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HESylation – a new technology for polymer conjugation to biologically active molecules

Modification of proteins and low molecular weight substances with hydroxyethyl starch (HES)

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HESylation – a new technology for polymer conjugation to biologically active molecules

Modification of proteins and low molecular weight substances with hydroxyethyl starch (HES)

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Michele Orlando

Geboren am 12. Dezember 1975 in Potenza (Italien)

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Alla mia famiglia.

The experiments for the present work were performed from September 2000 until July 2003 in the laboratories of the Biotechnologie-Gesellschaft Mittelhessen mbH (Bim GmbH) in Giessen under the supervision of Prof. Dr. Alfred M. Pingoud.

Dekan: Prof. Dr. Jürgen Mayer
Institut für Biologiedidaktik, FB 08
der Justus Liebig Universität Gießen
Karl-Glöckner-Straße 21c
D-35394 Gießen

Referent: Prof. Dr. Alfred M. Pingoud
Institut für Biologie, Chemie und Geowissenschaften, FB 08
der Justus Liebig Universität Gießen
Heinrich-Buff-Ring 58
D-35392 Gießen

Korreferent: Prof. Dr. Jürgen Hemberger
Fachbereich KMUB - Biotechnologie
der Fachhochschule Gießen-Friedberg
Wiesenstraße 14
D-35390 Gießen

"L’alto disio che mo t’infiamma e urge,
d’aver notizia di ciò che tu vei,
tanto mi piace più quanto più turge;
ma di quest’acqua convien che tu bei
prima che tanta sete in te si sazi"

(Dante Alighieri – Paradiso XXX, 70-74)

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Hilfsmittel verwendet habe. Die Arbeit wurde so oder in ähnlicher Form noch keinem anderen Prüfungsausschuss vorgelegt.

Gießen, 9. Oktober 2003

Michele Orlando

Curriculum vitae

Born: 12. December 1975

in: Potenza (Italy)

Education:

1989-1994	High school at Liceo Classico "Q. O. Flacco" Potenza (Italy) graduated with the note 60/60.
1994-1999	Study of Pharmaceutical Chemistry and Technology (CTF) at the "Federico II" University in Naples (Italy).
1997-1999	24 months of laboratory works in the department of Pharmaceutical Chemistry in collaboration with prof. G. Caliendo reseach group. Main topics of the researches were organic synthesis of pharmacologically active molecules and peptide synthesis.
23.07.1999	Master degree obtained discussing the thesis: "Synthesis and characterisation of new 1,2,3-benzotriazin-4-one-arylpiperazine derivatives as 5-HT_{1A} serotonin receptor ligands". Final note 110/110 summa cum laude.
since 05.2001	Ph.D. student in biochemistry at the "Justus Liebig" University of Giessen (Germany). The research works are performed in the laboratories of Biotechnologie Gesellshaft Mittelhessen mbH under the supervision of prof. dr. A. Pingoud.

Professional experience:

09.1999 – 12.1999	Training period at Biotechnologie Gesellschaft Mittehessen mbH (Giessen, Germany) supported by the Leonardo EU program.
01.2000 – today	Working at Biotechnologie Gesellshaft Mittelhessen mbH as researcher.

Patent applications:

03.2002	Kopplung von Proteinen an ein modifiziertes Polysaccharid *(Coupling of proteins to a modified polysaccharide)* International publication N°: WO03074087
03.2002	Kopplung niedermolecularer Substanzen an ein modifiziertes Polysaccharid *(Coupling of low molecular weight substances to a modified polysaccharide).* International publication N°: WO03074088
11.2002	Water-soluble prodrugs of Propofol. PCT N°: PCT18421
03.2003	Pharmaceutically active oligosaccharide conjugates. European patent application N°: EP27268

Publications:

2003	Highly water soluble derivative of Amphotericin B having the same antimycotic potential of the original drug. *(in preparation)*
2003	Increase in selectivity of Amphotericin B after conjugation with a biocompatible polysaccharide. *(in preparation)*

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3D	Three-dimensional
Abs	Absorption
AU	Absorption units
BCA	Bicinchoninic acid
BMS	Bristol Meyer Squibb
BSA	Bovine serum albumin
CDI	Carbonyl diimidazole
CE	Continuum electrostatic
CFU	Colony forming units
CMC	Critical micellar concentration
CRE	Creatinase
DCC	N,N’-dicyclohexylcarbodiimide
DIN	Deutsches Institut für Normung
DMAB	p-dimethylaminobenzaldehyde
DMAP	N,N-dimethylamino pyridine
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulfoxide
DS	Degree of substitution
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
ELISA	Enzyme-linked immunosorbent assay
EPR	Enhanced permeability and retention
FDA	Food and drug administration
GPC	Gel permeation chromatography

GP-FPLC	Gel permeation fast protein liquid chromatography
GP-HPLC	Gel permeation high pressure liquid chromatography
HES	Hydroxyethyl starch
HES_{xx}	Hydroxyethyl starch with a molecular weight of XXkD
HOBt	Hydroxybenzotriazole
HPMA	N-(2-hydroxypropyl)methylacrylamide
HSA	Human serum albumin
IC_{50}	Concentration needed to reach a 50% inhibition
LD_{50}	Dose that kills 50% of the test animals
LMW	Low molecular weight
LS	Light scattering
Lys	Lysine
MALDI	Matrix assisted laser desorption / ionisation
MEAD	Macroscopi electrostatics with atomic details
MIC	Minimal inhibitory concentration
Mn	Number average molecular weight
mPEG	Monomethoxy polyethylene glycol
MRI	Magnetic resonance imaging
MS	Molar substitution
MW	Weight average molecular weight
oxHES	Oxidised hydroxyethyl starch
$oxHES_{xx}$	Oxidised hydroxyethyl starch with a molecular weight of XXkD
PacM	Poly (N-acryloylmorpholine)
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffered saline

PDB	Protein data bank
PDI	Polydispersity index
PEG	Polyethylene glycol
peroxHES	Periodate oxidised hydroxyethyl starch
pfPh	Pentafluorophenol
pnPh	p-nitrophenol
PTM	Post-translational modification
PVP	Polyvinylpyrrolidone
RIA	Radio immunoassay
rIL-2	Recombinant interleukin-2
RPC	Reverse phase chromatography
RP-HPLC	Reverse phase high pressure liquid chromatography
RT	Room temperature
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TCA	Trichloracetic acid
TEA	Triethylamine
U	Units
UV	Ultraviolet
XO	Xanthine oxidase
λ	Wavelength

Zusammenfassung

Polymerkonjugate sind für die pharmazeutische Industrie von erheblichem Interesse, da sie sich für das “Drug delivery” von niedermolekularen Substanzen, aber auch von komplexeren Verbindungen wie Oligonukleotiden, Peptiden oder Proteinen einsetzen lassen. Der Schwerpunkt der Forschungsarbeiten liegt dabei auf der Modifizierung von Peptiden und Proteinen. Hauptgrund hierfür ist, dass diese aus therapeutischer Sicht zwar hochinteressant sind, aber auf Grund ihrer häufig unbefriedigenden pharmakokinetischen Eigenschaften wie geringe Stabilität, geringe Löslichkeit, immunogene Wirkung und geringe in vivo-Verweilzeiten Probleme in der klinischen Anwendung mit sich bringen.

Das Thema dieser Arbeit war die Entwicklung einer neuen Kopplungstechnologie, mit Hilfe derer ein Großteil der oben erwähnten Probleme gelöst werden könnte. Dazu wurde Hydroxyethylstärke, ein optimal biokompatibles, semi-synthetisches Polysaccharid als Konjugationspartner ausgewählt.

Die hier berichteten Experimente zeigen die Machbarkeit einer kovalenten Kopplung zwischen dem Polymer und einem Protein mit zwei unterschiedlichen Kopplungsstrategien, die entweder zu einer Amidbindung (Strategie A) oder zu einer Aminbindung (Strategie B) führten. Als Modellprotein für diese Machbarkeitsstudien wurde humanes Serumalbumin (HSA) eingesetzt.

Als Voraussetzung für die geforderte selektive Kopplung nach der Strategie A musste die Hydroxyethylstärke selektiv oxidiert werden. Zwei verschiedene Methoden zur selektiven Oxidation am reduzierenden Ende des Polysaccharids zur Carbonsäure mit sehr hohen Ausbeuten wurden etabliert. Bei der Kopplung des so oxidierten Polymers mit HSA wurden Ausbeuten von > 90% erzielt.

Im Alternativansatz B wurde das Polysaccharid unbehandelt eingesetzt und eine Kopplung über eine Schiff-Basen-Reaktion in Gegenwart eines selektiv reduzierenden Reagens erreicht. Mit dieser Strategie wurden Kopplungsausbeuten von 40-50% beobachtet.

Beide Reaktionswege wurden mit Polysacchariden unterschiedlicher molarer Masse im Bereich von 10 bis 130 kD durchgeführt.

Mit einem zweiten Modellprotein, dem Enzym Superoxiddismutase (SOD) konnte zudem gezeigt werden, dass die biologische Aktivität des Enzyms im konjugierten Zustand komplett erhalten wurde.

In Doppel-Immundiffusionstests konnte gezeigt werden, dass die HES-Protein Konjugate anscheinend eine geringere Reaktivität gegen Antikörper haben, welche das unmodifizierte Protein mit hoher Affinität binden. Wahrscheinlich maskieren die Polymerketten durch sterische Hinderung antigene Epitope auf dem Zielprotein, so dass die Bindung der Antikörper erschwert wird.

Die HES-Kopplungstechnolgie konnte weiterhin für die Konjugation von niedermolekularen Wirkstoffen adaptiert werden, deren breite klinische Anwendung durch pharmakokinetische oder toxische Probleme limitiert ist. Für die Modifizierung dieser Stoffe wurde eine elegante Methode gefunden, die ohne Aktivierungsreagentien auskommt. Das selektiv oxidierte Polysaccharid wird dabei durch Elimination eines Wassers in die Lactonform überführt. Die Lactonform stellt in wasserfreien Medien eine aktivierte Form der Carbonsäure dar, die in der Lage ist, mit einer Aminogruppe im Sinne einer Aminolyse zur stabilen Amidbindung zu reagieren.

Die beschriebene Reaktion wurde mit Amphotericin B (AmphoB) durchgeführt, ein sehr effektives Antimykotikum, das jedoch eine sehr geringe Wasserlöslichkeit besitzt, die als Ursache für zahlreiche, zum Teil schwere Nebenwirkungen wie Nieren- und Lebertoxizität angesehen werden. Das AmphoB-HES-Konjugat behält seine volle antimykotische Wirksamkeit und weist zudem eine, im Vergleich zum unmodifizierten AmphoB mehr als 1000-fach höhere Wasserlöslichkeit auf. Des weiteren gibt es Hinweise auf eine erhöhte Selektivität des Konjugats gegenüber Pilzmembranen im Vergleich zu Membranen der Wirtszelle. Das Konjugat wies eine unerwartet hohe thermische Stabilität auf. Bei Lagerung als wässrige Lösung unter Raumtemperaturbedingungen wurde nach mehr als 90 Tagen keine Abnahme der antimykotischen Aktivität gefunden.

Da diese neue Technologie von bedeutendem kommerziellen Interesse ist, wurden mit den in dieser Arbeit gewonnen Daten zwei Patentanmeldungen eingereicht, die Schutzrechte für die Kopplung in wässriger Phase und die Aminolyse des HES-Lactons in wasserfreien Medien erwirken sollen:

WO03074087 – Kopplung von Proteinen an ein modifiziertes Polysaccharid;

WO03074088 – Kopplung niedermolekularer Substanzen an ein modifiziertes Polysaccharid.

Abstract

Polymer conjugation attracts increasing interest in pharmaceutical industry for delivering drugs of simple structure as well as complex compounds such as oligonucleotides, peptides and proteins. However, by far the most active research field is peptide and protein conjugation, mainly because they are therapeutically interesting compounds with very unsatisfactory pharmacokinetics (e.g., low stability, solubility problems, immunogenicity and low residence time in the body).

The objective of this work was to develop a new conjugation technology, which might be able to overcome some of these limitations. This technology uses hydroxyethyl starch, a highly biocompatible semi-synthetic polysaccharide, as a polymeric carrier.

Our experiments demonstrated the feasibility of a covalent coupling between polymer and protein with two different coupling strategies yielding amide (approach A) and amine bonds (approach B), respectively. The protein chosen as a model for the optimisation phase was human serum albumin.

As prerequisite for the desired selective coupling according to approach A, the polysaccharide previously needs to undergo a selective oxidation process. Two different selective oxidation methods were optimised to obtain in high yield one unique carboxylic function per polymer chain. The coupling of the oxidised polymer to HSA according to approach A resulted in yields greater that 90%.

Approach B uses the polymer as such and the coupling exploits a Schiff's base reaction in presence of a selective reducing agent. This strategy gave yields around 40-50%.

Both these reactions were carried out with polymer chains of different size in the range of 10 – 130 kD.

Besides the chemical feasibility of these new conjugation reactions, it was moreover shown, by using a model enzyme (superoxide dismutase) as target protein, that it is possible to obtain a conjugate which still completely retains the functionality of the original protein after the chemical modification.

Furthermore the conjugates seem to have a much lower reactivity towards antibodies than the original proteins, as shown by a double immunodiffusion testsystem. Probably the polymer chains mask some antigenic epitops by sterically hindering the accessibility of the protein surface.

The coupling technology was furthermore adapted to the conjugation of drugs with low molecular weight, which present administration limits due to their pharmacokinetics. In

this case a “cleaner” coupling strategy was exploited. The selectively oxidised polysaccharide was obtained in the lacton form by simply eliminating a water molecule. The lacton being a reactive form of the carboxylic acid, yielded a stable covalent bond without the need of any other reagent than the polymer and an amino function of the the drug itself.

The conjugation was performed with Amphotericin B, an antifungal drug which is very effective but presents solubility problems that turn out to be the origin of many side effects. The hydroxyethyl starch-Amphotericin B conjugate, besides keeping intact the whole antimycotic potential of the drug, was found to increase the water solubility almost 1000-fold and showed evidences of a better selectivity in distinguishing the pathogen from the host. Moreover, the conjugate showed an unexpected stability in solution. After 90 days at room temperature the antimycotic potential was still the same compared to the original, non-modified drug.

Since this new technology is of considerable commercial interest, the data obtained in this work have lead to two patent applications:

WO03074087 – Coupling of proteins to a modified polysaccharide;

WO03074088 – Coupling of low-molecular weight substances to a modified polysaccharide.

1 Introduction

It has long been a dream in medicine and pharmacy to use peptides and proteins as drugs. Driving force for this interest is the ability of these substances to eliminate toxic or overproduced compounds from the body and to mimic endogenous hormones, cytokines and other effectors. Already in 1910 Paul Ehrlich was dreaming of “magic bullets” able to destroy the pathogen without harming the host. New therapies often need to interfere with biochemical pathways to compensate for the loss of equilibrium caused by the pathological effect and therefore have to involve delicate regulation mechanisms. In this respect proteins and peptides may be ideal candidates.

A therapy with protein-drugs needs to restore the physiological conditions in the same way a healthy system would do, in a way that the body can accept without having big troubles. By using this approach in the past, two major hurdles were evident for protein-drugs. First, there was a difficulty in obtaining sufficient amounts of material. Small peptides could be made by chemical synthesis, but larger molecules could only be the result of laborious extraction and purification processes from natural sources. The second problem was (and still is) how to deliver the molecules to the body ensuring that a significant portion of the administered dose is able to reach the desired target. Oral delivery of proteins remains impossible because proteins are routinely destroyed by the digestive system. Even injected proteins generally have a poor pharmacokinetic behaviour because of rapid renal excretion and proteolytic metabolism. As foreign entities they may additionally cause significant immunological reactions in the body. Finally, proteins are difficult to formulate because of their intrinsic instability.

The improvement of recombinant gene technology has enabled the rapid development of protein therapeutics. Cytokines and other biological response modifiers, thrombolytics, adhesion molecules, agonist and antagonist peptide fragments of growth factors, and their receptors, all have widespread applications. Clinicians today use a variety of proteins and peptides for diagnosing, monitoring, and treating disease states. These proteins and peptides provide new approaches to old dilemmas, but they raise new challenges as well.

The main difficulty with most of these therapeutic proteins is their non-human origin [1]. There are various methods of protein production available commercially, including bacterial, insect, fungal, and mammalian cell culture systems. However, they each suffer, to varying degrees, from two major limitations. First, many therapeutic proteins have specific configurations that are necessary for activity. In addition, they frequently require quite complex

post-translational modifications (PTM). Some proteins require glycosylation for activity or to ensure that they are not cleared from the circulation too quickly [2].

Bacterial expression systems cannot perform most of these modifications and can subtly alter the folding of the protein.

Yeasts and higher plants can make many of the simpler additions, but are limited in their ability to perform complex modifications.

One form of PTM found only in mammalian cells is γ-carboxylation, which is essential for the vitamin K dependent proteins, such as Factor IX, used to treat haemophilia B, and protein C, an anti-coagulant [2]. Although these proteins can be produced in mammalian cell culture systems, the levels of expression are very low and attempts to increase it have not been very successful.

Because of their production system, these proteins often have poor stability and short half-lives in vivo, and their repeated use leads to both an immunogenic response (formation of antibodies) and an antigenic response (reaction to specific antibodies). The body's enhanced immune response results in increased clearance of the protein, causing a vicious cycle of raising the dose, which enhances the immune reaction, which increases the clearance. The ultimate result is a lack of effectiveness. Investigators have, therefore, turned to proteins derived from human sources [3].

The development of biotechnology led to the large-scale production of human-derived proteins and less reliance on non-human sources. These proteins provoked fewer allergic reactions, but the problems of poor pharmacokinetics and sub-optimal pharmacodynamics remained. Most parenterally administered proteins are rapidly cleared from the circulation by the reticuloendothelial system, liver, kidney, and other organs. The rate of clearance depends on the ionic charge, the size of the molecule, attached carbohydrate chains, and the presence of cellular receptors for the protein. Plasma proteases or plasminogen activators cause degradation and rapid loss of biological activity, so achieving a clinical effect is still a problem [4].

Many approaches were suggested to enhance the protein delivery and to alleviate problems of immunogenicity, inactivation by plasma proteases, and poor pharmacokinetics. Sustained-release technologies were a first step. These technologies include protein entrapment in soluble and insoluble matrices such as liposomes, microspheres, hydrogels [3]. Problems associated with this kind of approach were, in particular, that all these particles were rapidly sequestered in the liver, spleen, kidneys and reticuloendothelial system, and some of those can also act as immunological adjuvants.

In principle, another possibility to avoid immunogenic reaction is to shield antigenic epitopes on a protein. This may be achieved by attaching an inert water-soluble polymer to the protein surface. With the soluble–polymer strategy one tries to mask all determinant sites on the protein molecule which cause the body to recognise it as foreign and thereby to protect the protein against the degradation by the host. Objectives include a stable linkage between the protein and the polymer, increased water solubility, low immunogenicity, prolonged half-life, and intact biological activity. Polymers studied include polyethylene glycol (PEG), dextran, albumin, styrene-maleic acid anhydride, and polyvinyl pyrrolidone.

1.1 Polymer protein conjugation

The rationale of polymer conjugation to proteins for pharmaceutical applications is: attachment of soluble and biocompatible polymers to improve the protein stability and the pharmacokinetic profile. As a matter of fact, one of the major drawbacks in the use of biologically active proteins in therapy is the common short body residence time of these molecules that are either rapidly removed by renal ultrafiltration or inactivated by the immune system or by plasma enzymes. After polymeric conjugation, the stability of a protein is generally enhanced because the polymer increases the drug's volume which protects it from enzymatic and hydrolytic degradation and shields its immunological epitopes. The polymer may also direct the bioconjugate to specific organs or districts in the body.

Many polymers have been studied for these applications, the most popular are dextran (a polyfunctional polymer) and monomethoxy-PEG (a mono-functional entity). Other mono-functional polymers with properties similar to mPEG have also been studied. Examples are, a new form of poly (N-vinyl pyrrolidone) [5] and poly (N-acryloyl morpholine) [6], both having a single activable end terminal residue (COOH or OH) per polymer chain. Poly (oxazolines) and poly (vinyl alcohol) are also mono-functional polymers that have gained some popularity due to their intrinsic properties. Both have been considered for protein modification [7, 8].

1.1.1 Dextran – protein conjugates

In the literature one can find reports on at least 20 polypeptides and proteins conjugated with polysaccharides, mainly dextrans. These involve streptokinase, insulin, pullulanase, lysozyme, α-amylase, β-amylase, trypsin, superoxide dismutase, β-glucosidase, plasmin, asparaginase, haemoglobin, chymotrypsin, carboxypeptidase, arginase and catalase [9].

