1}cm^{-1}$ for free *p*-nitroaniline (PNA). The reaction volume was always 1.0 mL. A Hanes-Woolfe plot was constructed and the kinetic parameters were calculated.

3.3.4.2 Determination of trypsin esterase activity using BAEE

The esterase activity of native and PEGylated-trypsin conjugates was determined at 25 °C using BAEE as substrate. Briefly, 20 μL of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv.) was added to a cuvette containing 3 ml of 0.25 mM BAEE in 67 mM sodium phosphate buffer, pH 7.6, then immediately mixed thoroughly by inversion. The increase in absorbance at 253 nm was measured using a Shimadzu UV 1601 Spectrometer (Kyoto, Japan) for approximately 10 min and the trypsin activity was calculated for a range of substrate concentrations (0.025–0.25 mM). One unit of esterase activity is defined as the amount of enzyme that hydrolyses 1.0 mmol of BAEE/min at 25 °C. (Bergmeyer, 1974).

3.3.5 Evaluation of thermal stability of PEG-trypsin conjugates

To test their thermal stability, native trypsin and the PEGylated conjugates were incubated in aqueous buffer (1.67 mM of 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$ for amidase activity and 0.25 mM of 67 mM sodium phosphate buffer, pH 7.6 for esterase activity) for 10 min at temperatures between 30 - 70 °C. Then the residual trypsin for amidase or esterase activity was assayed using BAPNA or BAEE as described above. The activity of samples was compared to that measured at 30 °C (Zhang, et al., 1999; Fernandez et al., 2002; Fernandez et al., 2003).

3.3.6 Evaluation of autolysis of PEG-trypsin conjugates

Native trypsin and the PEGylated-trypsin conjugates were incubated at 40 ºC in aqueous buffer (0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$ for amidase activity and 67 mM sodium phosphate buffer, pH 7.6 for esterase activity) for 180 min. Aliquots of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv.) (20 μL) were taken at different times and assayed for amidase or esterase activity using BAPNA or BAEE as described above.

3.4 Results and Discussion

3.4.1 Synthesis and characterization of the activated mPEG and PEG-trypsin conjugates

The succinoylation of mPEGs of different molecular weight was monitored by TLC. No free succinic anhydride was detected in the product, and the content of acid groups was 87-98 % by titration.

FT-IR analysis of the activated polymers was performed using a PerkinElmer Spectrum One FT-IR Spectrometer from PerkinElmer Life and Analytical Sciences with Spectrum v5.0.2 software from PerkinElmer Life and Analytical Sciences (Beaconsfield, UK). FT-IR of SA-mPEG showed disappearance characteristic of –OH moiety of mPEG at 3423 cm^{-1}, having ester characteristic at 1731 cm^{-1} and carboxyl group (-COOH) of succinoyl moiety at 1648 cm^{-1} (Figure 3.5a). ^{1}H-NMR showed succinoylated mPEG characters through the aliphatic protons of succinoyl moiety at 2.5-2.7 ppm (4H) and multiplet band of PEG protons at 3.5-4.2 ppm (Figure 3.5b).

The FT-IR spectra of CC-mPEG showed disappearance of the characteristic -OH moiety of mPEG at 3503 cm^{-1}, with the appearance of a characteristic C=N at 1718-1758 cm^{-1}. The skeleton vibration of 1, 3, 5-triazine ring at 804 and 1511-1544 cm^{-1} can also be seen (Figure 3.6a). ^{13}C-NMR showed 1, 3, 5-triazine ring at 172.1-174.2 ppm and carbon skeleton of PEG at 69.7-72.7 ppm (Figure 3.6b).

The FTIR spectrum of TC-mPEG showed disappearance of the characteristic –OH moiety of mPEG at 3485 cm^{-1}, with the appearance of a characteristic SO_2 at 1710 cm^{-1} and aromatic at 1398 cm^{-1} (Figure 3.7a). ^{1}H-NMR showed aromatic protons at 7.2-7.6 ppm and multiplet band of PEG protons at 3.4-3.8 ppm (Figure 3.7b).

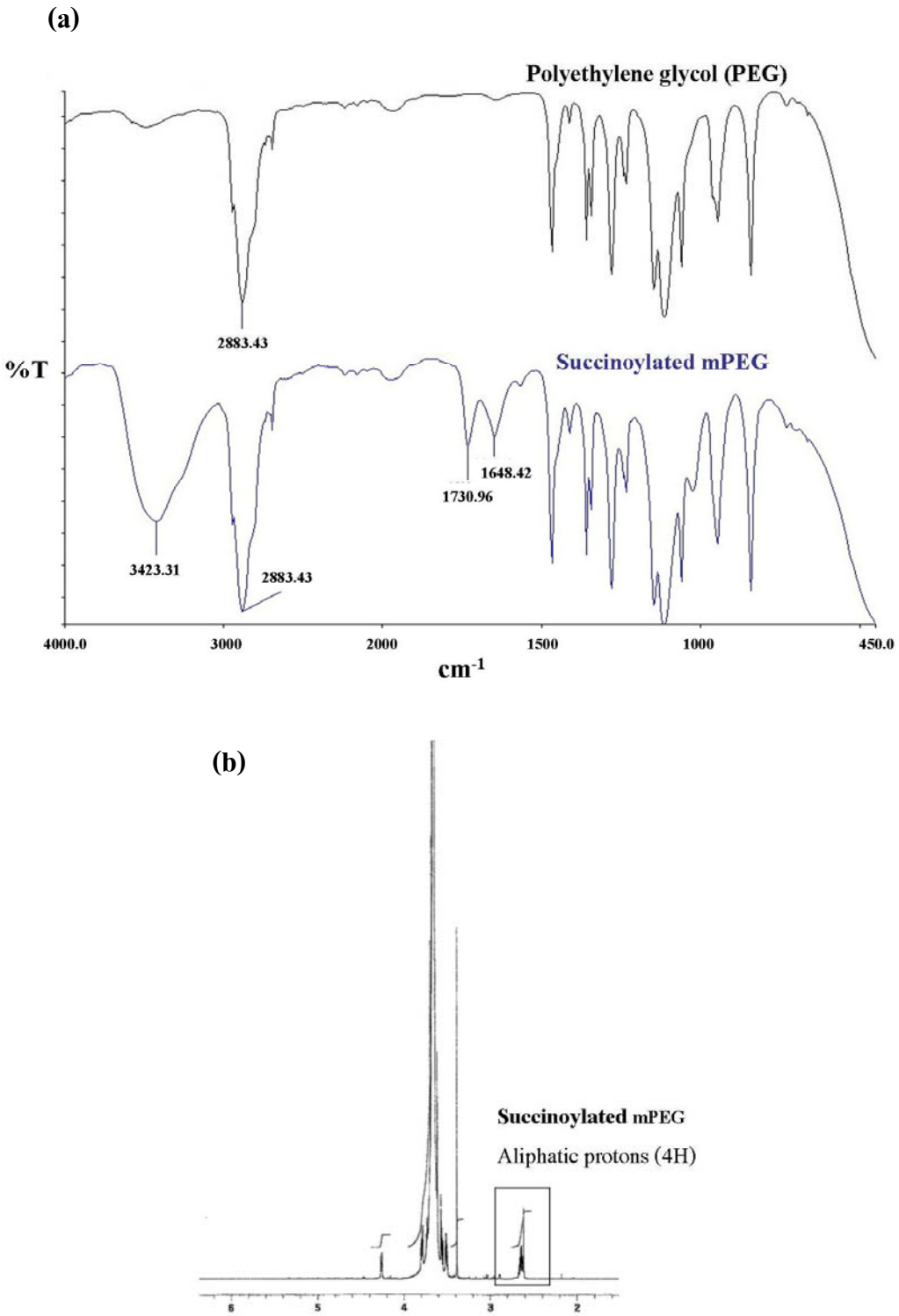


Figure 3.5 Characterization of succinoylated mPEG using panel (a) FT-IR spectroscopy and panel (b) ^{1}H-NMR.

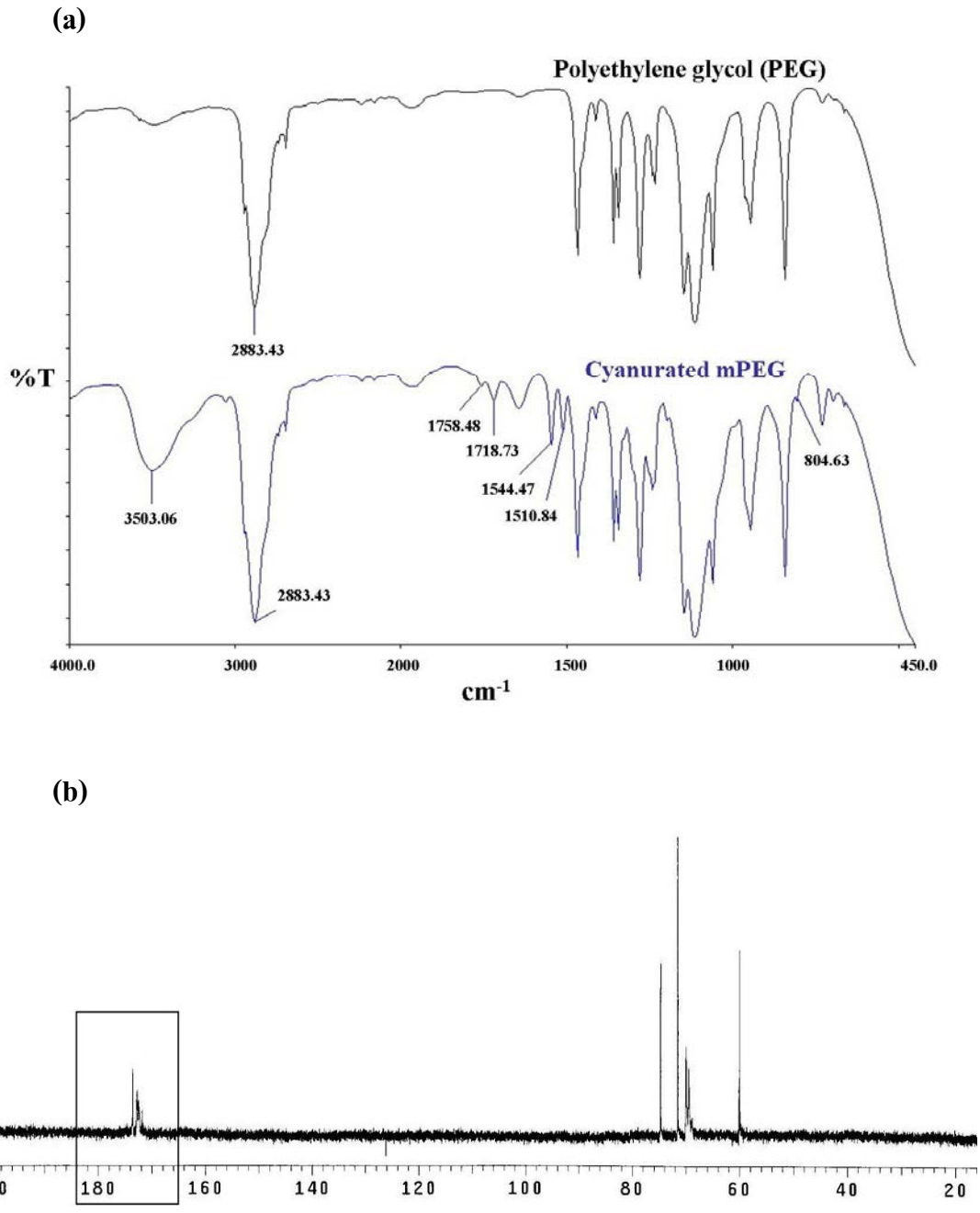


Figure 3.6 Characterization of cyanurate mPEG using panel (a) FT-IR spectroscopy and Panel (b) ^{13}C-NMR.

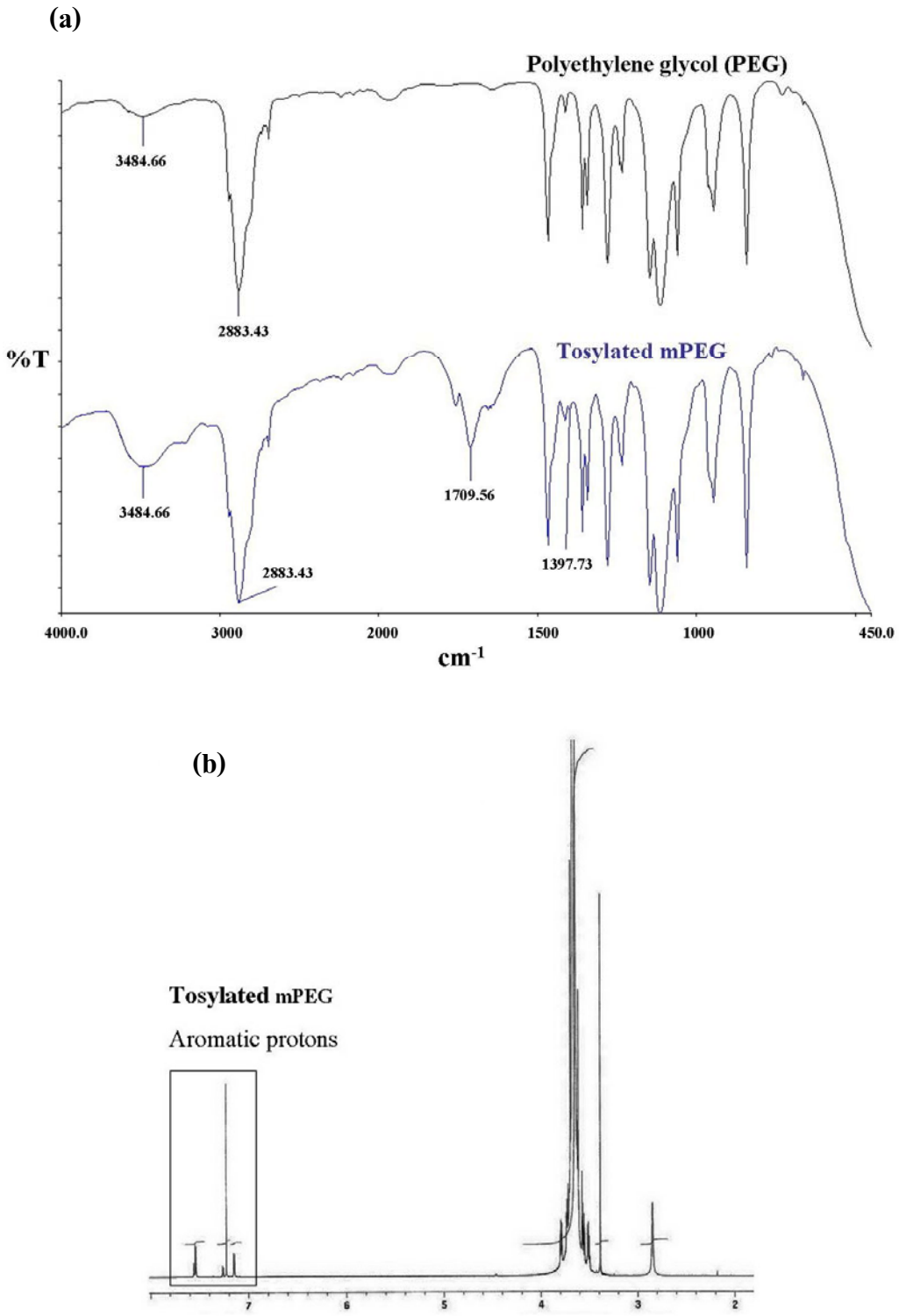


Figure 3.7 Characterization of tosylated mPEG using panel (a) FT-IR spectroscopy and Panel (b) ^{1}H-NMR.

These activated mPEG intermediates were then used to prepare the library of trypsin conjugates. All the PEGs of different molecular weight produced comparable levels of amino group substitution (Table 3.2). When experiments were carried out to ascertain the optimum ratio between trypsin: activated mPEG (reactions at molar ratios of 1:20, 1:40 and 1:60), the ratio of 1:20 gave the best highest degree of substitution, and adding increased amounts of the activated mPEG concentration did not produce a higher degree of substitution.

The TNBS assay indicated that the primary structure trypsin of the bovine pancreatic trypsin used here had 14 accessible LYS amino groups. After modification, it appeared that 4-9 mPEG molecules were covalently bound depending on the reaction conditions and specific mPEG used. This suggested that not all of the LYSs present were available for conjugation. Furthermore, the different reaction it has previously been suggested that conditions used may affect the degree of substitution and which LYSs are involved in conjugate. Murphy and O'Fagain (1996) used bovine trypsin and they found that only 8 of 14 LYSs were modified by mPEGs which were derivatized by acetic acid *N*-hydroxysuccinimide ester. Zhang and coworkers (1999) modified bovine trypsin using different molecular weight mPEGs (350, 750, 2000 and 5000 g/mol), each activated by NPC. They demonstrated in this case that 11 of the 14 LYS residues available were modified. This was a higher degree of modification than seen for bovine trypsin in this study. Gaertner and Puigserver (1992) also modified trypsin (unknown source) with PEG-*p*-nitrophenyl carbonate (10 molar excess over protein amino groups) of different molecular weight (350, 550, 750, 2000, 5000 and 8000 g/mol). They estimated that 12-14 PEG molecules were covalently attached to their trypsin, a protein that was initially shown to have 16 free LYS amino groups.

When all the mPEG derivatives used in this study are compared it can be seen that the degree of trypsin modification that was achieved was highest for TC-mPEG > CC-mPEG > SA-mPEG (Table 3.2). This is probably due to the fact that tosyl chloride is a better leaving group for conjugation under the condition used.

Table 3.2 Estimated number of amino groups substituted in trypsin[a]

Conjugate	Molar ratios of trypsin:PEG	mPEG 1100[b]	mPEG 2000[b]	mPEG 5000[b]
Trypsin-SA-mPEG	1:20	4.4 ± 0.7	4.6 ± 0.1	5.4 ± 0.1
Trypsin-SA-mPEG	1:40	5.3 ± 1.2	3.2 ± 0.2	4.8 ± 0.1
Trypsin-SA-mPEG	1:60	5.2 ± 0.8	2.6 ± 0.2	5.2 ± 0.1
Trypsin-CC-mPEG	1:20	8.5 ± 0.4	6.9 ± 0.1	7.6 ± 0.2
Trypsin-CC-mPEG	1:40	8.6 ± 0.1	5.2 ± 0.5	6.8 ± 0.1
Trypsin-CC-mPEG	1:60	8.5 ± 0.3	4.0 ± 0.3	4.8 ± 0.8
Trypsin-mPEG	1:20	9.1 ± 0.1	9.7 ± 0.1	8.5 ± 0.4
Trypsin-mPEG	1:40	8.9 ± 0.1	9.5 ± 0.1	7.0 ± 0.2
Trypsin-mPEG	1:60	9.0 ± 0.1	8.9 ± 0.1	7.0 ± 0.8

[a]It should be noted that native trypsin has ~ 11 amino groups in the TNBS assay.

[b]Data show mean ± SD (n = 3).

3.4.2 Enzyme activity of trypsin and PEG-trypsin conjugates measured using BAPNA

The enzyme activity and kinetic parameters obtained for both native trypsin and the PEGylated trypsin conjugates (measured using BAPNA as substrate) are summarized in Table 3.3. The enzyme activity of trypsin-SA-mPEG 5000, trypsin-CC-mPEG 5000 and trypsin-TC-mPEG 5000 were 62.4 %, 19.6 % and 16.8 %, respectively (SA > CC > TC) when performed in 0.1 M Tris-HCl containing 20 mM $CaCl_2$, pH 8.2 at 30 °C and using BAPNA (0.4–2.0 mM) as substrate. The trypsin-SA-mPEG conjugates contained less mPEG molecules than the other conjugates when the PEG used had a molecular weight of 5,000 g/mol, and they displayed a higher enzyme activity (rate of hydrolysis; mM/min) than the other conjugates at 30 °C (Figure 3.8a). Polymer molecular weight may also affect to enzyme activity. For example, the enzyme

activity of trypsin-SA-mPEG conjugate was reduced to 96.3 %, 71.4 % and 62.4 % when mPEG molecular weight of 1,100, 2,000 and 5,000 g/mol was used respectively, compared to native trypsin (Table 3.3). The velocity of trypsin-SA-mPEG 5000 conjugate was lower than the other conjugates when the mPEG used had a molecular weight of 1,100 and 2,000 g/mol (Figure 3.8b). This was likely due to the greater steric hindrance caused by the larger, bulkier polymer, or higher molecular weight of polymer blocking the trypsin active site that affected to be a lower velocity (Veronese, 2001).

Table 3.3 Enzyme activity and kinetic parameters of native and PEG-trypsin conjugates[a]

Compound	Activity[†] (%)	K_M[††] (mM)	V_{max}[††] (mM/min)	K_{cat}[††] (s^{-1})
Trypsin	100	0.95 ± 0.11	0.0198 ± 0.0003	0.79 ± 0.01
Trypsin-SA mPEG 1100	96.3 ± 8.0	1.40 ± 0.23	0.0191 ± 0.0061	0.76 ± 0.02
Trypsin -SA mPEG 2000	71.4 ± 7.9	0.84 ± 0.12	0.0142 ± 0.0021	0.57 ± 0.09
Trypsin -SA mPEG 5000	62.4 ± 6.0	0.77 ± 0.22	0.0113 ± 0.0019	0.45 ± 0.08
Trypsin -CC mPEG 1100	69.0 ± 5.8	0.55 ± 0.06	0.0137 ± 0.0011	0.55 ± 0.04
Trypsin -CC mPEG 2000	36.3 ± 10.8	0.44 ± 0.14	0.0072 ± 0.0022	0.29 ± 0.07
Trypsin -CC mPEG 5000	19.6 ± 5.6	0.31 ± 0.06	0.0039 ± 0.0011	0.16 ± 0.04
Trypsin-mPEG 1100	66.2 ± 7.3	0.94 ± 0.10	0.0131 ± 0.0054	0.53 ± 0.22
Trypsin-mPEG 2000	56.0 ± 1.7	0.80 ± 0.17	0.0111 ± 0.0003	0.44 ± 0.01
Trypsin-mPEG 5000	16.8 ± 2.3	0.67 ± 0.12	0.0033 ± 0.0004	0.13 ± 0.02

[a]In all cases the conjugates were prepared using a molar ratio of 1:20

[†]Data shown relate to the trypsin control; mean ± SD (n = 3)

[††]Estimated using the Hanes-Woolfe plot

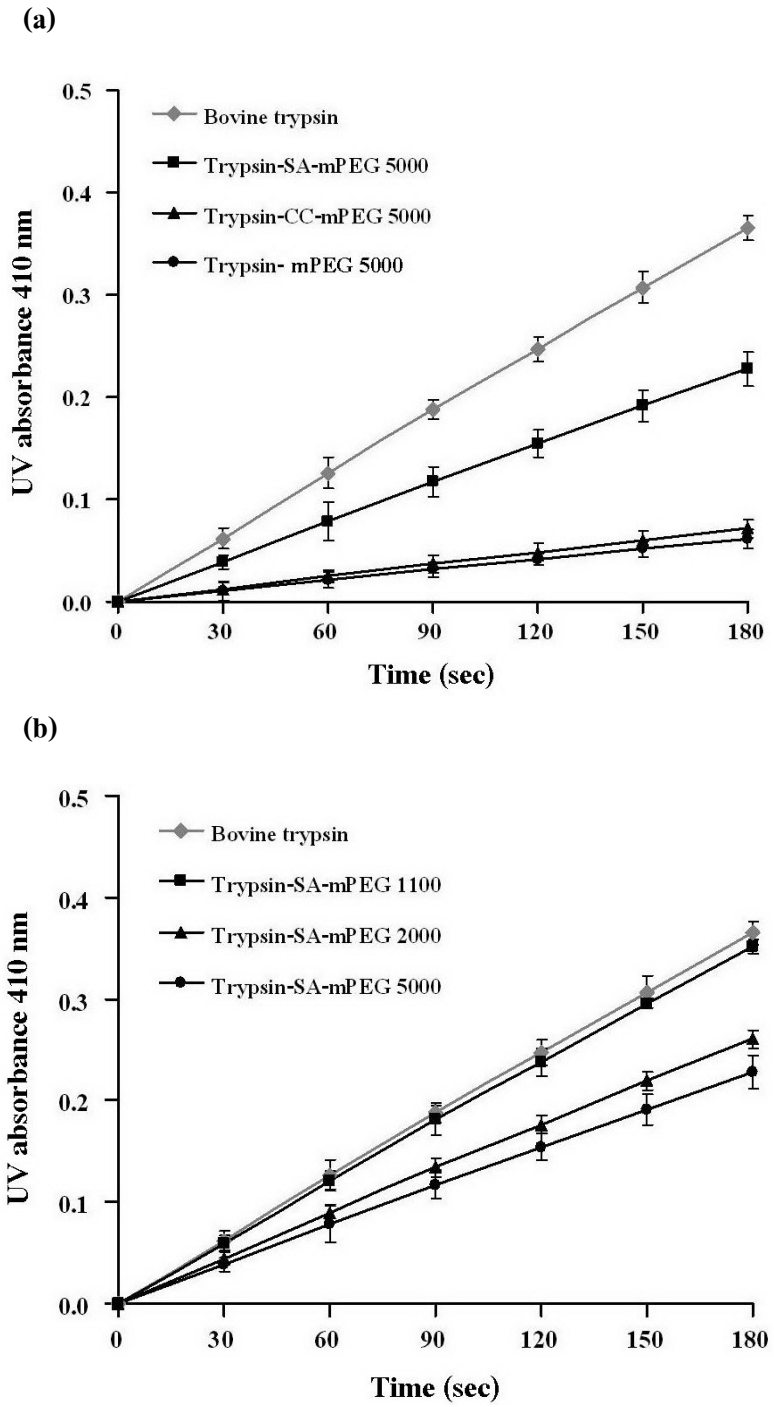


Figure 3.8 Comparison of the activity of native and PEG-modified trypsin conjugates. Panel (a) the PEG used had a molecular weight of 5,000 g/mol and panel (b) the SA-mPEG used had a molecular weight of 1,100, 2,000 and 5,000 g/mol. In each case 10 µg/ml protein equivalent was used (mean ± SD; n = 3).

All conjugates had lower activity than native trypsin. These results were in contrast to the those obtained by Gaertner and Puigserver (1992) who modified trypsin with PEG of different molecular weight (350, 550, 750, 2000, 5000 and 8000 g/mol), each activated by NPC. They found that all conjugates had a higher amidase activity (2-4 times) than native trypsin, while the esterolytic activity increase only slightly (1.5-fold). Zhang et al. (1999) who found that trypsin-mPEG 350 and trypsin-mPEG 750 showed lower amidase activity (80 % and 85 %, respectively) than native trypsin, while trypsin-mPEG 2000 and trypsin-mPEG 5000 showed enhanced amidase activity (140 % and 150 %, respectively) when mPEGs were activated by NPC. The alteration of enzymatic activity may result from the changed microenvironment of trypsin molecule caused by the PEG modification (Zhang et al., 1999).

In this studies the conjugates synthesized using higher molecular weight PEGs displayed a much lower K_M value indicating that they had higher affinity for the binding site of trypsin. Increasing molecular weight of mPEGs resulted in a decrease in the K_M and V_{max} values seen (Table 3.3). These observations were consistent with those of Gaertner and Puigserver (1992) who showed that the K_M value of their trypsin-PEG 5000 conjugate was reduced to 0.42 mM compared to native trypsin (0.80 mM). Their experiments were carried out in 0.05 M Tris-HCl buffer containing 0.02 M $CaCl_2$, pH 8.2 at 25 °C.

3.4.3 Thermal stability and autolysis of trypsin and PEG-trypsin conjugates

All mPEG-modified trypsins showed increased thermal stability compared to native trypsin (Figures 3.9 and 3.10). This was true at all temperatures studied (except 70 ºC) when both BAPNA (Figure 3.9) and BAEE (Figure 3.10) were used as substrates. The highest thermal activity was found when using PEG with molecular weight of 5,000 for all. T_{50} values (calculated from Figure 3.9) for the series of trypsin conjugates containing mPEG (molecular weight of 1,100, 2,000 and 5,000 g/mol) increased to 47.5, 49, and 51 °C, respectively, compared to native trypsin (47 °C). These observations were consistent to those of Zhang et al. (1999) who modified trypsin with mPEG of molecular weight of 350, 750, 2000 and 5000 g/mol activated by NPC and found that T_{50} values ranged from 47 to 66 °C. This effect may be due to the higher

protection capability of the higher molecular weight polymer chain (Gaertner and Puigserver, 1992; Zhang et al., 1999).

The time course of autolysis of native and trypsin conjugates at 40 °C was determined using both BAPNA and BAEE as substrates (Figure 3.11 and 3.12 respectively). Trypsin-modified with mPEG 5000 had the highest percentage residual activity for all types of linker when using either substrate to measure activity. The percentage residual activity measured for native trypsin, trypsin-SA-mPEG 5000, trypsin-CC-mPEG 5000 and trypsin-mPEG 5000 using BAPNA as substrate was 13 %, 37 % 50 % and 39 %, respectively (CC > TC > SA). Moreover, when using BAEE as substrate the percentage residual activity was 2 %, 8 %, 75 % and 38 %, respectively (CC > TC > SA).

These results indicated that all the trypsin-mPEG conjugates were less prone to autolysis compared to the native trypsin. In addition all conjugate showed similar autolysis patterns. Again, these results obtained were similar to those Zhang et al. (1999). They found that trypsin activity reduced to 20 % after a 90 min incubation at 60 °C, while the PEG modified trypsin retained 28 %, 36 %, 57 % and 63 % for trypsin-mPEG 350, trypsin-mPEG 750, trypsin-mPEG 2000 and trypsin-mPEG 5000, respectively.

These observations can explained if the steric hindrance caused by mPEG chains stabilizes the protein against autolysis (Murphy and O'Fagain, 1996; Zhang et al., 1999). The fact that the molecular weight of mPEG increased the conjugates was more stable supports this hypothesis. In fact, the half-life ($t_{1/2}$) of the PEG-trypsin conjugates was increased up to 4 fold compared to native trypsin (Table 3.4). Both amidase and esterase activity showed the same trend, but there were some differences seen for different linkers. Trypsin-modified with CC-mPEGs were more stable than conjugates prepared from the other linkers. This is perhaps simply because the cyanurate modified mPEG conjugate contained more chains and thus they were able to better protect the protein while SA-mPEGs and TC-mPEGs could contain less mPEG molecules resulting in less protection to autolysis (Veronese, 2001).

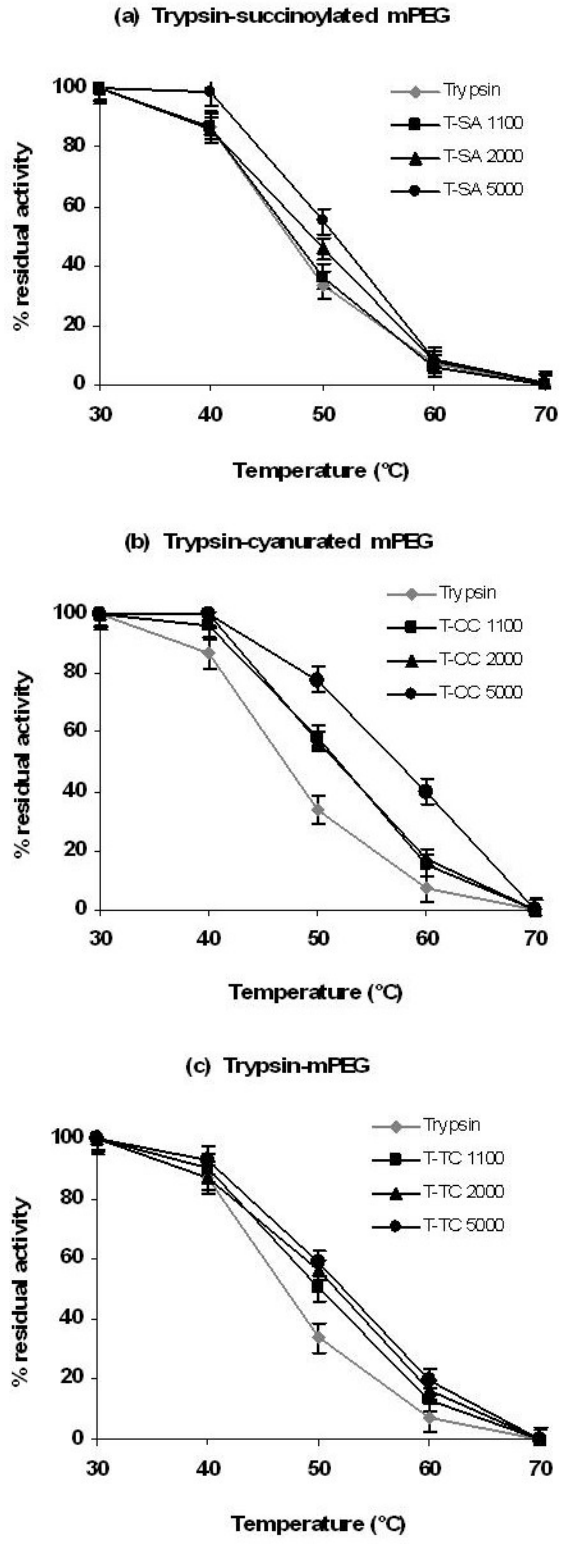


Figure 3.9 Effect of temperature on the activity of native and PEG-modified trypsin conjugates measured using BAPNA as a substrate (n = 3).

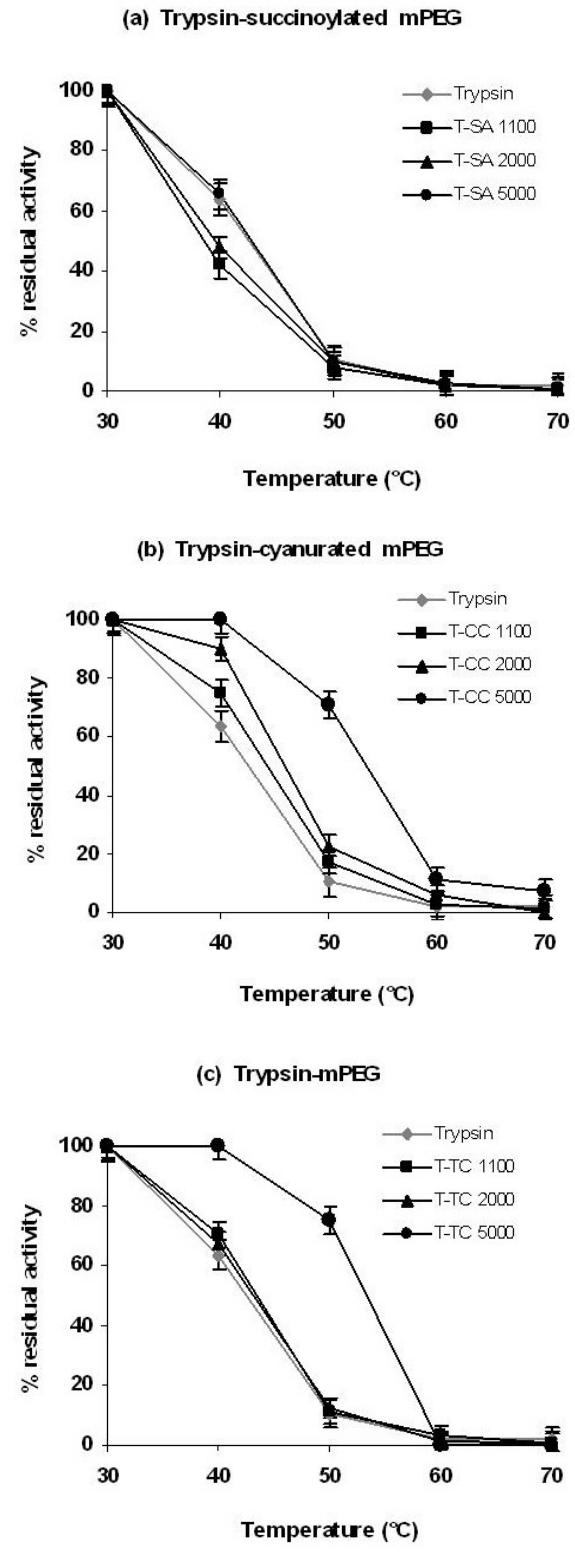


Figure 3.10 Effect of temperature on the activity of native and PEG-modified trypsin conjugates measured using BAEE as a substrate (n = 3).

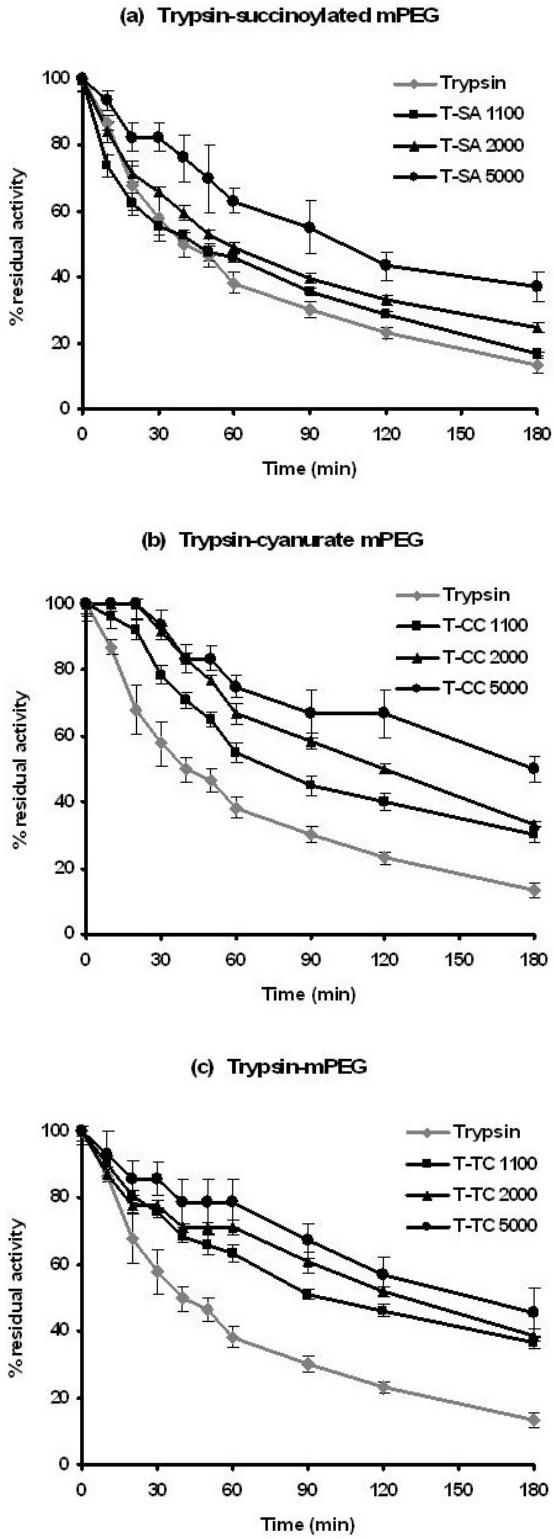


Figure 3.11 Autolysis of native and PEG-modified trypsin conjugates measured using BAPNA as a substrate (mean ± SD; n = 3).

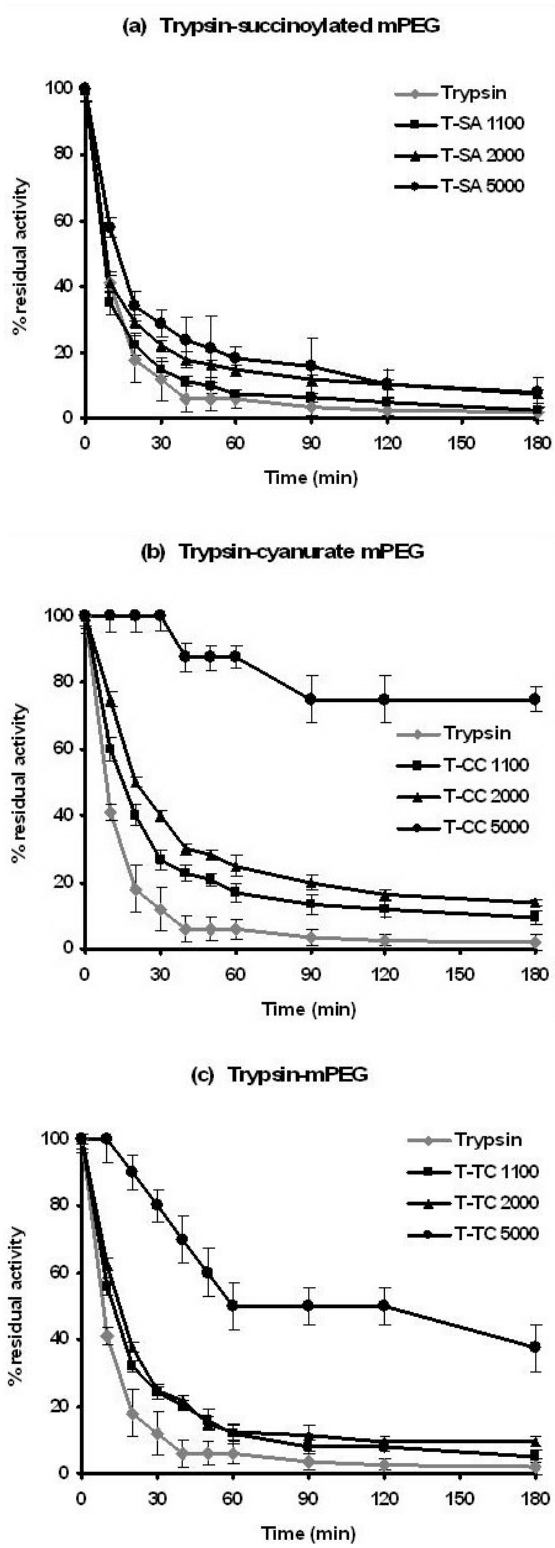


Figure 3.12 Autolysis of native and PEG-modified trypsin conjugates measured using BAEE as a substrate (mean ± SD; n = 3).

Table 3.4 Half-life ($t_{1/2}$) (min) of native and PEG-trypsin conjugates[a]

Conjugate	Substrate[b]							
	BAPNA				BAEE			
	Trypsin	mPEG 1100	mPEG 2000	mPEG 5000	Trypsin	mPEG 1100	mPEG 2000	mPEG 5000
Trypsin	41 ± 1	-	-	-	8 ± 1	-	-	-
Trypsin-SA-mPEG	-	45 ± 1	56 ± 1	102 ± 1	-	8 ± 1	9 ± 1	13 ± 1
Trypsin-CC-mPEG	-	73 ± 1	119 ± 2	180 ± 2	-	15 ± 1	20 ± 1	>180
Trypsin-mPEG	-	95 ± 2	127 ± 3	157 ± 3	-	12 ± 1	15 ± 1	60 ± 1

[a]Experiments were conducted at 40°C and the hydrolysis of BAPNA and BAEE was used to measure residual trypsin activity

[b]Data represent $t_{1/2}$ min (mean ± SD) (n=3)

The trypsin-CC-mPEG 5000 demonstrated the best thermal stability of all the conjugates (including changes in molecular weight of PEG). However, these are well known disadvantages of PEGs activated by cyanuric chloride and these are difficult to avoid. Cyanuric chloride is toxic and its degradation products may also be toxic (Delgado et al., 1992). Therefore, many suggested the PEGs activated by succinic anhydride or tosyl chloride were more suitable for protein modification. Although, trypsin-SA-mPEG 5000 was less stable than trypsin-CC mPEG 5000 conjugate, it still showed higher enzyme activity and greater thermal stability than native trypsin and trypsin-mPEG 5000 conjugate.

The linking chemistry is important for polymer conjugation. During conjugation, high molecular weight protein and polymers exhibit steric hindrance for the reaction (Khandare and Minko, 2006). Vaidaya et al. (1999) who investigated on varying chain length of linker (*p*-aminobenzamidine (PABA)) between *N*-isopropylacrylamide copolymers and trypsin. They found that the efficiency of bonding between polymer-PABA and trypsin increased when the linker length increased (Vaidaya et al., 1999). These results were consistent to this study that the linker length between trypsin and activated mPEG was longest for SA > CC > TC, but the degree of trypsin modification was highest for TC > CC > SA (Table 3.2). However, the highest degree of mPEG molecules on trypsin was avoided due to reducing trypsin activity cause by steric hindrance as describe earlier. Therefore, SA-mPEG was suggested to use for protein conjugation.

In thermal stability studies, all conjugates showed higher stability than native enzyme. However, for both PEG- and the other polymer-protein conjugates there have been few studies on thermal stability. Chemical modification of laccase with PEG (Mw 5,000 g/mol) yielded an enzyme derivative showing only minor changes in both secondary structure and enzyme activity, in comparison with the parent enzyme. PEG-laccase conjugate proved to be more stable, its T_{50} being 2 °C higher than free laccase. The results can be explained by entropy stabilization by PEG conjugation because of the restricted motion of some surface amino acid side chains, which results in a more stable active site (López-Cruz et al., 2006).

This increased stability could be explained by specific modification of lysine residues (remembering the substrate specificity of trypsin) as this would inherently be expected to

protect against autolysis, and/or the fact that the mPEG chains attached on the enzyme surface would anyhow cause non-specific steric hindrance (Murphy and O'Fagain, 1996). PEG conjugation may cause the restricted motion of some surface amino acid side chains, which results in a more stable active site (López-Cruz et al., 2006). Moreover, PEG modification could affect to electrostatic and hydrophobic properties of trypsin surface which play important roles in determining the thermal stability of protein (Zhang et al., 1999). However, the observation that the higher molecular weight PEGs produced greater trypsin stability suggest a major role for the latter mechanism. The increasing of thermal stability of modified trypsins could also result from PEG chains high mobility associated with conformational flexibility and water-binding ability, which formed a highly hydrogen-bonded structure, with polymer wrapped around the protein (Gaertner and Puigserver, 1992; Zalipsky, 1995; Zhang et al., 1999).

3.5 Conclusion

In this study, trypsin was successfully conjugated with mPEG 1,100, 2,000 and 5,000 g/mol. Although chemical modification of mPEG to trypsin decreased activity of trypsin, the modified trypsin conjugates containing the higher molecular mass mPEG showed higher affinity to binding site of enzyme with substrate, higher thermal stability, more stable against autolysis and had an increased $t_{1/2}$ compared to the native enzyme (mPEG 5,000 g/mol > 2,000 g/mol > 1,100 g/mol). Trypsin-SA-mPEG was the most suitable for protein conjugation than trypsin-CC-mPEG and trypsin-TC mPEG as described above. Therefore, succinoylated polymers will be used in further experiments for study the effect of polymer architecture.

In Chapter 4, globular polymer (e.g. β-CD and PAMAM dendrimer) was chosen to modify trypsin and investigate these trypsin conjugates properties compared to linear polymer (e.g. PEG).

CHAPTER 4

β-CYCLODEXTRIN-TRYPSIN AND PAMAM-TRYPSIN CONJUGATES: ENZYME ACTIVITY, AUTOLYSIS AND THERMAL STABILITY

4.1 Introduction

During the last few decades, several efforts have been devoted to the development of new methodologies for increasing functional stability of protein without affecting their biological behavior. In the previous studies it was shown that both PEG molecular weight and linking chemistry can influence the activity and thermal stability of a bound protein. Therefore, the aim of this study was to investigate these properties of trypsin conjugates of β-CD and PAMAM dendrimer compared to PEG conjugates which have linear or branched-lines architecture. Using β-CD and PAMAM dendrimer it was possible to study the effect of polymer architecture.

4.1.1 β-cyclodextrin and PAMAM dendrimer as model polymers

4.1.1.1 β-cyclodextrin (β-CD)

Historically, polysaccharide polymers (e.g. dextran) have been extensively used to prepare conjugates as already described in Chapter 1, section 1.2.3.1. β-CD is a family of cyclic non-reducing α-(1-4)-linked D-glucopyranoses composed of 7 glucose units in the chair conformation. The structure of these remarkable molecular receptors resembles a truncated annular cone with a central cavity. This is hydrophobic in nature and has the appropriate size to include a wide variety of hydrophobic guest compounds (Szejtli, 1998), a property that has been widely explored in the design of non-covalent drug delivery systems.

Previous studies showed that amino derivatives of β-CD can be coupled to aspartic and glutamic acid residues of proteins under mild conditions (Fernandez et al., 2002) and

using this approach, β-CD was conjugated to enzymes such as trypsin (Fernandez et al., 2002; Fernandez et al., 2003; Fernandez et al., 2004a), α-chymotrypsin (Fernandez et al., 2004b; Fernandez et al., 2005) and oxytocin (Bertolla et al., 2008). When trypsin from bovine pancreas was conjugated with mono-6-carboxyl-6-deoxy-β-CD, the modified enzyme contained about 6 mol of oligosaccharide per 1 mol of trypsin and the catalytic and stability properties of trypsin were improved after modification. When the thermal stability profile of the β-CD-trypsin conjugate was studied, it was found that T_{50} increased by 10-14°C. Also, the conjugates were more resistant to autolysis at pH 9.0, compared to native trypsin (Fernandez et al., 2004a).

α-Chymotrypsin from bovine pancreas has also been chemically modified with mono-6-amino-6-deoxy-β-CD. The conjugate contained about 2 mol of oligosaccharide per mol of protein and retained full proteolytic and esterolytic activity. The optimum temperature for α-chymotrypsin esterolytic activity was increased by 5° C after modification and its thermostability was enhanced by about 6 °C. Interestingly, the glycosylated enzyme became markedly more resistant to thermal inactivation at 50 °C and retained 70 % of the original activity when pre-incubated at pH 9.0 for 180 min. Complete inactivation was seen for the unmodified protease under these conditions (Fernandez et al., 2005).

A monofunctionalized carboxylic β-CD derivative conjugated to the *N*-side of the nonapeptide oxytocin which is important in myometrium contraction was studied pharmacologically. When added to isolated rat myometrium, the β-CD-oxytocin conjugate preserved the contracting activity of oxytocin (EC_{50} = 0.40 μM) and indeed it was 230-times higher than seen for free oxytocin (EC_{50} = 1.7 nm). Moreover, the frequency of contraction induced by the β-CD-oxytocin conjugate was also lower than that induced by oxytocin alone. Conjugation was able to improve the solubility, stability and biological activity of oxytocin while reducing its side-effects (Bertolla et al., 2008).

As mentioned above, a β-CD was chosen in this study and it was modified by succinoylation before conjugation to amino group of trypsin.

4.1.1.2 PAMAM dendrimer

PAMAM dendrimers are another interesting class of potential drug carriers. They have been extensively investigated since being introduced in the early 1980s (Tomalia et al., 1985) although still no PAMAM drug delivery system has been tested clinically. The PAMAM dendrimer has a well-defined structure and consists of a central core and branched monomers. At higher generation, it has a globular shape and a large number of end groups. The ideal dendrimer for drug delivery should exhibit a high aqueous solubility and high drug-loading capacity, biodegradability, low toxicity, favorable retention and biodistribution characteristics, specificity, and appropriate bioavailability (Wolinsky and Grinstaff, 2008). However, many synthetic chemistries used to prepare dendrimers can led to problems of toxicity (Duncan and Izzo, 2005).

PAMAM dendrimers can be classified by the native of this surface or terminal groups e.g. amine (-NH_2), carboxyl (-COOH) or hydroxyl (-OH) groups (Tomalia et al., 1985). PAMAM-NH_2 generation 3 (G3) and PAMAM-OH generation 4 (G4) have been widely used to conjugate many drugs. For example, 5-ASA (Wiwattanapatapee et al., 2003), ibuprofen (Kolhe et al., 2006), methotrexate (Gurdag et al., 2006), methylprednisolone (Khandare et al., 2005), paclitaxel (Khandare et al., 2006), penicillin V (Yang and Lopina, 2003), propanolol (D'Emanuele et al., 2004) and others (Table 1.8).

However, conjugation of PAMAM dendrimer to proteins or enzymes has been rarely reported. The interaction between PAMAM dendrimers of 4^{th} and 5^{th} generations and proteins such as human bovine serum albumin (Klajnert et al., 2003), erythrocyte membrane acetylcholine esterase (Klajnert et al., 2004), human and bovine serum albumin (Shcharbin et al., 2005a), and acetylcholinesterase (Shcharbin et al., 2006) has been studied. It was found that PAMAM dendrimers can affect enzyme activity, protein conformation, and binding properties (Klajnert et al., 2003; Klajnert et al., 2004; Shcharbin et al., 2005a; Shcharbin et al., 2005b; Shcharbin et al., 2006). Their interaction depended on pH and ionic strength. In the case of PAMAM (-NH_2) dendrimer G4, G5 and G6, a significant conformational change of the protein as well as changes in size were observed under varying solvent conditions. When the solution pH was decreased, the dendrimers increased in size (Maiti et al., 2005). Low generation (< G4)

PAMAM dendrimers have been utilized for drug delivery because they appear to have low or negligible toxicity and immunogenicity, favorable biodistribution, and less sensitive to conformational changes with variations in the pH (Roberts et al., 1996; Chen et al., 2000; Malik et al., 2000; El-Sayed et al., 2002).

In these studies, PAMAM-OH (G3) was chosen for conjugation to trypsin because: (i) PAMAM-OH has many hydroxyl group similar to β-CD, (ii) its hydroxyl groups can be modified using succinic anhydride to form a succinoylated PAMAM intermediate, and (iii) the molecular weight of PAMAM-OH (G3) was 6,941 g/mol which similar to the molecular weight of PEG used to prepare protein conjugates which was previously reported.

Again trypsin was chosen as the model protein to enable comparison with the studies in Chapter 3 and also the literature. The stabilization of bovine pancreatic trypsin by chemical modification with β-CD and PAMAM dendrimer has been studied. The polymers were functionalized by reacting with succinic anhydride to produce carboxylic end group which were bound to amino group of trypsin.

4.1.2 The specific technical aims of this study

Specifically the aims of this study were:

(1) To synthesize and characterize β-CD- and PAMAM-trypsin conjugates

(2) To determine trypsin activity of β-CD- and PAMAM-trypsin conjugates and calculate their kinetic parameters (K_M, V_{max} and K_{cat}) compared to native trypsin using BAPNA as a substrate.

(3) To investigate thermal stability and autolysis of trypsin of the conjugates compared to native trypsin and PEG-trypsin conjugates described in Chapter 3.

4.2 Materials

All chemicals used in this work were analytical grade and the equipment, compounds, general reagents and their suppliers were already given in Chapter 2 (section 2.2). The specific reagent and compounds used in this work were as follow. β-CD was purchased from Fluka

(USA) and PAMAM dendrimer (G3, -OH) was purchased from Aldrich (USA). Trypsin from bovine pancreatic and BAPNA were from Sigma (UK). BAEE and BSA from Sigma (Switzerland). Protein standards of bovine pancreas ribonuclease A (13,700 g/mol), chymotrypsinogen A (25,000 g/mol), bovine pancreas ovalbumin (43,000 g/mol), hen egg albumin (67,000 g/mol) and bovine serum blue dextran 2000 were also from GE Healthcare (UK). Prestained SDS-PAGE standards of broad molecular weight range (16.5-175 kDa), and TEMED were supplied by Bio-Rad (USA). Acrylamide (40 %) and bis-acrylamide (2 %) were from Amersham Biosciences (USA). Ammonium persulfate, brilliant blue R-250 and glycine (Electrophoresis Grade) were from USB Corporation (USA). Tris-HCl was supplied by Biomol (Germany) and isopropanol was from Lab-scan Asia, Thailand.

4.3 Methods

4.3.1 Succinoylation of β-CD and the PAMAM dendrimer G3 (-OH)

4.3.1.1 Succinoylation of β-CD (Figure 4.1a)

β-CD (0.5 g, 0.38 mmol) and succinic anhydride (0.5 g, 5 mmol) in anhydrous pyridine (10 mL) were mixed and stirred at room temperature for 4 h. The succinoylated β-CD was then precipitated by addition of 40 mL isopropyl alcohol. The precipitate was then washed 3 times with 10 mL isopropyl alcohol (Wolff et al., 2004) to yield 0.35 g of a white solid. The product was titrated to quantify the percentage carboxylic acid groups incorporated and further characterized by FT-IR and ^{1}H- NMR.

4.3.1.2 Succinoylation of PAMAM dendrimer (Figure 4.1b)

Methanolic PAMAM dendrimer G3 (-OH) solution (equivalent to 0.035 mmol) was first dried under a stream nitrogen to give a solid residue, and then dissolved in DMF (5 mL) and succinic anhydride (1.12 mmol) was added followed by DMAP (1.12 mmol). The reaction mixture was purged with N_2, sealed and stirred overnight at 50 °C, under N_2. It was then poured into vigorously stirred diethyl ether (1 L) and stirred for 10 h. The ether was removed by filtration under

vacuum. The remaining solid was dissolved in minimum amount of distilled water and poured into a dialysis tube (MWCO 3,500 g/mol) and dialyzed against distilled water for 48 h. The resulting solution was freeze-dried to yield succinoylated PAMAM (0.237 g) which obtained as a white solid. The acid groups were quantified by titration (using 1% bromothymol blue as indicator and NaOH as base) (Hreczuk-Hirst et al., 2001a). The product was characterized by FT-IR and ^{1}H-NMR.

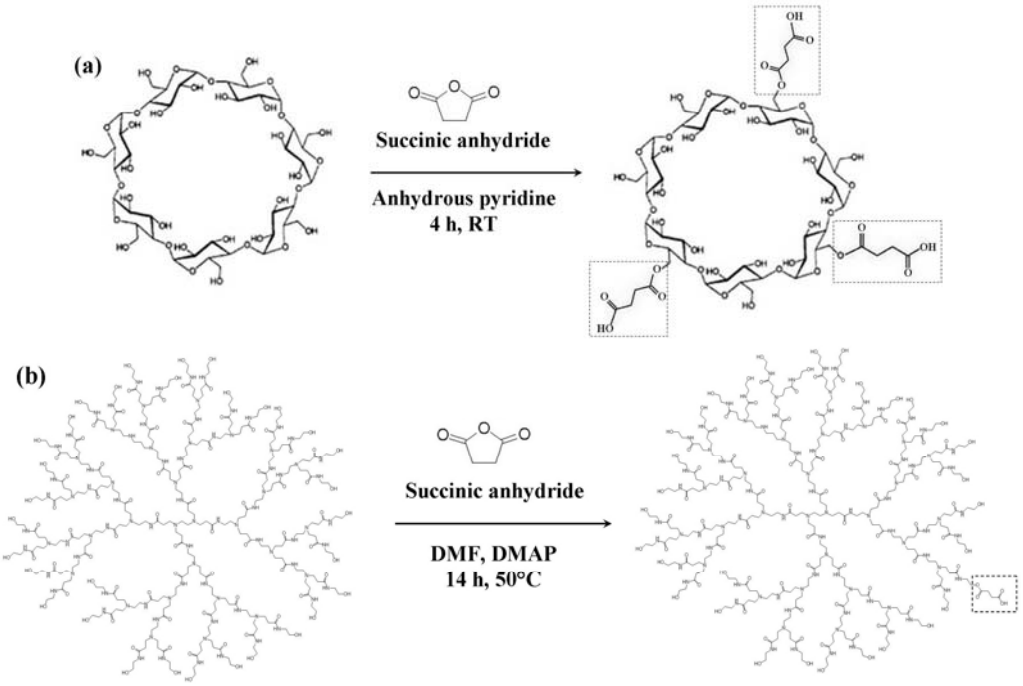


Figure 4.1 Reaction scheme for (a) succinoylation of β-CD and (b) succinoylation of the PAMAM dendrimer.

4.3.2 Synthesis of β-CD- and PAMAM-trypsin conjugates

4.3.2.1 Synthesis of β-CD-trypsin conjugates (Figure 4.2)

The succinoylated β-CD (103.0 mg) was dissolved in distilled water (2 mL). EDC (24.0 mg) was added, and the reaction mixture was stirred for 10 min. Then sulfo-NHS (29.4 mg) was added and the reaction mixture was stirred for a further 45 min. Trypsin (24.6 mg) was then added to the reaction mixture and the pH adjusted to pH ~ 8 by addition of NaOH dropwise. The reaction mixture was left stirring for 18 h at room temperature. The conjugate was

purified by FPLC, and the collected fractions were desalted using an Amicon Ultracel^{TM} tubing (molecular weight cut-off 10,000 g/mol) before freeze-drying to yield 65.4 mg of a white powder. The conjugate was characterized by SDS-PAGE electrophoresis, GPC, and FPLC. The Bradford protein assay was used to estimate the total protein content (Bradford, 1976) with BSA standards.

4.3.2.2 Synthesis of PAMAM-trypsin conjugates

Succinoylated PAMAM dendrimer (90 mg) was dissolved in 2 mL distilled water. EDC (14.3 mg) was added and the reaction mixture was stirred for 10 min. Then sulfo-NHS (16.6 mg) was added and the reaction mixture was stirred for a further 45 min. Trypsin (26.8 mg) was then added to the reaction mixture (Figure 4.2). The product was obtained as a white powder (45.6 mg). All other reaction conditions, purification and characterization were similar to that described above for β-CD conjugates.