Streptokinase was the first therapeutic enzyme to be conjugated to a polymer (dextran of 35-50kDa molecular mass) with significant therapeutic success. Since 1980, after its approval for clinical use in the treatment of cardio-vascular and ophtalmological pathologies caused by thrombosis, it has been produced in Russia, on a large scale, under the trade name of "Streptodekase". This streptokinase conjugate is characterised by long body permanence in humans where it can last for over three days. As a consequence, streptokinase may be administered in a single bolus instead of by continuous infusion as needed for the non-modified form, whose body permanence is of only a few minutes. Also the overall toxicity of streptokinase is decreased after dextran conjugation, as demonstrated by reduced hemorragic complications, rethromboses and allergic reactions [10].

Despite the important results obtained with Streptodekase, dextran has not gained general success in polymer derivatisation. The main reason is found in the relatively high incidence of severe anaphylactoid reactions. Dextran has been used for a variety of pharmaceutical preparations over the past 40-50 years. The wide use of dextrans has included purified native dextrans for plasma replacement/volume expansion, dextran-active conjugates, iron-dextran iron supplements, and dextran coated particles for MRI contrast agents. Whereas the majority of the patient population seems to tolerate dextran conjugates quite well, a minority reacts with severe anaphylactoid responses that in some cases resulted in death of the patient (as noted in the Physicians Desk Reference product information for "INFeD", Edition 51, pp. 2478, 1997). A second reason is the non-homogeneity of the product, because both dextran and proteins are poly-functional molecules and their coupling usually yields a complex and heterogeneous mixture of conjugates. This problem may be partly overcome by using mono-functional polymers [11, 12].

Only in certain specific cases are poly-functional polymers preferred to the mono-functional ones in protein modification. This happens when an increased conformational stability is needed as, for example, when the protein is to be used as biocatalyst in organic solvents. In this case the reticulated complex that is formed from the reaction between the two poly-functional entities often confers a higher stability towards denaturing environments.

1.1.2 Polyethylene glycol – protein conjugates

However, it was the development of the polyethylene glycol conjugation technology that provided the real breakthrough in enhancing the pharmaceutical properties of proteins. This technology is generally known as PEGylation. PEG offers several advantages as a polymer, because it has a linear structure and is hydrophilic (the oxygen in PEG forms hydrogen bonds with water molecules). Its quasi-random coil conformation and the high hydration level confers the PEG molecule a hydrodynamic radius corresponding to a three times bigger molecule [1, 3, 4]. PEG is non-charged and thus offers flexible bonding [13]. PEG is approved by the FDA in a variety of pharmaceutical preparations, such as some intravenous immune globulin formulations as well as in food and cosmetics. PEG has little toxicity when given orally or intravenously or when used as an epidermal preparation [3]. The theoretical benefits of coupling a protein to PEG include an increased half-life (which would have the greatest clinical effect), decreased immunogenicity, decreased antigenicity, increased resistance to proteolysis, and increased solubility [4].

PEG strands appear to protect a protein by causing steric hindrance. This shield effect block the recognition from the immune system. PEG also inhibits interaction with cell-associated receptors and enzymes that may degrade the protein. The result is an increase in protein half-life. The gain in molecular weight that PEG adds to the molecule also contributes to the increase of the residence time in blood by decreasing the glomerular filtration rate. It is believed that the strands of PEG form a shell around the protein masking the majority of the surface.

1.2 Benefits of polymer modification for protein pharmaceuticals

1.2.1 Increased plasma half-life

PEG modification has extended the plasma half-life of numerous proteins by a factor of 3- to 486-fold (tables 1.1 and 1.2) [14].

In general, the more polymer is attached per molecule, the greater is the extension of half-life [15-18], but the price paid may be reduced specific activity of the protein. Proposed mechanisms will be discussed below.

Table 1.1

Influence of polymer conjugation on proteins plasma half-life in humans

Protein	Native $t_{1/2}$ = (h)	Modified $t_{1/2}$ = (h)	Times increase
Asparaginase	20	357	17.7
Asparaginase	72	528	7.3
Glutaminase-asparaginase	< 0.5	72	> 144
Uricase	< 3	8	> 2.7
Superoxide dismutase	0.42	204	486

Table 1.2

Influence of polymer conjugation on protein plasma half-life in other species

Protein	Animal	Native $t_{1/2}$ = (h)	Modified $t_{1/2}$ = (h)	Times increase
Asparaginase	Mouse	< 6	96	> 16
Asparaginase	Rat	2.9	56	19.3
Asparaginase	Rabbit	20	144	7.2
Glutaminase-asparaginase	Mouse	2	24	12
Superoxide dismutase	Mouse	0.06	17	283
Lactoferrin (PEG-2000)	Mouse	0.05	0.25	5
Lactoferrin (PEG-4000)	Mouse	0.05	1	20
Streptokinase	Mouse	0.07	0.33	4.7
Plasmin-streptokinase cx	Mouse	0.05	0.22	4.4
Adenosine deaminase	Mouse	0.5	28	56
Interleukin-2	Rat	α 0.05 β 0.73	0.32 6.8	6.4 9.3
Bovine albumin	Rabbit	β 93	96	1.03

1.2.2 Reduced renal clearance

The cut-off point for glomerular filtration lies between albumin (mw 66,000), which is retained, and haemoglobin (mw 64,500), which is filtered (with some variation in the filtration of approximately 70-kDa proteins due to shape, charge, etc.). Plasma clearance of unmodified recombinant interleukin-2 (rIL-2) is essentially that predicted for a small protein cleared by glomerular filtration [19]. Experiments with nephrectomised animals support this. The decrease in systemic clearance rate (and associated increase in plasma half-life) of PEG-rIL-2 is not marked when the effective molecular size increases from 19.5 to 21 kDa, but drops largely between 66 to 70 kDa [19]. This effect is thus likely to be due largely to exclusion of the protein from glomerular filtration. However, since above the molecular size of 70 kDa the systemic clearance of PEG-rIL-2 does not drop to zero and it is further reduced by addition of more PEG, an additional clearance mechanism is anticipated. Knauff et al. [19] demonstrated degradation products in the urine with both rIL-2 and PEG-rIL-2. Degradation prior to filtration is unlikely, however, given the abrupt change in clearance as the 70-kDa threshold is exceeded, suggesting metabolism by cells lining the proximal tubule or other sites in the kidney.

Experience with haemoglobin modified with PEG and dextran is similar and consistent with reduced renal clearance [15, 20]. Such a modification, since it retains oxygen-carrying capacity, may allow the construction of blood substitutes. Superoxide dismutase is also cleared by the kidney and shows dramatic increase in half-life when modified (tables 1.1 and 1.2), which is even more marked than that for IL-2.

1.2.3 Reduced cellular clearance

Systematically administered proteins of over 70 kDa are cleared by the reticuloendothelial system or by specific cell-protein interactions. Polymer conjugation appears to reduce cellular clearance, irrespective of mechanism. For example, lactoferrin is cleared by interaction with carbohydrate receptors on hepatocytes [17]. Its half-life increases from < 3 min to 15 min for PEG-2000-lactoferrin and 60 min for PEG-4000-lactoferrin. In contrast to carbohydrate receptor clearance, α_2-macroglobulin-protease complexes are cleared through the reticuloendothelial system receptors for specific sequences of amino acids on the macroglobulin, exposed during complexation with the protease [17, 18]. PEG modification of α_2-macroglobulin-

trypsin complexes and PEG-streptokinase complexed to plasmin and α_2-macroglobulin showed significantly longer plasma half-life than unmodified complexes (although with the latter some reduced ability to complex to α_2-macroglobulin probably also contributes). In the case of α_2-macroglobulin-streptokinase, it has been shown via competition studies that when the PEG-modified complex is ultimately cleared, it is through the same mechanism as that for the unmodified complex [14].

1.2.4 Reduced proteolysis

An additional advantage of polymer modification is the increased resistance to proteolytic degradation (tables 1.3 and 1.4) [14]. The most widely studied model proteases include trypsin, chymotrypsin, and *Streptomyces griseus* protease. This resistance to proteolysis presumably reflects steric hindrance by the PEG strands, surrounding the protein and thereby preventing proteolytic attack. In the case of trypsin, which cleaves at the carboxy-site of lysine and arginine, the presence of PEG covalently attached to lysine represents a direct barrier to cleavage. The extent of modification may relate to the protective effect. However, no formal comparison has been made for a range of proteins to confirm this.

Other factors may contribute to this protective effect. For example, exposure of phenylalanine ammonia-lyase to its competitive inhibitor, cinnamic acid, enhanced the protective effect of PEG modification while not influencing the proteolytic degradation of the unmodified enzyme [21]. This is somewhat puzzling since it suggests that the latter does not merely impede a tryptic cleavage site in the active site region, but must exert some more subtle effect such as conformational change resulting in resistance to digestion of one of the more vulnerable sites in the PEG modified protein. This effect may be of benefit for in vitro as well as in vivo applications.

Table 1.3

Effect of polymer modification on proteolysis by trypsin

Protein	Lysines modified (%)	Proteolytic digestion (min)	Native Activity left (%)	Modified Activity left (%)
Catalase	43	40	0	95
Asparaginase	79	10	12	80
Streptokinase	23	10	50	50
Streptokinase	23	20	18	18
β-glucuronidase	60	20	16	83
Phenylalanine ammonia-lyase	40	10	17	34
*Phenylalanine ammonia-lyase	40	10	17	81

Table 1.4

Effect of polymer modification on proteolysis by other proteases

Protein	Lysines modified (%)	Proteolytic digestion (min)	Native Activity left (%)	Modified Activity left (%)
Protease: Chymotrypsin				
Catalase	43	60	30	98
β-glucuronidase	60	20	11	59
Protease: *Streptomyces griseus*				
Catalase	43	60	10	80
Phenylalanine ammonia-lyase	40	2	43	68
*Phenylalanine ammonia-lyase	40	2	43	79

** Phenylalanine ammonia-lyase associated with cinnamic acid, a competitive inhibitor of the enzyme.*

1.2.5 Reduced immunogenicity and antigenicity

Immunological responses limit the clinical use of many proteins. Even recombinant human proteins may be immunogenic, i.e., they have the ability to invoke antibody formation [22, 23]. Such antibodies can neutralise biological activity (if they bind to key epitopes), cause life-threatening hypersensitivity reactions, or increase clearance by the reticuloendothelial system.

PEG modification frequently reduces both the antigenicity and the immunogenicity of proteins (tables 1.5 and 1.6) [14]. Reduced antigenicity (i.e., the reduced ability to react with pre-existing antibodies) presumably reflects masking of antigenic determinants of the native protein by PEG chains. An unusual exception to reduced antigenicity is PEG-SOD, which reacted with antibodies against SOD to the same extent as the unmodified enzyme [24] (possibly in this case the antigenic determinant is far apart from a lysine residue).

Markedly reduced immunogenicity is frequently observed for polymer-modified proteins (table 6). With individual proteins, such as catalase and uricase, the protein is non-immunogenic only above a certain degree of modification (58% modification in uricase and 43% in catalase) [25, 26]. Thus, the mechanism at work may simply be the shielding of antigenic determinants by a material that is relatively immunologically inert, but may also in part result from avoidance of reticuloendothelial cells [27]. The route of administration may influence the apparent immunogenicity of the native and/or PEG-modified forms (e.g. PEG-1900-catalase, which was non-immunogenic via the intravenous route and modestly immunogenic via the intramuscular route [25]).

Reduced antigenicity and reduced immunogenicity contribute to prolongation of protein half-live. Multiple exposures to the unmodified protein may lead to enhanced clearance by the immune system. In many cases this could be avoided by administration of the PEG-modified protein (figure 1.1) [14].

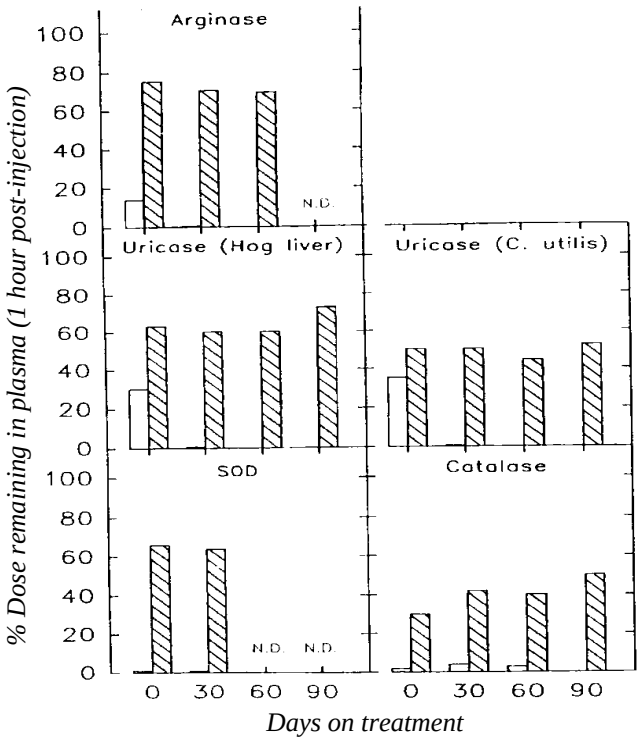


Figure 1.1: Fate of injected protein after single and repeated injections. Filled and open bars represent modified and unmodified proteins, respectively [14].

Table 1.5

Reduced antigenicity in polymer modified proteins

Modified protein	Antigenicity	Test system
Asparaginase	Abrogated	Precipitin
Adenosine deaminase	Abrogated	Immunodiffusion
Uricase	Abrogated	Immunodiffusion
Uricase	Abrogated	Precipitin
Superoxide dismutase	Reduced	Immunodiffusion
Catalase	Abrogated	Immunodiffusion
Catalase	Reduced	ELISA
Arginase	Abrogated	Immunodiffusion
Streptokinase	Reduced	RIA / neutralisation
Streptokinase	Abrogated	Precipitin
ß-glucuronidase	Reduced	Immunodiffusion / RIA
Trypsin	Abrogated	Immunodiffusion
Phenylalanine ammonia-lyase	Reduced	Precipitin / immunodiffusion
Bovine albumin	Abrogated	Immunodiffusion

In the rare cases where a PEG-protein elicit antibody formation, the antibodies produced show a weaker affinity compared to those produced against the native enzyme [21, 25].

There is some evidence of cross-reactivity between antibodies directed against polymer conjugated-proteins e.g., between anti-PEG-uricase and anti-PEG-SOD antibodies [28]. These antibodies, however, do not seem to recognise either PEG itself or the protein; the most plausible suggestion is that the region of the coupling moiety is recognised. Animals sensitised with antiserum to PEG-uricase prepared with the cyanuric chloride method showed a reduced passive anaphylaxis reaction when challenged with PEG-uricase prepared using the succinate method, which has a different coupling moiety [28].

Table 1.6

Reduced immunogenicity in polymer modified proteins

Modified protein	Antigenicity	Test system
Asparaginase	Reduced	Mice
L-Glutaminase – L-Asparaginase	Abrogated	Mice
Adenosine deaminase	Abrogated	Mice
Uricase	Abrogated	Mice
Uricase	Reduced	Rabbits
Catalase	Reduced	Rabbits
Catalase	Reduced	Mice
Superoxide dismutase	Reduced	Mice
Arginase	Abrogated	Mice
ß-glucuronidase	Reduced	Rabbits
Phenylalanine ammonia-lyase	Reduced	Rabbits
Bovine albumin	Abrogated	Rabbits

1.2.6 Increased solubility

An increased solubility is anticipated for any protein modified with PEG since PEG is highly water soluble by virtue of hydrogen bonding of water molecules at the ethylene oxide unit. Increased solubility has been documented for several proteins [26, 29]. This is of benefit for both the formulation and administration of proteins with limited solubility at physiological pH, like some monoclonal antibodies or IL-2 (which precipitates at pH 7 and requires either pH 9 or 10 µg SDS per mg of protein to render it soluble [29]). PEG-IL-2 is readily soluble at pH 7 even at 20 mg/ml.

Insolubility or very low water solubility is a disadvantage shared by many therapeutic proteins due to their expression system. Post-translational modifications missing and folding in a non-homologous organism are basically the reason for such problems. Scarcely soluble proteins tend to aggregate when administered and this may be the basis of phlebitis observed at infusion sites [30] and also of rapid clearance.

1.3 Problems and solutions in polymer-protein conjugation

The unusual properties that the polymer conveys to proteins are the basis of this successful technology. Nevertheless there are many relevant questions still open. It is important to remember the limits depending on the chemistry. Proteins often have solubility problems, they must be handled with particular care to avoid denaturation, precipitation and loss of activity. The coupling reaction ideally should involve chemical conditions, compatible with the protein properties.

A second problem is the analytical characterisation of the coupling product. As proteins and polymers are poly-functional entities the coupling reaction will lead to a really complex mixture of different isomers. Several protein molecules could bind the same polymer chain, several polymer chains could be attached on the same protein or finally a reticulated system could be built by cross-linking. A successful strategy, to overcome some of these problems, has been the use of a mono-functional polymer. The monomethoxylated form of PEG is generally used in protein conjugation, since its mono-functionality yields more defined products. In this way the cross-linkage problem is mainly solved.

But even with such polymers and mild chemical conditions, the polymer-protein conjugate is still keeping a low characterisation level. A common property of polymers is their polydispersity. The polydispersity index (PDI) is a measure of the molecular weight distribution around an average value. The higher the polydispersity, the worse is the quality of the material. In fact, working with polymers, it is not possible to exactly discriminate between chains of similar size. Figure 1.2 shows a typical profile for a polymer molecular weight distribution.

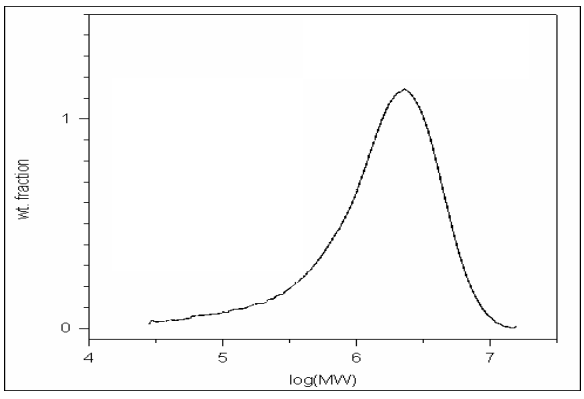


Figure 1.2: Typical Mw distribution of a polydisperse polymer.

As a consequence, polymer-protein conjugates possess the same polydisperse variation in molecular weight, that can only be calculated as an average. A related problem is the difficulty to fractionate the different entities obtained after coupling due to the presence of the polymer that greatly reduces the resolution in any chromatographic medium. This often impairs the purification of single species of conjugates as well as the establishment of the exact number of polymer chains linked to each protein.

Furthermore, the precise sites of polymer coupling along the protein's primary sequence are very difficult to identify. The long-debated problem of the exact quantification of the polymer chains linked to each protein can now be overcome by the use of a single amino acid spacer between polymer and protein. For example, when the unnatural amino acid, norleucine (but also β-alanine) is used as a spacer, amino acid analysis after acidic hydrolysis of the product allows one to calculate the number of bound polymer chains. In fact, this number corresponds to the amount of norleucine evaluated by using certain stable amino acids in the protein as internal reference standard [31]. This method has proven to be more accurate than the more common colourimetric [32, 33] and fluorimetric [34] ones that measure the amount of unmodified primary amines in the protein to calculate the modified ones by difference. On the other hand, all of these methods only give the average value of conjugation. With the more recent MALDI mass spectrometry, the pattern of the individual conjugated species could be obtained in some cases. Unfortunately, this technique cannot be considered quantitative, mainly because each mass species present in the sample is characterised by a different yield of extraction from the matrix in the ionisation process. Therefore, when a mixture of products is analysed, it is not possible to say whether the pattern which appears in the mass spectrum corresponds quantitatively to the composition present in the sample.

Another relevant parameter in the characterisation of the conjugate product is the presence of positional isomers. The number of possible targets on a protein surface are generally never completely modified. That would be the only case where the product would show a better homogeneity, but this in fact is not desirable because a completely modified protein will most probably lose its activity because of conformational changes. Generally the attachment only involves few amino acids of a polypeptide and, unless there is a relevant reactivity difference, the coupling does not take place always at the same site, generating isomers. Each one of these molecules even having the same number of polymer chains attached may show several activity profiles and a different reactivity against the immune system (see chapter 6).

The use of a bulky, branched polymer offers an elegant improvement of this situation. Such polymer does not have the same flexibility as a linear polymer and therefore not every

amino acid can be reached on the protein surface. This mechanism limits the modification sites in the polypeptide, allowing a conjugation to the more accessible amino acids residues only. This has been well demonstrated in α-interferon conjugation that, once modified with a linear polymer, gave rise to eleven positional isomers, whereas, with the branched polymer, only one or two lysine residues were modified [35-37].

A further problem in enzyme conjugation is the possible loss of activity related to the polymer attachment which can induce conformational changes or may involve amino acids located in the active site of the enzyme or in its close environment. This limitation is more relevant for enzymes acting on high molecular weight substrates, because their approach to the active site may be hampered by the hindrance of the polymer bound in close proximity. In some enzymes, the problem has been overcome by carrying out the conjugation in the presence of an enzyme substrate or a reversible inhibitor in solution. A modification of this approach that proved more efficient is to work in a heterogeneous state, in the presence of an active site inhibitor linked to an insoluble resin. Because of steric effects, the resin-inhibitor complex hinders the conjugation also at the active site surroundings [38].