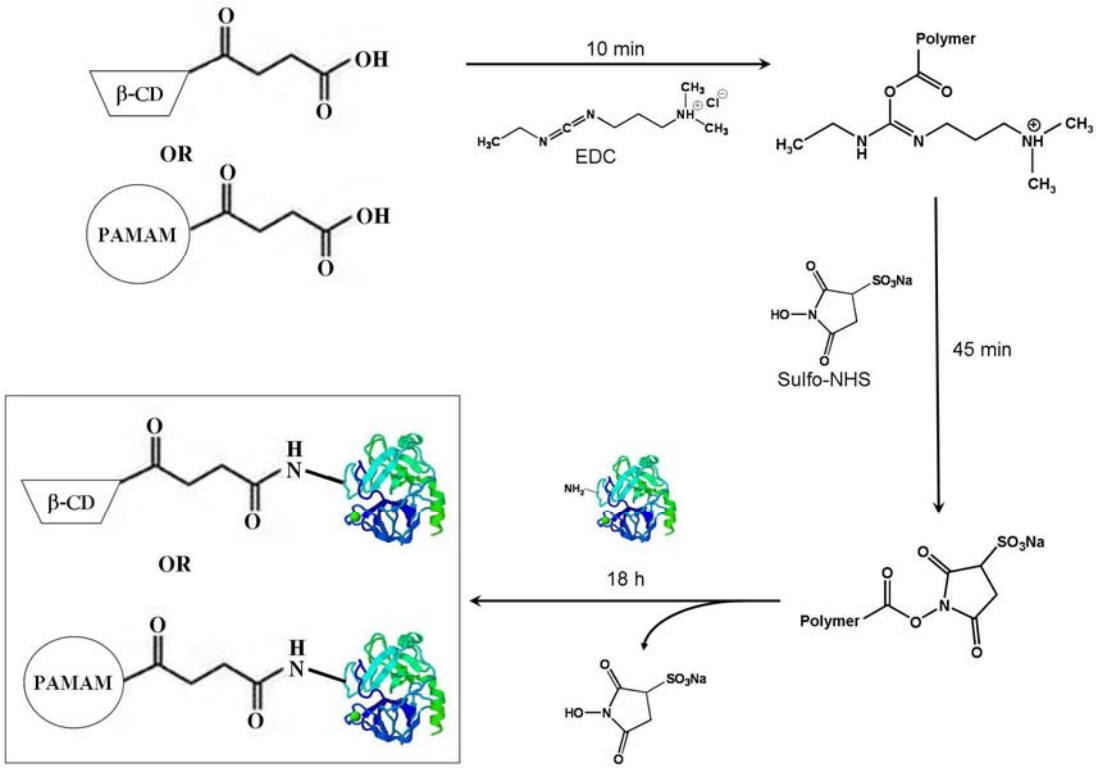


Figure 4.2 Reaction of β-CD-trypsin and PAMAM-trypsin conjugates.

4.3.3 Characterization of modified polymers and the β-CD- and PAMAM-trypsin conjugates

The methods used to characterize both the succinoylated β-CD and PAMAM dendrimer and the corresponding polymer-trypsin conjugates were the same as described in Chapter 2. Purification was achieved by include dialysis (section 2.2.1.1), Amicon Ultracel^{TM} centrifugal filters (section 2.2.1.3), freeze-drying (section 2.2.1.4), titration (section 2.2.2.1), FT-IR (section 2.2.2.2) and SDS-PAGE (section 2.2.3.3). Bradford protein assay was conducted using a Hewlett Packard 8452A Diode array spectrophotometer (Ontario, Canada) the same as described in Chapter 2 (section 2.2.3.2) to determine the total protein content of the conjugates. The Sephacryl S-100 HiPrep 16/60 High resolution FPLC column as described in Chapter 2 (Method I, section 2.2.1.2) was used to isolate the conjugate fraction and purify it from any unconjugated protein and low molecular weight impurities.

4.3.4 Quantitation of trypsin activity of β-CD- and PAMAM-trypsin conjugates using BAPNA as a substrate

The amidase activity of free and conjugated trypsin (8.3×10^{-5} mM) in Tris buffer (0.1 M containing 20 mM $CaCl_2$; pH 8.2) was measured by modified method using BAPNA as a substrate (Duncan et al., 2008). Briefly, BAPNA solution (stock solution 2 mg/mL in DMSO; 5-50 μL) was added to a quartz cuvette (1 mL) and made up to 980 μL with Tris buffer and equilibrated at 37 °C. To start the assay, free trypsin (stock solution 0.1 μg/μL in Tris buffer; 20 μL) or polymer-trypsin conjugates (20 μL; 2 μg/mL protein-equiv. in Tris buffer) was added to the cuvette and the solution was thoroughly mixed and measured the release of *p*-nitroaniline (PNA) at 410 nm (at 37 °C for 5 min) using a Shimadzu UV 1601 Spectrometer (Kyoto, Japan) (PNA ε_{410} = 8800 $M^{-1}cm^{-1}$). The kinetic parameters K_M, V_{max} and K_{cat} were derived from the raw data of trypsin activity against a range of substrate concentrations (0.023-0.23 mM) using a Hanes-Woolfe plot.

4.3.5 Evaluation of thermal stability of β-CD- and PAMAM-trypsin conjugates

Thermal stability was assessed as follows. Native trypsin and the polymer-trypsin conjugates were incubated in 0.1 M Tris-HCl (pH 8.2) containing 20 mM $CaCl_2$ for 10 min at temperatures between 30-70 °C (Zhang et al., 1999). Then the residual enzyme activity was assayed using BAPNA as described above. The activity of samples was compared to that measured at 30 °C.

4.3.6 Evaluation of autolysis of β-CD- and PAMAM-trypsin conjugates

To determine autolysis, native trypsin and polymer-trypsin conjugates were incubated at 40 ºC in 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$ for 180 min. Aliquots (20 μL) of free trypsin or β-CD- and PAMAM-trypsin conjugates (2 μg protein-equiv.) were removed at different times and assayed for retained amidase activity using BAPNA as described above.

4.4 Results and Discussion

4.4.1 Synthesis and characterization of succinoylated β-CD and succinoylated PAMAM dendrimer

FT-IR confirmed the presence of the characteristic ester peak at 1731 cm^{-1} in the succinoylated β-CD (Figure 4.3a) and ^{1}H-NMR spectrum of succinoylated β-CD confirmed aliphatic protons at 2.3-2.7 ppm (Figure 4.3b). FT-IR confirmed the presence of the characteristic ester peak at 1722 cm^{-1} in the succinoylated PAMAM (Figure 4.4a) and ^{13}C-NMR spectrum of the succinoylated PAMAM confirmed the carbonyl of the carboxylic and ester peak at 181.4 ppm and 176.3 ppm respectively (Figure 4.4b). When the degree of COOH substitution was determined by titration it was found that one molecule of succinoylated β-CD and succinoylated PAMAM contained 3 or 1.1 carboxylic groups respectively.

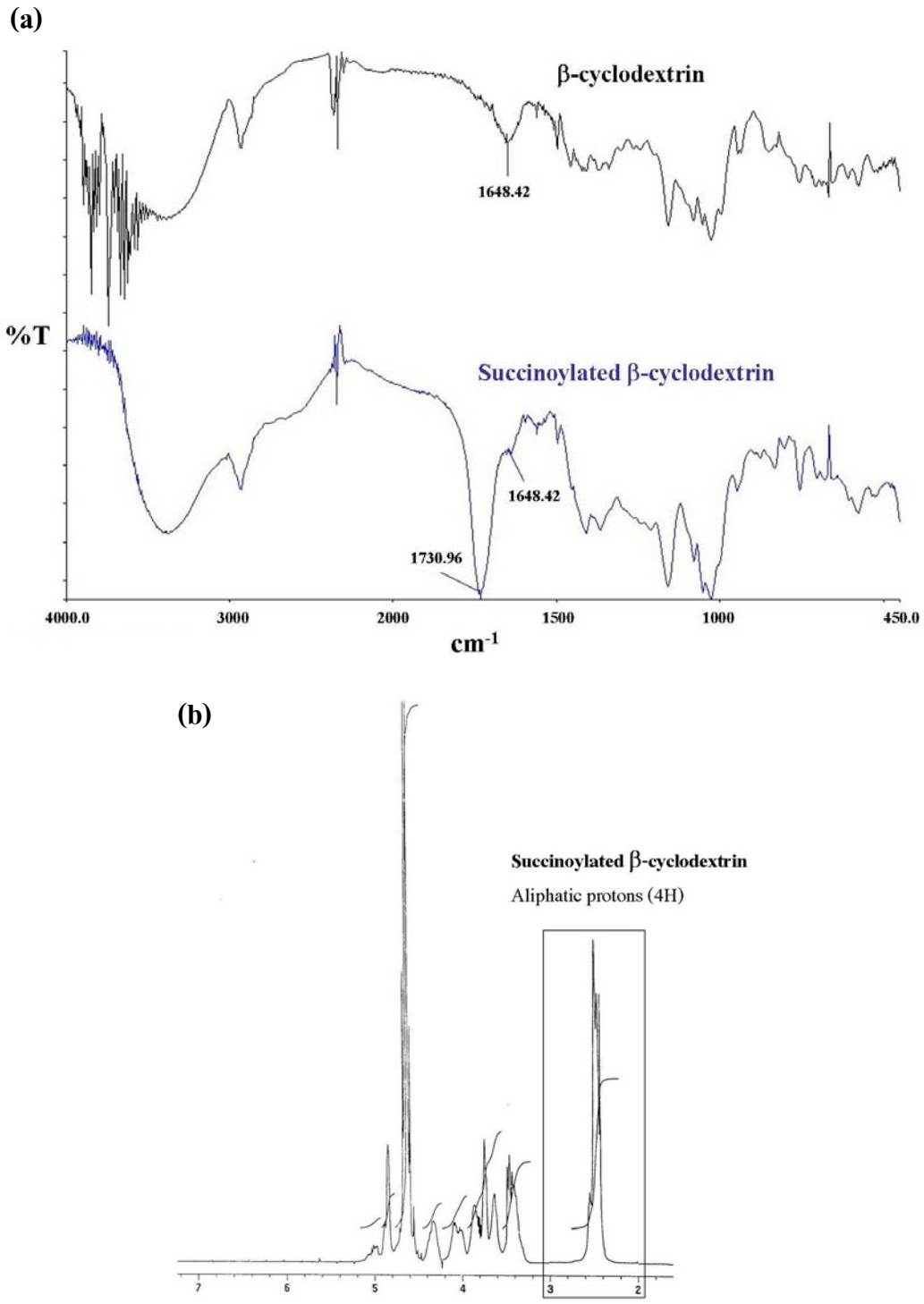


Figure 4.3 Characterization of succinoylated β-CD. Panel (a) FT-IR and panel (b) ^{1}H-NMR. The spectra are compared to their parent polymer.

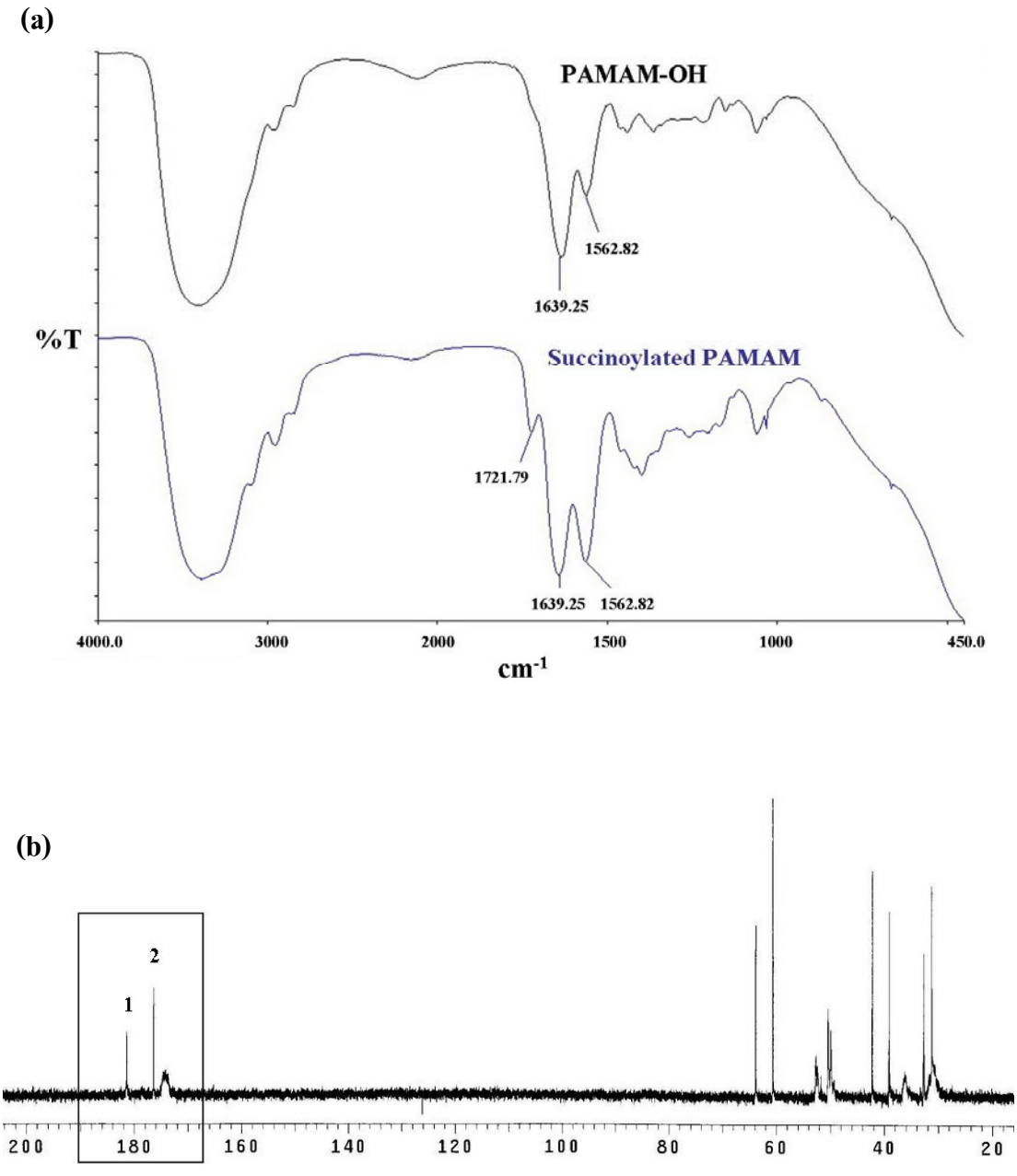


Figure 4.4 Characterization of succinoylated PAMAM. Panel (a) FT-IR and panel (b) ^{13}C-NMR spectrum; peak (1) is carbonyl of carboxylic at 181.4 ppm and peak (2) is carbonyl of ester at 176.3 ppm. The spectra are compared to their parent polymer.

4.4.2 Synthesis and characterization of β-CD- and PAMAM-trypsin conjugates

β-CD-trypsin and PAMAM-trypsin conjugates were prepared, and after purification by FPLC (Figure 4.5a) and SDS-PAGE revealed that no free trypsin was present in the purified conjugates (Figure 4.5b). The molecular weight of the conjugates predicted by FPLC given in Table 4.1. The β-CD- and PAMAM-trypsin conjugates contained 72 and 50 wt% total protein, respectively (Table 4.1).

Table 4.1 Characteristics of the β-CD, the PAMAM dendrimer and the β-CD- and PAMAM-trypsin conjugates

	Protein content (wt%)	**Mw (g/mol)**
Trypsin[†]	-	21,400[††]
β-CD	-	1,135[a]
β-CD-trypsin	72	29,000[††]
PAMAM-OH dendrimer	-	6,941[b]
PAMAM-trypsin	50	39,300[††]

[†]Trypsin from bovine pancreas (Mw 23,800 Da; 223 amino acids) (http://www.sigmaaldrich.com)

[††]Estimated using protein FPLC standards (bovine pancreas ribonuclease A, Mw 13,700; chymotrypsinogen A, Mw 25,000; bovine pancreas ovalbumin, Mw 43,000 and hen egg albumin, Mw 67,000).

[a]β-CD (Mw 1,135 g/mol) from Fluka (USA) (http://www.sigmaaldrich.com)

[b]PAMAM-OH dendrimer (Mw 6,941 g/mol) from Aldrich (USA) (http://www.sigmaaldrich.com)

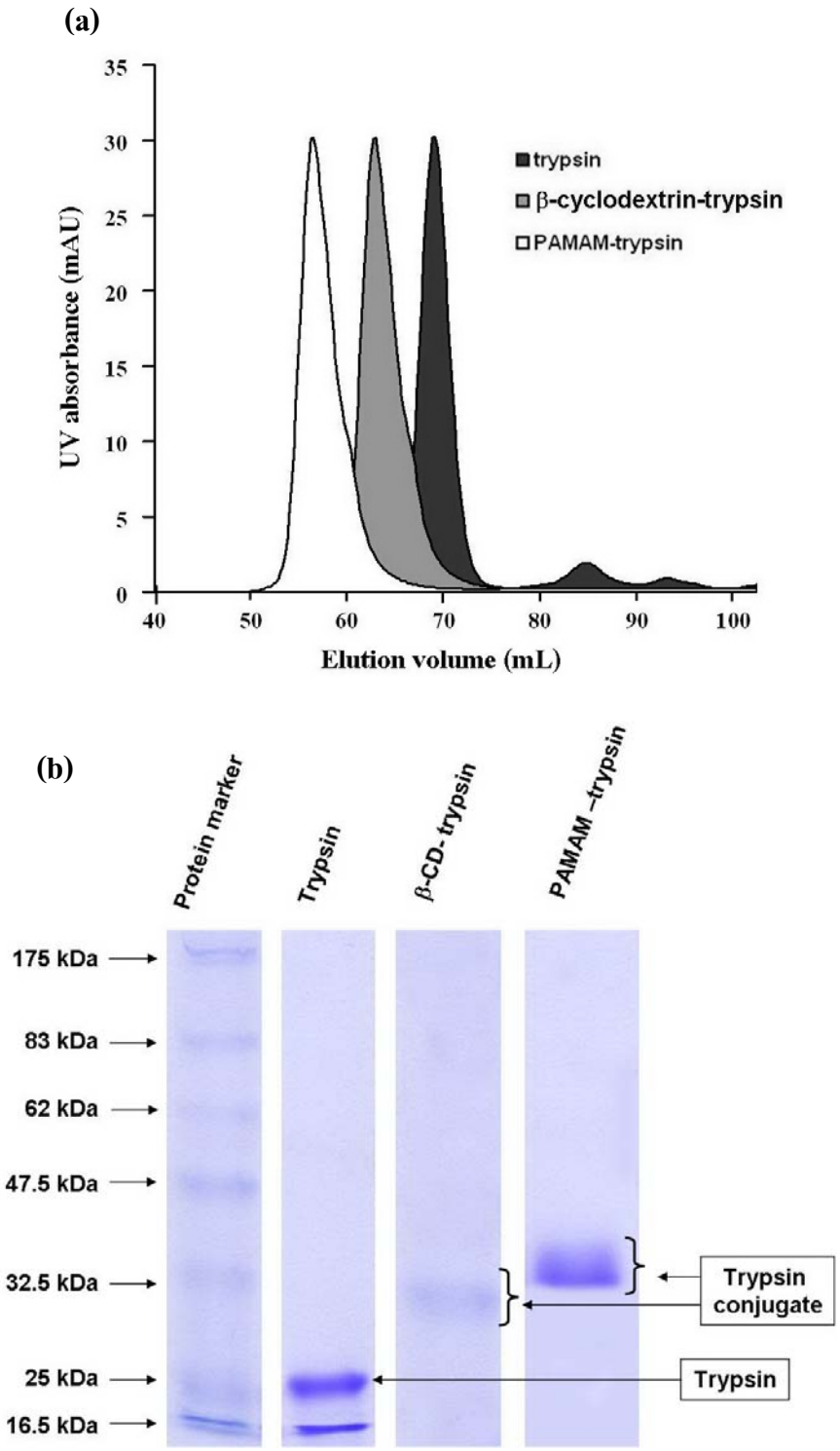


Figure 4.5 Characterization of β-CD- and PAMAM-trypsin conjugate. Panel (a) FPLC chromatogram, and panel (b) SDS-PAGE electrophoresis.

4.4.3 Enzyme activity of β-CD- and PAMAM-trypsin conjugates

V_{max} values can be affected by experimental conditions such as enzyme purity and concentration (Cornish-Bowden, 2004; Samoshina and Samoshin, 2005). For example, the K_m can be influenced by temperature and pH (Cornish-Bowden, 2004). However, the experimental conditions do not greatly affect the K_{cat}. Therefore, here it is possible to compare the K_{cat} values obtained both to those obtained in other studies and also between the conjugates studied in this thesis. The kinetic parameters measured and the percentage residual amidase activity of the polymer-trypsin conjugates and native trypsin were determined are summarized in Table 4.2.

The amidase activity of β-CD- and PAMAM-trypsin conjugate were reduced to 13.9 % and 29.2 %, respectively relative to the native trypsin control (Table 4.2) when performed in 0.1 M Tris-HCl containing 20 mM $CaCl_2$, pH 8.2 at 37 °C and using BAPNA (0.023-0.23 mM) as substrate. The β-CD- and PAMAM-trypsin conjugates showed a similar rate of hydrolysis (mM/min) at 37 °C and they displayed a lower enzyme activity than that seen for native trypsin (Figure 4.6). This result is consistent to PEG conjugates that study previously(Chapter 3).

Table 4.2 Enzyme activity of native trypsin and the β-CD- and PAMAM -trypsin conjugates[†]

Compound	Activity[†] (%)	K_M[††] (mM)	V_{max}[††] (mM/min)	K_{cat}[††] (s^{-1})
Bovine trypsin	100	0.29 ± 0.05	0.00150 ± 0.00019	0.30 ± 0.04
β-CD-trypsin	13.9 ± 0.7	0.15 ± 0.04	0.00037 ± 0.00002	0.04 ± 0.01
PAMAM-trypsin	29.2 ± 1.8	0.26± 0.02	0.00044 ± 0.00003	0.09 ± 0.01

[†]Data shown relate to the trypsin control; mean ± SD (n = 3)

[††]Estimated using the Hanes-Wolfe plot

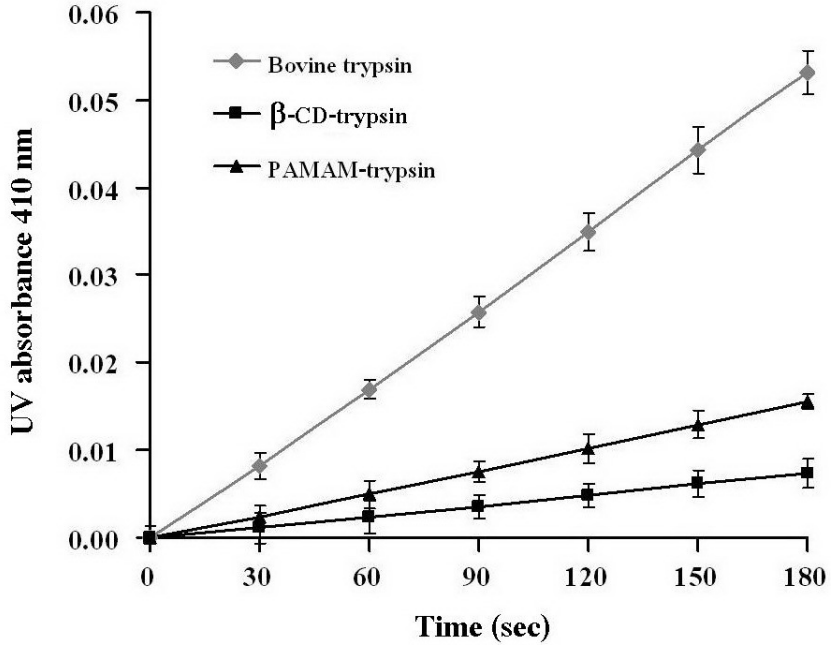


Figure 4.6 Comparison of the enzymatic activity of the β-CD- and PAMAM-trypsin conjugates and native trypsin. In each case 2 mg/mL trypsin equivalent was used (mean ± SD, n=3).

This observation is also similar to the previous studies where a reduction of trypsin activity was found after polymer conjugation (Zhang et al., 1999; Villalonga et al., 2000; Treetharnmathurot et al., 2008; Duncan et al., 2008). For example, the previous report of Villalonga et al. (2000), where enzyme activity was reduced following carboxymethylcellulose (CMC) conjugation. Trypsin from bovine pancreas was modified by the polyaldehyde derivative of CMC via reductive alkylation with $NaBH_4$. The modified trypsin retained 62% and 42% of esterolytic and proteolytic activity, respectively.

However, the results above were in contrast to other reports. Trypsin from bovine pancreas was chemically modified by monosubstituted amino derivative of α-, β- and γ-cyclodextrin. The specific esterolytic activity and the affinity of trypsin for substrate were improved (140-170 %) by the attachment of the cyclodextrin residues (Fernandez et al., 2002; 2003).

In this study, enzyme kinetic analysis made using the Hanes-Woolfe plot showed that the Michaelis constant (K_M) (Table 4.2) decreased after β-CD attachment compared to native

trypsin. The K_M value of the PAMAM-trypsin conjugate was slightly decreased to that seen for native trypsin, suggesting that apparent affinity is altered by polymer conjugation. The PAMAM-trypsin conjugate displayed a reduction in activity compared to trypsin (29.2 %) as described above. This is likely to be due to steric hindrance restricting access of the substrate to the active site.

All conjugates displayed V_{max} values that were ~3-4 fold lower, and the substrate turnover rate K_{cat} decreased ~3-7 fold lower (Table 4.2) than that of native enzyme.

4.4.4 Thermal stability and autolysis study of β-CD- and PAMAM-trypsin conjugates

A progressive decline in activity of the β-CD- and PAMAM-trypsin conjugates and native trypsin occurred with incubation time at all temperatures (30-70 °C) (Figure 4.7a, b, c).

The β-CD-trypsin conjugate was more stable than native trypsin but the PAMAM-trypsin conjugate was less stable than native trypsin at all temperatures (Figure 4.8a). After incubation at different temperature (30-70 °C) for 10 min, it has been found that the PAMAM-trypsin conjugate was inactivated at 50 °C while β-CD-trypsin conjugate and native trypsin were inactivated at 70 °C.

When the thermal resistance observed for trypsin, β-CD-trypsin, PAMAM-trypsin and trypsin-SA-mPEG 5000 conjugates, was expressed as T_{50} the value obtained were 47 °C, 55.5 °C, 44 °C and 51 °C respectively (β-CD-trypsin > trypsin-SA-mPEG 5000 > native bovine trypsin > PAMAM-trypsin conjugate). The T_{50} value for the β-CD-trypsin and trypsin-SA-mPEG 5000 conjugates was 8.5 °C and 6 °C, respectively, higher than native trypsin while the PAMAM-trypsin conjugate was 3 °C lower than seen for native trypsin. In previous reports by Murphy and O'Fagain (1996) described the covalent modification of trypsin with acetic acid *N*-hydroxysuccinimide ethyl ester and reported only an increase of T_{50} of 5 °C was obtained. Similar stabilization was also seen for trypsin modified with carboxymethylcellulose, where the T_{50} increased 7 °C under similar experimental conditions (Villalonga et al., 2000).

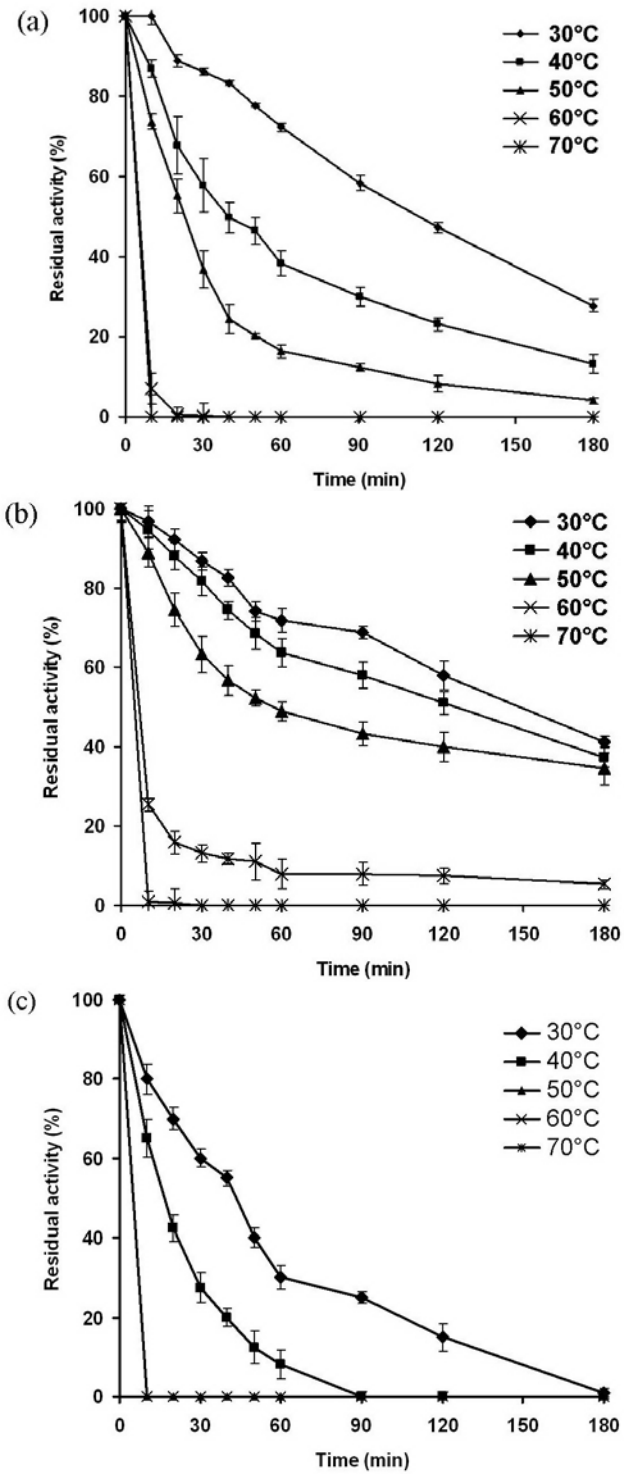
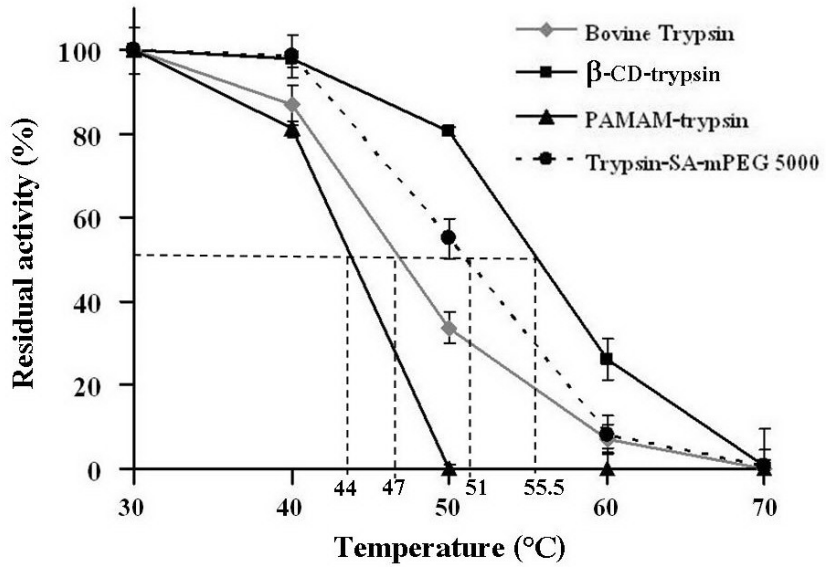


Figure 4.7 Effect of temperatures (30-70 °C) on the activity of (a) native bovine trypsin, (b) β-CD-trypsin conjugates and (c) PAMAM-trypsin conjugates. Activity was measured using BAPNA as a substrate after incubation for 180 min (mean; n = 3).

(a) Thermal stability (10 min incubation)



(b) Autolysis at 40 °C

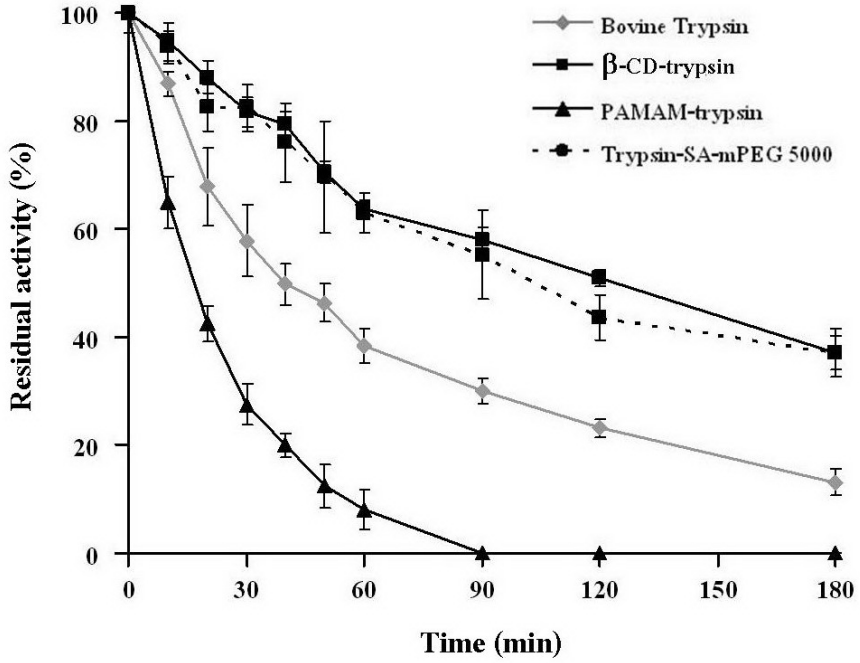


Figure 4.8 Comparison of thermal stability and activity of trypsin, and the β-CD-, PAMAM- and PEG-trypsin conjugates. Activity was measured using BAPNA as a substrate (mean; n = 3).

Fernandez et al. (2002) synthesized the conjugation of trypsin with the mono-6-amino-6-deoxy derivative of α-, β- and γ-CD. The T_{50} of CD-trypsin conjugates increased 14-17.2 °C. These CD conjugates were about 5.5-8.2 fold more resistant to autolytic degradation at pH 9.0 compared to native trypsin (Fernandez et al., 2002). Fernandez et al. (2003) also found a high increased in T_{50} for β-CD-trypsin conjugates of 12.5-14.5 °C. The differences in these results observed might have been due to the fact that Fernandez and coworkers (2003) used monosubstituted amino derivatives of β-CD to modify carboxylic groups in trypsin. However, here the succinoylated β-CD (carboxylic end group) reacts with amino group of trypsin.

While the β-CD-trypsin conjugate showed greater thermal stability than the PAMAM-trypsin conjugate, the lattes was less susceptible to autolysis at 40 °C than native trypsin (Figure 4.8b). The $t_{1/2}$ for autolysis (Table 4.3), all conjugates tested in Chapter 3 and here was in the order: β-CD-trypsin > trypsin-SA-mPEG 5000 > native bovine trypsin > PAMAM-trypsin conjugate.

Table 4.3 Half-life ($t_{1/2}$) of β-CD-, PAMAM- and PEG-trypsin conjugates from autolysis study[†]

Compound	**Half-life (min)**[††]
Trypsin	41 ± 1
β-CD-trypsin	125 ± 3
PAMAM-trypsin	17 ± 1
Trypsin-SA-mPEG 5000	102 ± 1*

[†]Experiments were conducted at 40 °C and the hydrolysis of BAPNA was used to measure residual trypsin activity

[††]Data represent $t_{1/2}$ min (mean ± SD) (n=3)

*Data taken from Treetharnmathurot et al. 2008

The possible structures of the β-CD- and PAMAM-trypsin conjugates are shown in Figure 4.9 and the characteristics of β-CD- and PAMAM-trypsin conjugates were summarized in Table 4.4. The average number of molecule of β-CD and PAMAM dendrimer per 1 molecule of trypsin was 6.7 and 2.6 mol respectively.

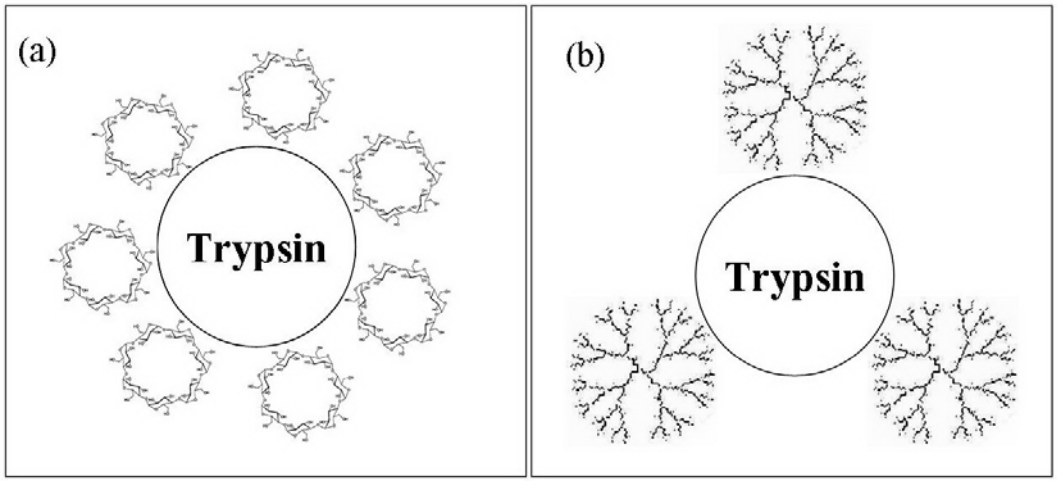


Figure 4.9 Possible structures of (a) β-CD-trypsin and (b) PAMAM-trypsin conjugates

Table 4.4 Comparison between β-CD and PAMAM-OH dendrimer used in this study

	β-CD	**PAMAM-OH**
Molecular weight (g/mol)	1,135[a]	6,941[b]
Shape	truncate	spherical
Number of -OH groups in a polymer	21: 1	32: 1
Degree of carboxyl substitution after succinoylation	3: 1	1: 1
Polymer: trypsin ratio	6.7: 1[†]	2.6: 1[†]

[†]Data calculated from Table 4.1

[a]β-CD (Mw 1,135 g/mol) from Fluka (USA) (http://www.sigmaaldrich.com)

[b]PAMAM-OH dendrimer (Mw 6,941 g/mol) from Aldrich (USA) (http://www.sigmaaldrich.com)

The fact that the β-CD-trypsin conjugate was more stable than PAMAM-trypsin conjugate, suggesting that β-CD enhances protection from autolytic attack due to steric hindrance by wrapping around protein better than PAMAM dendrimer. This characteristic was also suggested for the formation of PEG conjugate of Oupicky et al. (1999) and is supported by the results of PEG-trypsin conjugates in Chapter 3, here it was demonstrated that longer obtained chain with PEGs more increased thermal stability and protected trypsin from autolytic attack probably by wrapping around protein. These polymers could protect and stabilize trypsin by formation of hydrogen-bonding between the hydroxyl groups of the polymers e.g. PEGs (Treetharnmathurot et al, 2008). It has been shown previously that the glycosidic –OH groups of poly(glucose) polymers can create hydrogen-bonding to protect and stabilize proteins (Mislovicova et al., 2006; Treetharnmathurot et al, 2009).

Interestingly, the PAMAM-trypsin conjugates displayed lower thermal stability, and were less stable against autolysis. Moreover, they had a lower $t_{1/2}$ for autolysis than other conjugates and native trypsin (Table 4.3). Trypsin was less achieve after conjugated with PAMAM dendrimer. This might be explained by (i) the lower steric hindrance of PAMAM structure due to both less PAMAM molecules in the conjugate and potentially their more rigid structure, (ii) modification of essential functional groups in the trypsin structure that could interfere with trypsin activity (Oupicky et al., 1999) or (iii) the chemical modification of the trypsin with PAMAM dendrimer causes some degree of aggregation (Kang et al., 2005).

As mentioned before, the size and conformation is known to change for higher generation (> G4) PAMAM dendrimers with pH conditions. Size increases when the solution pH decreases (Maiti et al., 2005). However, in this study it was found that the lower generation (< G4) PAMAM dendrimers was less sensitive to conformational changes with variations in the pH (Chen et al., 2000; El-Sayed et al., 2002). Therefore, it is likely that the conformation or size of PAMAM dendrimer (G3) in this study would not be affected when varying pH of the medium.

4.5 Conclusion

In summary, it was found that the type, size and shape of the polymers used had an important role in activity and stability of conjugated trypsin for further development. β-CD has the potential for protein conjugation as it can stabilize and protect a protein from degradation.

The β-CD- and PAMAM-trypsin conjugates were synthesized and had a trypsin content of 72 wt % and 50 wt %, respectively. The β-CD- and PAMAM-trypsin conjugates showed lower K_M, V_{max} and K_{cat} than native enzyme. The amidase activity of trypsin in β-CD- and PAMAM-trypsin conjugates reduced to 13.9 % and 29.2 %, respectively, compared to the native trypsin. Both native trypsin and the conjugates lost activity with the time of incubation at temperatures 30-70 ºC. All conjugates here and trypsin-SA-mPEG 5000 taken from Chapter 3 displayed their thermal stability in order to: β-CD-trypsin > trypsin-SA-mPEG 5000 > native bovine trypsin > PAMAM-trypsin conjugate. The β-CD-trypsin conjugate displayed higher thermal stability, greatest $t_{1/2}$ (125 ± 3) and was also more stable against autolysis.

In Chapter 5, biodegradable polysaccharides (e.g. dextrin) or branched polymer (e.g. ST-HPMA homopolymer) are interesting for protein conjugation and they were chosen to modify trypsin. Their trypsin activity and thermal stability were determined and compared to linear polymer (e.g. PEG) and hyperbranched polymer (e.g. β-CD and PAMAM (-OH) dendrimer).

CHAPTER 5

DEXTRIN-TRYPSIN AND ST-HPMA-TRYPSIN CONJUGATES: ENZYME ACTIVITY, AUTOLYSIS AND THERMAL STABILITY

5.1 Introduction

The effect of conjugate of linear PEGs (different molecular weight and with different linking chemistries) and polymers (having same 3D architecture of β-CD and PAMAM (-OH) dendrimer) on the activity and thermal stability of trypsin has already been reported in Chapters 3 and 4, respectively. In the next two Chapters, this was a more towards polymers, dextrin and a ST-HPMA that were of particular interest to develop further as therapeutic conjugates. In Chapter 6 the synthesis and biological properties of RNase conjugates of these polymers is described. Here, the main aim of this study was again to examine the effect of polymer (for dextrin also molecular weight) on protein activity, autolysis and thermal stability again using trypsin as a model. Dextrin-protein (Duncan et al., 2008; Hardwicke et al., 2008; Ferguson and Duncan, 2009) and ST-HPMA-protein conjugates (Ulbrich et al. 2000) were proposed as potential new therapeutics. They have been synthesized and studied only in protein activity. However, their autolysis and thermal stability studies have never been studied. Here, the dextrin- and ST-HPMA-trypsin conjugates were synthesized by the coupling reaction using conventional conjugation chemistry. The polymers used were dextrin of low molecular weight (~ 8,100 g/mol) and high molecular weight (~ 61,000 g/mol) and ST-HPMA homopolymer of molecular weight ~10,100 g/mol.

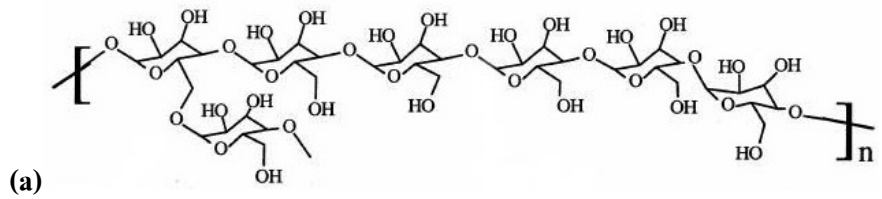
5.1.1 Dextrin as a potential protein carrier

Dextrin (α-1,4 poly(glucose) (Figure 5.1a) was selected as the model polymer for conjugation. It has been approved by FDA for use as a peritoneal dialysis solution in the trade

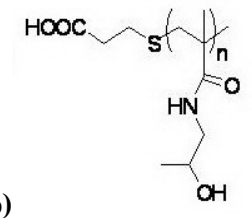
name of Icodextrin® (Mistry and Gokal, 1994). Dextrin was used to generate novel bioresponsive protein conjugates for the PUMPT concept (Duncan et al., 2008) as previously described in Chapter 1, section 1.2.5.2. PUMPT is generally applicable to protein and peptide therapeutics having various kinds of pharmacological targets, e.g. enzymes, cytokines, growth factors and antibodies. The recent studies have successfully applied the dextrin-α-amylase strategy for the delivery of rhEGF for use in a polymer therapeutic design to promote wound healing (Hardwicke et al., 2008), and for PLA_2 as an anticancer agent (Ferguson and Duncan, 2009).

5.1.2 HPMA as a potential protein carrier

A significant number of HPMA copolymer-anticancer drug conjugates progressed into clinical trial (Duncan, 2006). More recently, these non-biodegradable, synthetic polymers have also been used to create protein conjugates using either a multifunctional HPMA copolymer intermediate, or single point attachment using ST-HPMA (Duncan, 2005; Oupicky et al., 1999; Ulbrich et al., 2000). This work has been described earlier (Chapter 1, section 1.2.5.4). ST-HPMA-COOH of molecular weight 11,000 g/mol (Figure 5.1b) was selected for protein conjugation here to comparison with the natural biodegradable polymer, dextrin of molecular weights 8,100 and 61,000 g/mol and the PEGs, β-CD and PAMAM dendrimer studied earlier.



(a)



(b)

Figure 5.1 Structure of (a) dextrin and (b) ST-HPMA-COOH

5.1.3 Polymer functionalization methods used

In the past many methods have been used to functionalize polysaccharide in order to create an active intermediate suitable for conjugation to proteins (Figure 5.2). Polysaccharide activation could be achieved by: (a) *periodate oxidation* for functionalization of pullulan, dextran and chitosans (Bruneel and Schacht, 1993a; Cheung et al., 2005; Vold and Christensen, 2005), (b) *cyanogen bromide activation* (Kost and Goldbart,1997; Schnaar et al., 1977), (c) *chloroformate activation* using 4-nitrophenyl chloroformate to modify pullulan (Bruneel and Schacht, 1993b), (d) *succinoylation* using succinic anhydride to incorporate ester groups of pullulan and dextran (Bruneel and Schacht, 1994; Groff et al., 1982), and (e) *monotosylation* which is considered one of the most effective reactions to activate one hydroxyl group of cyclodextrin (Fernandez et al., 2003).

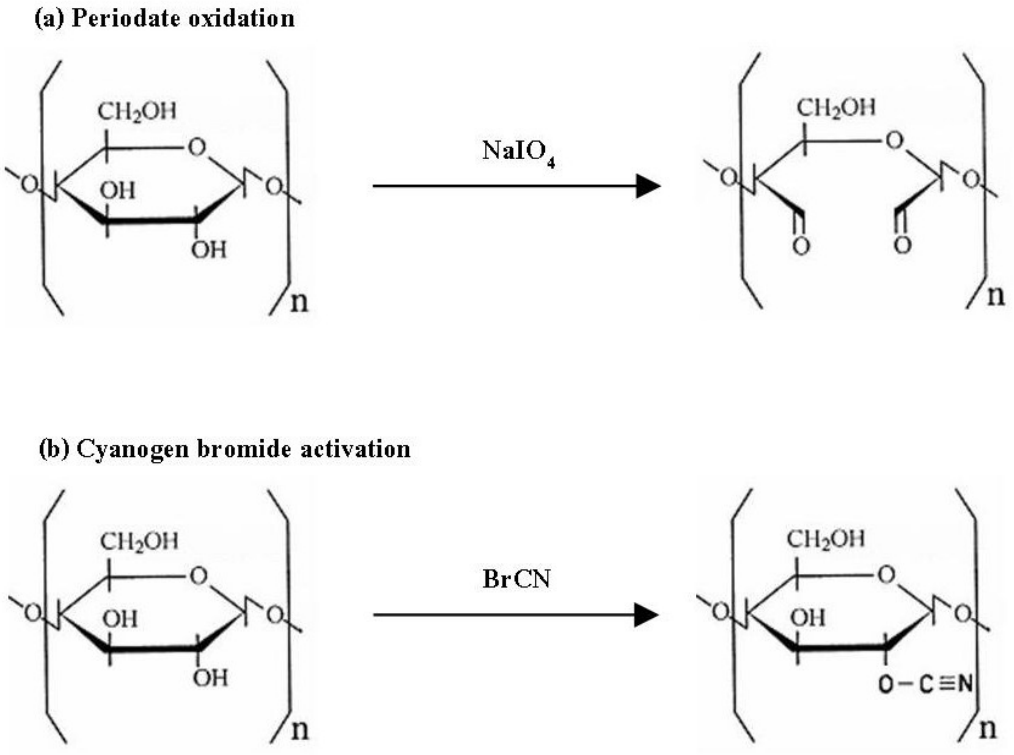


Figure 5.2 Methods used for polysaccharide functionalization. Panel (a) periodate oxidation, (b) cyanogen bromide activation, (c) chloroformate activation, (d) succinoylation, and (e) tosylation.

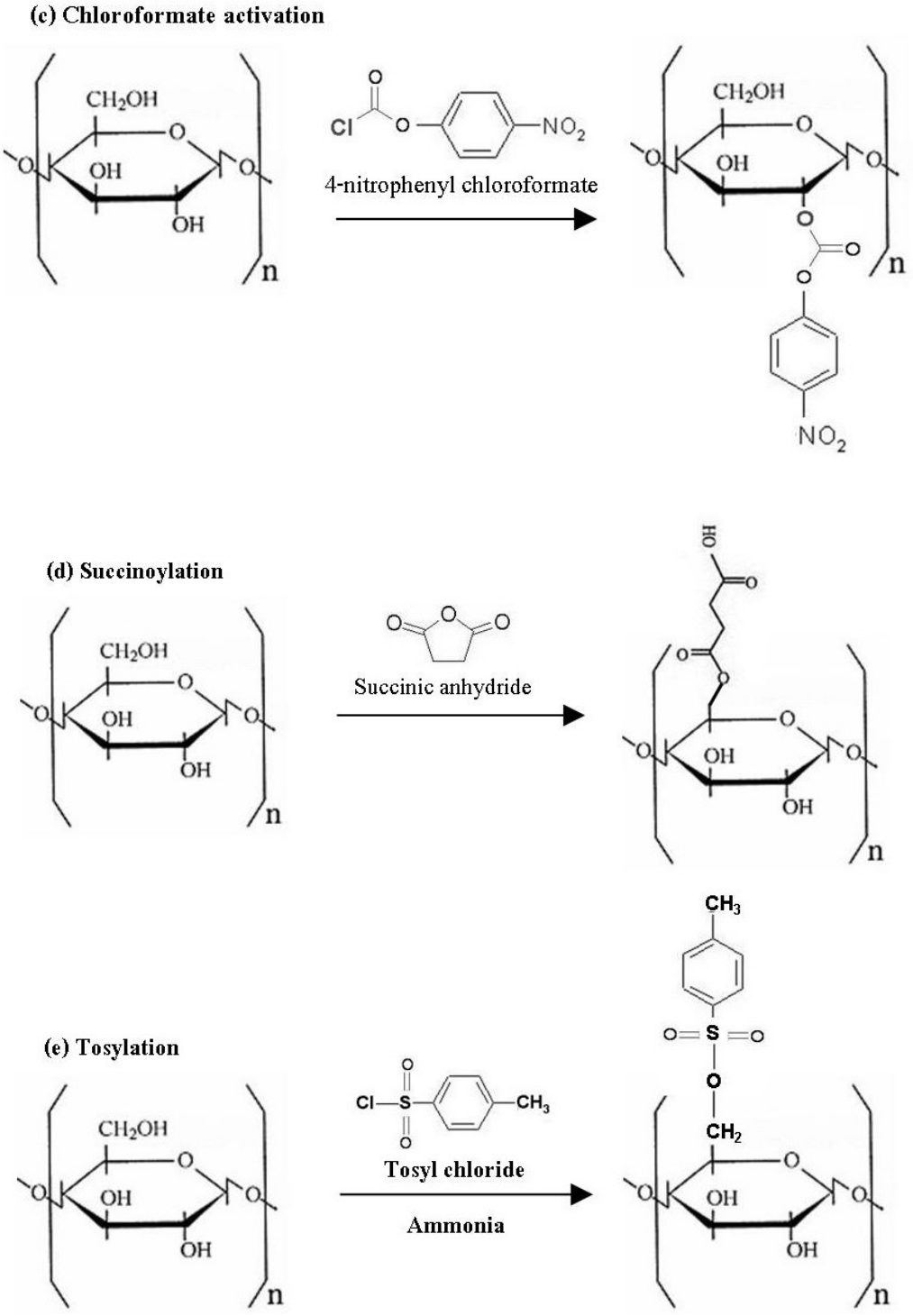


Figure 5.2 Contd. Methods used for polysaccharide functionalization. Panels (a) periodate oxidation, (b) cyanogen bromide activation, (c) chloroformate activation, (d) succinoylation, and (e) tosylation.

The polymer used for protein conjugation should be functionalized via a hydroxyl, amine or carboxyl group which present on the polymer before attachment to the protein. The most popular site for modification is the ε-amino group on a lysine residue (LYS) or the *N*-terminal (α-*N*) amino acid groups (Harris and Chess, 2003; Veronese and Morpurgo, 1999). The ε-amino group on LYS and α-*N* terminus can react efficiently with a number of functional groups, including aldehyde (Figure 5.3a) and activated carboxylic acids (Figure 5.3b). The reaction of LYS and/or the α-N terminus with aldehydes results in the reversible formation of a Schiff base (imine), which can be further reduced with $NaCNBH_3$ to form a secondary amine linker. The main disadvantage of this method is a two-step procedure requirement. Moreover, aldehyde modification method is often even more challenging than that of carboxylic-acid-terminated polymers (Thordarson et al., 2006).

Numerous methods can be used to activate carboxylic acids for the reaction with α-*N* terminus of LYS. These include *N*-hydroxysuccinimide (NHS) activation which is often the method of choice (Veronese and Pasut, 2005). Using NHS the activation of a carboxylic acid terminated polymer is uncomplicated, but the selectivity of the reaction yield is quite low (Roberts et al., 2002). Site-specific protein conjugation methods for a 1:1 ratio of polymer: protein have been developed to avoid random conjugation and to produce a well-defined products (Thordarson et al., 2006; Roberts et al., 2002).

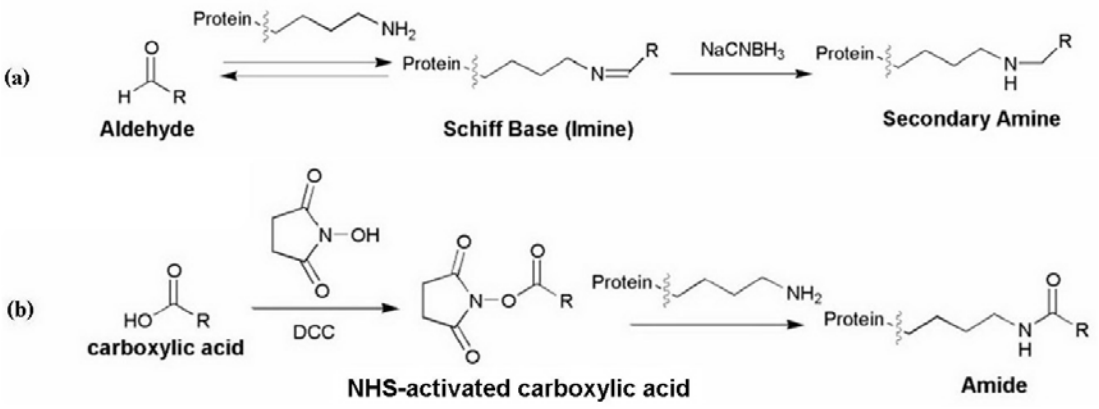


Figure 5.3 Lysine modifying reactions. Panel (a) aldehyde modification and (b) carboxylic acid modification (from Thordarson et al., 2006).

In many cases this is advantage. However, a multifunctional succinoylated dextrin (activated by NHS) has been widely used for protein conjugation. In first studies dextrin-trypsin and dextrin-melanocyte stimulating hormone conjugates were synthesized and use to study α-amylase activation in the context of the new approach called **P**olymer-masking-**U**n**M**asking-**P**rotein **T**herapy (PUMPT) (Duncan et al., 2008). Dextrin-rhEGF conjugates for wound repair (Hardwicke et al., 2008), and dextrin-phospholipase A_2 for cancer therapy (Ferguson and Duncan, 2009) were also synthesized using this method.

In this study, succinoylation was chosen for dextrin functionalization because (i) it introduces a carboxylic group enable for conjugation to amino groups in protein, (ii) uses relatively non-toxic reagent, (iii) the reaction condition can be easily reproduced (Hreczuk-Hirst et al., 2001b) and (iv) as mentioned above it is being used widely to create therapeutic conjugates. Conjugation was achieved using EDC followed by sulfo-NHS. The linker obtained from this reaction is relatively stable (Bruneel and Schacht, 1994; Groff et al., 1982). The ST-HPMA-COOH could be directly conjugated to trypsin, again via EDC and sulfo-NHS activation as described below.

5.1.4 The specific technical aims of this study

Specifically the aims of this study were:

(1) To synthesize and characterize dextrin I-, dextrin II- and ST-HPMA-trypsin conjugates

(2) To determine trypsin activity of dextrin I-, dextrin II- and ST-HPMA-trypsin conjugates compared to native trypsin using BAPNA as a substrate.

(3) To study trypsin structure before and after polymer conjugation using circular dichroism spectroscopy

(4) To calculate the kinetic parameters (K_M, V_{max} and K_{cat}) and compare to native trypsin and PEG-, β-CD and PAMAM-trypsin conjugates described in Chapters 3 and 4.

(5) To investigate thermal stability of trypsin of the conjugates compare to native trypsin and PEG-, β-CD and PAMAM-trypsin conjugates described in Chapters 3 and 4.

5.2 Materials

All chemicals used were of analytical grade and a detailed list of the specific equipment used and compounds used, the general reagents and their suppliers were already given in Chapter 2 (section 2.2). The specific reagent and compounds used in this work were as follow:

Dextrin, type I from corn starch (~8,100 g/mol) was from Sigma (UK) and dextrin II (~61,000 g/mol) and semitelechelic HPMA copolymer (~10,100 g/mol) were donated by the Centre for Polymer Therapeutics, Cardiff University. Trypsin from porcine pancreas (type 1X-S, lyophilized powder), and BAPNA were purchased from Sigma (UK).

The equipment used for conjugate analysis, purification and characterization have been previously described in Chapter 2, section 2.1.2.

5.3 Methods

5.3.1 Succinoylation of dextrin (Figure 5.4)

Dextrin I (molecular weight 8,100 g/mol) (1 g) was dissolved in DMF (10 mL), and succinic anhydride (91.3 mg) was added followed by DMAP (40 mg) as catalyst. The reaction was purged with nitrogen, sealed and left to stir for 14 h at 50 °C, under nitrogen. The reaction mixture was poured into vigorously stirred diethyl ether (1 L) and stirring continued for another 10 h. The ether was removed by filtration under vacuum and the remaining solid was redissolved in a minimum amount of distilled water. The resulting solution was poured into a Spectra/por regenerated cellulose dialysis membrane (MWCO 2 kDa) and dialyzed against double distilled water (6 x 3 L) for 48 h. The resulting solution was freeze-dried using a Flexi Dry FD-1.540 freeze dryer (FTS systems, USA) to yield succinoylation dextrin I (615.6 mg) which obtained as a white powder. The acid groups were quantified by titration with standard solution 5.125×10^{-5} M NaOH using 1% bromophenol blue as indicator. The product was also characterized by FT-IR and GPC (Hreczuk-Hirst et al, 2001).

Succinoylated dextrin II was prepared similarly but using dextrin II (molecular weight 61,000 g/mol) (1 g) in DMF (10 mL) and succinic anhydride (182.5 mg) with DMAP (80 mg). Purification was performed using a Spectra/por regenerated cellulose dialysis membrane (MWCO of 10,000 g/mol). The product was obtained as a white powder (775.1 mg).

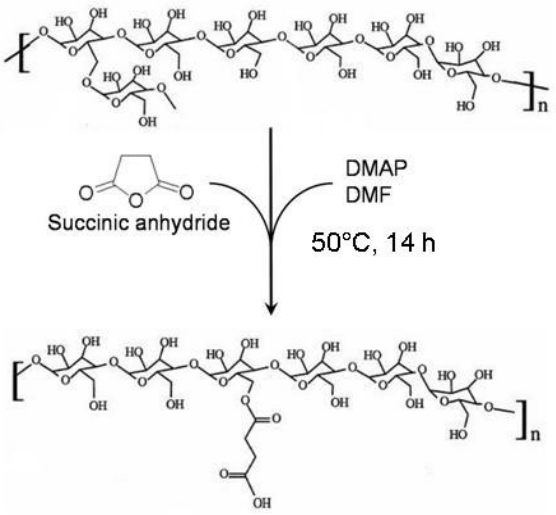


Figure 5.4 Reaction scheme for synthesis of succinoylated dextrin

5.3.2 Synthesis of dextrin-trypsin and ST-HPMA-trypsin conjugates

5.3.2.1 Synthesis of dextrin I-trypsin conjugate

The succinoylated dextrin I (101.5 mg) was dissolved in distilled water (2 mL). EDC (15.9 mg) was added and the reaction mixture was stirred for 10 min. Then sulfo-NHS (16.8 mg) was added and the reaction mixture was stirred for a further 45 min. Trypsin (49.2 mg) was added to the mixture and the pH was adjusted to pH ~ 8 by dropwise addition of 5.125 x 10^{-5} M NaOH, and the reaction mixture left stirring for 18 h at room temperature (Figure 5.5). The product was obtained as a white powder (34.5 mg). The conjugate was purified by FPLC. The collected fractions were desalted using a vivaspin 6 centrifugal filter of MWCO 30,000 g/mol) before freeze-drying. The conjugate was characterized by SDS PAGE electrophoresis, GPC, and FPLC. The Bradford protein assay (Bradford, 1976) was used to estimate the total protein content with BSA standards.