Another method to principally protect the active-site area is the use of a more hindered polymer such as a branched polymer. The hindrance of the branched polymer prevents or reduces the entrance into the cleft of the active site as demonstrated in asparaginase and uricase modification [39] with a branched PEG derivative [40, 41]. A branched polymer ensures also a higher protection from proteases and from the immune system at the same substitution rate. It also masks a much higher surface portion than a linear polymer, therefore a lower numbers of chains are needed to hide the same protein surface. Moreover, as the probability to lose the enzyme activity is related to the substitution rate it is evident that a modification with a branched polymer promises to be more effective in keeping the enzyme activity intact.

1.4 Polymer modification of small drugs

Polymer conjugation is also considered to be relevant for non-protein compounds. Already in the early sixties Ushakov [42] reported conjugation of antibiotics (penicillin) to water-soluble polymers. Also in Russia ten yeas later dextran modification of kanamycin, tetracycline and ampicillin were developed for clinical application. In recent years the use of water-soluble natural and synthetic polymers has received increasing attention. Many kinds of polymers were

combined with a wide spectrum of antitumour drugs to improve their pharmacokinetic profile, body distribution and pharmacological efficacy, respectively. There are nowadays at least seven polymer drug conjugates that have entered phase I/II clinical trial as anticancer agents. These include N-(2-hydroxypropyl)methylacrylamide (HPMA) copolymer-doxorubicin, HPMA copolymer-paclitaxel, HPMA copolymer-camptothecin, PEG-camptothecin, polyglutamic acid-paclitaxel, HPMA copolymer-platinate, and also HPMA copolymer-doxorubicin conjugate bearing additionally galactosamine (specific for liver targeting) [43].

The term "polymer drug conjugate" includes polymer matrices which incorporate the drug as a part of the polymeric backbone, polymers bearing chemotherapeutic units attached to polymer side chains distributed along the polymer backbone, or drugs attached as terminal groups of the polymer backbone. Generally, a covalent attachment is preferred. The conjugated drug molecule has a largely increased size which modifies its pharmacokinetic behaviour. Macromolecules, unlike low molecular weight molecules, cannot enter cells by diffusion, and their uptake by cells is restricted to receptor-mediated transport and passive or active endocytosis [44]. In an endocytic process, macromolecules enter the cell in small vesicles called endosomes. They subsequently fuse with lysosomes containing a variety of enzymes effective in an environment with low pH. The efficiency of lysosomotropic drug delivery depends on the proper choice of the polymer, the drug and the chemistry of conjugation. The drug can be released from the polymer by passive hydrolysis [45], by more specific enzymatic release [46-48], or by pH-controlled release [49]. All these strategies generate a conjugate which somehow acts as prodrug, it merely plays the role of targeting the drug to the desired location.

Another possibility is that the conjugate itself could act as a drug, in other words, that the modified drug keeps its efficacy after the conjugation and does not need a cleavage from the polymer to exert its therapeutic action. For drugs whose targets lie on the outside of the cells (membrane lipids, membrane proteins, membrane receptors, etc.) or as free molecules in the blood stream this approach can be predicted to be successful. For drugs which need to penetrate the cell membrane for being effective, this approach will be considered only as a second choice.

1.5 Advantages in preparation of bioconjugates with low molecular weight drugs

One of the main achievements obtained by coupling low molecular weight drugs to polymers is the modification of the drug's pharmacokinetic profile and, as a consequence, of its bioavailability.

In the bioconjugation technology with high molecular weight polymers described here, several factors act together to affect the pharmacokinetics and, therefore the bioavailability of the drug. Among them the following appear to be relevant:

1. The coupling to a water soluble polymer ensures an improved water solubility. This property allows drugs with application field limited to the topical use to extend its spectrum and to be administered systemically for internal diseases. In fact, an increase in solubility entails that the therapeutic concentration can be reached in water without any surfactant or other substances which may harm the patient or may cause intolerance responses from the organism. Moreover, the water solubility often reduces the drug toxicity which is related to aggregation, accumulation in specific organs or district, precipitation and other phenomena which are typical in the application of poorly soluble molecules;

2. The reduction of renal excretion, due to the large volume of the macromolecular conjugate. This reduction generally occurs when the threshold of serum albumin volume is reached. It is to be noted that polymers often have a hydrodynamic volume much higher than a protein having the same molecular mass. For example for PEG, the critical point of the albumin hydrodynamic volume is reached at a molecular mass of about one third of the albumin;

3. In some cases, polymer coupling was demonstrated to promote a targeted delivery of drugs to body sites characterised by an increased capillary permeability as, for example, inflamed tissues. This phenomenon is thought to be the basis of the so-called "enhanced permeability and retention" (EPR) effect. EPR allows the specific localisation of a drug at the level of cancer tissue thanks to the higher permeability of blood capillaries in that area, accompanied by a reduced lymphatic drainage. Both these phenomena permit the accumulation of the drug-polymer at the site of the tumour tissue through a process similar to ultrafiltration [50];

4. Besides the modification of the pharmacokinetic profiles, the macromolecular characteristics of the bioconjugates are responsible for the exploitation of a totally new pathway

for the drug's entrance into the cell that can only be based on adsorption- or receptor-mediated endocytosis [44]. This new pathway has been exploited even more thoroughly by the design of specific linkage arms between polymer and drug. This linkage has to be stable in blood while cleavable only intracellularly, because of the acidic environment of endosomes or by means of the rich enzymatic machinery of the lysosomes. Sometimes the drug-polymer conjugate possesses intrinsic activity and the hydrolysis step is not needed to obtain a therapeutic effect.

1.6 Objective of the present work

Polymer conjugation is of increasing interest in pharmaceutical chemistry for delivering drug of simple structure or complex compounds such peptides, enzymes and oligonucleotides. However by far the most active research field is polypeptide and protein conjugation, for the two following reasons: first of all because a greater number of therapeutically interesting compounds can now be produced by genetic engineering in large quantity and, secondly, because these products are difficult to administer to patients for several inherent drawbacks.

At the moment the most widely used conjugation technology is PEGylation. Although it represents a historical breakthrough in pharmaceutical technology this strategy shows several inherent limitations, mostly related to the polymer and the chemistry used. Both these aspects will be discussed in more detail in the next chapters. It has taken 20 years for PEGylation to become a standard technique, in part this was due to the time required to improve protein manufacturing, but also it has been necessary for the organic- and polymer- chemistry of PEG to mature. Of course there has been tremendous progress in the understanding of protein structure and properties, and of the PEGylation effect. Obviously this experience gained with PEG conjugation also revealed the limits of this approach. Such limits, although not severe enough to prevent PEGylated molecules from being approved by the FDA and reaching the market, are responsible for several failures (complete or partial loss of activity, relevant aliquots of immunogenicity left, scarcely characterised coupling products, etc.) which have in the last years contributed to reinforce the scepticism and cool down the enthusiasms and hopes raised around such revolutionary strategy. The researchers in the field are trying to fill this structural gap by using new PEG derivatives, anyway the range for improvement does not appear very wide.

As a natural alternative to the synthetic polymer PEG and its derivatives one might consider polysaccharides for use as water soluble, biocompatible polymers.

The drawbacks of polysaccharides in bioconjugation are essentially the following:

- higher immunogenicity than PEG;
- rapid degradation rate in vivo;
- poly-functionality due to unselective activation methods.

The aim of this work is the development of a new strategy of polymer conjugation using a polysaccharide for a highly selective coupling with protein, peptides and low molecular weight drugs. The unique properties of this polysaccharide (inert towards the immune system, very slow hydrolysis in vivo), together with a new selective activation chemistry allow overcoming the above mentioned common drawbacks. As polysaccharide for this work we have chosen hydroxyethyl starch (HES).

2 Hydroxyethyl starch

Hydroxyethyl starch is a semi-synthetic derivative of amylopectin, the highly branched starch component. Starch is the major carbohydrate reserve in plant tubers and seed endosperm where it is found as granules [4], each typically containing several million amylopectin molecules accompanied by a much larger number of smaller amylose molecules. The commonly used sources are maize, wheat, potato, tapioca and rice. Amylopectin (without amylose) can be easily isolated from 'waxy' maize starch whereas amylose (without amylopectin) is best isolated after specifically hydrolysing the amylopectin with pullulanase [1].

2.1 Structural unit

Starch consists of two types of molecules, amylose (normally 20-30%) and amylopectin (normally 70-80%). Both are polymers of α-D-glucose units in the $^4C_1$ chair conformation. In amylose these are linked α-(1→4)- whereas in amylopectin about 5 % of glucose is also linked α-(1→6)-, forming branch-points (see figure 2.1).

The relative proportions of amylose to amylopectin and α-(1→6)- branch-points both depend on the source of the starch, e.g. amylomaizes contain over 50% amylose whereas "waxy" maize has almost none (~3%) [2, 3].

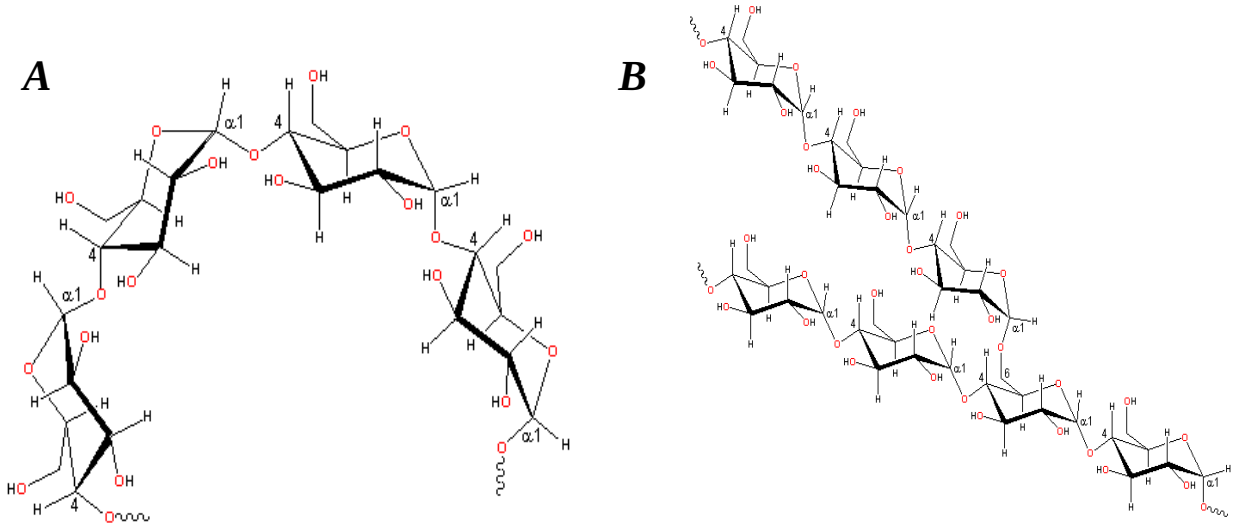


Figure 2.1: Partially representative structure of amylose (**A**) and amylopectin (**B**).

2.1.1 Amylose

Amylose molecules consist of single mostly-unbranched chains with 500-20,000 α-(1→4)-D-glucose units dependent on source (a very few α-(1→6) branches and linked phosphate groups may be found, but these have little influence on the molecule's behaviour) [4]. Amylose can form an extended shape (hydrodynamic radius 7-22 nm [5]) but generally tends to wind up into a rather stiff left-handed single helix (see figure 2.2) or form even stiffer parallel left-handed double helical junction zones [6]. Single helix amylose behaves similarly to the cyclodextrins by possessing a relatively hydrophobic inner surface that holds a spiral of water molecules, which are relatively easily lost to be replaced by hydrophobic lipid or aroma molecules. It is also responsible for the characteristic binding of amylose to chains of charged iodine molecules (e.g. the polyiodides; chains of I_3^- and I_5^- forming structures such as I_9^{3-} and I_{15}^{3-}; note that there is no interaction with neutral I_2 molecules) where each turn of the helix holds about two iodine atoms and a blue colour is produced due to donor-acceptor interaction between water and the electron deficient polyiodides.

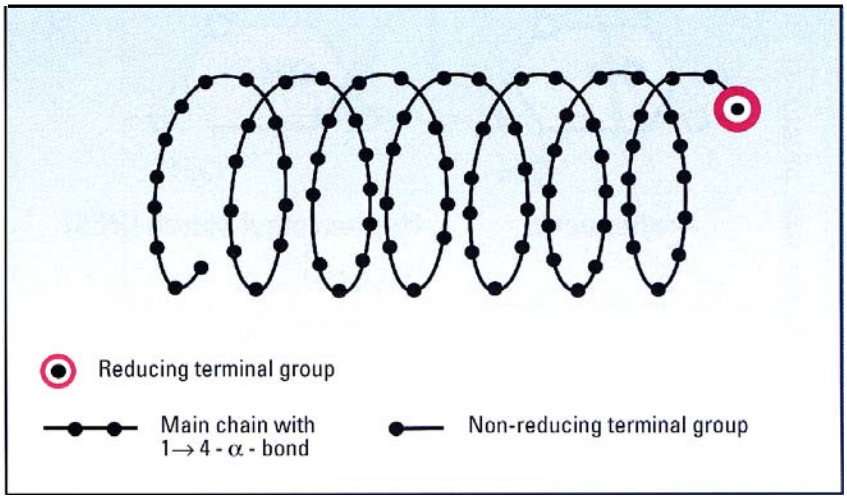


Figure 2.2: Schematic structure of the amylose molecule.

2.1.2 Amylopectin

Amylopectin is formed by non-random α-(1→6) branching of the amylose-type α-(1→4)-D-glucose structure (see figure 2.3). This branching is determined by branching enzymes that leave each chain with up to 30 glucose residues. Each amylopectin molecule contains about a million residues, about 5% of which form the branch points. There are

usually slightly more “outer” unbranched chains than “inner” branched chains. There is only one chain containing the single reducing group.

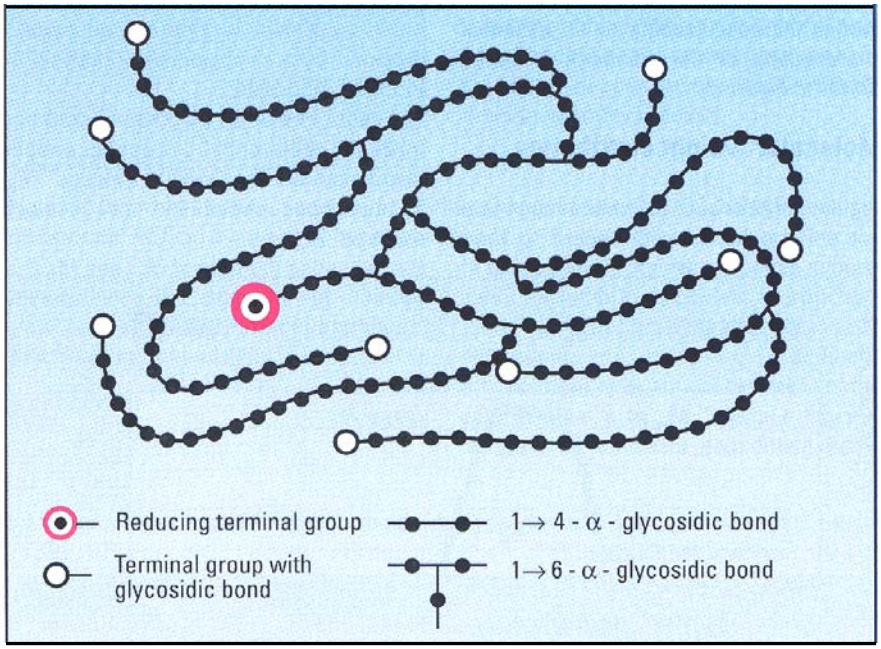


Figure 2.3: Schematic structure of the amylopectin molecule.

Each amylopectin molecule builds up a compact structure with an hydrodynamic radius of 21-75 nm [5]. The molecules are oriented radially in the starch granule and as the radius increases so does the number of branches required to fill up the space, with the consequent formation of concentric regions of alternating amorphous and crystalline structure.

In the figure 2.4 below:

A - shows the essential features of amylopectin.

B - shows the organization of the amorphous and crystalline regions (or domains) of the structure generating the concentric layers that contribute to the “growth rings” that are visible by light microscopy.

C - shows the orientation of the amylopectin molecules in a cross section of an idealized entire granule.

D - shows the likely double helix structure taken up by neighboring chains and giving rise to the extensive degree of crystallinity in granule. There is some debate over the form of the crystalline structure but it appears most likely that it consists of parallel left-handed helices with six residues per turn.

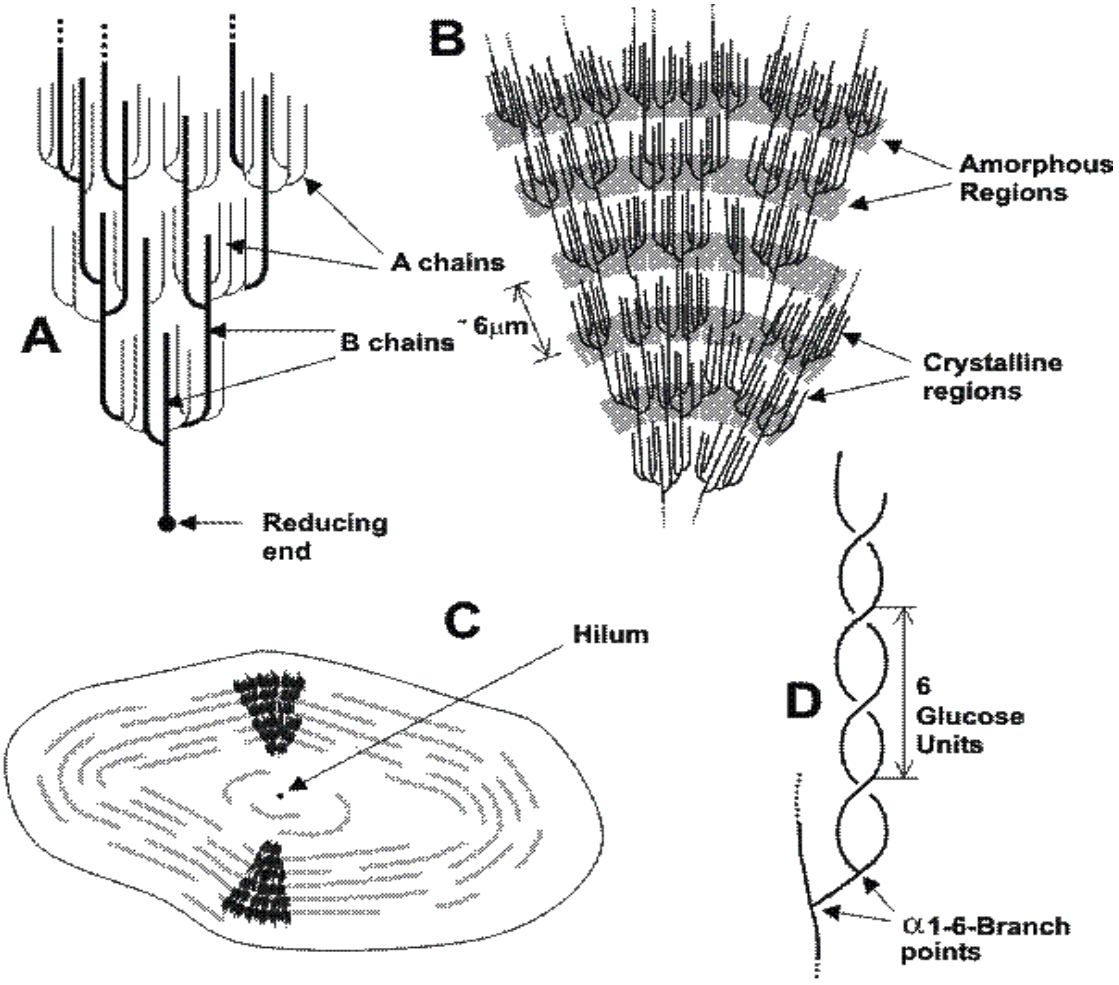


Figure 2.4: Structural organisation of a starch granule.

2.2 Preparation of hydroxyethyl starch

As starting material “waxy” maize is normally used because it naturally contains more than 95% of amylopectin. The production process implies three steps: hydrolysis, hydroxyethylation and ultrafiltration. The complete procedure is described in US Patent 5218108. By the hydrolytic pre-degradation and the ultrafiltration step the molecules are selected according to the desired size. The hydroxyethylation reaction allows to widen the molecule with the consequence of an increased hydrodynamic volume, a decreased viscosity and a reduced degradation rate. In fact, the unmodified amylopectin is very rapidly digested in the bloodstream and has an average residence time of only about 10 minutes. The final product is HES, available in different molecular weights and degrees of substitution. The following scheme resumes the most significant production steps.