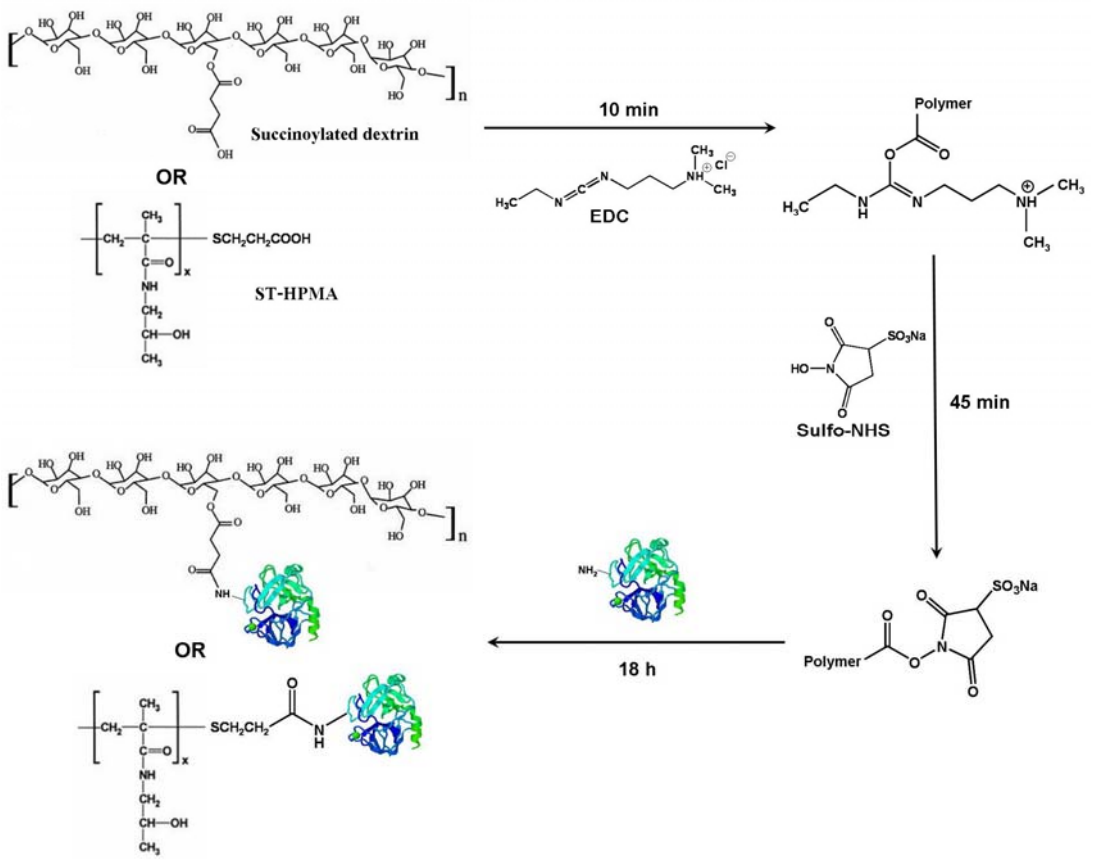


Figure 5.5 Reaction scheme for synthesis of dextrin-trypsin and ST-HPMA-trypsin conjugates

5.3.2.2 Synthesis of dextrin II-trypsin conjugate

In this case succinoylated dextrin II (154.8 mg) in distilled water (2 mL) was used. The reaction was conducted as described above, but in this case using EDC (21.7 mg), sulfo-NHS (22.4 mg) and trypsin (41.2 mg) (Figure 5.5). The product was obtained as a white powder (30.0 mg). All the other reaction conditions, purification and characterization were similar to the procedure described above.

5.3.2.3 Synthesis of ST-HPMA-trypsin conjugate

ST-HPMA-COOH (203.9 mg) which prepared by Lucile Dieudonné was dissolved in distilled water (2 mL). EDC (21.6 mg) was added and the reaction mixture was stirred for 10 min. Sulfo-NHS (24.8 mg) was then added and the mixture was stirred for a further

45 min, after that trypsin (42.2 mg) was then added (Figure 5.5). The product was obtained as a white powder (178.1 mg). Further reaction, purification and characterization process were conducted as described above.

5.3.3 Purification and characterization of dextrin- and ST-HPMA-trypsin conjugates

Succinoylated dextrin I and succinoylated dextrin II were characterized by titration (section 2.2.2.1) and FT-IR (section 2.2.2.2). The synthesized polymer-trypsin conjugates were purified by one or more of the following methods: Dialysis (section 2.2.1.1), FPLC fractionation (section 2.2.1.2) and Vivaspin 6 centrifugal filters (section 2.2.1.3). The final products were obtained by freeze-drying process (section 2.2.1.4).

Free and total protein content of the dextrin I-, dextrin II- and ST-HPMA-trypsin conjugates were determined. In order to identify the presence of free protein in the conjugate, samples were analyzed using SDS-PAGE (section 2.2.3.3) and the total protein content of the conjugates was determined using Bradford assay as described in Chapter 2 (section 2.2.3.2).

Their purity and molecular weights of the obtained products were analyzed as previously described in Chapter 2, section 2.2.1.2. Moreover, visual analysis of the chromatogram was carried out to determine the presence of free protein in the polymer-protein conjugate, to estimate the molecular weight of the polymer-protein conjugates and also to identify the presence of any degradation products as described in Chapter 2, section 2.2.3.4. GPC was also used to estimate their molecular weights and to determine their polydispersity. The method used has already been described in Chapter 2, section 2.2.2.3.

5.3.4 Determination of trypsin amidase activity

The release of *p*-nitroaniline (PNA) was measured at 410 nm (at 37 °C for 5 min) using a Cary1G UV-Vis spectrophotometer (Varian, Australia) to determine the amidase

activity of native and trypsin conjugates using BAPNA as a substrate according to Chapter 4, section 4.3.4. The kinetic parameters K_M, V_{max} and K_{cat} were derived from the raw data of trypsin activity against a range of substrate concentrations (0.023-0.23 mM) using a Hanes-Woolfe plot.

5.3.5 Evaluation of thermal stability and autolysis of dextrin- and ST-HPMA-trypsin conjugates

Thermal stability and autolysis were assessed as previously described in Chapter 4, sections 4.3.5 and 4.3.6 respectively. Then the residual enzyme activity was assayed using BAPNA (0.23 mM) as described above.

5.4 Results and Discussion

5.4.1 Synthesis and characterization of dextrin- and ST-HPMA-trypsin conjugates

Reactive polymeric intermediates were successfully prepared. The ST-HPMA with a carboxylic acid end-group was synthesized by Lucile Dieudonné using 3-mercaptopropionic acid (MPA) as the chain transfer agent (Dieudonne, 2008). The reaction yield was ~70%. FT-IR, ^{1}H-NMR and ^{13}C-NMR, and GPC confirmed the absence of impurities (Data not shown). GPC indicated that ST-HPMA had a Mw = 10,100 g/mol, Mn = 7,000 g/mol and a polydispersity of 1.50.

In the case of the succinoylated dextrin, FT-IR analysis of the polymers was performed using an AVATAR OMNI-sampler 360 FT-IR from Nicolet Instrument Corporation (USA) with EZ OMNIC E.S.P. 5.2 software for data analysis. FT-IR also confirmed the presence of the ester peak characteristic at 1720 cm^{-1} in both the succinoylated dextrin I and dextrin II intermediates (Figure 5.6a). ^{1}H-NMR qualitatively confirmed the incorporation of succinoyl groups (Figure 5.6b). These were indicated by peaks at 2.6 – 2.8 ppm.

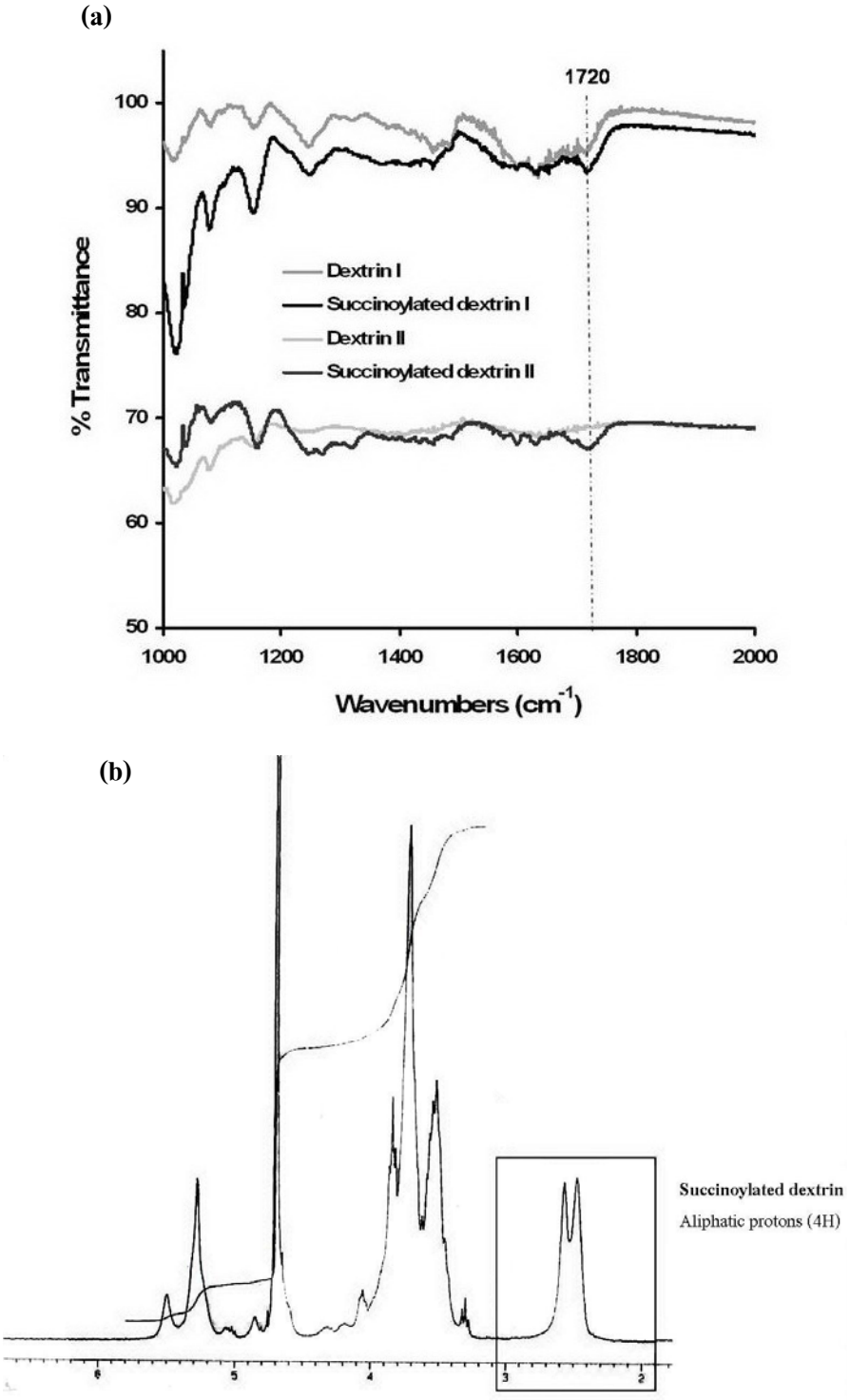


Figure 5.6 Characteristics of dextrin and succinoylated dextrin. Panel (a) FT-IR of dextrin and succinoylated dextrin and panel (b) ^{1}H NMR (D_2O) spectrum of succinoylated dextrin.

On GPC, the succinoylated dextrin eluted faster than dextrin (Figure 5.7). This is almost certainly due to the increased radius of the extended COOH-containing polymer chain compared to parent dextrin rather than a significantly increased polymer molecular weight after succinoylation.

Semi-telechelic polymers such as PEG and ST-HPMA form a single covalent attachment after conjugation bringing advantages of minimal protein cross-linking and improving product homogeneity (Lu et al., 1998). In this study, a molar ratio of polymer : trypsin of 1:1 and gentle reaction conditions were chosen with hope of generating conjugates containing one trypsin per polymer chain. Although the multifunctional dextrin conjugates would probably have more than one polymer attachment sites per trypsin molecule, this is desirable in the context of PUMPT where the aim is to envelope the protein prior to conjugate activation.

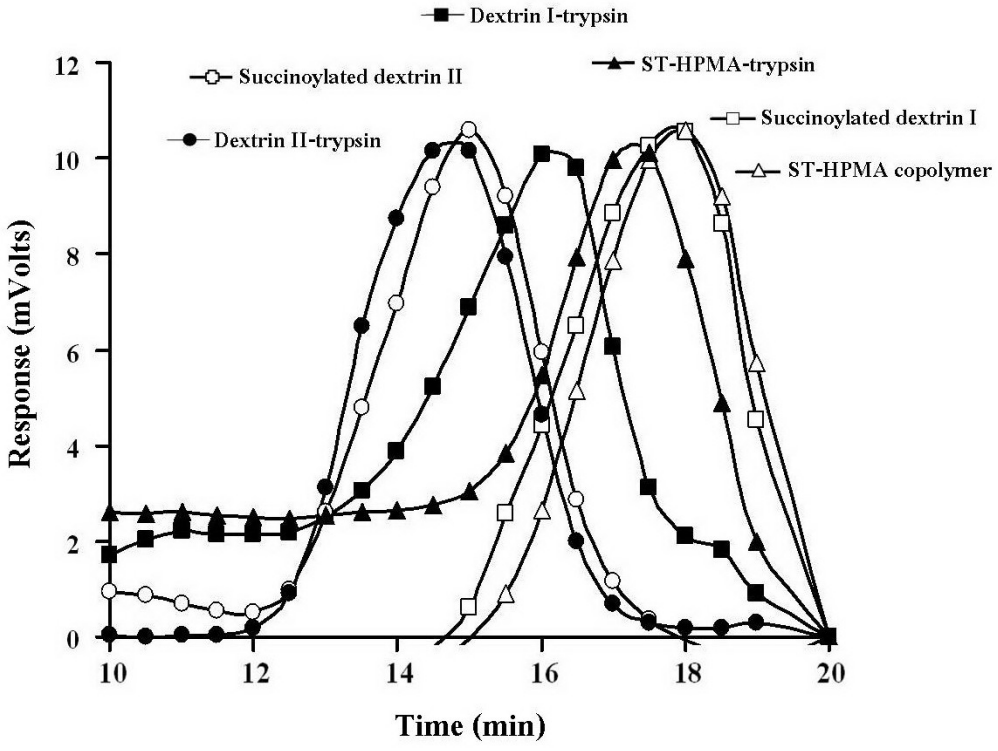


Figure 5.7 GPC elution of succinoylated dextrins, ST-HPMA homopolymer and their trypsin conjugates.

Titration result of succinoylated dextrins revealed a degree of modification of 8.8 mol% and 16.7 mol% respectively (Table 5.1). The Bradford assay was chosen to estimate total protein content using BSA as standard using using a Sunrise UV-vis absorbance plate reader (Tecan, Austria). The resultant dextrin I-, dextrin II- and ST-HPMA-trypsin conjugates contained 54, 17 and only 3% by weight of total proteins, respectively (Table 5.1).

All the conjugates synthesized here were heterogeneous (from the polydispersity data of the polymeric intermediates in Table 5.1). Although the GPC characterization was not quantitative, the estimated molecular weight and the trypsin content of the dextrin conjugates (Table 5.1) suggested a ~1:1 or 1:2 polymer chains to protein content.

Table 5.1 Characteristics of dextrins, ST-HPMA homopolymer and polymer-trypsin conjugates

Compound	**Acid groups (mol%)**	**Protein content (%)**	**Mw**[†]	**Mn**[†]	**Polydispersity (Mw/Mn)**
Trypsin[††]	-	-	23,475[††]	-	-
Dextrin I	-	-	8,100	5,200	1.6
Succinoylated dextrin I	8.8	-	11,200	7,000	1.6
Dextrin I-trypsin	-	54	40,400	22,000	1.9
Dextrin II	-	-	61,000	36,000	1.6
Succinoylated dextrin II	16.7	-	73,800	40,000	1.9
Dextrin II-trypsin	-	17	87,400	51,000	1.7
ST-HPMA copolymer	-	-	10,100	7,000	1.5
ST-HPMA-trypsin	-	3	17,500	14,200	1.2

[†]Estimated using GPC and pullulan standards

[††]Porcine pancreatic trypsin, 223 amino acids (from http://www.expasy.ch/tools/protparam.html)

GPC (Table 5.1) suggested that the ST-HPMA-trypsin conjugate apparently had lower molecular weight than native trypsin. The recent studies have been using small-angle neutron scattering (SANS) for characterization (data not shown) have suggested that the polymer wraps closely around the compact protein structure but it is still surprising that the hydrodynamic of the conjugate volume on GPC showed be apparently smaller.

The molecular weight of polymer-trypsin conjugates are most difficult to estimate due to the difference in conformation of polymer standards in solution. Pullulan was chosen as the most appropriate GPC standard in this study because it is a typical polysaccharide which similar to the structure expected of dextrin and could display a random coil in solution. The globular structure of proteins makes them unsuitable to estimate the molecular weight of polysaccharides, but of course would be most appropriate to analyze trypsin (Gilbert, 2007). However, FPLC chromatogram using protein standards clearly showed that the ST-HPMA-trypsin conjugates had a molecular weight that was higher than native trypsin (Fig 5.8a). During synthesis of the trypsin conjugates, a 1:1 molar ratio of polymer: protein was used. FPLC was proved as a useful tool to separate conjugate from free trypsin using an ÄKTA FPLC system (Amersham Pharmacia Biotech, UK) with a Superdex HR 10/30 SEC column (Fig 5.8a). Dextrin II-trypsin conjugate contained no detectable free trypsin (on SDS-PAGE), whereas, the dextrin I- and ST-HPMA-trypsin conjugates contained 3.65 % and 2.3 % free trypsin, respectively (Fig 5.8b).

When the products were analyzed by SDS-PAGE gels, it was found that the conjugates showed broad polydispersity compared to that of molecular weight markers and trypsin. Moreover, as would have been expected the dextrin conjugates had a higher polydispersity of (Mw/Mn = 1.6 -1.9) (Table 5.1) than the ST-HPMA-trypsin conjugate. The latter displayed a relatively low molecular weight (perhaps due to the low trypsin loading) and very low polydispersity of this polymer (Mw/Mn = 1.2). In fact, the polydispersity of this conjugate was lower than seen for the parent polymer (Mw/Mn = 1.5) suggesting either preferential conjugation of lower molecular weight polymer chains or an unusual conformation of the conjugate (polymer wrapped around the protein).

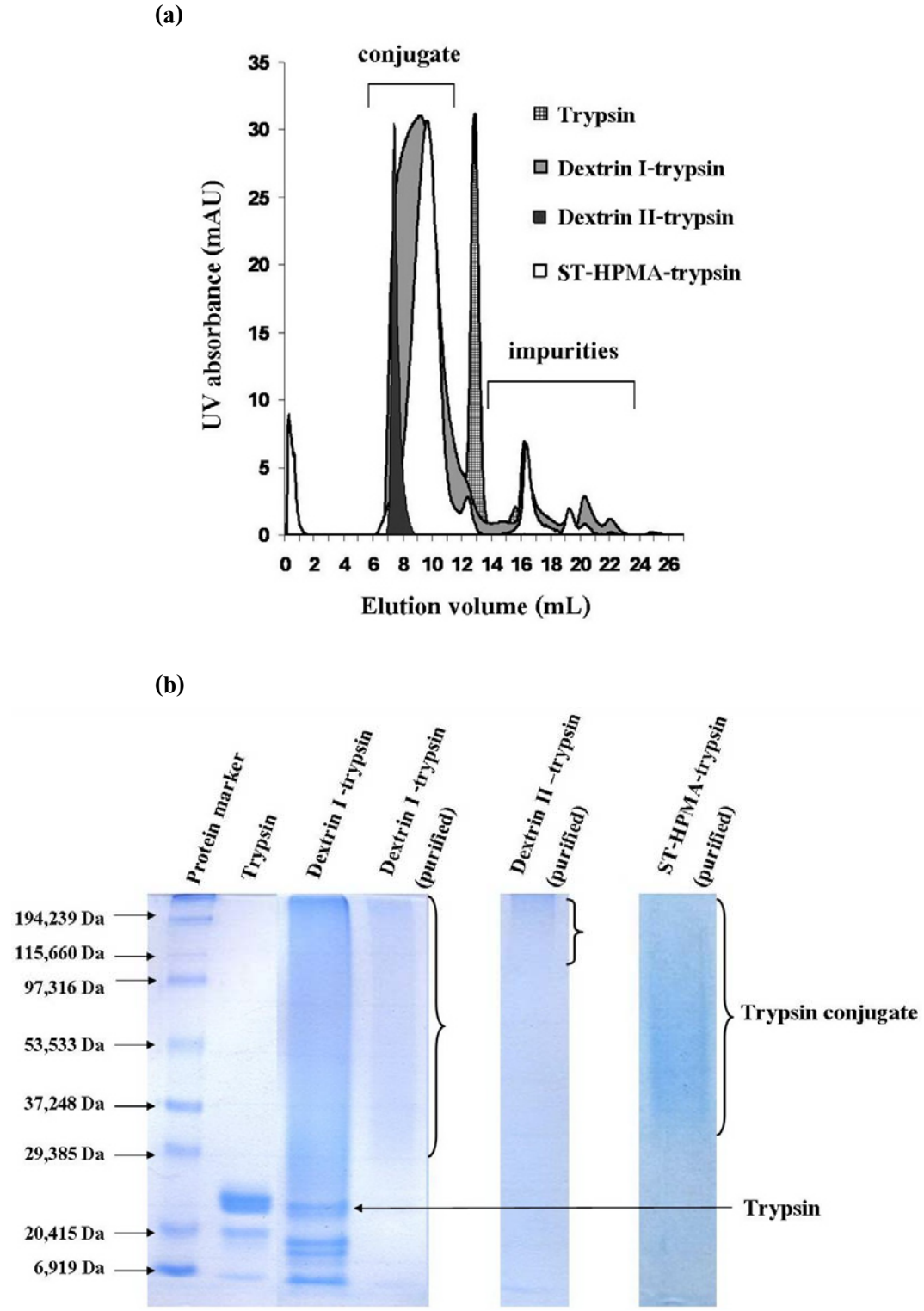


Figure 5.8 Characterization of dextrin I-, dextrin II- and ST-HPMA-trypsin conjugates. Panel (a) FPLC of trypsin and conjugates and panel (b) SDS-PAGE of trypsin and conjugates.

Circular dichroism spectroscopy was used to study the protein structure before and after conjugation. Dextrin II and succinoylated dextrin II showed a completely unstructured conformation, whereas the spectra obtained for trypsin and dextrin II-trypsin conjugate showed a negative minimum at 208 nm (Fig 5.9). The spectrum of the dextrin II-trypsin conjugate was very similar to that observed for trypsin, but it had a slightly decreased amplitude of the 208 nm. These results indicated that the dextrin conjugation did not have any significantly effect on trypsin structure.

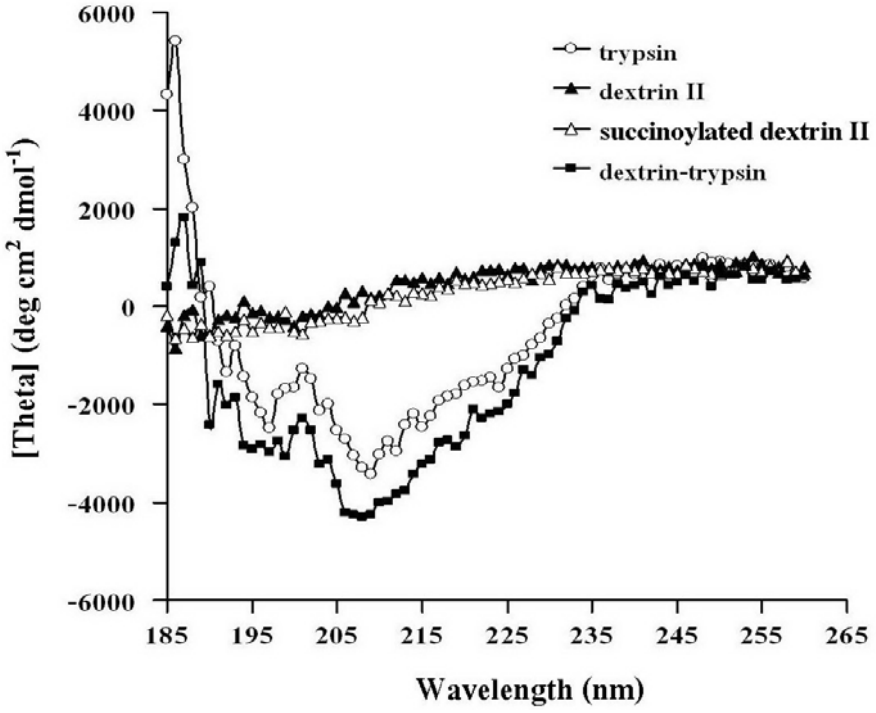


Figure 5.9 Circular dichroism spectroscopy of dextrin II, trypsin, succinoylated dextrin II and the dextrin II-trypsin conjugate.

5.4.2 Enzyme activity of dextrin- and ST-HPMA-trypsin conjugates

When experiments were conducted at a single enzyme substrate/enzyme concentration, all three conjugates showed a similar decreased in trypsin activity (13.8 - 18.6 %) relative to the trypsin control (Figure 5.10; Table 5.2).

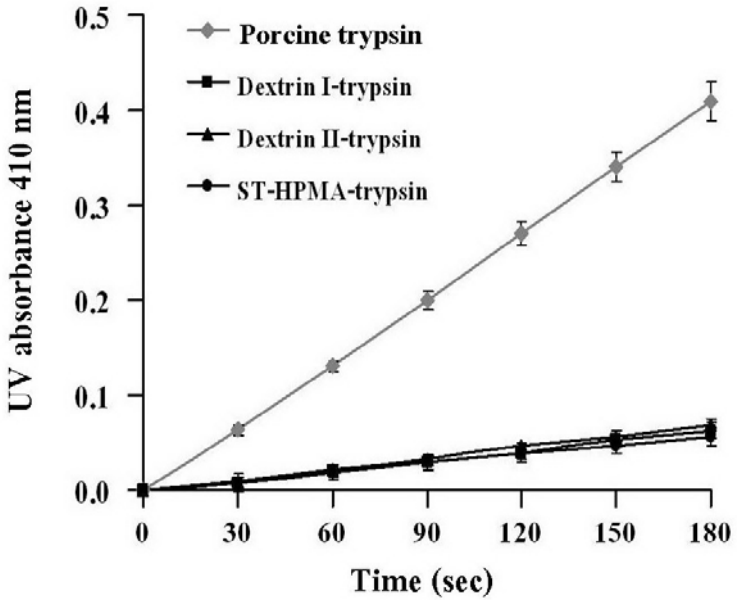


Figure 5.10 Comparison of the enzymatic activity of native trypsin and the dextrin I-, dextrin II- and ST-HPMA-trypsin conjugates. In each case 2 mg/mL trypsin equivalent was used (mean ± SD, n=3).

Table 5.2 Enzyme activity and kinetic parameters of native trypsin, dextrin I-trypsin, dextrin II-trypsin and ST-HPMA-trypsin conjugates

Compound	Activity† (%)	K_M †† (mM)	V_{max} †† (mM min^{-1})	K_{cat} †† (s^{-1})
Porcine trypsin	100	0.122 ± 0.031	0.0228 ± 0.0041	4.56 ± 0.83
Dextrin I-trypsin	15.1 ± 0.2	0.131 ± 0.005	0.0037 ± 0.0001	0.69 ± 0.01
Dextrin II-trypsin	18.6 ± 3.8	0.105 ± 0.062	0.0037 ± 0.0007	0.85 ± 0.17
ST-HPMA-trypsin	13.8 ± 1.6	0.136 ± 0.021	0.0033 ± 0.0004	0.63 ± 0.07

†Data shown relate to the trypsin control; mean ± SD (n = 3)

††Estimated using the Hanes-Wolfe plot.

This was consistent with the reduction in trypsin activity reported by others following polymer conjugation (Zhang et al., 1999; Villalonga et al., 2000; Treetharnmathurot et al., 2008) and as mentioned earlier it is usually attributed to modification of functional groups close to the active site resulting in steric hindrance, and/or refolding of the protein chain upon polymer attachment (Oupicky et al., 1999). Recent studies with dextrin-protein conjugates have confirmed that incubation with α-amylase results in protein unmasking be generating 20-100 % of the original activity, e.g. for dextrin-trypsin, dextrin-melanocyte stimulating hormone (MSH), dextrin-recombinant human epidermal growth factor (rhEGF), and dextrin-phospholipase A_2 conjugates (Duncan et al., 2008; Hardwicke et al., 2008; Ferguson and Duncan, 2009).

In the case of dextrin-rhEGF conjugate, succinoylated dextrin (~85,000 g/mol; 19 mol% succinoylation) was conjugated with rhEGF. The conjugate synthesized contained ~16%wt rhEGF and <1% free protein. It exhibited increased stability towards proteolytic degradation by trypsin and the clinically relevant enzyme neutrophil elastase. The dextrin component was degraded on addition of α-amylase leading to sustained release of free rhEGF over time (52.7% release after 168 h). Dextrin conjugation reduced rhEGF bioactivity, however, exposure to physiological concentrations of α-amylase triggered dextrin degradation and this led to protein unmasking with restoration of bioactivity to the level seen for unmodified rhEGF (Hardwicke et al., 2008).

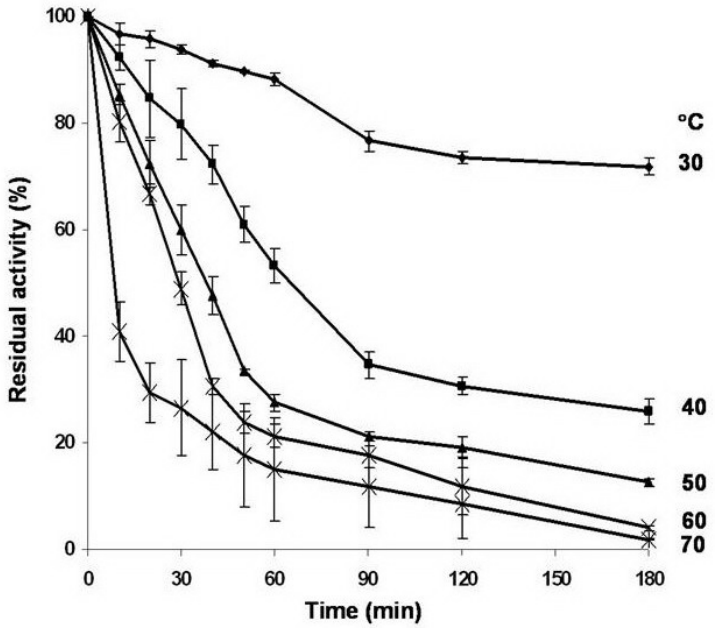
Dextrin-PLA_2 conjugates which containing 3-9%w/w PLA_2 and <1% free PLA_2 were synthesized using succinoylated dextrin (~51,000 g/mol; 23.2 mol% succinoylation). The PLA_2 activity of the conjugates was reduced to ~36% compared to free PLA_2. However, it was possible to regenerate all of the PLA_2 activity (114%) by incubation of the conjugate with α-amylase for polymer degradation. Moreover, the conjugate demonstrated significant toxicity towards human breast (MCF7) and murine melanoma (B16F10) cancer cells, comparable to the free enzyme (Ferguson and Duncan, 2009). Enzyme activity was regenerated with time in the presence of α-amylase. These observations suggest that dextrin-rhEGF and dextrin-PLA_2 conjugates have pharmacologically potential for further development as bioresponsive nanomedicines for tissue repair and anticancer therapy, respectively.

The porcine pancreatic trypsin rate constants, K_M, V_{max} and K_{cat} were again calculated using the Hanes-Wolfe plot (Table 5.2). The values obtained were differed from values reported by Johnson et al., 2002 for the same enzyme. For example, the K_{cat} of 40.09 s^{-1} (Table 5.2) is significantly higher than that the value of 2.89 s^{-1} estimated in their study using the least squares fit of the Michaelis-Menten equation to the reciprocal data using LUCENZIII, a computer software programme. It is well known that the analytical methodology and experimental conditions used, such as temperature and pH can affect K_M values. Enzyme purity and enzyme concentration can also cause variability in V_{max} values (Cornish-Bowden, 2004; Samoshina and Samoshin, 2005). Although Johnson et al. (2002) also used *L*-BAPNA as substrate, they performed their experiments at 35 °C (not 37 °C as used in our experiment), and using a different range of enzyme and substrate concentrations and also different incubation buffers. In these studies, although the K_M values obtained for free and conjugated trypsin were similar suggesting the substrate had a similar trypsin affinity after conjugation. Both the V_{max} and K_{cat} values estimated for the conjugates were reduced after conjugation, with the V_{max} values ~6-7 fold lower, and the substrate turnover rate K_{cat} values decreased by ~5-7 fold (Table 5.2).

5.4.3 Thermal stability and autolysis study of dextrin- and ST-HPMA-trypsin conjugates

The activity of both free trypsin and the trypsin conjugates progressively decreased with increasing incubation time at temperature between 30-70 °C (Figure 5.11). Trypsin, dextrin I-trypsin and ST-HPMA-trypsin conjugates were completely inactivated when incubated at 70 °C for 180 min (Figure 5.11a, b, d). Dextrin II-trypsin, however, still had a little of activity after incubation at this temperature for 180 min (Figure 5.11c).

(a) Porcine trypsin



(b) Dextrin I-trypsin conjugate

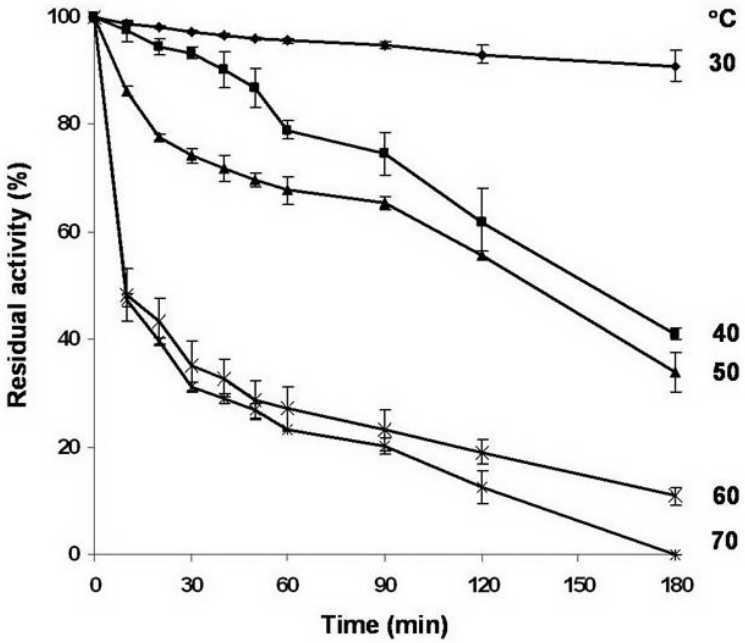
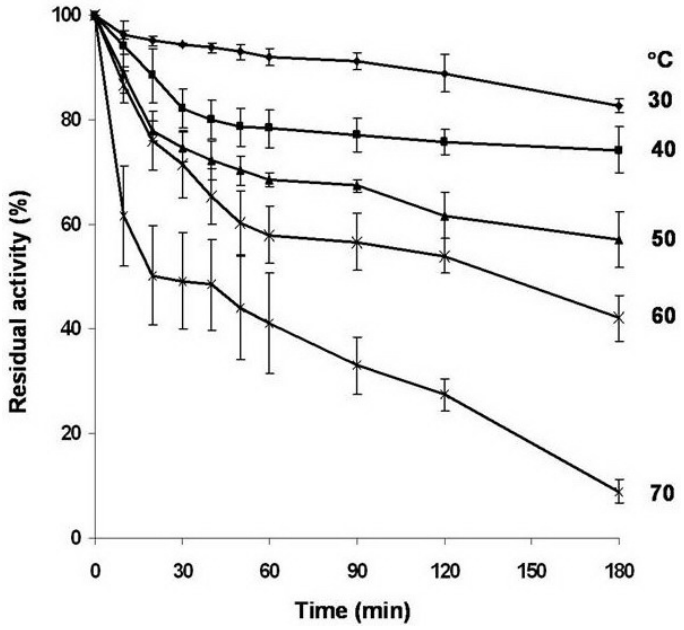


Figure 5.11 Thermal stability of trypsin and dextrin I-, dextrin II- and ST-HPMA-trypsin conjugates during incubation over 180 min at temperatures between 30-70 °C. The results represent mean ± SD (n=3).

(c) Dextrin II-trypsin conjugate



(d) ST-HPMA-trypsin conjugate

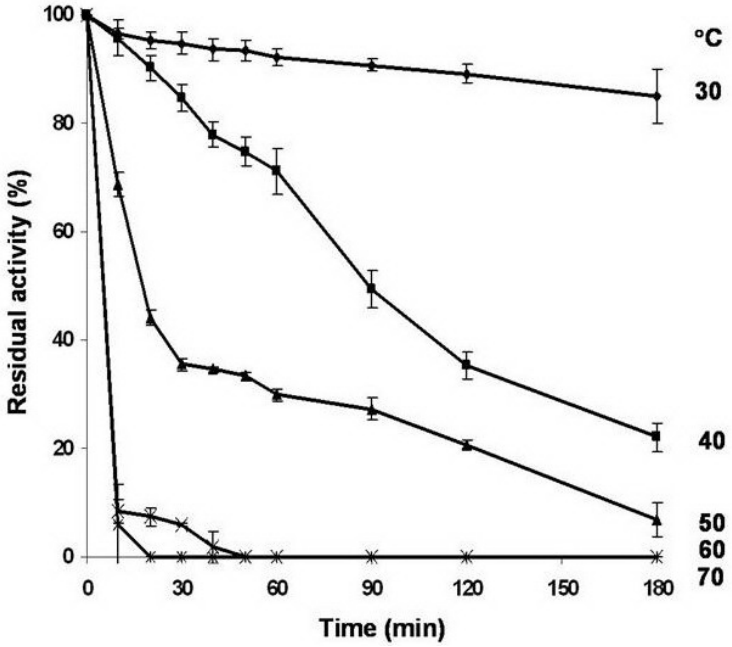


Figure 5.11 Contd. Thermal stability of trypsin and dextrin I-, dextrin II- and ST-HPMA-trypsin conjugates during incubation over 180 min at temperatures between 30-70 °C. The results represent mean ± SD (n=3).

When incubated for 10 min at each temperature (30–70 °C), the dextrin II-trypsin conjugate was more stable than the other conjugates and native trypsin (Figure 5.12a). At lower temperatures (up to 50 °C) the dextrin I- and ST-HPMA-trypsin conjugates were more stable than native trypsin, but they were, however, less stable at higher temperatures (50–70 °C) (Figure 5.12). The T_{50} values estimated for trypsin, dextrin I-trypsin, dextrin II-trypsin and ST-HPMA-trypsin conjugates were 68 °C, 60 °C, >70 °C, and 53 °C, respectively (Figure 5.12). The dextrin II-trypsin conjugate clearly demonstrated the best stability in both assays. Its higher T_{50} compared to native trypsin was consistent with the studies of Murphy and O'Fagain (1996) which showed that covalent modification of trypsin with acetic acid *N*-hydroxysuccinimide ethyl ester (AANHS) increased the T_{50} by 5 °C. Similarly, Villalonga et al. (2000) reported a T_{50} increase of 7 °C after carboxymethylcellulose conjugation (using the same assay experimental condition). However, the different between dextrin I and II clearly showed the important of polysaccharide molecular weight.

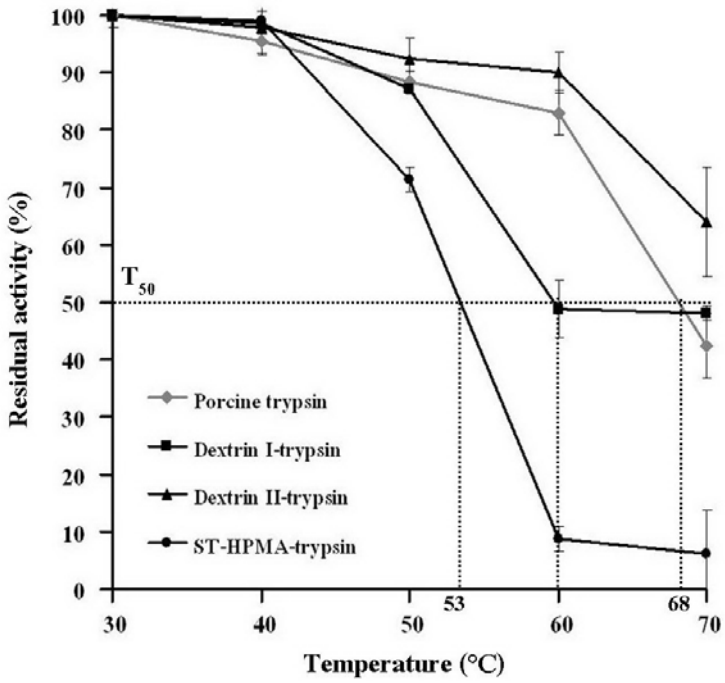


Figure 5.12 Comparison of thermal stability (10 min incubation) and activity of trypsin, and the dextrin I-, dextrin II-, and ST-HPMA-trypsin conjugates. Activity was measured using BAPNA as a substrate (mean ± SD, n=3).

Dextrin I-, dextrin II- and ST-HPMA-trypsin conjugates studied here were less susceptible to autolysis at 40 °C than native porcine trypsin (Figure 5.13). Again the dextrin II-trypsin conjugate showed the greatest stability, and moreover, they also showed better stability than the PEG-, β-CD- and PAMAM-trypsin conjugates which studied previously (Chapter 3 and 4).

The half-life ($t_{1/2}$) calculated from the time course for autolysis (Table 5.3) indicated an order of stability:- dextrin II conjugate > dextrin I conjugate > ST-HPMA conjugate > β-CD conjugate > trypsin-SA-mPEG 5000 > porcine trypsin > bovine trypsin > PAMAM conjugate. Interestingly, porcine trypsin had a higher $t_{1/2}$ than bovine trypsin. This result suggested that different source of trypsin may affect to its activity.

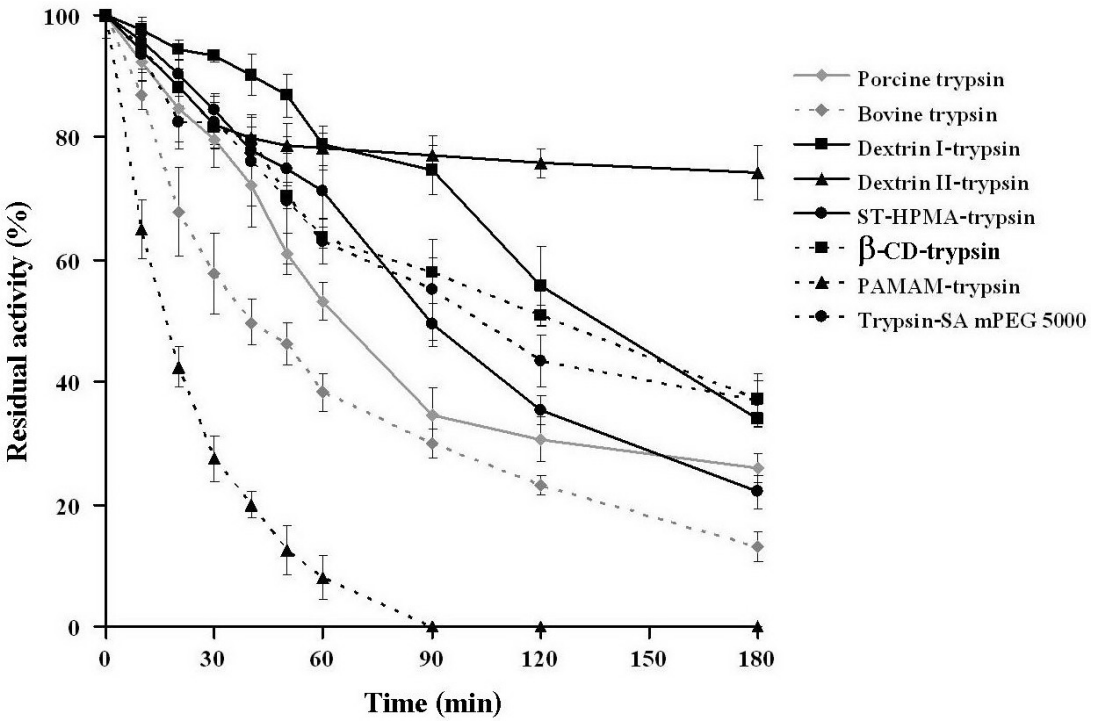


Figure 5.13 Comparison of autolysis at 40 °C and activity of trypsin, and the dextrin I-, dextrin II-, ST-HPMA-, β-CD-, PAMAM- and PEG-trypsin conjugates. Activity was measured using BAPNA as a substrate (mean ± SD, n=3).

Table 5.3 Half-life ($t_{1/2}$) (min) of native trypsin, dextrin- and ST-HPMA-trypsin conjugates[†]

	Half-life (min)[††]	**Half-life Ratio**
Porcine trypsin	65 ± 1	-
Dextrin I-trypsin	135 ± 2	2.08
Dextrin II-trypsin	> 180	>2.77
ST-HPMA-trypsin	89 ± 1	1.37
Bovine trypsin	41 ± 1	-
Trypsin-succinoylated mPEG[a]	102 ± 1	2.48
β-CD -trypsin[b]	125 ± 3	3.05
PAMAM-trypsin[b]	17 ± 1	-

[†]Experiments were conducted at 40 °C and the hydrolysis of BAPNA was used to measure residual trypsin activity

[††]Data represent $t_{1/2}$ min (mean ± SD, n=3)

[a]Data taken from Treetharnmathurot et al., 2008

[b]Data taken from Chapter 4

All the conjugates studied here showed both increasing in thermal stability (at most temperatures) and better stability to autolysis than native trypsin (Figure 5.12, Figure 5.13 and Table 5.3). The higher molecular weight of dextrin might be expected to provide enhanced protection from autolytic attack due to steric hindrance caused by the larger and bulkier polymer chain. It is also known that the glycosidic –OH groups can create hydrogen-bonding to protect and stabilize proteins (Mislovicova et al., 2006). Interestingly, the ST-HPMA-trypsin conjugates displayed lower thermal stability at high temperatures, and had a lower $t_{1/2}$ for autolysis than dextrin I-trypsin conjugate even though these polymers were of similar molecular weight. This may also reflect the better protective properties of the poly (glucose) polymer.

Although dextrin and β-CD are biodegradable polysaccharide, dextrin could be able to stabilize trypsin better than β-CD (Table 5.3). Dextrin I- and dextrin II-trypsin conjugates showed the higher thermal stability, autolysis and $t_{1/2}$ than β-CD-trypsin conjugate. This might be explained that dextrin is a branched, long chain polysaccharide and displayed more flexible compared to β-CD cyclic polysaccharide (Veronese, 2001). Moreover, dextrin contained a higher number of –OH groups which hydrogen-bonding was produced to protect proteins (Mislovicova et al., 2006).

5.5 Conclusion

In conclusion, under specific conditions both dextrin and ST-HPMA were able to increase stability of conjugates trypsin. The dextrin II-trypsin conjugate was also most stable than previously reported PEG-trypsin conjugates (Treetharnmathurot et al., 2008), β-CD- and PAMAM-trypsin conjugates (Chapter 4). This simple study underlines the potential of higher molecular weight dextrin II for protein conjugation, not only in the context of protein masking with subsequent regeneration of activity (PUMPT) but also as new biodegradable, polymer-protein conjugates. After initial studies on dextrins of different molecular weight (Duncan et al., 2008), the higher molecular weight of dextrin (~51,000 and 85,000 g/mol) was used by Ferguson and Duncan (2009) and Hardwicke et al. (2008) respectively- not only best pharmaceutical, controlled release profile but would be expected to give the best formulation properties.

CHAPTER 6

DEXTRIN-RNASE A AND ST-HPMA-RNASE A CONJUGATES: EFFECT OF POLYMER ON CYTOTOXICITY

6.1 Introduction

The earlier studies showed that trypsin activity of conjugated enzyme decreased after conjugation either PEGs (Chapter 3), β-CD and PAMAM dendrimers (Chapter 4) and dextrin and ST-HPMA homopolymer (Chapter 5). However, all polymer-trypsin conjugates (except PAMAM dendrimer conjugate) significantly improved the thermal stability of trypsin (Chapters 3-5). Here it was considered important to evaluate a potentially therapeutic polymer-anti-cancer protein conjugate. For these preliminary studies, it was decided to utilize dextrin and ST-HPMA homopolymer and synthesize conjugates with the anti-cancer enzyme ribonuclease A (RNase A). Dextrin and ST-HPMA homopolymer have been widely used to develop polymer therapeutics for protein delivery (Chapter 5).

6.1.1 Potential cancer therapeutics

Cancer is a major cause of mortality and worldwide incidence of cancer continues to increase, largely owing to the aging population. The treatment with small molecule drugs such as cisplatin, mitomycin, doxorubicin or 5-fluorouracil, alone or in combination, often results in side effects such as temporary diarrhea, nausea, loss of hair or reduced resistance to infection. Long-term impacts on heart, lung, kidney or bone marrow sometimes occur (Arnold and Ulbrich-Hofmann, 2006). The target specificity of antibodies, which selectivity bind to tumor-associated antigens presented on the cell surface, has the potential to reduce the systemic toxicity of the drugs, radionuclides or protein toxins from plants or bacteria. However, so further approach to targeting has not been realized. The major problem of antibody toxin conjugate is the

powerful non-specific toxicity of the toxin and the immunogenicity of the foreign proteins (Rybak and Newton, 1999). The development of milder toxins that are immunologically tolerated by humans is suggested as one possibility to solve these problems. Ribonucleases can be considered as toxins because of their ability to degrade RNA and to cause cell death (Ilinskaya and Makarov, 2005). Living cells contain approximately 20 exo- and endoribonucleases. They process RNA into mature forms and regulate the RNA turnover. RNases also participate in RNA metabolism, cell maturation, control of physiological cell death, promotion of blood vessel formation and also in host defense against RNA viruses. If exogenous RNases enter a cell they are likely to kill the cell by degrading the RNA, impairing protein biosynthesis and causing apoptosis (Arnold and Ulbrich-Hofmann, 2006).

6.1.2 Ribonuclease A as an anticancer therapeutic enzyme

The three sources of ribonuclease that have been most widely studied and compared the cytotoxicity are bovine pancreatic ribonuclease A (RNase A), bovine seminal (BS-RNase) and northern leopard frog (Onconase) (Raines, 1998). Their cytotoxicity has been described and compared, see Chapter 1, section 1.2.6.2. The basis of structural and functional properties of RNase A has been investigated since 1955 (Ledoux et al., 1955) and RNase A has been a popular model system in studies of enzymology and protein chemistry, and many studies focused on the variants and homologues that have remarkable biological activities and potential medicinal applications (Leland and Raines, 2001; Matousek, 2001).

Several homologues of RNase A are endowed with natural cytotoxic activity. For example, BS-RNase and Onconase are cytotoxic to cells in culture (mammalian cell lines), and they are able to evade cytosolic RIs or resistant to RIs (Kim et al., 1995) (see Chapter 1, section 1.2.6.2 for review). Unlike BS-RNase, the monomeric molecule of RNase A can efficiently cleave rRNA, but its catalytic activity was shown to be totally inhibited by RI (Klink and Raines, 2000). Moreover, it has been found that RNase A is not cytotoxic when bound tightly by RI due to protein-protein interaction (Kim et al., 1995).

When considering RNase as a therapeutic it is important to consider how it would gain cytosolic access. It is possible that highly cationic RNases adsorb to the negatively charged cell surface by electrostatic interaction, resulting in the efficient internalization into the cell, either by endocytotic or by passive through the plasma membrane. In theory, if the RNase exhibits a low affinity for RI it should show potent cytotoxicity. BS-RNase is a homodimeric protein with more than 80% sequence identity to RNase A. BS-RNase is more toxic than RNase A because it not only has low affinity for RI due to its unique dimeric structure, but also is more cationic than RNase A (Futami et al., 2001). Interestingly, when RNase A and Onconase were delivered via the transferrin receptor, both constructs showed equal cytotoxicity. It indicated that not RI evasion, but the mechanism of cellular uptake was also the limiting factor (Arnold and Ulbrich-Hofmann, 2006). The internalization pathway for RNase delivery to the cytosol has not yet been fully elucidated (Haigis and Raines, 2002). The general model for the mechanism of the cytotoxic action of RNases includes the interaction of the enzyme with the cellular membrane, internalization, translocation to the cytosol, and degradation of RNA (Leland and Raines, 2001).

In previous studies, several *in vitro* assays were used to determined ribonuclease activity (Table 6.1). These include measurement of anti-tumor activity, embryotoxic activity, fluorescence assay of RNA oligomers fluorescein substrate, measurement of immunosuppressive activity and determination of cytotoxicity using MTT assay. Here a UV-vis spectroscopy assay using yeast RNA as a substrate was chosen to measure the *in vitro* RNase enzyme activity as it is a simple method based on the change of RNA absorbance at 300 nm as it is degraded (Kunitz, M., 1946). The method is described later in section 6.3.4.

6.1.3 Studies on RNase A delivery systems

To try and develop improved delivery systems RNases have been chemically modified or conjugated to various molecules in order to reduce RI affinity and/or to improve their cellular uptake. RNase A has been chemically modified with ethylenediamine (Futami et al., 2001) and polyethylenediamine (Futami et al., 2005) to try to use cationic charge to promote cell

entry. However, it is known that such polycationic are themselves incredibly cytotoxic (Arnold and Ulbrich-Hofmann, 2006).

Amidation of the carboxyl groups in RNases by carbodiimide reaction is another way to change the net charge of the RNase. It can be undertaken systematically because there are several amines can be modified. It was postulated that the interaction of the RNase with RI would sterically hinder by such carboxyl group modification and also enhanced cellular uptake might be observed (Futami et al., 2001).

RNases have been coupled to many types of polymer with different molecular weight as reviewed in Table 1.13. Conjugation of BS-RNase or RNase A with HPMA or PEG produced conjugates that showed anti-tumor activity (Table 6.2). For example, PEG-RNase A conjugates were prepared via amide bond and when delivered systemically in mice they showed increased anti-tumor activity compared to free RNase A. This was attributed to decreased renal clearance time, reduced proteolytic degradation, and impaired binding of the conjugation to RI (Matousek et al., 2002). In this study, dextrin I, dextrin II and ST-HPMA homopolymer were chosen to prepare RNase A conjugates and their *in vitro* cytotoxicity was studied.

6.1.4 Rationale for the selection of B16F10 cells for the biological assay

Various *in vitro* cell models have been used to measure RNase cytotoxicity (Table 6.3) and the activity of RNase conjugates (Table 6.4). These include UKF-NB-3 (cells derived from evans stage 4 NB), ML-2 (human myeloid leukaemia cells), CV-1 (cell from African green monkey fibroblasts) and B16F10 (murine melanoma cells). These previous studies showed that ribonuclease conjugates were not cytotoxic in UKF-NB-3 (Michaelis et al., 2002) and ML-2 cells (Soucek et al., 2002; Pouckova et al., 2004; Matousek et al., 2004; Ulbrich et al., 2000), but were cytotoxic in CV-1 and B16F10 (Gilbert, 2007). In the preliminary experiments reported here the B16F10 melanoma cell model was used to study the cytotoxicity of RNase A and the polymer-RNase A conjugates.

Table 6.1 Examples of *in vitro* assays used to measure ribonuclease activity

Type of assay	Measurement	Cell line (seeding density)	Reference
Anti-tumor activity	Cell uptake of radioactivity [6-^{3}H]-thymidine	ML-2 (2×10^{5} cells/mL)	Matousek et al., 2003 Matousek et al., 2004
Embryotoxic activity	Monitored development stage of embryos culturing in CZB media by stereomicroscope	Embryos from C57/BL6 mice	Ulbrich et al., 2000 Matousek et al., 2003 Matousek et al., 2004 Pouckove et al., 2004
Fluorescence assay	Fluorescence spectroscopy at 490 and 515 nm using an RNA oligomer-fluorescein substrate	NA	Daly et al., 2005 Lee and Raines, 2005
Immunosuppressive activity	Cell viability of radioactivity [6-^{3}H]-thymidine	Human lymphocytes in a MLC	Soucek et al., 1999 Ulbrich et al., 2000 Soucek et al., 2002 Matousek et al., 2003
MTT cytotoxicity assay	Absorbance of formazan crystals at 550 nm and calculated cell viability	UKF-NB-3 cells (2×10^{4} cells/mL)	Michaelis et al., 2002

ML-2 = human myeloid leukaemia cells; MLC = a mixed lymphocyte culture; UKF-NB-3 = cells derived from evans stage 4 NB;

NA = not applicable

Table 6.2 Examples of composition, bond type and molecular weight of polymer-ribonuclease conjugates

Polymer (Mw, g/mol)	Protein	Conjugates (g/mol)	Bond type and site of conjugation	Reference
PEG 20,000 g/mol	RNase A	(i) 34,600 (ii) 54,600	Amide linkage of N-terminal of RNase A and mPEG-propionaldehyde	Daly et al., 2005
PEG: (i) 5,000 g/mol (ii) 22,000 g/mol	BS-RNase RNase A	ND	Amide linkage of lysine of RNase and PEG-*N*-hydroxysuccinimide ester	Matousek et al., 2002
PEG	RNase A	ND	Amide linkage of lysine of RNase A and PEG-*N*-hydroxysuccinimide ester	Matousek et al., 2004
PEG 22,00 g/mol	BS-RNase	ND	Amide linkage between lysines of BS-RNase and mPEG-succinimidyl propionate	Michaelis et al., 2002
ST-HPMA: (i) 2,940 g/mol (ii) 7,250 g/mol (iii) 19,470 g/mol	BS-RNase	ND	Amide linkage between lysines of BS-RNase and succinimidyl ester of HPMA	Oupicky et al., 1999
HPMA: (i) 15,000 g/mol (ii) 23,900 g/mol	Bs-RNase RNase A	ND	Amide linkage between lysines of RNase and succinimidyl ester of HPMA	Pouckova et al., 2004

Contd.

Table 6.2 Examples of composition, bond type and molecular weight of polymer-ribonuclease conjugates (Contd.)

Polymer (Mw, g/mol)	Protein	Conjugates (g/mol)	Bond type and site of conjugation	Reference
HPMA: (i) 15,000 g/mol (ii) 23,900 g/mol	RNase A	(i) 82,000 (ii) 486,000	(i) Amide linkage between lysines of RNase A and succinimidyl ester of HPMA (star-shape structure) (ii) Multiple RNase A conjugated to biodegradable Gly-Phe-Leu-Gly spacers of HPMA copolymer backbone (classic structure)	Soucek et al., 2002
HPMA 15,000 g/mol	BS-RNase	95,000	Amide linkage between lysines of BS-RNase and succinimidyl ester of HPMA	Soucek et al., 2002
HPMA: (i) 13,000 g/mol (ii) 17,900 g/mol	BS-RNase	(i) 60,000 (ii) 67,000	(i) Amide linkage between lysines of BS-RNase and succinimidyl ester of HPMA (star-shape structure) (ii) Multiple RNase A conjugated to biodegradable Gly-Phe-Leu-Gly spacers of HPMA copolymer backbone (classic structure)	Ulbrich et al., 2000

BS-RNase = Bovine seminal ribonuclease; RNase A = ribonuclease A; PEG = Polyethylene glycol; ND = Not determined

HPMA = Poly[*N*-(2-hydroxypropyl)methacrylamide]; ST-HPMA = Semitelechelic *N*-(2-hydroxypropyl)methacrylamide

Table 6.3 *In vitro* cell models used to determine ribonuclease cytotoxicity

Cell line	Seeding density (cells/mL)	Ribonuclease	IC_{50} (µM)	Cytotoxic	Reference
K-562	5×10^4	BS-RNase	1.3 ± 0.1	Yes	Lee and Raines, 2005
		RNase A	NA	No	Lee and Raines, 2005
		Onconase	0.2 ± 0.1	Yes	Lee and Raines, 2005
ML-2	2×10^5	RNase A	NA	No	Pouckova et al., 2004
					Soucek et al., 2002
		BS-RNase	2	Yes	Matousek et al., 2003
			ND		Ulbrich et al., 2000a
			ND		Pouckova et al., 2004
		Onconase	0.4	Yes	Matousek et al., 2003
UKF-NB-3	2×10^4	BS-RNase	2.18 ± 0.18	Yes	Michaelis et al., 2002

K-562 cells = cells derived from chronic myelogenous leukaemia (human); ML-2 = human myeloid leukaemia cells;

UKF-NB-3 = cells derived from evans stage 4 NB; ND = not determined; NA = not applicable

Table 6.4 *In vitro* cell models used to study polymer-ribonuclease conjugates cytotoxicity

Cell line	Seeding density (cells/mL)	Conjugates	Cytotoxic	Reference
UKF-NB-3	2×10^4	PEG-BS-RNase	No	Michaelis et al., 2002
ML-2	2×10^5	HPMA-RNase A	No	Soucek et al., 2002 Pouckova et al., 2004
		HPMA-BS-RNase	No	Ulbrich et al., 2000 Pouckova et al., 2004
		HA-RNase A PEG-RNase A HA-PEG-RNase A	No	Matousek et al., 2004
CV-1	2.5×10^4	HA-RNase A	Yes	Gilbert, 2007
B16F10	2.5×10^4	HA-RNase A	Yes	Gilbert, 2007
	4×10^4	Dextrin I-RNase A	Yes	Data shown here
		Dextrin II-RNase A	Yes	Data shown here
		ST-HPMA-RNase A	Yes	Data shown here

UKF-NB-3 = cells derived from evans stage 4 NB; ML-2 = human myeloid leukaemia cells; CV-1 cells = African green monkey fibroblasts; B16F10 cells = murine melanoma

6.1.5 The aims of this study

To summarize the aims of this study were:

(1) To synthesise dextrin I-, dextrin II- and ST-HPMA-RNase A conjugates.

(2) To study RNase A structure before and after polymer conjugation using circular dichroism spectroscopy

(3) To determine polymer-RNase A conjugates activity and compare it to free RNase A activity using yeast RNA as an enzyme substrate

(4) To determine the *in vitro* cytotoxicity of free RNase A and polymer-RNase A conjugates using the MTT assay and B16F10 melanoma cells.

6.2 Materials

All chemicals used in this study were of analytical grade and a detailed list of the compounds, general reagents, equipment and their suppliers, was previously described in Chapter 5. The compounds, general reagents, equipment and their suppliers used here to determine RNase activity and for cell culture were detailed in Chapter 2, section 2.1.1.5.