Hydroxyethylation of starch using ethylene oxide or 2-chloroethanol has been common practice for production of colloidal plasma volume expanders. The field of volume substitution (e.g. in case of hemorrhagic shock) or hemodilution (e.g. for arterial occlusive disease, Fontaine II B, III) is today inconceivable without the use of colloidal plasma substitutes. For both these indications, of the exogenous plasma substitutes (starch, gelatines, dextran, albumin), hydroxyethyl starch (HES) has found the greatest acceptance in recent years.

The lower disturbance of coagulation [7-9] and the clearly reduced incidence of serious anaphylactoid reactions [10] compared with dextran are responsible for the good acceptance of hydroxyethyl starch in the field of volume replacement and hemodilution. The modification allows a longer vascular half-life, slower degradation rate by α-amylase and slower clearance than unmodified starch [11]. It improves moreover the rheological parameters of starch itself enhancing its hydration degree and consequently reducing the viscosity [5].

The factor considered particularly favourable here is the low colloid osmotic pressure of starch solutions compared with dextrans. With regard to the kidney, the lower urine viscosity involves a lesser risk of a decrease in functional activity. In the area of hemodilution, in addition to the reduction of the hematocrit, the reduction of plasma viscosity in particular has proven to be a therapeutically effective principle of HES-induced rheological improvement.

2.3 Characterisation of hydroxyethyl starch

Hydroxyethyl starches used as plasma expanders have different molecular weights (Mw) and substitution degrees (MS and DS) as well as different substitution patterns.

Due to the use of the natural starting raw material, amylopectin, and the production process in which to a certain extent cleavage of the polymer chains is necessary, the hydroxyethyl starch is not present as a molecularly homogeneous substance with defined molecular weight but as a mixture of molecules of different size which are also differently substituted by hydroxyethyl groups. The characterisation of such mixtures requires the aid of statistically determined magnitudes [12]. To denote the *weight average molecular weight*, the mean molecular weight **Mw** is used. This value is generally obtained with the help of a light-scattering detection which gives the average molecular weight among all components of a polydisperse mixture.

The average value **Mn** indicates the *number average molecular weight* currently used for calculating the real number of moles in a specimen. This value is measured by its effect on osmotic pressure using a membrane osmometer.

There are two differently defined substitution degrees for defining the substitution by hydroxyethyl groups. The substitution degree **MS** (molar substitution) is defined as the average number of hydroxyethyl groups per anhydroglucose unit. It is determined from the total number of hydroxyethyl groups in a specimen, by ether splitting and subsequent quantitative determination of ethyl iodide and ethylene, which are thereby formed.

In contrast, the substitution degree **DS** (degree of substitution) is defined as the proportion of the substituted anhydroglucose units of all anhydroglucose units. It can be determined from the measured amount of the unsubstituted glucose after hydrolysis of a specimen. It follows from these definitions that Ms is normally greater than DS. In the case where only monosubstitution is present, i.e. each substituted anhydroglucose unit carries only one hydroxyethyl group, MS equals DS.

Today it is well known that besides molecular weight and degree of substitution [13] the **C_2 / C_6 ratio** [14] is also of great importance. Hydroxyethyl groups can be attached to the –OH in C_2, C_3, and C_6 of the glucose moieties. It is known that α-amylase breaks down hydroxyethyl starches in the sense that only glycosidic bonds of unsubstituted anhydroglucose units are split. Additionally, hydrolysis by α-amylase is more retarded by substitution on C_2 than on C_6. This means that the higher the degree of substitution and the C_2 / C_6 ratio, the greater is the delay in breakdown and elimination of hydroxyethyl starch. In this respect, only

hydroxyethyl starches having a high C_2 / C_6 ratio or being highly substituted are used for pharmaceutical purposes.

2.4 Pharmacokinetic behaviour of hydroxyethyl starch

Thus, these factors not only play an important role in the incidence of side effects, especially in the modulation of coagulation, but also in the determination of vascular persistence and its fate in the body after administration. The pharmacokinetic behaviour in case of polymers and colloids becomes exceptionally complicated because of the polydispersity. In principle distribution, degradation and elimination are determined by the physicochemical properties (Mw, Ms, Ds and C_2 / C_6 ratio). The main factors determining the HES-pharmacokinetics are the renal clearance (related to the polymer size) and the degradation rate by α-amylase (in relation with the extent of substitution) [11, 15].

After intravascular degradation, HES is almost exclusively eliminated by the kidney. As soon as by enzymatic degradation the size-limit is reached (60-70kD) the glomerular filtration removes the polymer from blood the stream [16-18]. To a certain extent moreover also free glucose can be delivered which will be metabolised by the normal biochemical pathways. Gastrointestinal elimination does not play any role in the elimination process [19, 20].

The table 2.1 [23] shows the rate of renal excretion for the most common species of HES already available on the market. The denomination HES 130/0.4 indicates hydroxyethyl starch with a MW of 130kD and a DS of 0.4.

Renal excretion of starting material (%) after:			
	6 hours	*12 hours*	*10 days*
HES 130/0.4	~50	~55	up to ~70
HES 70/0.5	~50	~55	up to ~70
HES 200/0.5	~30	~45	up to ~65
HES 200/0.62	~15	~20	up to ~45
HES 450/0.7	~10	~15	up to ~35

Table 2.1: Relation between rate of renal elimination and molecular weight / degree of substitution of the most common HES species.

Repeated administrations moreover do not result in the accumulation of low degree of substitution and low molecular weight HES in the plasmatic compartment. This may become relevant only for HES 200/0.62 and for HES 450/0.7. The cells of the reticule-endothelial system have also been reported as accumulation sites [21, 22]. Only sensations of itching match the deposit in tissues, which is absolutely reversible and dose-dependent. Anyway these results were obtained administering doses required for plasma substitution or blood volume restoring. The amount of material administered in case of therapy with HES-modified proteins or drugs is supposed to be significantly smaller.

2.5 Toxicology of hydroxyethyl starch

Because of its molecular structure and its similarity with human glycogen, hydroxyethyl starch offers a promising basis for an exceptionally low toxicological potential. Nevertheless, this polymer has been submitted to the standard testing procedures [23] required for its therapeutic category, which consisted of:

- acute toxicity;
- subacute, chronic and subchronic toxicity;
- embryonic / foetal and perinatal toxicity;
- damage of fertility and of the descendant;
- mutagenicity;
- antigenicity;
- local tolerance.

All tests, even when performed administrating doses up to 4-5 times higher than the today approved ones, gave negative results, confirming the extraordinary properties of HES which possesses no toxic, antigenic, mutagenic or haemolytic potential, and in addition is very well locally tolerated.

3 Chemistry of polymer-drug conjugation

3.1 General considerations

The technique of coupling polymers to proteins originated in the 1950s and 1960s with investigations of protein structure and function by site-directed chemical modification. These studies led to so-called “gentle chemistry” for protein manipulation. Important were also the discoveries made in 1970s that enzymes could be covalentely linked to insoluble matrices for biocatalytic application.

The chemistry of bioconjugation is an extensive one, it combines classic chemical methods with biochemical protocols. Different approaches may be used depending on the properties of the bioactive molecule to be modified (a small organic compound, a protein, a glycoprotein or a nucleic acid) and of the ones of the ligand to be coupled (a coloured, fluorescent or enzymatic probe, a monofunctional polymer such as PEG or a polyfunctional one such as polysaccharides or polyacrylates). Despite the large variability that characterises this field of chemistry, a common feature shared by the reactions used in bioconjugation is the fact that all of them must be carried out under mild conditions, so that the structure, and therefore the biological activity is not disrupted.

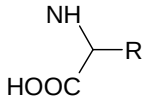
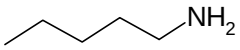
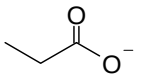
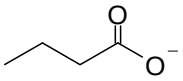
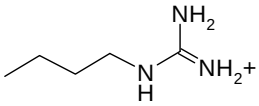
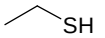
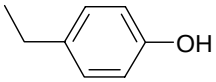
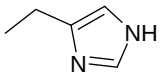
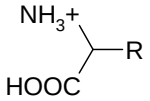
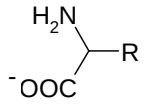
As a preliminary consideration it may be important to note that, generally, both the drug molecule and the polymer are not reactive by themselves and a preliminary step of activation is usually needed before coupling. Furthermore, since the drug molecule often carries more than one functional group, it is the polymeric moiety that is generally (although not always) transformed into a reactive agent that is then coupled to the drug. In some cases, it may be necessary to introduce a new functional group in either drug or polymeric molecule. This may be achieved either through a specific chemical reaction that transforms a functional group into the desired one [1], through the use of bifunctional reagents (add examples) [2] or, as in the case of proteins, through genetic recombinant techniques that allows the introduction of an amino acid with the desired reactivity, as, for example, the thiol function of cysteine [3] or amino group of lysine [4].

3.2 Functional groups of the drug

3.2.1 Proteins

Among the 20 amino acids found in peptides and proteins, up to seven may be derivatised more or less easily at their side chains. All of these amino acids possess functional residues that in the appropriate environment may act as nucleophiles, **table 3.1** summarises the main characteristics of these amino acid side chains, their structure and their pK_a values. The terminal amino and carboxylic functions are also suitable as possible sites for conjugation. Due to the nucleophilic properties of these residues, the most common reactions that take place in protein conjugation are nucleophile-to-electrophile attacks. The nucleophilicity of each amino acid residue may be modulated by changing the environmental pH since only when the pH is close or above the residue's pK_a, can nucleophilic attack take place. As a consequence, the order of nucleophilicity for the major groups in proteins, provided that each unprotonated form is more nucleophilic than its protonated parent, can be summarised as follows [5-9]:

Table 3.1:
Structure and pKa value of protein amino acid residues [11].

Amino acid	Structure	pKa
		
Lysine		9.3 – 9.5
Aspartic acid		3.7 – 4.0
Glutamic acid		4.2 – 4.5
Arginine		> 12
Cysteine		8.8 – 9.1
Tyrosine		9.7 – 10.1
Histidine		6.7 – 7.1
a-carbonyl; C-terminus		2.1 – 2.4
a-amine; N-terminus		7.6 – 8.0

R-S$^-$ > R-NH$_2$ > R-COO$^-$ = R-O$^-$

Although according to this rank order, cysteine thiols are the most reactive groups in proteins, this amino acid in free form is generally quite rare, because the majority is involved in disulfide bridges. When present, it often plays an important role in catalysis or in binding, therefore conjugation at such site most probably will disturb the protein function. For these reasons the main target in protein bioconjugation is the ε-lysyl amino residue. The latter

amino acid is present with high frequency in proteins (up to about 10% of the overall amino acids [11]) and, because of its hydrophilicity, mainly distributed on the protein surface, therefore easily accessible for bioconjugation. Nevertheless, in some cases, cysteines have been successfully used as anchoring sites after being introduced into the protein, by recombinant gene techniques [3], in positions that are known to be not essential for the protein's biological activity.

Carboxyl carrying amino acids are considered as targets of coupling only when the modification of the more reactive lysines is known to impair the biological activity of the protein. The reason why these amino acids are considered as a second choice is because it is difficult to avoid intramolecular cross-linking with the amino groups of other protein molecules.

In glycoproteins, the sugar moiety can also be used as the target for polymer conjugation. Sugars may carry a few possible sites for modification such as hydroxyl groups or, in some special cases, primary amines, carboxylates or phosphates. Furthermore, vicinal-hydroxyl groups can be easily oxidized by periodate, leading to two reactive formyl residues. It may be interesting to mention also a quite different strategy, useful for a site specific PEGylation, that was used for the conjugation of the growth hormone releasing factor, a polypeptide of 29 amino acids normally obtained by a solid phase synthesis. In this case, side chain PEGylated amino acids (lysine or aspartic acid) were directly introduced at the desired level of the sequence during the synthetic preparation of the peptide [10]. This strategy is actually applicable to any low molecular weight peptide that can be obtained by synthesis.

3.2.2 Small organic molecules

The main sites of polymer attachment in a small organic molecules share similar characteristics with those described for protein in the sense that they often possess nucleophilic residues. However, they must be devoid of critical relevance for the molecule's biological activity to be considered as sites of conjugation. Therefore, a polymer is generally attached to a small drug through its —OH, —NH_2 or —COOH groups and as in the case of proteins, these, or other residues, if not directly available in the molecule, may be introduced through a desired spacer by means of an intermediate synthetic step. In case that the conjugate product is desired to retain its drug activity, the modification, in contrast to the prodrug approach, should not directly involve the pharmacophoric part of the molecule.

3.2.3 Polymeric moieties

There are many polymers used in the preparation of bioconjugates for pharmaceutical application. Because of their application in the biomedical field, they all share the common properties of being highly hydrated, non-toxic, non-immunogenic and of having a molecular weight sufficiently low to allow, when they are not biodegradable, filtration (although slow), through the kidney. The two most common polymers presently employed are PEG, dextran and HPMA but others are also being used, namely, poly(lysine), poly(aspartic acid) or other poly(amino acids) poly(vinyl alcohols), poly(acrylates), poly(N-vinyl pyrrolidine) and poly(N-acryloyl morpholine), or copolymers of the same.

From a general point of view, these polymers can be divided into two main groups, poly- and mono-functional ones. In bioconjugation, as already mentioned, the choice of a mono- or a poly-functional polymer mainly depends on the type of drug that has to be modified and, generally, mono-functional polymers (mPEG, PVP, PacM derivatives, etc.) are preferred in the modification of poly-functional or high molecular weight bioactive molecules (such as proteins). On the other hand, poly-functional polymers are mostly used for the modification of small mono-functional drugs, where the risk of cross-linking does not exist and a more favourable drug/polymer ratio is often desired.

Besides the different structure in their polymeric backbone, the polymers used in bioconjugation have only a limited number of functional residues that are normally exploited in the coupling reaction. Also in this case, the most common anchoring groups are —COOH, —OH and —NH_2. These groups must be activated in order to react with the desired drug molecule and many mild activation methods are presently available. Of course, the chemistry of binding can also be planned the other way round, so that it is the drug molecule to be activated first. The strategy is determined by the best conditions for preserving the drug activity.

Special attention must also be paid to the 3D structure of the polymers, their hydrophobic/hydrophilic balance, their flexibility and biodegradability. All of these factors are important in dictating the fate of the drug in vivo, the rate of elimination from blood and the protection from degradation, both in vivo and in the pharmaceutical formulation. As general rules, it may be important to remember that:

1. C-C backbones are degraded only in rare cases in the body;
2. Highly hindered polymers (as, for example, the polysaccharides), greatly protect from chemical and enzymatic degradation;

3. Hydrated and flexible polymers (like PEG) are slowly eliminated from the kidney: for a similar reason, they give rise to entanglement of ultrafiltration membranes;

4. Hydrophobic polymers are more frequently localised in organs;

5. High molecular weight polymers may localise in specific sites because of the EPR effect, from where they are captured by the cell through an endocytic mechanism;

6. The degradation of polymers inside the cell may occur by means of the action of specific, very active lysosomal enzymes or because of the acidic endosomal environment;

7. Often the polymer, especially when its mass is higher than that of the drug, dictates the in vivo behaviour of the conjugate.

According to these general parameters, an ideal polymer for bioconjugation should be a polysaccharide with the following properties:

- low in vivo degradation rate
- high molecular weight
- biocompatibility
- well known pharmacokinetic behaviour
- possibility to predict and condition its in vivo behaviour
- mono-functional chemical reactivity

3.3 Generally used coupling strategies

In case of protein based drugs, the vast number of attempts and all the different linking reactions share the usual preference to “pre-activate” the polymer instead of the protein. This choice is mainly due to chemistry reasons and also to cost problems, because the polymers are, in general, much cheaper than proteins or peptides. This kind of chemistry yields moreover much cleaner reaction and a better defined coupling product.

Among the most common strategies one can distinguish between selective coupling and non-selective coupling approaches. Selective activation is defined as a procedure which results in one unique specific reactive site per polymer molecule, while a non-selective one allows more than one reactive site on the same polymer chain, with an increased risk of cross-linkage and a reduced chance of a clear definition of the coupling product. In fact, the non-selective reactions represent somehow the history of bioconjugation. Having been used firstly

for immobilisation of proteins on insoluble matrices, e.g. for affinity chromatography, they were applied as first choice also for linkage with soluble polymers.

The figures below show the most common non–selective and selective coupling activation strategies. The blue residue always represents the polymer side while the green residue is indicating the target (protein, peptide or low molecular weight drug) side.

Among the non-selective strategies the CNBr and the periodate activation are reported.

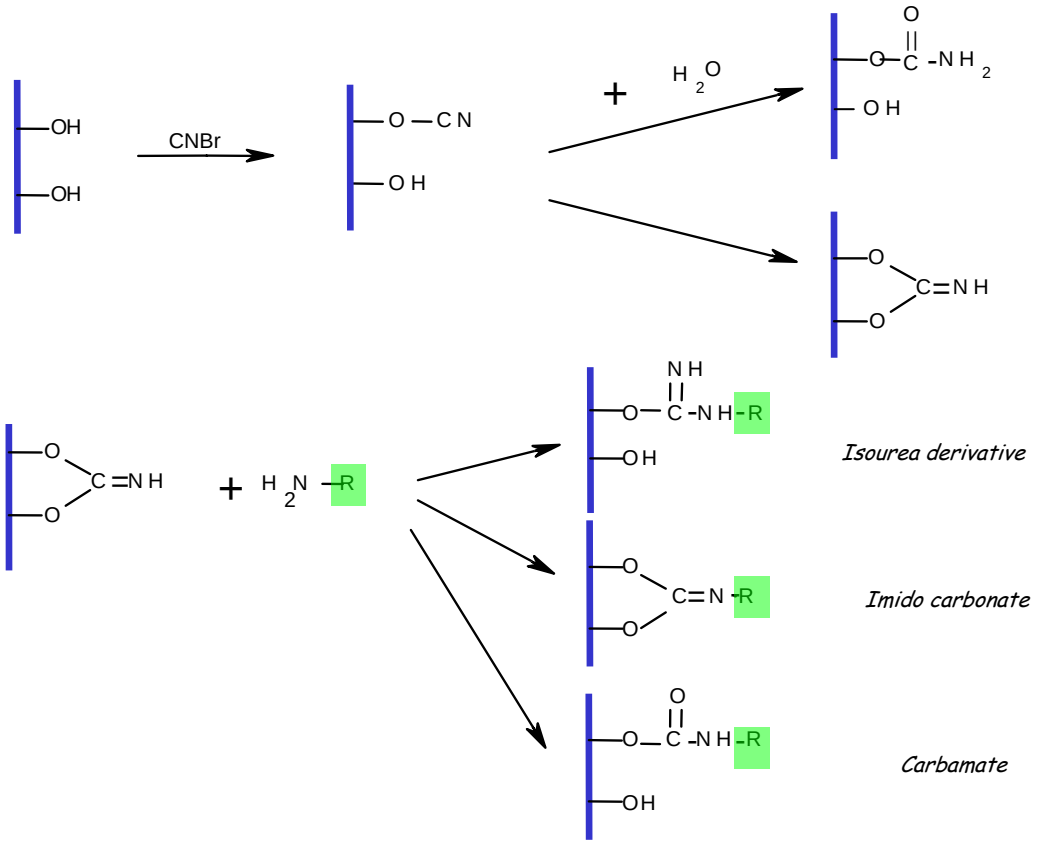


Figure 3.1: Non-selective conjugation: activation with CNBr.

They are currently used with polysaccharides and yield many reactive sites per polymer chain. CNBr is moreover reported to play a role in deactivating enzymes. The product is very often a complex mixture of all possible coupling combinations, many times it even results in a reticulated system with very high molecular weight. This is often not compatible with pharmaceutical requirements.

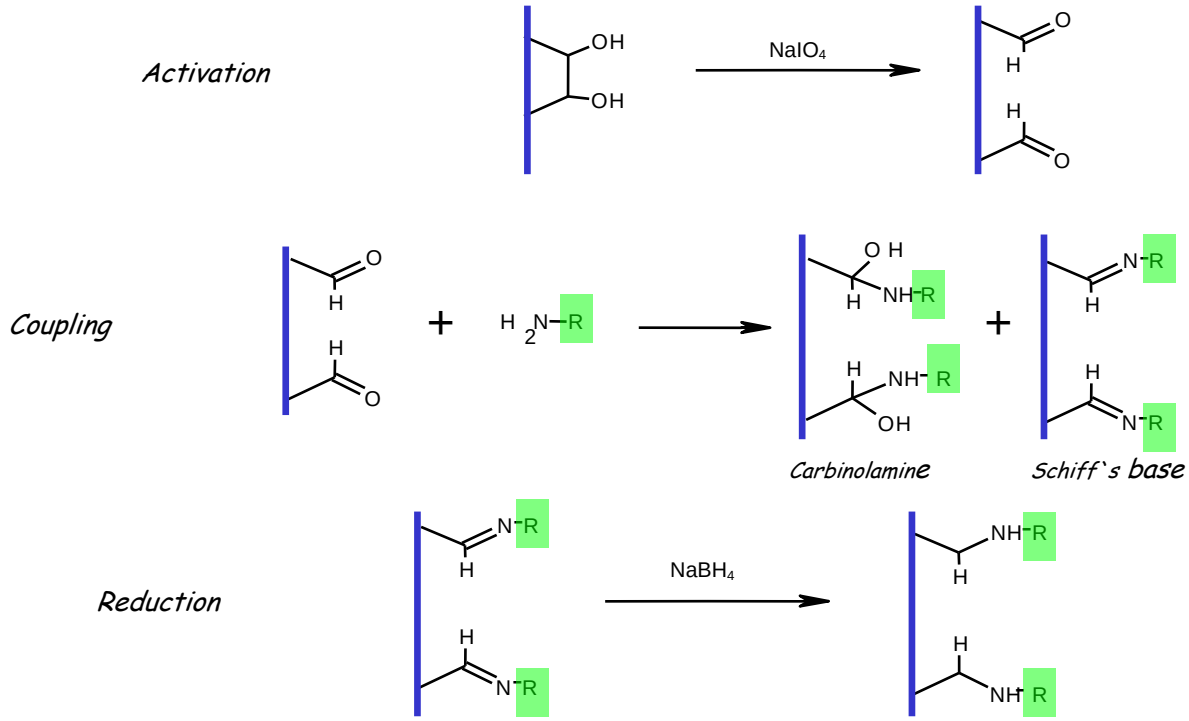


Figure 3.2: Non-selective conjugation: activation with $NaIO_4$.