The equipment used for conjugate analysis, purification and characterization have been previously described in Chapter 2, section 2.1.2. Also the cell culture equipment was described in Chapter 2, section 2.1.2.5.

6.3 Methods

6.3.1 Succinoylation of dextrin

Dextrin I and dextrin II were succinoylated as described in Chapter 5, section 5.2.2.1 (Figure 5.1). The methods used to characterize the succinoylated dextrin were previously described in Chapter 2. They included titration (section 2.2.2.1), FT-IR (section 2.2.2.2) and GPC (section 2.2.2.3).

6.3.2 Synthesis of polymer-RNase A conjugates

6.3.2.1 Synthesis of dextrin I-RNase A conjugate

The reaction scheme is shown in Figure 6.1. Succinoylated dextrin I (52.1 mg) was dissolved in distilled water (2 mL). EDC (8.5 mg) was added and the reaction mixture was stirred for 10 min. Then sulfo-NHS (9.1 mg) was added and the mixture was stirred for a further 45 min. RNase A (13.9 mg) was added drop wise, and the pH measured and adjusted to pH ~8 with NaOH before leaving, under stirring for 18 h at room temperature. The conjugate prepared was then purified by FPLC. The collected fractions were further purified (remove salts) using a vivaspin tube (MWCO 30,000 g/mol). The resulting solution was freeze-dried to yield a white powder (33.4 mg). The methods used to characterize the final product were previously described in Chapter 5. The total protein content of the conjugates was measured using a Bradford assay (section 5.3.3.1). To characterize the polymer-RNase A conjugate, the basic methods for SDS-PAGE (section 2.2.3.2), FPLC (section 5.3.3.2) and GPC (section 5.3.3.3) and were also described in Chapter 5.

6.3.2.2 Synthesis of dextrin II-RNase A conjugate

To prepare the dextrin II-RNase A conjugate (Figure 6.1), succinoylated dextrin II (50.4 mg) in distilled water (2 mL) was used. The reaction was conducted as described above but using EDC (7.5 mg), sulfo-NHS (7.7 mg) and RNase A (7.4 mg), and the product was obtained as white powder (59.8 mg). All other reaction conditions, purification and characterization were as described above.

6.3.2.3 Synthesis of ST-HPMA-RNase A conjugate

ST-HPMA-COOH (50.4 mg) was dissolved in distilled water (2 ml). EDC (6.5 mg) was added and the reaction mixture was stirred for 10 min. Then sulfo-NHS (7.3 mg) was added and the reaction mixture was stirred for a further 45 min. RNase A (7.6 mg) was then

added. The product was obtained as white powder (40.5 mg). The reaction conditions, purification and characterization were conducted as described above (Figure 6.1).

6.3.3 Determination of ribonuclease A activity

The RNase A activity of native enzyme and the polymer-RNase A conjugates was measured using yeast RNA as a substrate (Kunitz, M., 1946); Briefly, 5 mg/ml RNA (100 μL) and 50 mM acetate buffer, pH 5.0 were added to a 1 mL cuvette. Then, free RNase A or polymer-RNase A conjugates (20 μL) (2 μg protein-equiv. in 50 mM acetate buffer, pH 5.0) were added and the solution was thoroughly mixed before equilibration at 37 °C for 10 min. Absorbance was measured using a Cary1G UV-Vis spectrophotometer (Varian, Australia) at 300 nm, and one unit of activity defined as the amount of enzyme that catalyses 1 μmol RNA/min. The RNase A activity was calculated using a range of substrate concentrations 0.05-0.5 mg/mL. The reaction volume was always 1.0 mL. A Hanes-Woolfe plot was again constructed, and used to calculate the kinetic parameters.

6.3.4 MTT assay to characterize cell growth and cytotoxicity

General cell culture techniques including maintenance and seeding of B16F10 cells has been described in Chapter 2, section 2.2.4. The MTT assay of cell viability measures the formation of formazan crystals (blue) caused by the reduction of MTT by mitochondrial dehydrogenase (Mosmann, 1983) (described in Chapter 2, section 2.2.4.5). This assay was chosen to characterize cell growth and also used to evaluate the cytotoxicity of the polymer-RNase A conjugates using MTT assay. To adjust the growth curve, the cell line B16F10 melanoma cells were seeded a density of 1×10^4 or 4×10^4 cells/mL (100 μL) in a 96 well plate as described earlier (Chapter 2, section 2.2.4.5.1). The cytotoxicity of samples (parent polymer, RNase A, polymer-RNase A conjugates or a medium control) towards B16F10 melanoma cells was measured as described in Chapter 2, section 2.2.4.5.2. All experiments were performed during the exponential growth phase of cell cycle.

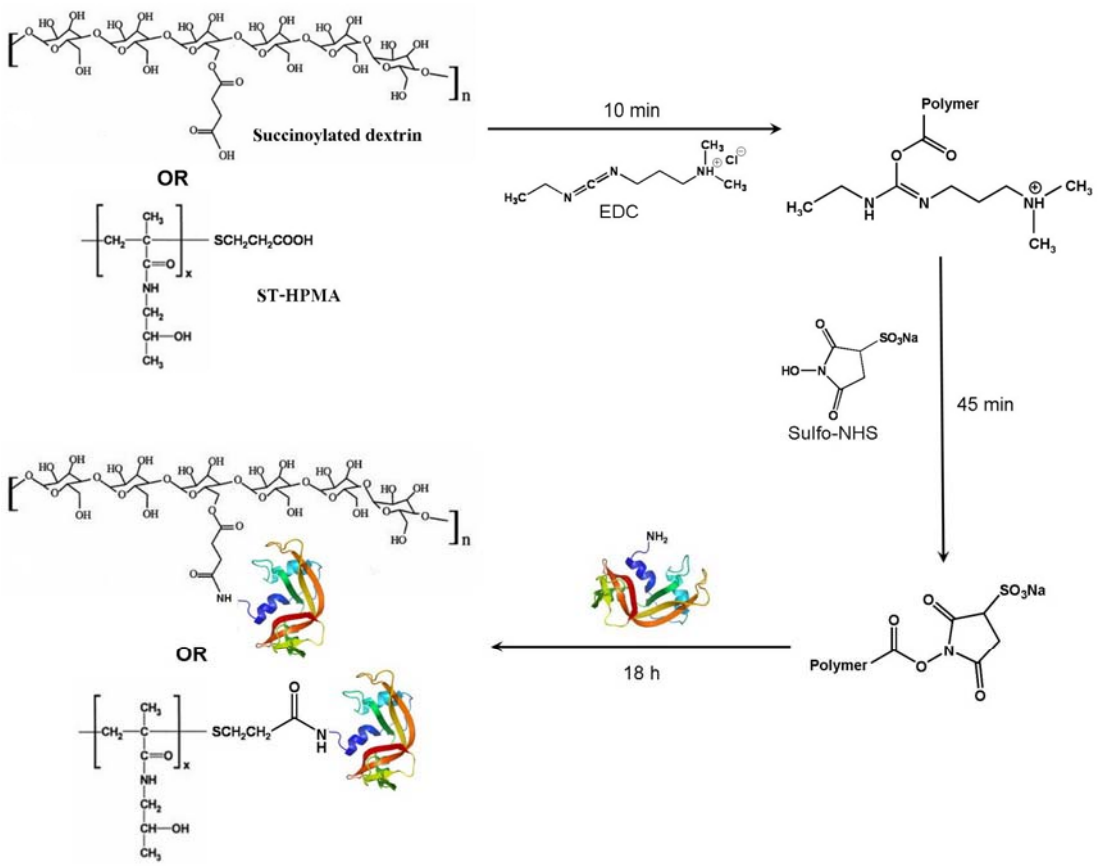


Figure 6.1 Reaction scheme for synthesis of polymer-RNase A conjugate

6.4 Results and Discussion

6.4.1 Synthesis and characterization of succinoylated dextrin

As described in Chapter 5, succinoylated dextrin I and succinoylated dextrin II were successfully synthesized. The acid groups were quantified by titration against 5.125 x 10^5 M NaOH solution and using bromothymol blue as an indicator. The degree of succinoylated dextrin I and succinoylated dextrin II modification was 8.8 mol% and 16.7 mol%, respectively. The products were characterized by FT-IR and GPC. FT-IR showed characteristic peaks of ester at 1720 cm^{-1}. ^1H qualitatively confirmed the incorporation of succinoyl groups that indicated by peaks at 2.6 – 2.8 ppm. These data were already reported in the previous chapter.

6.4.2 Preparation, purification and characterization of dextrin- and ST-HPMA-RNase A conjugates

Multifunctional polymers can cause cross-linking during reaction with proteins, and together with harsh coupling conditions this can also lead to decreased activity (Lu, et al., 1998). Thus, in this study both succinoylated dextrin I, and dextrin II and the ST-HPMA were used to modify RNase A. These polymer-RNase A conjugates were purified and characterized by FPLC (Figure 6.2a) and SDS-PAGE electrophoresis (Figure 6.2b). All the conjugates contained no detectable free RNase A after purification, however, the broad conjugates bands did indicate high polydispersity compared to that of molecular weight of protein markers and trypsin. The heterogeneity of the conjugates was probably caused by the differences in the degree of protein modification together with the starting polydispersity of the polymers used (Oupicky et al., 1999).

All polymer-RNase A conjugates were also characterized by GPC (Figure 6.3) and this method was used to get an approximate quantitation of the molecular weight and degree of polydispersity (Mw/Mn) (Table 6.5). Polymer-RNase A conjugates would be expected to elute earlier than an unbound polymer chain due to their larger size. The polydispersity of RNase A conjugates containing either dextrin I or dextrin II (Mw/Mn = 1.5-1.9) was higher than seen for the ST-HPMA conjugate (Mw/Mn = 1.3). These observations were similar to those seen for the trypsin conjugates containing the same polymers that were described in the previous Chapter 5 where the dextrin-trypsin conjugates also had a higher polydispersity than the ST-HPMA-trypsin conjugate. Again the ST-HPMA conjugate had a low protein loading.

As described in Chapter 5 the circular dichroism spectra of dextrin II and succinoylated dextrin II showed a completely unstructured conformation. In contrast the dextrin II-RNase A conjugate and RNase A showed a negative minimum at 208 nm and the spectra obtained were very similar (Figure 6.4), although, the dextrin II-RNase A conjugate had a slightly reduced amplitude at the 208 nm. These results were consistent to the previous study of polymer-trypsin conjugates (data shown in Chapter 5) and suggested that RNase A structure was not significantly changed by the dextrin conjugation.

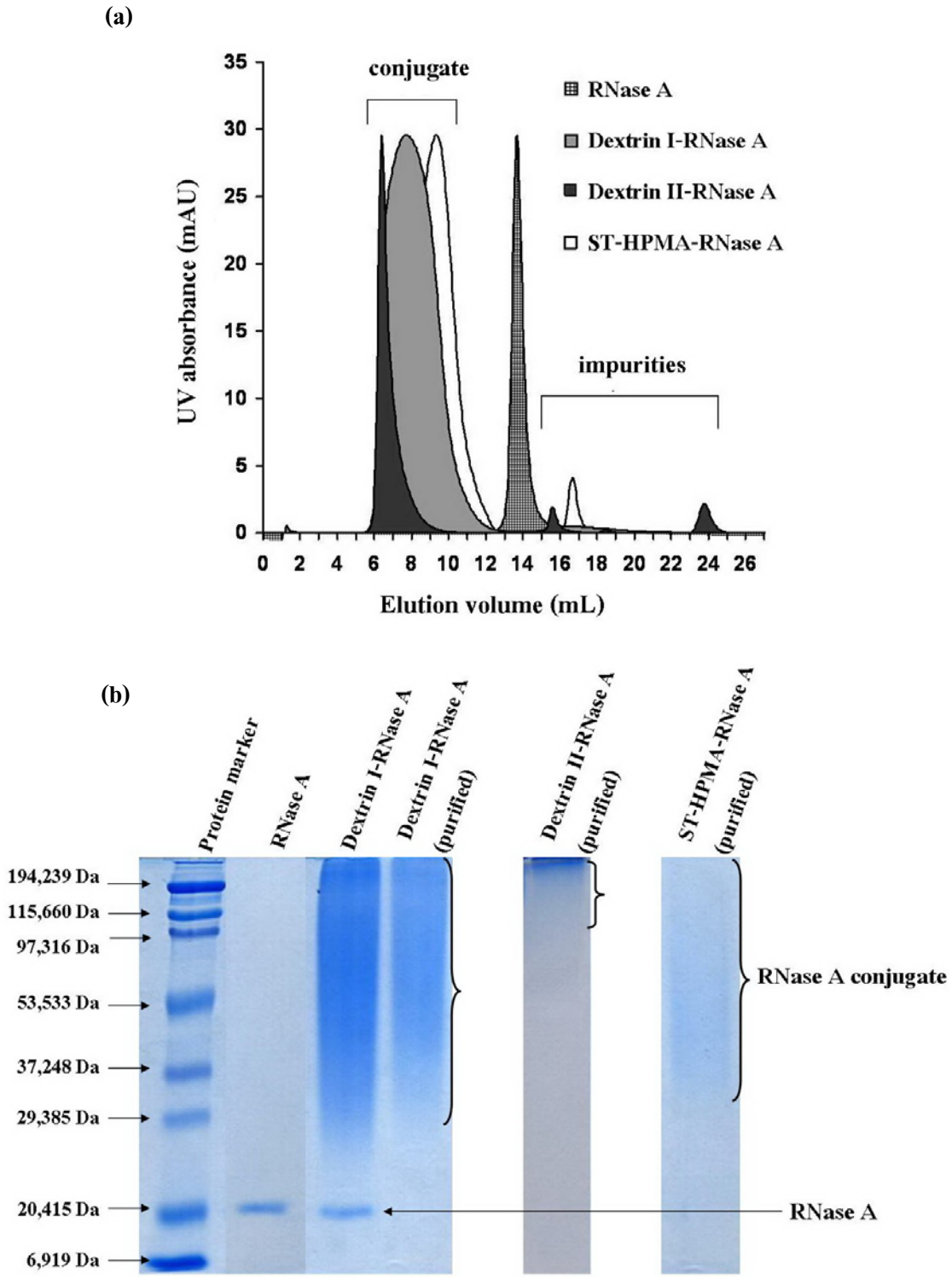


Figure 6.2 Characterization of polymer-RNase A conjugates. Panel (a) FPLC of RNase A and conjugates, and panel (b) SDS-PAGE of RNase A and conjugates.

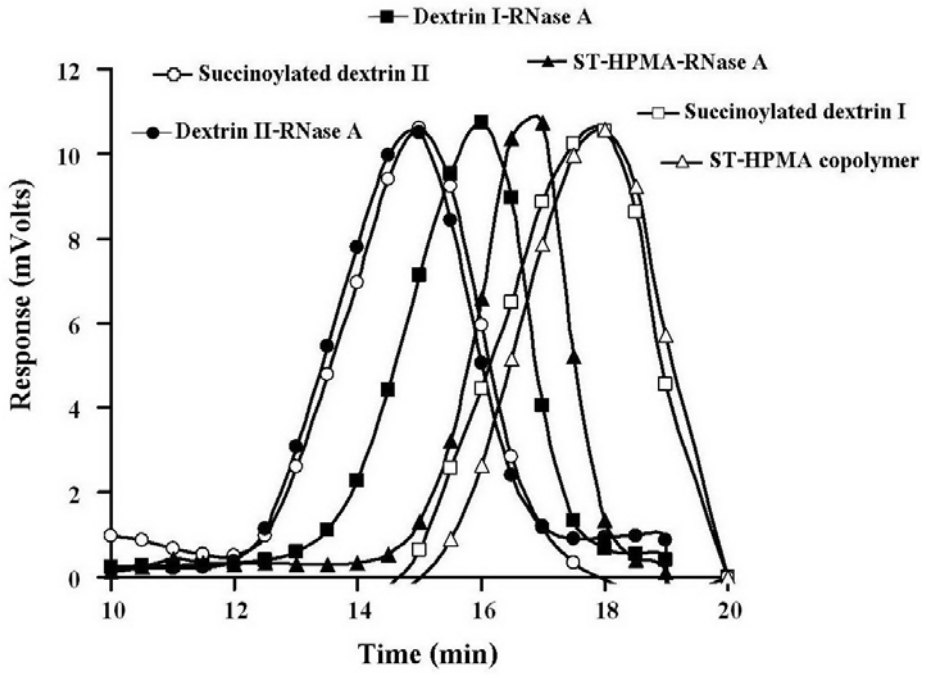


Figure 6.3 GPC elution of succinoylated dextrins, ST-HPMA copolymer and their RNase A conjugates.

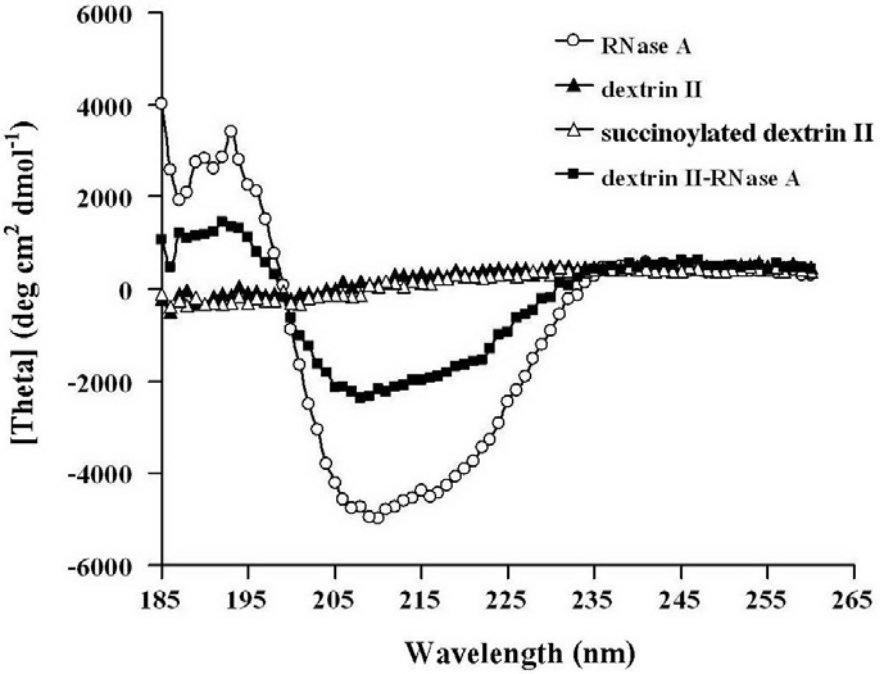


Figure 6.4 Circular dichroism spectroscopy of dextrin II, RNase A, succinoylated dextrin II and the dextrin II-RNase A conjugate

The total protein content of the conjugate was 29, 48 and 7 %, for dextrin I-RNase A, dextrin II-RNase A and ST-HPMA-RNase A conjugates, respectively (Table 6.5) compared to the protein content for trypsin conjugate containing the same polymers (Chapter 5) which was 54, 17 and 3 %, respectively. These observations suggested that ST-HPMA conjugate had lower protein content.

This RNase A content was also comparable to that reported for other polymer-ribonuclease conjugates For example, 16 % protein content of a HA-RNase A conjugate (Gilbert, 2007), 30 % protein content of a PEG-BS-RNase conjugate (Michaelis et al., 2002), 33 % protein in a poly(HPMA)-BS-RNase conjugate (Ulbrich et al., 2000) and 28-34 % protein in another poly(HPMA)-RNase A conjugate (Soucek et al., 2002).

Table 6.5 Characteristics of polymer and polymer-RNase A conjugates

Compound	Acid groups (mol%)	Protein content (%)	Mw[†]	Mn[†]	Polydispersity (M_w/M_n)
RNase A[††]	-	-	13,690[††]	-	-
Dextrin I	-	-	8,100	5,200	1.6
Succinoylated dextrin I	8.8	-	11,200	7,000	1.6
Dextrin I-RNase A	-	29	40,800	26,100	1.6
Dextrin II	-	-	61,000	36,000	1.6
Succinoylated dextrin II	16.7	-	73,800	40,000	1.9
Dextrin II-RNase A	-	48	95,900	62,100	1.5
ST-HPMA copolymer	-	-	10,100	7,000	1.5
ST-HPMA-RNase A	-	7	19,200	14,700	1.3

[†]Estimated using GPC and pullulan standards

[††]Porcine pancreatic RNase A, 124 amino acids (http://www.expasy.ch/tools/protparam.html)

6.4.3 RNase A activity

The kinetic parameters (K_M, V_{max} and K_{cat}) obtained for native RNase A and the polymer-RNase A conjugates (measured using yeast RNA as substrate in a range of concentration 0.05–0.5 mg/ml, at 37 °C) are summarized in Table 6.6. Enzyme kinetic analysis using the Hanes-Woolfe plot indicated that Michaelis constant value (K_M) was slightly decreased by polymer conjugation. Maximum velocity (V_{max}) of the conjugates was ~2-8 fold lower than seen for free enzyme and the substrate turnover rate (K_{cat}) was reduced to ~2-7 fold (Table 6.6).

All conjugates displayed lower activity than native RNase A with dextrin I-RNase A, dextrin II-RNase A and ST-HPMA-RNase A conjugates reducing RNase A activity to 59.2 %, 13.7 % and 52.7 %, respectively (Figure 6.5). This reduction of ribonuclease activity was also found by the other groups. For example, when BS-RNase was conjugated with HPMA (Oupicky et al., 1999). In that case 67 % of the amino groups of protein were modified by HPMA and the conjugate retained 84% enzyme activity compared to native enzyme.

Here, it was observed that the dextrin II-RNase A conjugate was less active than dextrin I-RNase A and ST-HPMA-RNase A conjugates. This is not surprising as the higher molecular weight dextrin II in the dextrin II-RNase A conjugate would be expected to mask RNase A activity more than other conjugates. The steric hindrance of this high molecular weight dextrin could restrict RNase A interaction with the substrate and/or bind to RNase A's active site. The higher molecular weight of polymer might mask enzyme activity more than lower molecular weight (Duncan, 2008).

As discussed previously, dextrin has been used for protein masking in other studies such as dextrin-trypsin, dextrin-MSH, dextrin-rhEGF, and dextrin-PLA_2 conjugates. The activity of these proteins was reduced up to 10-30 % depending on the protein and molecular weight of the dextrin. Nevertheless protein activity was regenerated to 20-100% by triggering dextrin degradation with addition of α-amylase (Duncan et al., 2008; Hardwicke et al., 2008; Ferguson and Duncan, 2009).

Table 6.6 Enzyme activity and kinetic parameters of native RNase A and RNase A conjugates

Compound	Activity† (%)	K_M†† (mM)	V_{max}†† (mM min^{-1})	K_{cat}†† (s^{-1})
RNase A	100	0.271 ± 0.017	0.0076 ± 0.0003	0.867 ± 0.038
Dextrin I-RNase A	59.2 ± 4.0	0.239 ± 0.025	0.0039 ± 0.0003	0.514 ± 0.034
Dextrin II-RNase A	13.7 ± 0.2	0.162 ± 0.005	0.0010 ± 0.0001	0.119 ± 0.002
ST-HPMA-RNase A	52.7 ± 1.3	0.154 ± 0.007	0.0039 ± 0.0001	0.457 ± 0.011

†Data shown relate to the RNase A control; mean ± SD (n = 3)

††Estimated using the Hanes-Wolfe plot

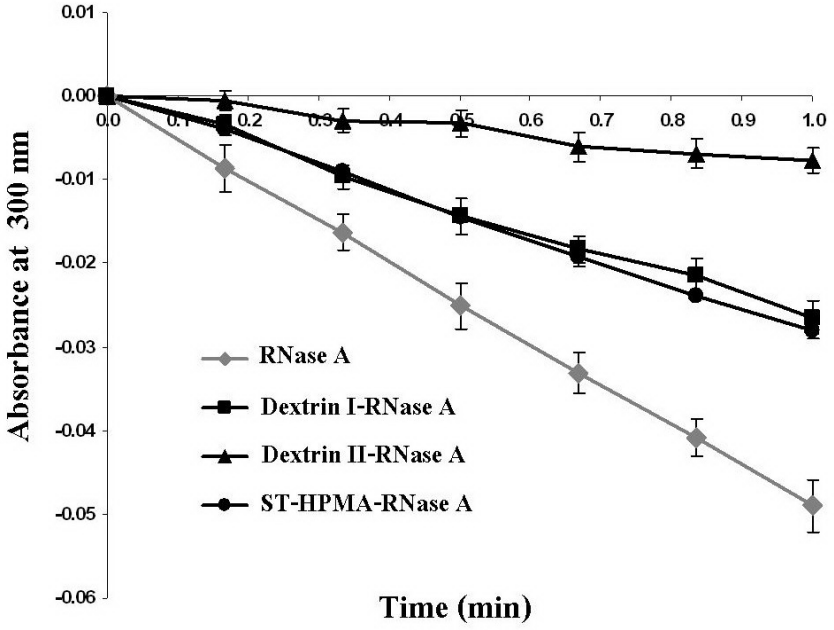


Figure 6.5 Comparison of enzyme activity of free RNase A and polymer-RNase A conjugates. In each case 2 mg/mL RNase A equivalent was used (mean ± SD, n = 3).

6.4.4 B16F10 melanoma cell growth and cytotoxicity of dextrin- and ST-HPMA-RNase A conjugates

B16F10 melanoma cell growth curves were obtained over 8 days after cells were seeded at a density of $1x10^4$ and $4x10^4$ cells/mL (Figure 6.6). The initial rate of cell growth depended on the seeding density. The higher density ($4x10^4$ cells/mL) produced exponential graph within 72 h. As this was the suitable period for performing cytotoxicity experiments, this seeding density ($4x10^4$ cells/mL) was chosen for further cytotoxicity tests in these studies.

Although native RNase A was not cytotoxic towards ML-2 or K-562 cells in previously studies (Pouckova, et al., 2004; Lee and Raines, 2003), here RNase A was cytotoxic towards B16F10 melanoma cells when used at a concentration of more than 1 mg/mL. Moreover, all polymer-RNase A conjugates were cytotoxic to B16F10 melanoma cells (Figure 6.7). Dextrin I-RNase A, dextrin II-RNase A and ST-HPMA-RNase A conjugates significantly reduced cell viability to lower 50 % at a concentration of 5 mg/mL, 5 mg/mL and 1 mg/ml RNase A equivalent, respectively after incubation at 37 °C for 72 h. The cytotoxic of conjugates to B16F10 melanoma cells showed as follow: ST-HPMA conjugates > dextrin I conjugates ≥ dextrin II conjugates.

This suggested that the polymer conjugates protect RNase A from binding tightly with RI in cytosol and this in some way promotes cytosolic access. The result was improved cytotoxicity (Suzuki, et al., 1999; Newton, et al., 2001; De Lorenzo et al., 2002) The ST-HPMA-RNase A conjugate was the most effective in terms of anti-tumor activity, it was note worthy that the ST-HPMA itself did not inhibit proliferation of B16F10 melanoma cells.

Modification of biologically active proteins with hydrophilic polymers has been shown to result in an improved resistance to proteolytic degradation (Oupicky et al., 1999). Therefore, dextrin I, dextrin II and ST-HPMA-COOH were chosen to modify RNase A in the present study. All conjugates showed *in vitro* anti-cancer effects in B16F10 melanoma cell. These results were similarly to the previous experiments of other ribonucleases.

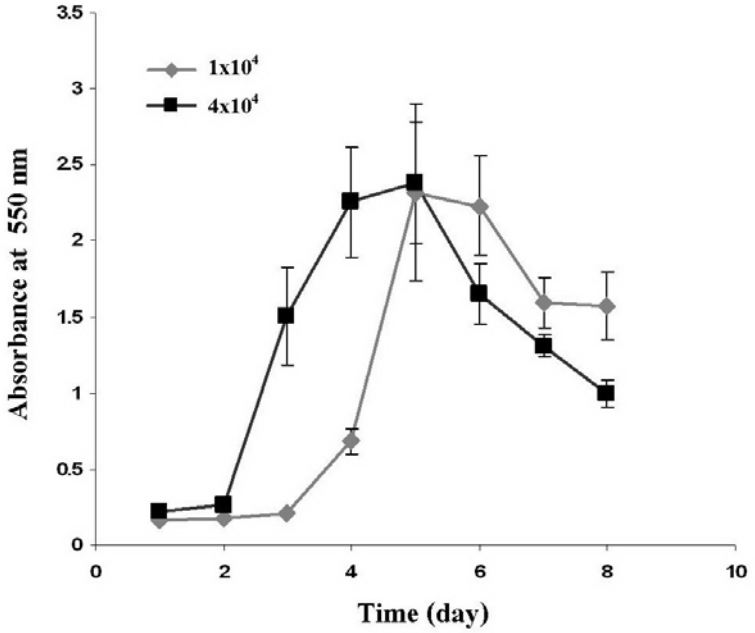


Figure 6.6 Typical growth curve for B16F10 murine melanoma cells over 8 days at seeding density of 1 x 10^4 and 4 x 10^4 cells/mL. Data shown represents the mean (n=18) ± SD.

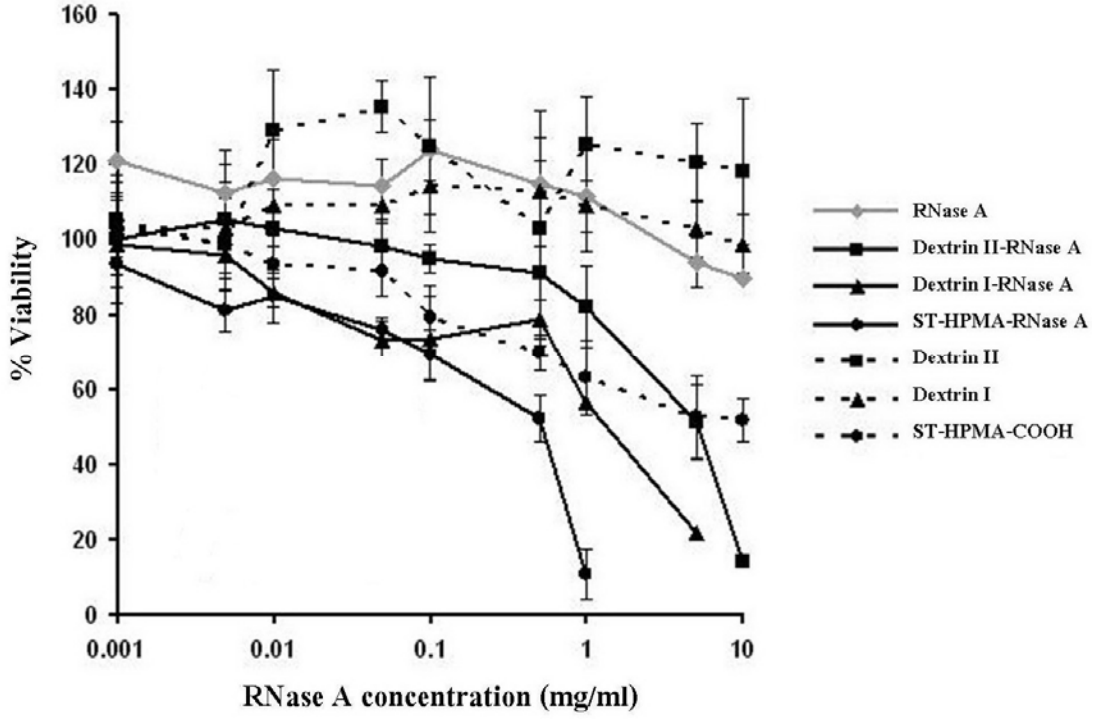


Figure 6.7 *In vitro* cytotoxicity of free RNase A and polymer-RNase A conjugates. Data shown represents the mean (n=18) ± SD.