The selective activation approaches have been widely used in PEGylation. The authors generally distinguish between alkyl- and acyl- PEGylation, depending on which kind of bond is used for the polymer anchoring.

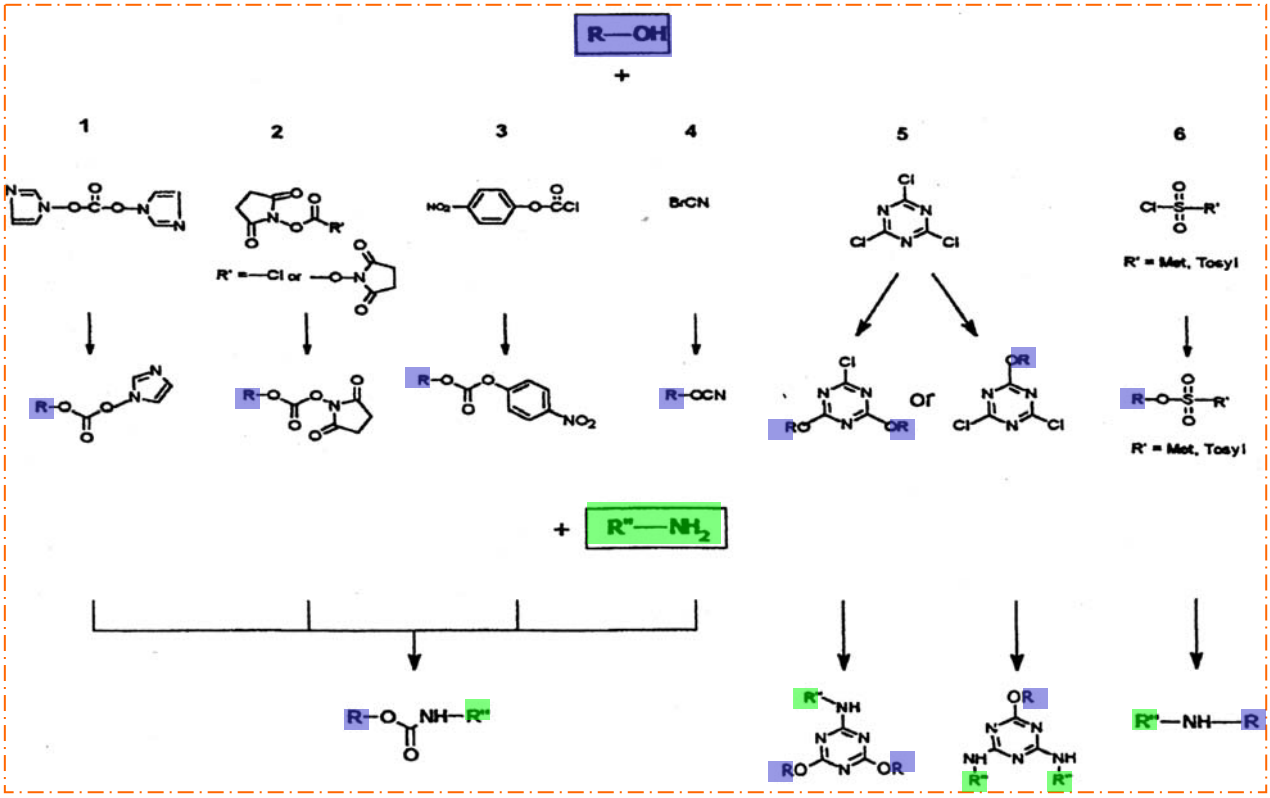


Figure 3.3: Selective conjugation: modification with mPEG-OH [11].

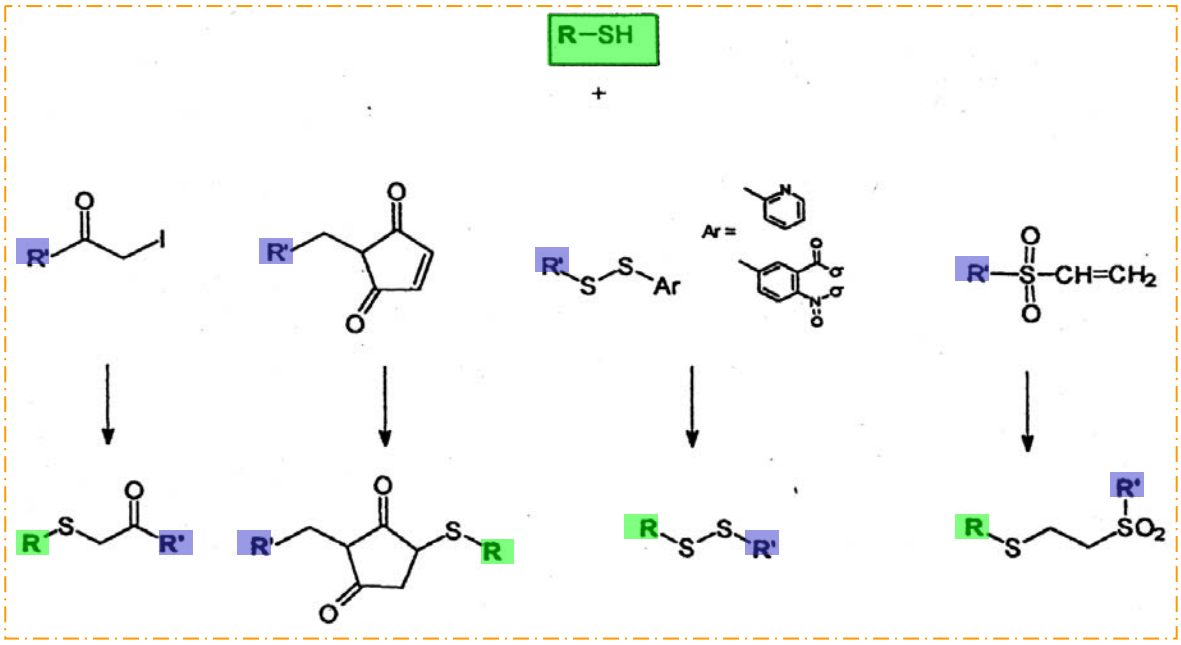


Figure 3.4: Selective conjugation: modification with mPEG-SH [11].

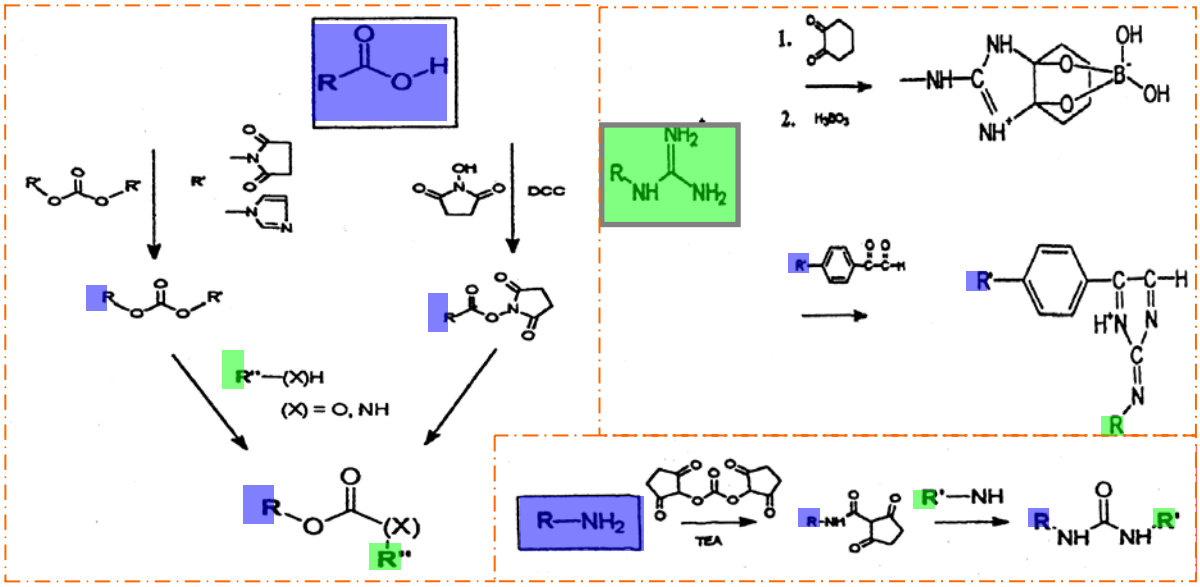


Figure 3.5: Selective conjugation: modification with mPEG–COOH, mPEG–NH_2 and specific coupling to Arg [11].

3.4 Preferred coupling strategies in HESylation

An HES molecule possesses an single aldehyde group (see Chapter 2). The different reactivity of such group allows to perform selective coupling reactions even with this polysaccharide. The aldehyde can be either used as such, or, by mild oxidation, transformed to a carboxyl acid with an enhanced spectrum of possible reactions.

The aldehyde as such can react with an amino function yielding a Schiff's Base (imine) which has to be reduced to a more stable secondary amine. The reduction is usually performed by $NaBH_4$. Due to the size of the reagents and to the presence of only one reactive group on such big molecule, the reaction needs a relative long time (24h-48h) to reach its optimal equilibrium; in this case $NaBH_4$ is not preferred because its reducing power is too strong and it would reduce the aldehyde groups before the equilibrium is reached. $NaBH_3CN$ unlike $NaBH_4$ is very selective and, at the appropriate pH conditions, is able to discriminate between imine and aldehyde, reducing only the former and leaving the aldehydes intact to reach equilibrium.

Nevertheless the transformation of the aldehyde to carboxylic acid was preferred. Since there is very limited literature available on successful selective oxidation of polysaccharides, the development and the optimisation of such oxidation step was also part of the objective of this thesis.

In organic solvents a much more elegant alternative may be used. The carboxyl acid can be transformed to a lacton simply by eliminating a water molecule. The dehydration is achieved by lyophilisation. The lacton form of carboxylic acid is already active per se and can spontaneously react with amino functions yielding an amide (aminolysis of lactones) [12]. The great advantage is the possibility to work in the absence of other molecules except the polymer and the target (generally a peptide or a small drug). This avoids the occurrence of side reactions due to the presence of other carboxyl groups than the one on the polymer chain. Moreover it greatly reduces purification problems.

This chemistry although not new, has been somehow ignored in the field of polysaccharides until now. Although polysaccharides own some unique properties, very promising in bioconjugation, as mentioned in the second Chapter, they have been so far considered only as second choice after synthetic polymers (PEG) because of their very low selectivity. Once this selectivity problem is solved, the polysaccharides, and particularly HES, have got all qualifications to be promising candidates in the future of bioconjugation.

4 Materials and methods

4.1 Materials

4.1.1 Devices

Besides the standard equipment for chemical reactions the following devices were used:

HPLC instruments:

- Dionex DX-500 System
- Dionex P580 quaternary pump for low pressure gradient
  ASI-100 (auto sampler)
  UVD-340 U (UV-Diode array detector)
  STH-585 (column oven)
- Dionex P580 binary pump for high pressure gradient
  ASI-100 (auto sampler)
  UVD-340 U (UV-Diode array detector)
  STH-585 (column oven)
- Merk – Hitachi L-6210 HPLC pump

HPLC column:

- Shodex GF510 HQ (Gel filtration)
- Phenomenex BioSep-SEC-S 3000 (Gel permeation)
- Nucleosil 100-5 C_{18} 150/4.6

FPLC column:

- Pharmacia Superose 6 HR 10/30 (Gel permeation)
- Pharmacia Superose-12 HR 10/30 (Gel permeation)

UV-Vis Spectrometer:

- Varian Cary 100 BIO

Fluorimeter:

- Varian Cary Eclipse

Freeze-drier:

- Christ BETA 1-8

Electrophoresis and blot equipment: Biorad Miniprotean 3

4.1.2 Chemicals

High purity standard chemicals as well as the proteins and peptides used in this work were purchased from the companies: SIGMA, Aldrich, Merk, Fluka, Acros Organics. The starting HES_{10}, HES_{25} and HES_{130} material was provided from FRESENIUS KABI (Friedberg - Germany) according to the terms of a co-operation project. HES_{70}, HES_{200} and HES_{450} was moreover provided from Serum-Werk Bernburg AG (Bernburg – Germany).

Specialised chemicals were from the following sources:

Chemical name	Mol. weight	Purchased from
Amphotericin B	924.1	ALPHARMA
Mepartricin	1141.4	SPA s.p.a.
$NaBH_4$	37.8	SIGMA-ALDRICH
$NaBH_3CN$	62.8	ACROS
HSA	66.7 kD	SIGMA-ALDRICH
CRE	2 x 46 kD	SIGMA-ALDRICH
SOD	2 x 32.5 kD	ROCHE
EDC	191.7	SIGMA-ALDRICH
DCC	206.3	SIGMA-ALDRICH
CDI	162.1	ACROS
p-nitrophenol	139.1	SIGMA-ALDRICH
Pentafluorophenol	184.1	SIGMA-ALDRICH
HOBt	135.1	SIGMA-ALDRICH
DMAP	122.2	ACROS
TEA	101.2	ACROS

Kits and ready-to-use assays:

-	GPC column calibration kit:	# H2899	SIGMA
-	Glycan detection kit (DIG):	# 1142372	Roche – Boeringer
-	SOD assay kit – WST:	# S311	Dojindo Molecular Technologies, Inc.
-	BCA protein assay:	# 23225	Pierce

Erythrocyte concentrate solutions were offered by the Transfusion Centre of the University Clinic at the Justus Liebig University in Giessen (Germany).

4.2 Methods

4.2.1 Chemical syntheses

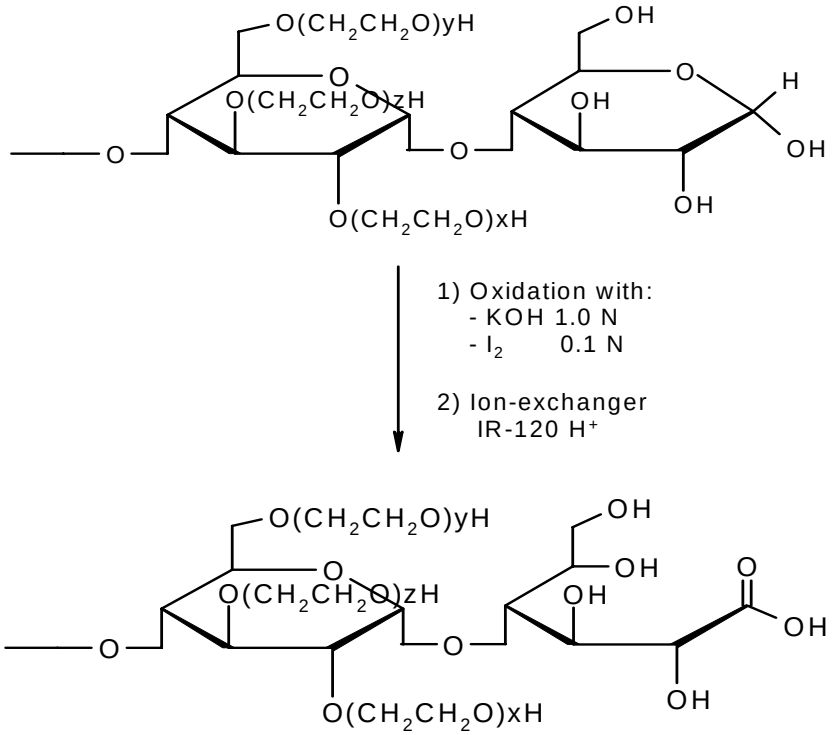
All following methods are the result of an optimisation procedure partially described in the chapters “Results”. Unsuccessful attempts were omitted. All procedures involving HES, apply to all above mentioned HES species, but are usually described only for one type.

4.2.1a HES oxidation

The oxidation of the saccharide’s reducing ends is known to require mild reaction conditions. The two ways we chose to perform this are described in details in the following section:

1) Oxidation of HES with Iodine in alkaline solution

Theory: The reducing end of HES is susceptible to oxidation with halogens in basic solution. In the case of iodine, the effective oxidant is sodium hypoiodite, generated in the reaction system. Since such molecule (IO^-) is known to be unstable and convertible into the corresponding iodate and iodide, small amounts of iodine and sodium hydroxide were successively added to the reaction solution in order to yield the oxidant in a limited amount in the reaction system, which oxidises effectively the reducing end groups of HES.



Description:

	Amount	Mn (D)	mMol	Ratio
HES_{130}	10.0 g	$42.6x10^3$	0.24	**1**
I_2 sol. 0.1 N	14.0 ml	---	1.4	**~6**
NaOH sol. 1.0 N	2.8 ml	---	2.8	**~12**
H_2O (to dissolve)	12 ml			

In a round bottom flask 10 g of HES_{130} were dissolved in distilled water (12 ml). Thereafter 2 ml of I_2 sol. 0.1 N were added. A 2 ml pipette containing 2 ml NaOH sol. 1.0 N was then connected to the flask using a two ways connector, and the NaOH solution was dropped in, once every four minutes (each drop having the volume of ~20 µl). After adding almost 0.2 ml of NaOH sol. 1.0 N the solution started to become clear again, then we the second 2 ml portion of iodine sol. 0.1 N had to be added. At the end of this process we had inserted a total amount of 14 ml of I_2 sol. 0.1 N and 2.8 ml of NaOH sol. 1.0 N.

The reaction was then stopped and dialysed for two days against distilled water. The final solution is allowed to pass through a cation exchanger resin (IR-120 H^+) and then lyophilised. Finally 75% - 85% of the material is recovered.

Alternative to the dialysis, which is not so suitable for small oligosaccharides being oxidised, another procedure was performed where, at the end of the reaction, the solution was directly passed through the cation exchanger IR-120 H^+, and then the filtrate was incubated in presence of silver carbonate overnight in order to eliminate the excess of iodine/iodide. Thereafter the filtrate has to pass once more through the cation exchanger before being lyophilised. The degree of oxidation, determined on the lyophilised product with the "BCA reducing sugar assay" (see the section "Analytic methods" in this chapter), was normally around 70-75%.

2) Oxidation with metal ions (Cu^{++}) in alkaline solution

Theory: This reaction can be performed using the oxidising power of metal ions as Ag^+ or Cu^{++}. The reaction follows this stoichiometric equation:

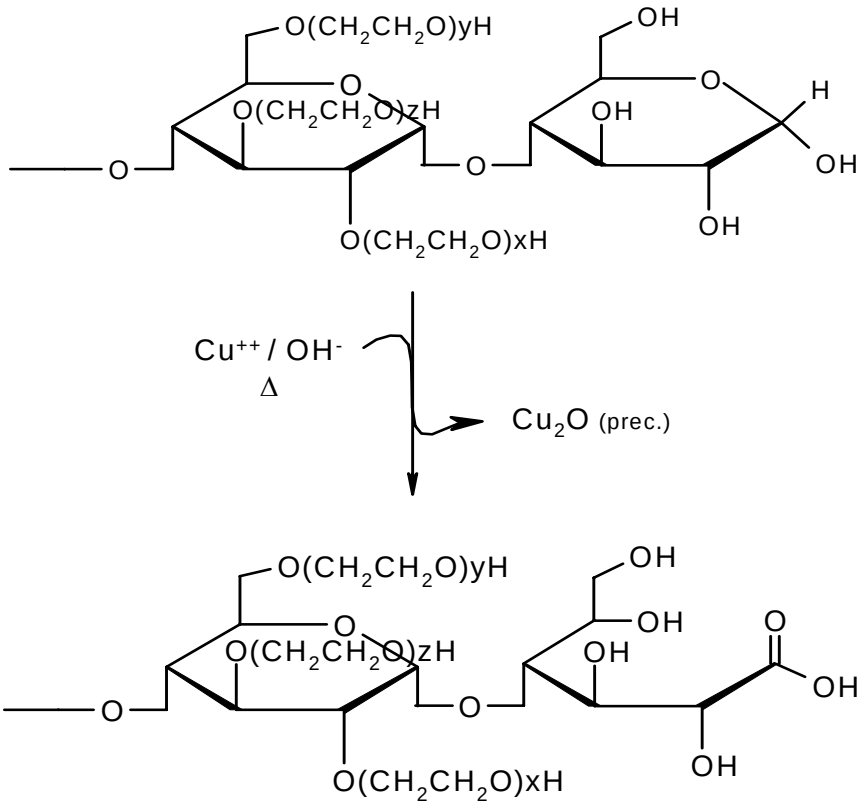
$$2Cu^{++} + –CHO + 2OH^- \longrightarrow 2Cu^+ + –COOH + H_2O$$

$$2Ag^+ + –CHO + 2OH^- \longrightarrow 2Ag + –COOH + H_2O$$

We decided to do it using copper hydroxide. In this case the equation becomes:

$$2Cu(OH)_2 + –CHO \longrightarrow Cu_2O\ (s) + –COOH + 2H_2O$$

Since the cupric ion is not stable in basic solution (it would precipitate as hydroxide), tartrate was present as stabiliser which is able to keep Cu^{++} in solution even under extremely basic conditions.



Description:

	Amount	Mn (kD)	mMol	Ratio
HES_{130}	10.0 g	42.6	0.24	**1**
Fehling's reagent (Cu^{++}) *(preparation see below)*	2 x 10.0 ml	---	2.00 (Cu^{++})	**8.33**
H_2O	10 ml			

Firstly a solution was prepared where 0.24 mmol of hydroxyethyl starch (MW=130 kD) were dissolved in 10 ml distilled warm water. When the temperature had reached about 70-80°C the first amount of 10 ml Fehling's reagent (320 mg of Cu^{++}), was added to the first solution in a 100 ml round bottom flask and stirred magnetically. The solution became blue. Thereafter the temperature was increased up to 100°C and the reaction colour started to change firstly to pale green and then to brick red. It took about 10 min. The reaction was allowed to run under stirring conditions at the same temperature for 20 more minutes, then stopped and the temperature was let to decrease to 4-5°C (slowly). The solution was filtered or centrifuged to completely remove the reddish precipitate (Cu_2O).

In the case the filtrate did not become blue the reaction was not complete. So the solution must be warmed up again, 10 ml more of Fehling's reagent have to be added and the previously described procedure repeated.

After filtration the solution, still blue, was shaken for one hour on 20 g cation exchanger IR-120 H^+ (~50 times excess).

Thereafter the solution was acidified to pH 3-4 by adding HCl 1 M and, with the help of a strong vacuum pump, the carbonate was eliminated as CO_2. Then the solution was dialysed against distilled water over a day to eliminate the tartrate and other undesired salts. Before the final lyophilisation the solution was passed once more through the cation exchanger resin.

We finally obtained 7.8 g of oxidised product (yield = ~78%) The oxidation degree determined on the lyophilised product with the "BCA reducing sugar assay" (see the section "Analytic methods" in this chapter), was normally found to be higher than 95%.

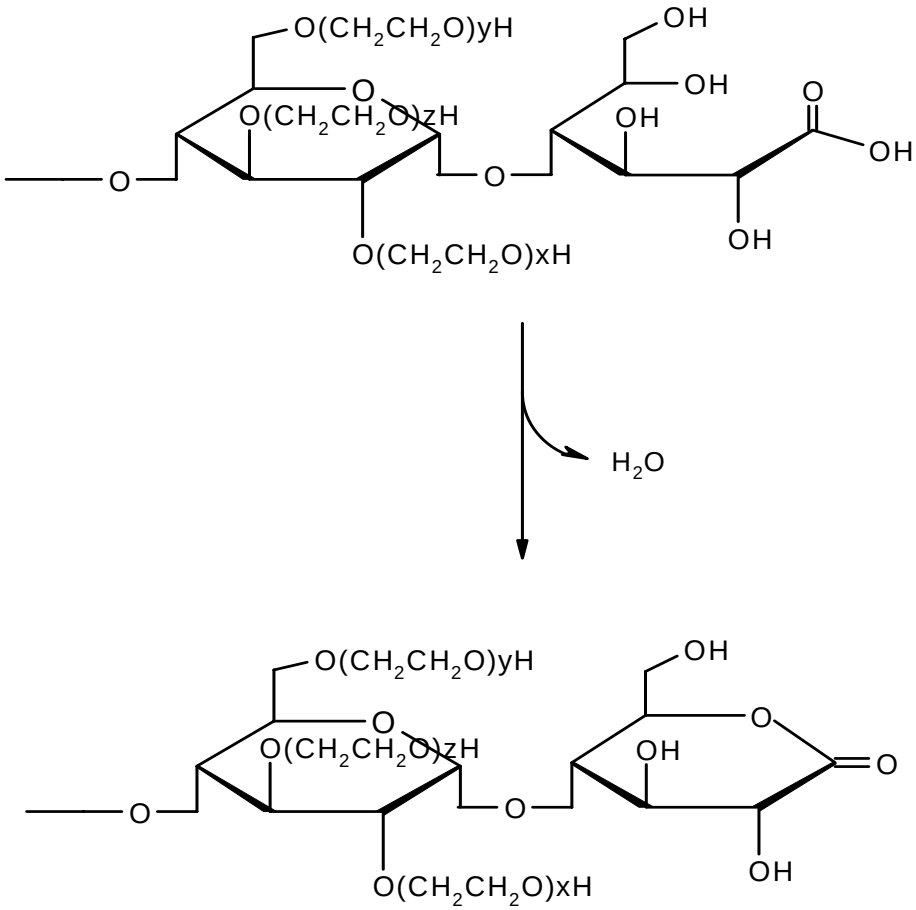
Fehling`s reagent preparation:

	Amount	**MW (D)**	**Mol**	**Ratio**
Rochelle salt	15.0 g	282.23	$5.3x10^{-2}$	**1.66**
Na_2CO_3 anhydrous	15.0 g	105.99	0.141	**4.42**
NaOH sol. 0.1 N	20 ml	---	$2.0x10^{-3}$	**$6.25x10^{-2}$**
$CuSO_4$ anhydrous	5.114 g	159.60	$3.2x10^{-2}$	**1**
H_2O	100 ml + 40 ml			

Dissolve completely the sodium-potassium tartrate and the sodium carbonate in 100 ml water, and then add 20 ml of a 0.1 N sodium hydroxide solution. In a separate vessel prepare the solution of cupric sulphate in 40 ml water. After complete dissolution the two solution are mixed and the "Fehling`s reagent" is ready to be used.

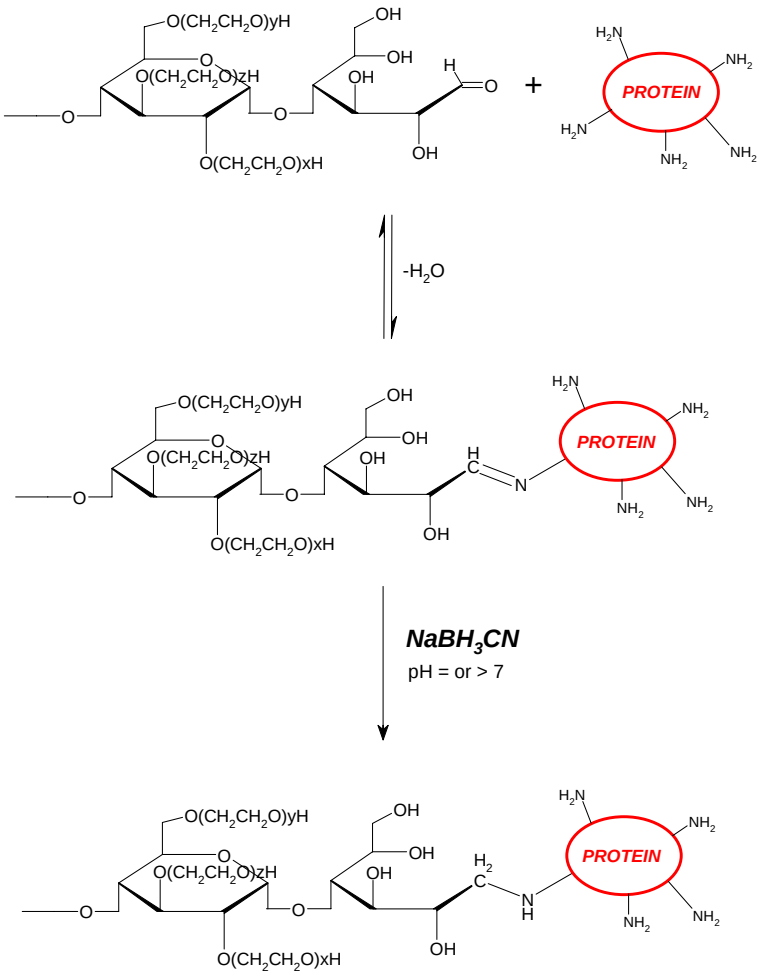
4.2.1b Lactonisation of oxidised HES (oxHES)

Each oxidation method yields a carboxylic acid either in the salt or in the acid form. The latter can easily be lactonised by removing one water molecule. The simplest way to do it is by lyophilisation. The samples dissolved in distilled water were firstly frozen with liquid nitrogen and then lyophilised for 20-24h at 0.200 mBar. The resulting lacton is stable in anhydrous organic solvents but is promptly hydrolysed to the acid form as soon as traces of water are present.



4.2.1c HES coupling to a model protein (HSA) in presence of $NaBH_3CN$

Theory: In this classic Schiff's base reaction with final reduction, a stable secondary amine was obtained. The use of cyanoborohydride is needed because of the long reaction time. This reagent is pH selective and, at basic pH, can discriminate between aldehydes and imines, reducing only the latter. Previous attempts using $NaBH_4$ resulted in a very low reaction yields (<10%) due to the concomitant reduction of the aldehyde groups.



The most recent and optimised version of the method, with an yield higher than 65%, is reported below.

Description:

	Amount	MW (D)	Mol	Ratio	
HES_{130}	32.8 mg	$43.287x10^3$	$7.57\ 10^{-7}$	**25**	**1**
HSA	1.89 mg	$66.0x10^3$	$2.86\ 10^{-8}$	**1**	
$NaBH_3CN$	1.0 mg	62.84	$1.6\ 10^{-5}$		**20**
Phosph. buffer pH ~ 8	400 µl				

The protein was first dissolved in the buffer solution and then the polymer was added. After complete dissolution of both the $NaBH_3CN$ solution was inserted in the reaction vessel.

The solution was left at room temperature under stirring condition for ~3 days. Then the reaction was stopped and the solution analysed by GP-HPLC. The coupling product was also checked by SDS-PAGE and glycan detection, to proof the presence of polysaccharides.

In the purification step the residual $NaBH_3CN$ was eliminated by dialysis and the uncoupled HES by ion exchange chromatography.

4.2.1d oxHES coupling to a model protein (HSA) in presence of water soluble carbodiimide (EDC)

Theory: The carbodiimide is a strong carboxyl acid activator. The usual reaction scheme implies the activated acid to react directly with a nucleophile (-SH, $-NH_2$, -OH). In water the suggested mechanism is a bit more complicate. As shown in the figure 4.1 the preferred mechanism (among all different possibilities) is the reaction with a second carboxyl acid to yield an anhydride (8) which rapidly reacts with the nucleophile.

Three possible combinations of anhydrides have to be considered:

1) symmetric anhydride (two polymer molecules reacting together);
2) asymmetric anhydride (a polymer molecule reacts with a carboxylic acid of a protein);
3) pseudo-symmetric anhydride (two carboxylic acid of proteins reacting together).

Among these options the first and the second ones seem to be the most likely to happen. Of course the third possibility cannot be excluded, but there should be scarce probability that two carboxylic acid from proteins get close enough to react between them selves, due to the highly hindered protein structure. In the third option is also included the case of intra-molecular activation. In such case the reactive function should also react with an intern nucleophile which is supposed to be much closer than the others. Nevertheless a protein-protein cross-linking cannot be completely excluded because the option of a nucleophile that directely reacts with the active acid (4) still remains, although it is demonstrated to happen much more slowly than the other one.

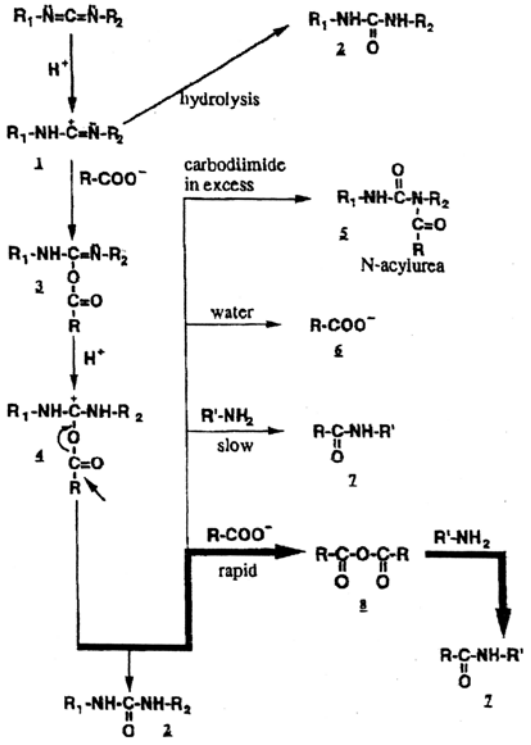
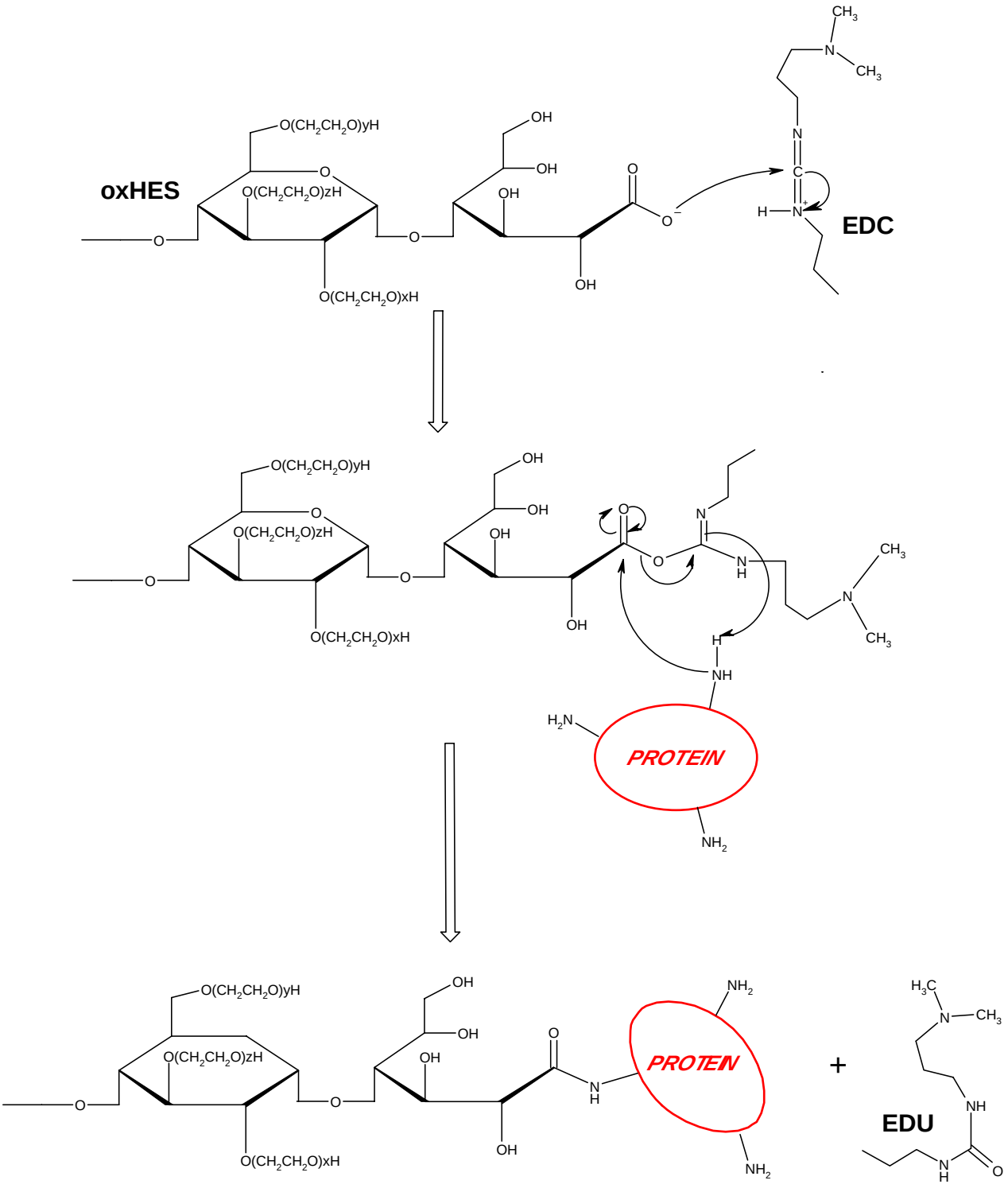


Figure 4.1: Reaction mechanism of carbodiimide in aqueous medium.

The real limit of this reaction are the “burning cycles” of EDC. The activated acid forms (4 or 8) can also be hydrolysed in water and return to the starting condition (6) after consuming an EDC molecule. Due to this problem an excess of carbodiimide is needed.



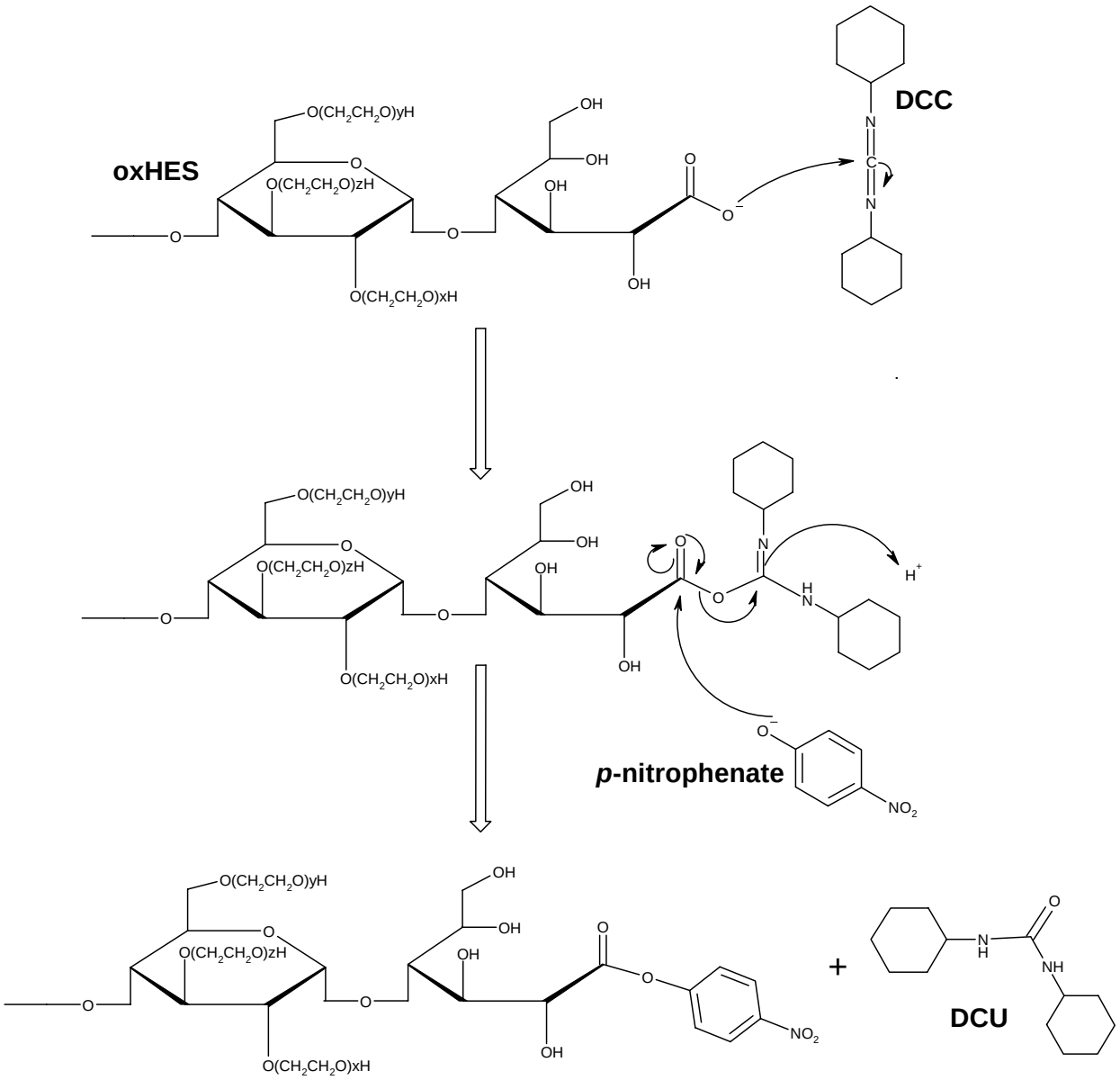
Description:

	Amount	MW (D)	Mol	Ratio	
oxHES$_{130}$	130 mg	43.287x10^{3}	3.0 10^{-6}	**3**	**1**
HSA	100 mg	66.0x10^{3}	1.5 10^{-6}	**1**	
EDC	200 mg	191.7	1.0 10^{-3}		**333**
Water	10 ml				

In a round bottom flask 130 mg of oxHES$_{130}$ (oxidation degree ~ 100%) and 100 mg of HSA were dissolved in 5 ml of distilled water. As soon as the solution became clear 200 mg of EDC were added in three portions dissolved in a volume of 5-10 ml, with the help of a dropping funnel during one hour. Every four hours a new portion was added. After the third addition the reaction was left for further 16 h under moderate stirring conditions. Thereafter the reaction was stopped and the solution was dialysed against distilled water overnight in a dialysis membrane with a cut-off of 4-6 kD. The solution was finally lyophilised.