For example, HPMA-BS-RNase conjugate was well preserved and the proteolytic stability was improved compared to free BS-RNase. These results illustrated that HPMA modification could prevent BS-RNase from enzymatic degradation and resistance to the action of the RI (Oupicky et al., 1999; Ulbrich et al., 2000). Another experiment was carried out to synthesize and evaluate the properties of HPMA conjugates (Soucek et al., 2002; Pouckova et al., 2004) and PEG conjugates (Matousek et al., 2002) from RNase A, an enzyme that does not exhibit any marked cytotoxic *in vitro* and *in vivo*. The anti-tumor activity of HPMA- and PEG-RNase A conjugates were increased compare to native RNase A. PEG chains and HA were also used to conjugate to the ribonuclease (RNase A and BS-RNase) and showed aspermatogenic and anti-tumor activity *in vivo*. The cleavage mechanism of the amide bond formed between those two ribonucleases and PEG remained uninvestigated and unclear *in vivo* (Matousek et al., 2004).

However, some explanations of the mechanisms of action of polymer-ribonuclease conjugates have been described. When polymer conjugated to ribonuclease, the polymer might protect RNase A with low biological against the action of RI and change it into a cytotoxically active substance. The polymer might prolong the conjugates in blood circulation therefore, the possibility to reach the site of action of ribonuclease was sustained. The cytotoxic effects could be more effective due to a less binding between the conjugates and antibodies (Matousek et al., 2002).

Following on these preliminary polymer-RNase A cytotoxicity studies, future work should be accomplished to understand the mechanism of action of these RNase A conjugates e.g. cellular uptake, binding and the release of the RNase A into cytosol where it may induce apoptosis. It was hoped that conjugation to polymer might increased RNase A tumor targeting by the enhanced permeability and retention (EPR) effect and improved stability of enzyme. The study of masking and reinstating dextrin-RNase A activity should also be examined.

6.5 Conclusion

Anti-cancer conjugates were successfully synthesized using ribonuclease A (RNase A) conjugated to dextrin I, dextrin II and ST-HPMA-COOH for development of therapeutic conjugates. The polymer-RNase A conjugates were investigated on cytotoxicity and prevention of proliferation in B16F10 cells for anti-cancer activity. RNase A alone was not cytotoxic (less than 1 mg/mL) whereas ST-HPMA-RNase A conjugates (1 mg/mL RNase A equivalent) was the most effective on cytotoxicity.

CHAPTER 7

GENERAL DISCUSSION

7.1 Summary of the key results of this thesis

Protein modification with polymers has been extensively investigated to tailor the various application needs such as PEG, polysaccharides or phospholiplid polymers for enhancing activity (Gaertner and Puigserver, 1992; Matsushima et al., 1996) or improving thermal stability of enzyme (Zhang et al., 1999; Fernandez et al., 2002; Miyamoto et al., 2005).

PEGylation is well established as a tool to improve the properties of biotech drugs including proteins, peptides and aptamers (Ryan et al., 2008). The resulting conjugates have therapeutic and formulation advantages, and also with the potential to be cost-effective and even cost-saving (Eldar-Lissai et al., 2008; Gerkens et al., 2007). With more and more clinical applications realized, studies on PEG-protein conjugates continue to grow exponentially. The biological properties of novel conjugates, improved chemistry for conjugate synthesis (Kinstler et al., 2002; Brocchini et al., 2006), the effect of PEG molecular weight and branching on physicochemical properties and biodistribution (Fee et al., 2007), and new techniques for purification and/or conjugate characterization (Na et al., 2008; Hardy et al., 2008) have recently been reported. PEG chains exist as relatively unperturbed random-coil domains adjacent to the consistent with previous report on the structure of PEGylated lysozyme (Daly et al., 2004) and PEGylated ribonuclease A (Daly et al., 2005). In spite of this success, PEG can also have disadvantages (Gaberc-Porekar et al., 2008), not least the fact that this is a non-biodegradable polymer. For both PEG- and the other polymer-conjugates there have been disappointingly few studies on thermal stability and other formulation issues (e.g. Tattini et al., 2005; López-Cruz et al., 2006). This has brought increasing interest in alternative polymers such as poly(2-ethyl 2-oxazoline) (Mero et al. 2008), poly(vinylpyrolidone) (Kaneda et al., 2004) and dextrin (Duncan et al., 2008; Hardwicke et al., 2008; Ferguson and Duncan, 2009).

Polysaccharides have been increasing in the use for application as biodegradable polymers in drug delivery. The application of dextran, pullulan and mannan for drug delivery has been reviewed (Mehvar, 2003). The particular application of polysaccharides such as HA has also been thoroughly reported for colon site-specific delivery (Jain et al., 2007). Furthermore, there remains interest in the potential application of dextran and dextrin as polymeric backbones for protein delivery, with recent studies investigating the effects of their conjugation such as dextran-ovalbumin conjugate (Choi et al., 2005), dextran-BSA conjugate (Jung et al., 2006), dextrin-trypsin and dextrin-MSH conjugate (Duncan et al., 2008), dextrin-rhEGF conjugate (Hardwicke et al., 2008) and dextrin-PLA_2 (Ferguson and Duncan, 2009).

The data obtained in this thesis described the effect of polymer type and architectures on the biological activities and properties of polymer-protein conjugates such as stability, autolysis rate and half-life. In Chapter 3-5, PEG-, β-CD-, PAMAM-, dextrin- and ST-HPMA-trypsin conjugates have been designed to stabilize and prolong half-life of trypsin. The results showed that all polymers used in this study, except PAMAM dendrimer, could improve thermal stability and increase half-life of the conjugated trypsin. However, all polymer conjugates were possible to mask trypsin activity.

The ability to reduced trypsin activity on conjugation could be affected by architecture of polymer (including type and shape, molecular weight/size and number of –OH group), number of conjugating bonds/linkers, and location of polymer conjugation. The different between the five polymers; PEG, β-CD, PAMAM dendrimer, dextrin and ST-HPMA, was major in term of both structure and molecular weight. They affected to the thermal stability (Figure 7.1) and half-life of conjugated trypsin (Table 7.1).

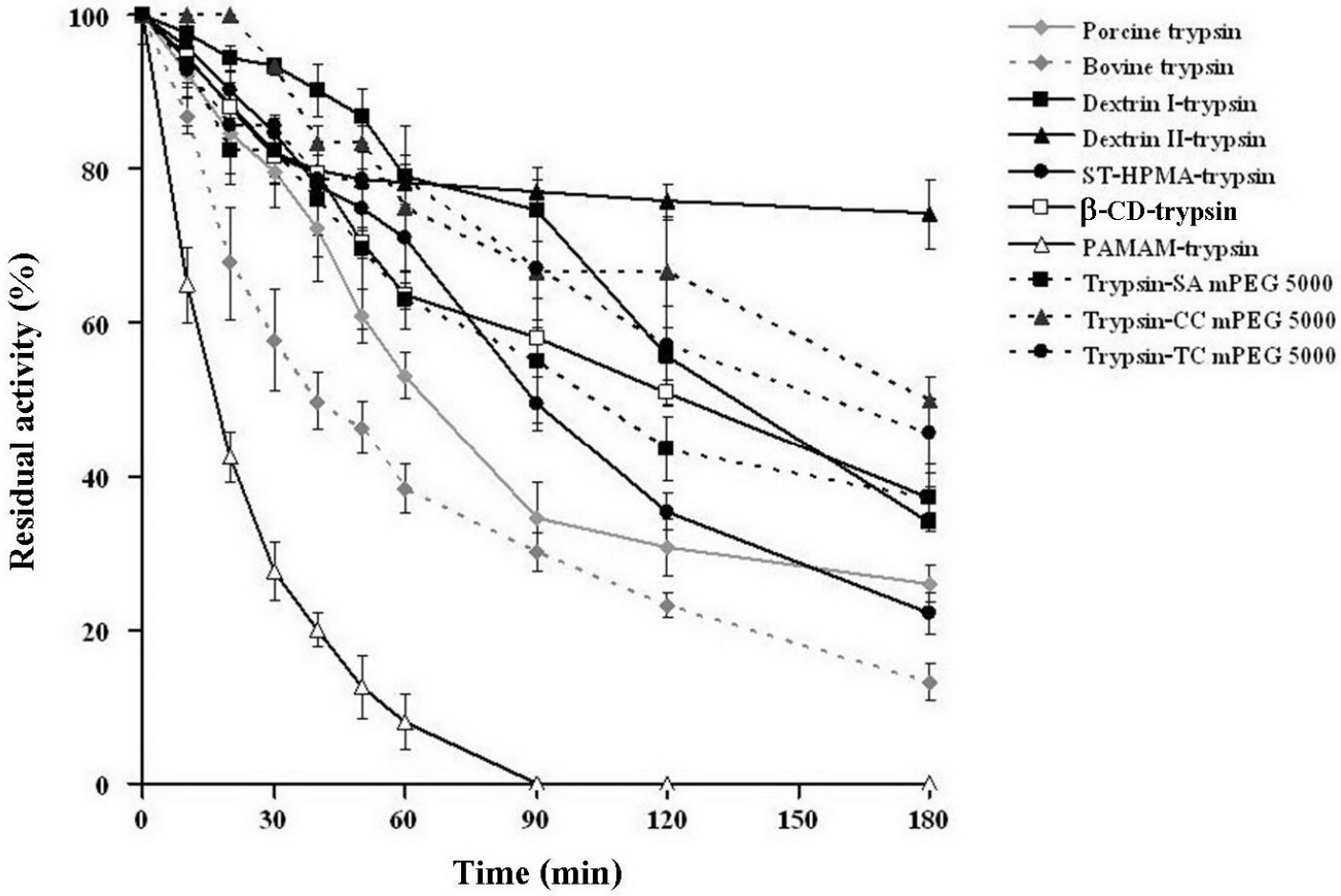
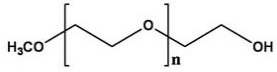
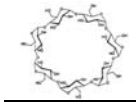
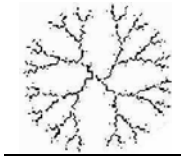
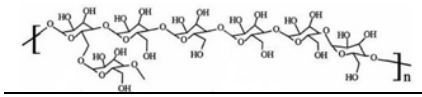
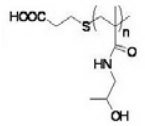


Figure 7.1 Comparison of Thermal stability and activity of trypsin, and the dextrin I-, dextrin II-, ST-HPMA-, β-CD-, PAMAM- and PEG-trypsin conjugates at 40 °C. Activity was measured using BAPNA as a substrate (mean ± SD, n=3).

Table 7.1 Comparison of physicochemical and biological properties of polymer-trypsin conjugates among polymers used in this study

Polymer	Shape	Mw (g/mol)	Linker	Polymer:linker	Trypsin:Polymer	Activity (%)	Half-life ratio
mPEG 	Linear	5,000	Succinoylated	1:1[a]	1:5[c]	62.4 ± 6.0[f]	2.48[f]
			Cyanurate	1:1[a]	1:5[c]	19.6 ± 5.6[f]	4.39[f]
			Tosylated	1:1[a]	1:7[c]	16.8 ± 2.3[f]	3.83[f]
β-cyclodextrin 	Truncate	1,135	Succinoylated	1:3[b]	1:7[d]	13.9 ± 0.7[f]	3.05[f]
PAMAM dendrimer 	Spherical	6,941	Succinoylated	1:1[b]	1:3[d]	29.2 ± 1.8[f]	0.41[f]
Dextrin 	Branch	8,800	Succinoylated	8.8 mol% [a]	1:1[e]	15.1 ± 0.2[g]	2.08[g]
		61,000		16.7 mol%[a]	1:1[e]	18.6 ± 3.8[g]	> 2.77[g]
ST-HPMA homopolymer 	Branch	10,100	Succinoylated	-	1:1[e]	13.8 ± 1.6[g]	1.37[g]

Ratio of polymer: linker was calculated by either titration[a] or ^{1}H-NMR[b].

Ratio of trypsin: polymer was predicted from either TNBS assay[c], FPLC standard curve[d] or GPC standard curve[e]

Trypsin activity and half-life of the conjugates were measured and compared to native trypsin either bovine trypsin[f] or porcine trypsin[g].

7.1.1 Architecture of polymer and its molecular weight

Polymeric architecture, molecular weight and molecular charge play important roles in delivering drug. This is especially true when the polymeric materials have linear, branched and graft architectures. In addition, such polymers exhibit high steric hindrance for prolong the half-life of therapeutic proteins in the circulatory system by increase molecular size which reduced kidney clearance, masking of protein surface and decrease proteolytic degradation (Abuchowski et al., 1977; Harris and Zalipsky, 1997).

7.1.1.1 Type and shape

In this study, the residual trypsin activity of polymer-trypsin conjugate was measured using BAPNA as a substrate after incubation at 30 °C for 10 min. The β-CD-, PAMAM-trypsin conjugates (Chapter 4) and the dextrin- and ST-HPMA-trypsin conjugates (Chapter 5) showed lower residual activity than PEG-trypsin conjugates (Chapter 3). That means globular (e.g. β-CD and PAMAM dendrimer) or branched (e.g. dextrin and ST-HPMA) polymers could mask trypsin activity better than linear polymer (e.g. PEG). The $t_{1/2}$ was calculated from the time course for autolysis at 40 °C for 180 min. The $t_{1/2}$ of conjugates showed as follow: dextrin II conjugate > dextrin I conjugate > ST-HPMA conjugate > β-CD conjugate > trypsin-SA-mPEG 5000 > porcine trypsin > bovine trypsin > PAMAM conjugate. Therefore, the globular/branched polymer-trypsin conjugates (except PAMAM-trypsin conjugate) displayed higher thermal stability, more stable to autolysis and longer $t_{1/2}$ than that of linear form. These results were consistent to the previous reports as described below.

Branched PEG-protein conjugates have been found to be more effective than their linear counterparts for the same total PEG adduct molecular weight due to a longer circulation $t_{1/2}$ of the branched form in vivo. This behavior has been attributed to a more effective masking of the protein surface but also to a larger effective size (Harris et al., 1999) or hydrodynamic volume of the branched PEGylated protein (Veronese et al., 1997). It has been found that there was no difference in the hydrodynamic volume or viscosity radii of proteins

PEGylated with linear and branched form of PEG, regardless of PEGylation extent. So, the higher stability of branched PEGylated protein was not explained by size difference and likely resulted from more effective masking of the protein surface when compared with linear PEGylated protein (Fee, 2007). Furthermore, its "umbrella-like" structure of branched PEGylated protein is more effective in protecting proteins from proteolysis, in the approach of antibodies and in reducing immunogenicity (Veronese, 2001) (Figure 7.2).

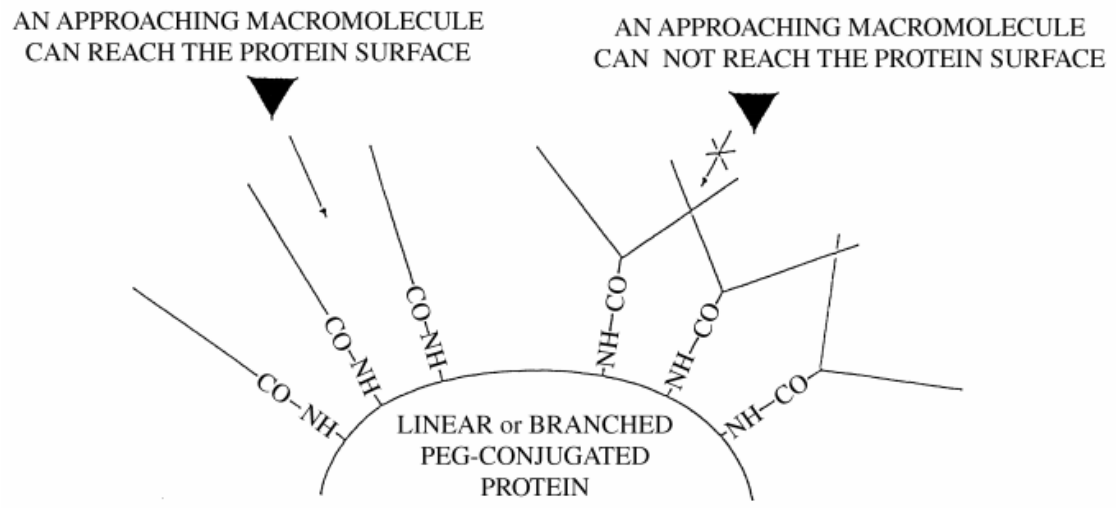


Figure 7.2 The "umbrella-like" structure of branched PEG compared to linear PEG of the same size (From Veronese, 2001).

The same mechanism that prevented the approach of proteolytic enzymes or antibodies to PEGylated protein could also reject a substrate from the protein active site. This mainly occurs with enzymes possessing high molecular weight substrates such as peptides, proteins, polysaccharides and also in the receptor-binding of PEGylated molecules (Veronese , 2001). The loss of biological activity during conjugation may be improved by conjugating with biodegradable polymer (Duncan et al., 2008; Hardwicke et al., 2008; Ferguson et al, 2009).

Conventional linear polymers (e.g. PEG) only have functional groups at one or two ends of the chains which are randomly coiled an entangled with others, resulting in low reactivity of conjugation. Dendritic polymers including dendrimers (e.g. PAMAM dendrimer) and

hyperbranched polymer (e.g. hyperbranched aromatic polyamide (HBPA)), have a highly branched structure which providing a spherical molecular shape and a high density of functional groups at the peripheral (Ge et al., 2007).

The HBPA (Mw ~150,000 g/mol) was conjugated to lipase. Each HBPA molecule could be covalently attached with a maximum of 5-6 lipase molecules. The conjugated lipase had an approximate K_M to the native lipase while V_{max} was increased by 20% for the hydrolysis of *p*-nitrophenylpalmitate. Moreover, the conjugated lipase exhibited a significantly higher stability at high temperature (Ge et al., 2007). These results were contrast to the data obtained from PAMAM-trypsin conjugate in this thesis (Chapter 4).

The K_M value of PAMAM-trypsin conjugate was similar to native trypsin but its V_{max} had reduced compared to native trypsin. The PAMAM-trypsin conjugate displayed lower thermal stability, less stable against autolysis and had a shorter $t_{1/2}$ for autolysis than other conjugates and native trypsin. It might be explained by a less steric hindrance of PAMAM structure due to a less number of PAMAM molecules in the conjugate that was inefficiency protect trypsin due to its rigid structure, modifying at essential functional groups could interfere the active site of trypsin (Oupicky et al., 1999) or the chemical modification of the trypsin with PAMAM dendrimer may cause some degree of aggregation (Kang et al., 2005).

7.1.1.2 Molecular weight

Three different molecular weights of PEG (1,100, 2,000 and 5,000 g/mol) were used in this study (Chapter 3). Trypsin conjugates which used PEG 5,000 g/mol had a greater half-life than trypsin conjugates which used PEG 1,100 and 2,000 g/mol and native trypsin when compared in the same of linker (Table 3.4). This result was parallel to another experiment. Comparison between dextrin I (low Mw; 8,100 g/mol) and dextrin II (high Mw; 61,000 g/mol) (Chapter 5), dextrin II-trypsin conjugate showed higher thermal stability, better stability to autolysis and higher half-life than dextrin I-trypsin conjugate (Table 5.3). It might be expected that the higher molecular weight provide the longer polymer chain affected to more steric hindrance which could envelop the protein surface from proteolytic degradation (Gaertner and

Puigserver, 1992; Zalipsky, 1995; Zhang et al., 1999; Treetharnmathurot et al., 2008). However, there are no relationship between molecular weight and the reduction of enzyme activity in this study.

7.1.1.3 The number of –OH groups

Previous study reported that polysaccharide modification could improve enzyme stability because the glycosidic –OH groups can create hydrogen-bonding to protect and stabilize proteins. The hydrophilic polysaccharide can prevent the enzyme from direct interaction with distorting non-conventional media by coating on the enzyme surface, so some hydrophobic pockets might be masked by the hydrophilic polysaccharide (Mislovicova et al., 2006). In this study, among biodegradable polysaccharide-trypsin conjugates (Chapter 4 and 5), dextrin II-trypsin conjugate showed the greatest thermal stability and longest half-life than dextrin I- and β-CD-trypsin conjugates due to higher number of –OH groups caused by the larger polymer chain.

Interestingly, the ST-HPMA-trypsin conjugates (Chapter 5) displayed lower thermal stability at high temperatures, and had a lower half-life for autolysis than dextrin I-trypsin conjugate even though these polymers were of similar molecular weight. This may also reflect the better protective properties of the poly (glucose) polymer.

7.1.2 The type of linker and the number of conjugating bonds

Besides polymer architecture and size, the type of linker between the protein drug and a polymer carrier and the number of conjugating bonds should be concern for improving the stability of conjugated protein as studied in Chapter 3. Specific linkers are often used to control the rate of BAPNA hydrolysis. The results from Chapter 3 showed that the type of linker affected to the number of conjugating bonds (Table 7.1). The number of conjugating bonds when used succinoylated mPEG, cyanurate mPEG and tosylated mPEG for coupling to 1 molecule of trypsin were 5, 5 and 7 molecules, respectively. This might be because of the more reactive leaving group of tosylated mPEG. However, the number of conjugating bonds did not directly

relate to enzyme activity and stability of the conjugates (as shown in Table 7.1) but it was a factor which should be concern.

Biodegradable linker should be the one of development of polymer-protein conjugate. The design of degradable polymer-protein linkages was to maximize the return of protein biological activity. Although, there are no new degradable linkers have been described specifically for polymer-protein conjugation but some biodegradable linkers for drug delivery have been mentioned. An established example was the tetrapeptide linker GLY-PHE-LEU-GLY, which was specifically cleaved by the lysosomal thiol-dependent protease cathepsin B in the lysosome. This tetrapeptide linker was popularized by the successful design of HPMA copolymer-GLY-PHE-LEU-GLY-doxorubicin conjugates (Duncan, 2003). Other examples of biodegradable linkers have included cathepsin B and D degradable peptide linker which used to link between chelated radiometal and the antibody for decreasing hepatic radiation dose. The radioimmunoconjugates could improve the therapeutic index for radiotherapy given in combination with bone marrow support (DeNardo et al., 2003). Acid-labile imine linkers which incorporated onto a polyethylenimine (PEI) backbone could be rapidly degraded into nontoxic low molecular weight PEI in acidic condition. Therefore, acid-labile PEIs may be useful for the development of a nontoxic polymeric gene carrier (Kim et al., 2005). A peptidyl (GLY-ILE-VAL-GLY-PRO-LEU) linker was utilized in a dextran-methotrexate conjugate to achieve tumor-targeted delivery of chemotherapeutics. The peptide linker has been optimized to allow drug release in the presence of tumor-associated enzymes included matrix-metalloproteinases-2 and matrix-metalloproteinases-9 (Chau et al., 2006).

7.1.3 Location of polymer conjugation

The therapeutic impact of PEGylation depends upon the location of the PEG modification and the structure of the conjugates. The beneficial effect of PEGylation has been described as originating from a shell of PEG chains surrounding the protein (Greenwald et al., 2003). PEGylation of biologically active agents has historically been accomplished by covalent conjugation to available primary amines of lysine residues. Significant loss of biological activity

frequently resulted as a consequence of conformational change or steric hindrance (Veronese, 2001). However, the succinoylated polymers used in this study would be randomly attached to lysine amino groups on the trypsin molecule. The enzyme activity would be reduced due to steric hindrance of polymer which preventing access of substrates to the active site caused by the polymer conjugation near the active site (Oupicky et al., 1999).

All conjugates contained PEG which is a non-biodegradable polymer, even though limitations of PEG are well known. PEGylation chemistry is frequently reviewed as it remains a key area of nanomedicines development (Veronese and Pasut, 2005). Site-specific conjugation has become a leading technology in development of polymer-protein conjugation. A maleimide-based protocol for thiol site-specific conjugation has been prepared by Manjula and coworkers (2003). Bovine hemoglobin has a naturally occurring very reactive cysteine at position 93, which has been targeted for site-specific conjugation to PEG-maleimide through a carbamate linkage. It has been found that increasing of hemoglobin molecular size by conjugation to PEG would decrease its undesirable vasoactive properties. The thiol specific conjugation to PEG-maleimide has also been accomplished by Natarajan and coworkers (2005). Single chain Fv antibody fragments (scFv) have been recombinantly produced in a vector which added an unpaired cysteine near the scFv carboxy terminus, thus providing a specific site for thiol conjugation.

Yamamoto and coworkers (2003) have applied the phage display system to create fully bioactive lysine-deficient mutant tumor necrosis factor-α (TNF-α) and carried out site-specific PEGylation for improving therapeutic potency. It has been found that this *N*-terminus mono-PEGylated mutant TNF-α showed higher bioactivity *in vitro* and greater anti-tumor therapeutic potency than randomly mono-PEGylated wild type TNF-α. Furthermore, the unique site-specific conjugation of PEG has been achieved to the lysine at position 18 in salmon calcitonin, using a one-pot synthetic strategy protecting other susceptible amines with 9-fluorenylmethoxycarbonyl (FMOC). This strategy showed the only target isomer without any amino acid sequence change in salmon calcitonin and also had a usefulness for increasing production yield (Youn et al., 2007).

Whereas, in a most recently study, Brocchini and coworkers (2006) developed a protocol that enables the conjugation of PEG to the disulfide bonds found naturally in protein. The irreversible denaturation of protein or alteration of the protein sequence did not occur. This new method of conjugation can be site-specific PEGylated without destroying the tertiary structure of protein molecule or abolishing its biological activity. UnPEGylated protein can be recovered and recycled. Therefore, disulfide bridging PEGylation is a cost-effective process for making a homogeneous product and also offers the potential to make PEGylated biopharmaceuticals as cost effective medicines (Brocchini et al., 2008).

7.2 The factors affected to activity and stability of polymer-protein conjugates

7.2.1 The factors affected to enzyme activity

Covalent linkage of a large polymer to a globular protein such as trypsin or ribonuclease A could have affected on steric hindrance, its conformation and potentially altered the intrinsic flexibility of polypeptide chain by dampening specific motions. The reduction of enzyme activity on polymer conjugation could be described by (Oupicky et al., 1999):

(1) The protein structure was denatured during chemical modification

(2) The modification of essential functional groups may alter the construct of active site and make the enzyme less effective.

(3) The modification of functional groups close to the active site resulting in steric hindrance and preventing access of substrates to the active site.

(4) The polymer attachment may cause refolding of the protein chain or the conformational transition of enzyme requested to accomplish the catalysis.

Although, the undesirable effects of PEGylation are often attributed to steric hindrance by PEG chains blocking the access to active site for molecular recognition as described above, but the effects of polymer on protein structure and dynamics is poorly understood that making the production of active biomaterials a largely unpredictable process. Gonnelli and

Strambini (2009) have extensively investigated the possible effects of the length of polymer chain, markedly between 5 kDa and 20 kDa of PEG, on the conformation and internal flexibility of polymer-protein conjugates. They found that the length of PEG chain did not perturb on the protein conformation and internal dynamics which correlated to structural flexibility, over a wide temperature range. Tagging protein molecules (e.g. enzymes, immunoglobulins or therapeutic polypeptides) with PEG of various molecular weights can be quite safe and need not lead to adverse effects on their biological function. This conclusion supported the notion that any loss of activity of PEGylated proteins was most likely associated to steric hindrance of recognition sites. However, Veronese et al (2007) have reported that the PEGylation of the granulocyte colony stimulating factor (G-CSF) has led to increased aggregation a result explained by subtle variation in protein conformation with exposure of hydrophobic residues.

7.2.2 The factors affected to thermal stability

The stabilization effect of polymer conjugation which covalently bound to trypsin may be explained as followed:

(1) Polymer conjugation caused the microenvironmental change in protein surface of polymer-protein conjugates by formation of a hydration layer around the protein (Venkatesh and Sundaram, 1998; Zhang et al., 1999).

(2) The electrostatic and hydrophobic properties were important for describing the stabilization effect of polymer-protein conjugates. Shielding or masking with large polymers may reduce the non-polar groups on enzyme surface (Mozhaev et al., 1990; Zhang et al., 1999). Increasing the hydrophilic properties of the protein surface also slightly increased the affinity of the modified enzyme for the substrate (Gaertner and Puigserver, 1992).

(3) Within the polymer-protein conjugates, it might occur several intramolecular cross-linking which increased the rigidity by reinforcing of enzyme structure. This reinforcement could restrain the unfolding of the protein molecule (Wong and Wong, 1992).

(4) The polymers displayed a “cage” like effect on the enzyme by bracing the molecule together therefore, the active site of conjugated enzyme has sufficient mobility whereas

the surrounding area was sequestered to become more rigid (Venkatesh and Sundaram, 1998). Juszczak et al (2002) have studied the tertiary conformation of hemoglobin conjugated with PEG. They also concluded that this effect arise from the osmotic impact of a large, close PEG molecule enveloping the surface of the protein leading to more compact, presumably more rigid protein configuration.

(5) Chemical modification at LYS residue as a specific site could prevent autodegradation of enzyme itself (Venkatesh and Sundaram, 1998).

7.3 Suggestions for future studies

All polymer-trypsin conjugates (except PAMAM conjugate) significantly improved the thermal stability and prolonged the $t_{1/2}$ of trypsin. The results obtained from this research were useful for formulation development of protein drug. Therapeutic enzyme using as an active ingredient must be stable, retain biological activity and avoid protein denaturation after formulation process. Thermal stability of therapeutic enzyme is a good indicator to predict activity of enzyme. In these studies, the dextrin II-trypsin conjugate showed better stability compared to the native trypsin and other trypsin conjugates when tested at high temperature. These can be expected that dextrin II conjugate might keep their enzyme activity during formulation process for better quality products. The dextrin II-RNase A conjugates also showed cytotoxicity and prevention of proliferation in B16F10 melanoma cell line, while previously no cytotoxicity has been demonstrated *in vitro* study for the native RNase A.

Dextrin II is interesting to use as bioresponsive polymer-protein conjugates that show the potential to be applied to selective and site-specific delivery for further development of polymer-protein conjugates. The study of masking and reinstating dextrin II-RNase A activity should be examined to proof the PUMPT concept. Following the preliminary cytotoxicity study, future work should be accomplished to understand the mechanism of action of these RNase A conjugates e.g. cellular uptake, binding and the release of the RNase A into cytosol where it may induce apoptosis. It was hoped that conjugation to polymer might increased RNase A tumor targeting by the EPR effect and also improved stability of enzyme.

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VITAE

Name Miss Benjaporn Treetharnmathurot

Student ID 4653003

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Pharmacy (second class honors)	Prince of Songkla University	2003

Scholarship Awards during Enrolment

- Scholarship from the Thailand Research Fund under the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0025/2547), 2003-2009.
- Prince of Songkla University grant, 2005-2007.
- Excellence Academic Support Fund of Faculty of Pharmaceutical Sciences, Prince of Songkla University, 2008.

List of Publications and Proceedings

List of Publications:

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