4.2.1e oxHES pre-activation in organic solvent with DCC

Theory: This reaction is a classic ester bond formation in presence of DCC as activator. The reaction is performed in a polar but non-protic organic solvent, as DMSO, DMF or N-methylpyrrolidone, able to dissolve the polymer and alcohol. The target molecule is preferably p-nitrophenol or pentafluorophenol, although active esters have also been synthesised with N-hydroxysuccinimide or with HOBt. The active esters are supposed to be moderately stable in water because they are thought to be used in aqueous solution. With this strategy we overcame the problem of side reactions due to unspecific –COOH activation with EDC in water. As an example oxHES$_{70}$ with p-nitrophenol is reported here.



Description:

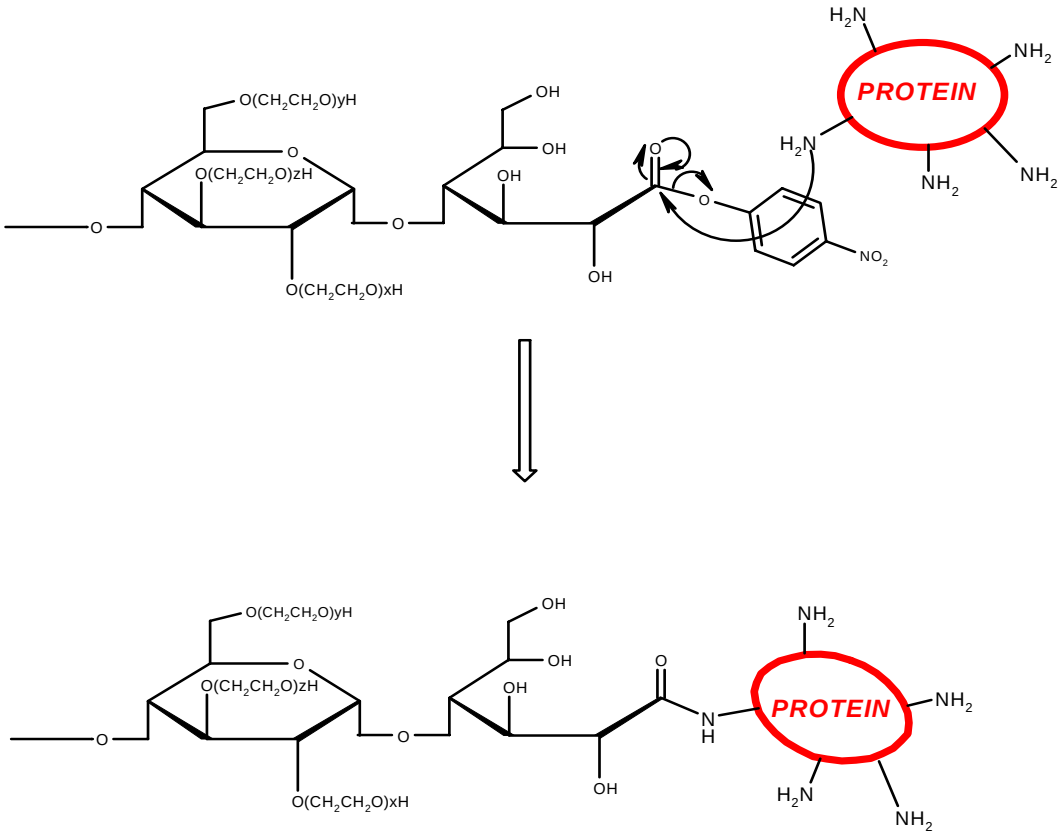
	Amount	MW	Mol	Ratio
oxHES$_{70}$	500 mg	18.667 kD	$2.7x10^{-5}$	**1**
p-nitrophenol	95 mg	139.1 D	$6.7x10^{-4}$	**~25**
DCC	276 mg	206.3	1.34×10^{-3}	**50**
DMAP	33 mg	122	$2.7x10^{-4}$	**10**
N-methylpyrrolidone	3.0 ml			
DMSO anhydrous	1.5 ml			

The oxHES was dissolved in 2 ml N-methylpyrrolidone and 1.5 ml DMSO previously dried over molecular sieve. Thereafter p-nitrophenol was added to the reaction vessel and the solution became yellowish. This was followed by the addition of DMAP and only finally DCC in a single addition. The reaction is allowed to run for 24 h at room temperature under moderate stirring conditions. The final solution looked yellow.

The purification process was realised by adding acetone (30 times excess), and incubating the precipitate in the cold for 3 h. Thereafter the solution was filtered and the precipitate washed with 30 ml of MeOH in two portions and finally with 50 ml of acetone. The precipitate was dried with the help of a vacuum pump to eliminate remaining traces of acetone.

4.2.1f Pre-activated oxHES (p-nitrophenyl) coupling to a model protein (HSA)

Theory: The p-nitrophenyl ester of oxHES is relatively stable in water and is particularly indicated for coupling in aqueous solution. As active ester it is able to react only with markedly nucleophilic functions which are for instance the ε-amino groups of lysines.



Description:

	Amount	MW	Mol	Ratio
activated-oxHES$_{70}$	500 mg	18.667 kD	2.7x10^{-5}	**1**
HSA	33 mg	122	2.7x10^{-4}	**10**
Water	400 µl			

Activated HES$_{70}$ and HSA were dissolved simultaneously in distilled water in the same reaction vessel at room temperature under magnetic stirring. The pH was confirmed to be in the neutral range or eventually slightly basic (in case of lower pH values some drops of TEA could be added). 20 min after the addition the solution became slightly turbid, and the reaction ran for 17 more hours. The final solution was still a bit turbid. After stopping the reaction the solution was dialysed overnight against distilled water and finally lyophilised.

4.2.1g oxHES coupling to a model protein in a DMF/water mixture with CDI

Theory: In order to overcome both the problems of possible inter-molecular protein cross-linkage and the "burning cycles" consuming the activator, the reaction has been performed in an organic solvent based mixture. It has surprisingly been found that most proteins are soluble in DMF when a relatively small amount of water (even less then 10%) is present. HES being completely soluble in pure DMF this allowed a pre-activation step followed by a conjugation step where the activated polymer solution is added to the protein dissolved in a DMF/water mixture.

Description:

	Amount	MW	Mol	Ratio	
oxHES$_{25}$	400 mg	14350 (Mn)	2.8x10^{-5}	**~40**	**1**
HSA	50 mg	67000	7.5x10^{-7}	**1**	
CDI	45 mg	162.15	2.8x10^{-4}	**~400**	**10**
TEA	1.0 ml	101 *(d=0.72)*	7.1x10^{-6}	**~10**	**~0.3**
DMF	4.0 ml				
Water	1.0 ml				

400 mg of oxHES$_{25}$ were dissolved in 3.0 ml DMF and then the temperature was decreased to 0°C. One ml of TEA was then added, and after few minutes also the CDI. The solution was incubated for 45 min at the same temperature under stirring.

In a separate vessel 50 mg of HSA were dissolved in 1.0 ml of water and 1.0 ml of DMF. The solution was chilled in an ice bath. Then the activated-HES solution was added to the protein solution drop by drop during a time period of 75 min, thereafter the reaction was allowed to proceed at 0°C until three hours and at 25°C overnight.

The solution is finally diluted with a 30 times volume of distilled water (millipore quality), dialysed over two days to completely eliminate DMF and the activation by-products, and lyophilised.

4.2.1h oxHES coupling to creatinase in a DMF/water mixture with CDI

Theory: The same reaction was repeated with a functional protein (enzyme) to check the influence of the coupling on the activity of the protein. As functional protein creatinase (CRE) has been chosen. The polymer was pre-activated, as previously described in the coupling to HSA, in pure DMF with CDI at low temperature.

Description:

	Amount	MW	Mol	Ratio	
oxHES$_{25}$	75 mg	14350 (Mn)	5.2x10^{-6}	**40**	**1**
CRE	6 mg	46000	1.3x10^{-7}	**1**	
CDI	8.5 mg	162.15	5.2x10^{-5}	**400**	**10**
TEA	0.4 ml	101 *(d=0.72)*	2.9x10^{-6}	**~20**	**~0,5**
DMF	800 µl				
Water	200 µl				

75 mg of oxHES$_{25}$ were dissolved in 600 µl DMF and then the temperature was decreased to 0°C. 400 µl of TEA were then added, and after few minutes also the CDI. The solution was incubated for 30 min at the same temperature under stirring.

In a separate vessel 6 mg of CRE were dissolved in 200 µl of water and 200 µl of DMF. The solution was chilled in an ice bath. Then the actHES$_{25}$ was added slowly with the help of a dropping funnel during 60 min, thereafter the solution was allowed to proceed at 0°C until three hour. During the run the solution remained clear.

The solution was finally diluted with a 30 times volume of 0.05 M phosphate buffer (pH 7.8) containing EDTA 0.1 mM, dialysed over two days against the same buffer and lyophilised.

4.2.1i oxHES coupling to superoxide dismutase in a DMF/water mixture with CDI

Theory: As the previous attempt using creatinase did not yield a functional product because of the low stability of the enzyme in DMF, several enzymes were checked for their stability in DMF, and superoxide dismutase (SOD) was found to keep the original activity after restoring physiological conditions. oxHES was as in the previous case, pre-activated in pure DMF and then added to the protein dissolved in a DMF/water mixture.

Description:

	Amount	MW	Mol	Ratio	
oxHES$_{25}$	75 mg	14350 (Mn)	5.2x10^{-6}	**42**	**1**
SOD	4 mg	32500	1.2x10^{-7}	**1**	
CDI	8.5 mg	162.15	5.2x10^{-5}	**430**	**10**
TEA	0.4 ml	101 *(d=0.72)*	2.9x10^{-6}	**23**	**~0.5**
DMF	800 µl				
Water	200 µl				

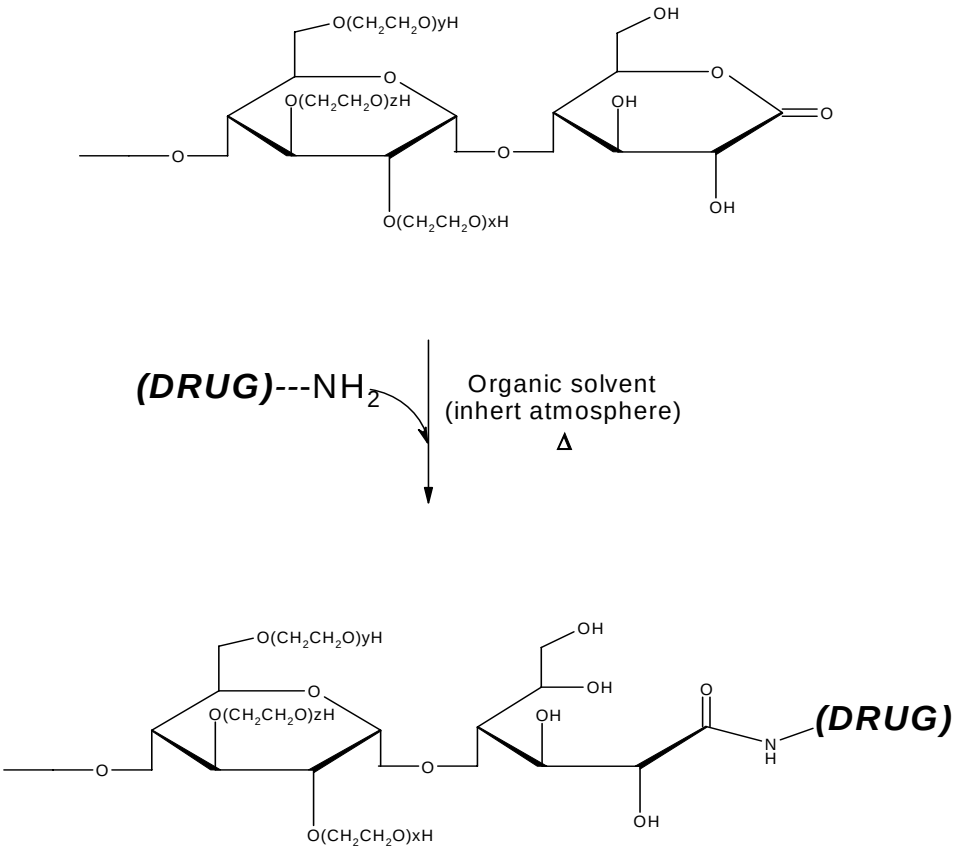
75 mg of oxHES$_{25}$ were dissolved in 600 µl DMF and then the temperature was decreased to 0°C. 400 µl of TEA were then added, and after few minutes also the CDI. The solution was incubated for 30 min at the same temperature under stirring.

In a separate vessel 4 mg of SOD were dissolved in 200 µl of water and 200 µl of DMF. The solution was chilled in an ice bath. Then the actHES$_{25}$ was added slowly with the help of a dropping funnel during 60 min, thereafter the solution was allowed to proceed at 0°C until three hour. During the run the solution remained clear.

The solution is finally diluted with a 30 times volume of 0.05 M phosphate buffer (pH 7.8) containing EDTA 0.1 mM, dialysed over two days against the same buffer and lyophilised.

4.2.1j oxHES coupling to Amphotericin B without activator

Theory: The oxidised form of HES can be easily transformed into the corresponding lacton by dehydration. The lyophilised product has a very low water content and if dissolved in an anhydrous organic solvent will keep the lacton form. The reaction we have used is generally regarded as: aminolysis of a lacton. The lacton is already a kind of reactive species and can undergo nucleophilic attack by amines resulting in an amide bond.



Description:

	Amount	MW (D)	Mol	Ratio
oxHES$_{130}$	2000 mg	43.287×10^{3}	4.7×10^{-5}	**~ 4**
Amphotericin B	10.0 mg	924.1	1.1×10^{-5}	**1**
anhydrous DMSO	3.0 ml			

The oxHES was dissolved in 2 ml of DMSO previously dried on molecular sieves. The temperature was increased up to 70°C and the solution degassed with a vacuum pump and N_2 was inserted in the flask to compensate the vacuum. In a separate vessel 10 mg of Amphotericin B were dissolved in 1 ml of DMSO and added to the oxHES solution. The reaction was allowed to run in dark for 24 h at the same temperature under moderate stirring conditions, then stopped. The coupled product was precipitated with 30 ml of acetone, washed 2 more times with acetone and once with methanol. The product was finally lyophilised. The yield, calculated spectrometrically, was found to be between 75% and 95%.

4.2.1k oxHES coupling to Mepartricin without activator

Theory: The same kind of strategy has been used to couple another polyene macrolide antibiotic having a free primary amino function. Mepartricin is very similar to amphotericin B also concerning the chemical – physical characteristics which allows the use of DMSO as solvent for both the reagents.

Description:

	Amount	MW (D)	Mol	Ratio
oxHES$_{130}$	2000 mg	43.287×10^{3}	4.7×10^{-5}	**~ 4**
Mepartricin	13.0 mg	1141.4	1.1×10^{-5}	**1**
anhydrous DMSO	3.0 ml			

The oxHES was dissolved in 2 ml of DMSO previously dried on molecular sieves. The temperature was increased up to 70°C and the solution degassed with a vacuum pump and N_2 was inserted in the flask to compensate the vacuum. In a separate vessel 13 mg of Mepartricin were dissolved in 1 ml of DMSO and added to the oxHES solution. The reaction was allowed to run in dark for 24 h at the same temperature under moderate stirring conditions, then stopped. The coupled product was precipitated with 30 ml of acetone, washed 2 more times with acetone and once with methanol. The product was finally lyophilised. The yield, calculated spectrometrically, was found to be between 75% and 95%.

4.2.2 Biological and microbiological tests

4.2.2a Determination of antimicotic activity

Theory: In order to determine the antimicotic activity left after the conjugation of Amphotericin B (or other antifungal drugs) with $oxHES_{130}$ a test using the Bouillon dilution method was carried out in 96 well microtiter plates according to E DIN 58940 Medical microbiology – Susceptibility testing of pathogens to antimicrobial agents, part 84: Microdilution – Special requirements for testing of fungi against antifungal agents. Results are given as MIC (Minimal Inhibitory Concentration). The MIC is the lowest concentration where no visible fungal growth can be detected in the tested sample.

Test Conditions:

Strain: *Candida albicans* DSM 11943
Inoculum: *C. albicans* $5 * 10^4$ CFU/ml in high resolution medium (Oxoid)
Incubation temperature: 30°C
Sample volume: 100 µl
Test volume: 200 µl (100 µl sample + 100 µl inoculum)

Procedure: After dissolving the freezedried coupled product in PBS (Phosphate buffered saline) the solution was filtered through a sterile non-pyrogenic filter (Whatman, 13 mm syringe filter, polysulfone, 0.2 µm) and subsequently a MIC test was carried out. Then the filtered solution was stored under exclusion of light at a temperature of 25°C and MIC was determined every 10 days for 90 days. The desired MIC value according to DIN 58940 lies between 0.125 and 1.0 µg/ml.

4.2.2b Creatinase (CRE) activity test

Theory: A unit of CRE is defined as the amount of enzyme which produces 1 µmol of urea per min under the conditions described below.

Reagents:

1. Potassium phosphate buffer, 0.3 M; pH 7.7 (reagent A);
2. Creatine solution, 0.1 M (reagent B);
3. *p*-Dimethylaminobenzaldehyde (DMAB) solution 0.87%:
   dissolve 2.0 g of DMAB in 100ml of ethanol (99.5%) and add 15 ml of conc. HCl and 115 ml of distilled water. (reagent C);
4. Enzyme dilution buffer: potassium phosphate buffer 10 mM, pH 8.0 and 2.5 mM of 2-mercaptoethanol (reagent D).

Sample: dissolve the lyophilised enzyme to a volume activity of 1.4-2.8 U/ml with ice-cold reagent D immediately before the measurement.

Procedure:

1. Pipette the following reagents into a test tube.
   0.1 ml reagent A
   0.8 ml reagent B
2. Equilibrate at 37°C for about 5 min.
3. Add 0.1 ml of the sample and incubate for 10 min at 37°C.
4. Add 2.0 ml of reagent C and allow to stand for about 30 min at 25°C.
5. Read the absorbance at 435 nm.

(The blank solution is prepared by reversing the sequence of addition of sample and reagent C)

4.2.2c Superoxide dismutase (SOD) – activity test

The SOD activity was measured using an assay kit commercially available from Dojindo Molecular Technologies, Inc.. The principle is an indirect measurement using a water soluble derivative of nitroblue tetrazolium (WST-1) which produces a water soluble formazan dye upon reduction with the superoxide anion (O_2^-).

The rate of the reduction with O_2^- is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD, as shown in the figure below.

Therefore, the IC_{50} (50% inhibition activity) can be determined by a colorimetric assay detecting the formazane derivative.

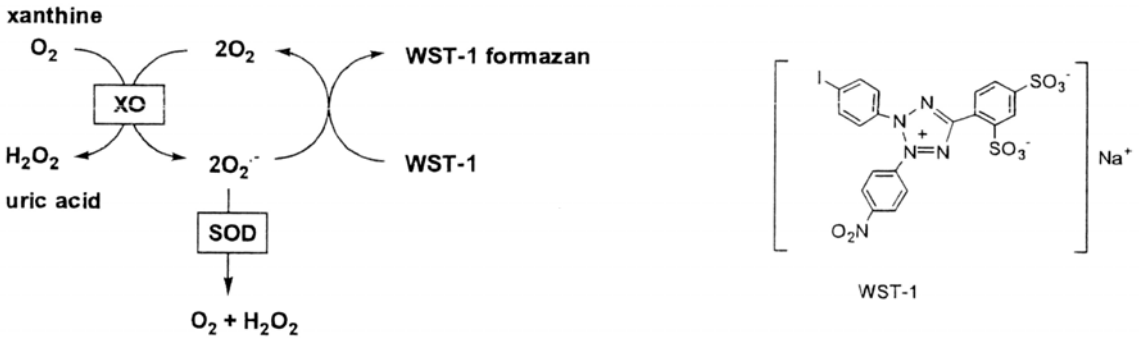


Figure 4.2: Principle of the SOD assay kit and chemical structure of the water soluble tetrazolium salt WST-1.

4.2.2d Haemolytic activity determination

Theory: In order to determine the haemolytic potential of Amphotericin B derivatives, the sample were incubated in presence of erythrocytes at 37°C for a certain time and at the end the content of free haemoglobin in solution was determined spectrometrically.

Reagents:

1. Erythrocytes concentrate solution from blood bank;
2. PBS buffer pH 7.4 (dilution buffer).

Procedure: An erythrocyte concentrate solution was diluted 1:1000 with PBS solution (this dilution was found to give the most convenient absorption in case of 100% haemolysis rate). The sample, diluted to the desired concentration (1.0 µg/ml), was applied to the erythrocyte dilution and after mixing the solution was incubated at 37°C respectively for one and two hours. Thereafter the sample was centrifuged at 14000g for 10 minutes to eliminate entire cells as well as residual cell membranes and the supernatant analysed with the spectrometer at 415 nm. The value was given as percent of the total haemolysis. The 100% haemolysis rate was defined as the absorption value at 415 nm obtained after freezing and thawing 5-times the erythrocytes dilution.

4.2.3 Analytical methods

4.2.3a Determination of HES oxidation degree (according to Somogyi) [1]

Theory: This method uses the oxidising power of copper ions (Cu^{++}) to measure how many aldehyde groups are left in a known amount of oxidised polysaccharide (according to the procedures previously described). The Cu_2O precipitate is reoxidised with a measured excess of iodine which is finally retrotitrated with thiosulfate. Every analysis needs a blank sample and a complete non-oxidised sample. The degree of oxidation is obtained by comparison among the three values.

Procedure: Prepare three test tubes containing respectively 5 ml water (blank), 300 mg of HES_{130} (non-oxidised) and 300 mg of $oxHES_{130}$. Add to each one 5 ml of the stabilised alkaline copper solution and after sealing, let the tubes cook in boiling water for 45 min. After cooling the samples down to room temperature 1.5 ml of a 2.0 N H_2SO_4 solution can be inserted and the tubes shaken for 5 min. If needed, more sulphuric acid can be added, until the sample has an acidic pH. After a further shacking few drops of starch indicator have to be added together with few drops of phenol red and then this solution can be titrated using $Na_2S_2O_3$ solution.

$$\text{Oxidation degree} = \frac{\text{Vol}_{\text{tiosulph}}\ \text{Sample} - \text{Vol}_{\text{tiosulph}}\ \text{Non-oxidised}}{\text{Vol}_{\text{tiosulph}}\ \text{Blank} - \text{Vol}_{\text{tiosulph}}\ \text{Non-oxidised}} \times 100$$

4.2.3b Determination of HES oxidation degree (BCA test) [2]

Theory: This method also uses the oxidising power of copper ions (Cu^{++}) to measure how many aldehyde groups are left in a known amount of oxidised polysaccharide (according to the procedures previously described). The reduced copper (Cu^{+}) does not precipitate as oxide but is caught by a chelating agent (bicinchoninic acid) which keeps it in solution. BCA (bicinchoninic acid) has a characteristic absorption maximum at 560 nm when bound with Cu^{+}. The sensibility of this method is much higher than that of Somogyi and also the reproducibility of results is much

increased. A calibration curve with increasing sugar amounts was prepared to calculate the amount of an unknown sample.

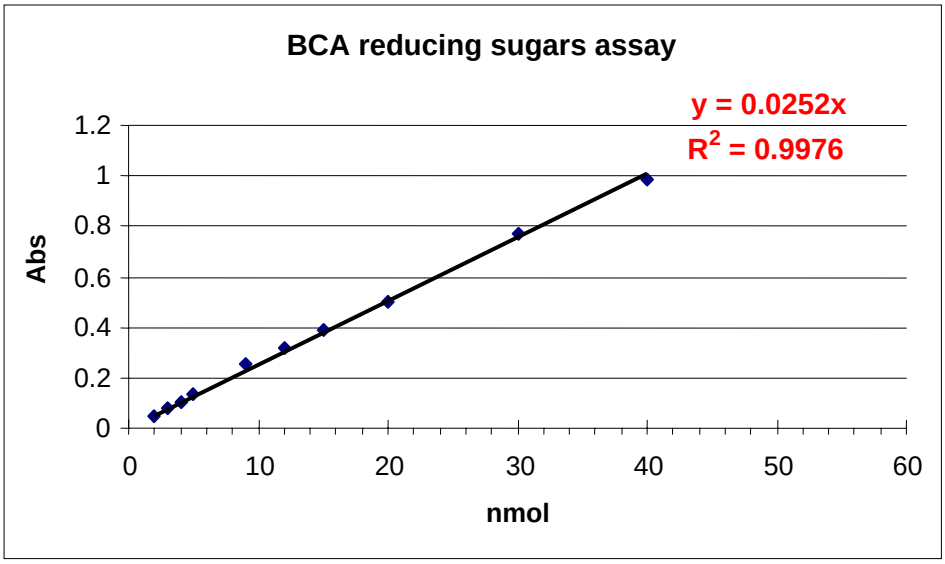
Reagents:

1. Solution A: Bicinchoninic acid (sodium salt) 194.2 mg
   Na_2CO_3 5.43 g
   $NaHCO_3$ 2.42 g
2. Solution B: Cupric sulphate anhydrous 79.8 mg
   L-serine 126.2 mg

Procedure: Mix equal volumes of assay solutions A and B to prepare the assay reagent, and keep it in dark. Then mix 1.6 ml of this reagent with 0.4 ml of sugar solution in a small test tube, and prepare a blank by mixing 1.6 ml of reagent and 0.4 ml of water in another test tube.

With the help of a thermomixer at 1400 rpm the tubes were kept for 30 min at 80°C, always under light protection. Afterwards, the test tubes were allowed to cool for 30 more minutes in the dark and finally their absorption was read at 560 nm. The linearity range is between 2 and 40 nmol of reducing end groups in 0.4 ml of solution. The calibration with glucose gave the following relation:

$$Abs_{sample} - Abs_{blank} = 0.0252 \times (\text{nmol of glucose})$$



4.2.3c Determination of sugar content[3]

Theory: This method may be used to detect the total neutral sugar content of soluble sample of glycoproteins, proteoglycans, and other materials that contain this type of sugars.

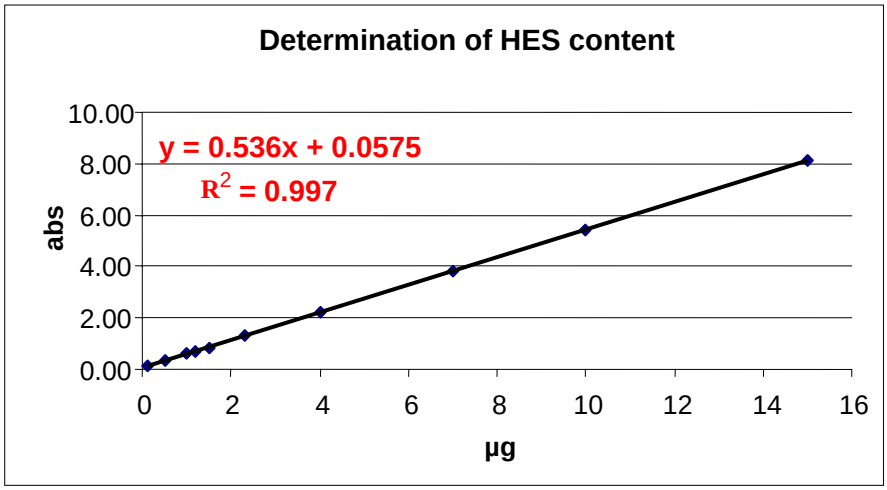
This assay does not require prior hydrolysis step is very sensitive and can detect oligo- and polysaccharide amounts in solution. As the reaction requires strong acid conditions the polymer is completely hydrolysed to its monomers. Therefore the colour development is not in relation with the moles of polysaccharide but with the number of monosaccharide units really present in the sample.

In fact, two samples equal in weight, containing the same polysaccharide in two different molecular weights will give the same absorption intensity.

Reagents:

1. Phenol solution (80% by weight).
2. Sulphuric acid, reagent grade 95.5%

Procedure: Put 400 µl of the solution to be tested in a tube and add 25 µl of 80% phenol to it. Then mix for a couple of seconds. A blank sample is also required, using 400 µl water instead of the polysaccharide solution. To both test tubes add 900 µl of concentrated sulphuric acid, shake the test tubes, and put them in a 20°C water bath exactly after 5 seconds. After 30 min in the water bath the sample's absorption is measured at 487 nm and 489 nm in a spectrometer. The samples are usually prepared and analysed in triplicate. Accurate timing is absolutely required. Sensitivity turned out to be very good, the linear range was between 0.1 µg and 15 µg. Calibration with HES_{200} gave the following relation: Abs = 0.0575 + 0.536 x (µg of HES) with an excellent R^2 of 0.997.



4.2.3d Determination of protein content (BCA)

The total protein concentration of the coupling product was measured using a ready-to-use assay kit commercially available from Pierce. The Pierce BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of proteins.

This method combines the well-known reduction of Cu^{++} to Cu^{+} by protein in an alkaline medium (the Biuret reaction [4]) with the highly sensitive and selective colorimetric detection of the cuprous cation using a unique reagent containing bicinchoninic acid.

The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water soluble complex exhibit a strong absorbance at 562 nm that is nearly linear with the increasing protein concentrations over a broad working range (20-2000 µg/ml).

The macromolecular structure of the protein, the number of peptide bonds and the presence of four particular amino acids (cystein, cystine, tryptophan and tyrosine) are reported to be responsible for colour formation with BCA [5]. Accordingly, protein concentrations are generally determined and reported with reference to standards of a common protein such as bovine serum albumine (BSA).

4.2.3e Gel permeation chromatography(GPC)

This technique was used in order to characterise the coupling product in both, proteins and small drugs conjugation. The column was usually calibrated with proteins having known molecular weight and then the calibration curve has been used to define the increased size of the coupling product. GPC was used with the FPLC as well as with HPLC equipment.

The GP-HPLC was useful in monitoring the reaction run because of a much shorter analysis time while with FPLC columns larger amounts of proteins could be purified. Gel permeation chromatography was also used to monitor the coupling with Amphotericin B but finally the preferred analytical method became RP-HPLC.

An example of typical analysis parameters are:

Buffer:	300 mM	NaCl
	10 mM	Na^+ phosphate (pH = 7.5)
	0,01 %	Triton
Flow rate:	0.75-1.0 ml min^{-1}	
Sample volume:	5 – 100 µl	

4.2.3f Reverse phase chromatography (RPC)

RPC with C_{18} columns was mainly used to detect the coupling product in Amphotericin B conjugation and the formation of the activated oxHES. Moreover this technique was applied to assess the purity of the conjugate and to detect the presence of non-coupled Amphotericin B traces after the purification step. By RP-HPLC analysis was also defined the chemical stability in water of the coupling product, by checking the free Amphotericin B content of samples after a discrete incubation time, by different temperatures.

An example of typical analysis parameters are:

Buffer:	$CH_3COO^- NH_4^+$ / acetonitrile (3:1)
Flow rate:	0.5 – 0.75 ml min^{-1}
Sample volume:	5 – 100 µl

4.2.3g Ion exchange chromatography

This technique allowed us mainly to purify HES-coupled proteins from non-coupled proteins. Non-HESylated proteins displayed a higher tendency to bind to cation exchanger column than their HESylated counter parts. The rational behind this effect is that by coupling HES to an ε-NH_2 function of lysine one positive charge will be lost so that the modified protein binds much weaker to a negatively charged support.

Therefore this method could be used also as analytic to discriminate among coupled proteins having a different modification rate, meaning proteins carrying a different number of polymer chains on them selves.

4.2.3h SDS-Polyacrylamide gel electrophoresis

This technique was done in a standard fashion as described in "Arbeitsmethoden der Biochemie" Pingoud/Urbanke, Ed. Walter de Gruyter (1997). SDS-PAGE has been used to detect the increase in the size of proteins due to conjugation. Staining was performed with Comassie blue or with silver, in the case a higher sensitivity was required, according to the standard protocols.

4.2.3i Native gel electrophoresis

It was essentially used in case of proteins that are naturally occurring as multimers. The technique was exactly applied as described in "Arbeitsmethoden der Biochemie" Pingoud/Urbanke, Ed. Walter de Gruyter (1997).

4.2.3j Silver stain for carbohydrate

Theory: A sensitive detection of carbohydrate groups following PAGE could be also done by silver staining after a brief periodate oxidation of the carbohydrates [6]. The methods exploits the reducing potential of the aldehyde groups yielded by periodate oxidation to reduce the silver ions to silver metal. The original method, that uses formaldehyde to develop the colour, will eventually stain proteins as well, but under the conditions outlined below (after our optimisations) there should be only selective detection of carbohydrates. After silver staining the gel can be cleared and stained for protein with Colloidal Coomassie Blue G-250.

Reagents:

1. Periodate reagent (prepare fresh just before use, under low light conditions):
   150 ml water
   4 ml 7% acetic acid containing 25% isopropanol
   1.05 g periodic acid
2. Silver stain reagent (prepare fresh just before use, the solution must be clear before and after use)
   28 mL NaOH (0.1 N)
   2.5 mL NH_4OH (> 25%)
   0.5 g $AgNO_3$ in 5 ml water
   115 ml water
3. 7% acetic acid

Procedure:

- Fix gel with 12% TCA for 1-15 hr using agitation throughout.
- Wash with water, then with 7% acetic acid.
- Decant acetic acid from the gel and add fresh periodate reagent (use low light).
- Treat gel for 15 min RT with orbital agitation under dark fabric.
- Wash gel: 4 x 250 ml over 30 min with agitation under dark fabric.
- Add stain, and agitate until the bands start to become visible.
- Rinse gel with water 4 x 250 ml, 5 min each until the pH becomes neutral.
- Store in distilled water at 4-8°C.

4.2.3k Precipitation of glycoconjugates with ammonium sulfate

Theory: Over the years, the “salting out” with ammonium sulphate has been used as an initial step in many protein purification schemes. Proteins but also glycoconjugates will precipitate out from a solution at a critical concentration of salt. Pilot experiments served to determine the optimal salt concentrations to use for a particular application.

This method allows a quick and efficient initial purification step for proteins from a crude sample. The pH has to be controlled using a buffer (50 mM Tris, pH 7.4). Ammonium sulphate was

always added as dry powder slowly to the stirred solution of the sample at 4°C. Most proteins are very stable when stored at -20 or -70^{o}C as an ammonium sulphate precipitate if kept in sealed tubes. When needed the pellet can be re-dissolved in water and dialysed. The dialysed product could be finally lyophilised and stored as solid powder at low temperature.

Reagents:

1. Ammonium sulphate (molecular biology grade)
2. 1 M Tris buffer, pH 7.4

Procedure:

- Measure A_{280nm} of the sample and adjust to 20 mM Tris, pH 7.4.
- To a known volume of sample (e.g. 1 L) add solid ammonium sulphate slowly while stirring (the amount of ammonium sulphate has to be adjusted so that the protein of interest will stay in solution).
- Stir well until dissolved and let stand on ice for 30-60 min.
- Centrifuge the sample at 5,000 x g for 15 min.
- Decant the supernatant and measure the volume.
- To the supernatant add solid ammonium sulphate slowly while mixing (the amount of salt has to be sufficient to precipitate the protein of interest).
- Stir on ice for 30-60 min.
- Centrifuge the sample as before.
- Decant and discard the supernatant and save the precipitate.
- The precipitate can be stored at -20 or -70^{o}C in sealed tubes for several months with no loss of activity.
- When needed, re-dissolve the pellet in a small volume of water and dialyse against buffer.

4.2.3l Western blotting and glycan detection with antibodies

Theory: Western blotting is also a standard procedure in biochemistry, the description of this method is available in "Arbeitsmethoden der Biochemie" Pingoud/Urbanke, Ed. Walter de Gruyter (1997). We used this method to transfer proteins after a coupling reaction, to a nitrocellulose membrane in order to identify the HES-modified proteins among the non-coupled ones. The identification is performed using a glycan-specific enzyme immunoassay.

Vicinal hydroxyl groups in sugars of glycoconjugates are oxidised to aldehyde groups by mild periodate treatment (in solution or on the filter). The spacer linked steroid hapten digoxigenin is then covalently attached to these aldehyde via hydrazide group. Digoxigenin labelled glycoconjugates are subsequently detected in an enzyme immunoassay using a digoxigenin specific antibody conjugated with alkaline phosphatase. This method can detect glycoproteins in a range of 0.1 - 10 µg.

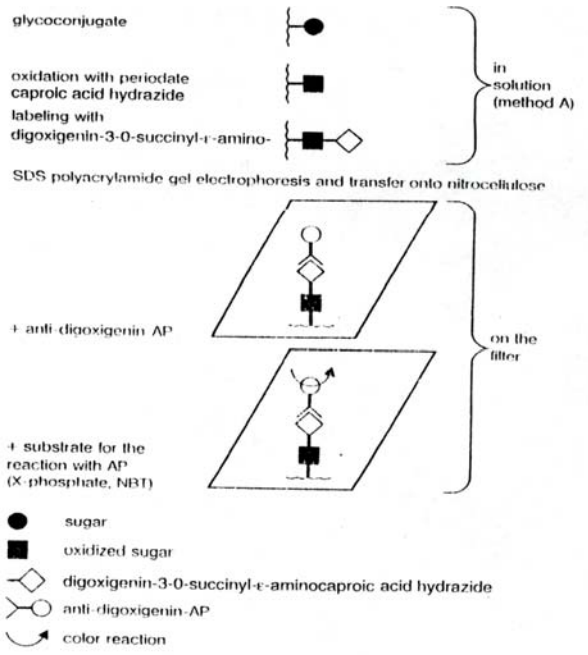


Fig. 4.3 – Glycan detection enzyme immunoassay

Procedure: The proteins were oxidised in solution with sodium metaperiodate and labelled with digoxigenin before separation on SDS-PAGE. These samples were subsequently resuspended in the SDS sample buffer and subjected to gel electrophoresis. Finally the proteins were transferred to a nitrocellulose carrier by Western blotting. The nitrocellulose filter was allowed to incubate for at least 30 min in a blocking solution containing no glycoproteins in order to saturate all unspecific binding site on the filter and avoid unspecific adsorption from the antibody. After several washing steps the filter was incubated with the anti-digoxigenin antibody and immersed in the staining solution containing a special substrate which was converted to a grey coloured molecule by the alkaline phosphatase conjugated on the antibody.

4.2.3m UV–Visible spectroscopy

UV–Visible spectroscopy has been used to follow enzyme kinetics and to determine Km and Vmax of modified enzymes. Moreover this method was also applied to define the content of Amphotericin B, as well as other UV-absorbing molecules, in the coupled product, in order also to calculate the effective yield. Therefore a calibration curve for Amphotericin B (as it is not water soluble) was performed in DMSO. Then a measured amount of HES coupled Amphotericin B (also soluble in DMSO) has been read at the same wavelength in order to find out the exact amount of drug present in the sample. The result has been confirmed twice by reading different amounts of coupled drug.

4.2.3n Fluorescence spectroscopy

This technique allowed the monitoring of the conformational changes occurring in the protein three-dimensional structure after polymer anchoring. Excited proteins are known to fluoresce because of the presence of some particular amino acids. Of the 20 amino acids generally found in proteins, the aromatic phenylalanine, tyrosine and tryptophane are the only ones of sufficient fluorescence intensity to be measured directly in solution, among them tryptophane has by far the highest intensity. The fluorescence of proteins due to these residues is a highly specific and sensitive tool for studying structure and conformation because the emission depends on the chemical neighbourhood of the amino acid residues. Phenomena of denaturation or structural modification can be followed by comparing the fluorescent emissions before, during, and after the coupling reaction. Since it is a non-invasive technique, fluorescence does not interfere with a sample, moreover the excitation light levels required to generate a fluorescence signal are quite low.

The fluorescence of the aromatic residues varies in somewhat unpredictable manner in various proteins. Comparing to the unfolded state, the quantum yield may be either increased or decreased by the folding. Accordingly, a folded protein can have either greater or less fluorescence than the unfolded form. The wavelength of the emitted light is a better indication of the environment of the fluorophore. Tryptophan residues that are exposed to water, have maximal fluorescence at a wavelength of about 340-350 nm, whereas totally buried residues fluoresce at about 330 nm.

4.2.3o Immunodiffusion

Theory: These are techniques in which antibody is allowed to encounter antigen by diffusion only. Many variations of the basic techniques have been developed [7]. A buffered agar gel is prepared and antigen and antibody loaded into separate wells bored in the agar about 0.5-2 cm apart. After incubation for 12-48 h antibody-antigen reconition is detected by the formation of an immunoprecipitation line between the wells. An advantage is that the concentration gradients of antigen and antibody are automatically formed by the diffusion process. Immunoprecipitation will occur somewhere between the wells providing that equivalence concentration is obtained at some point in the overlapping antigen and antibody gradient.

General procedure:

1. prepare 1% (w/v) agar solution by heating 1 g agar in 100 ml PBS-azide in a boiling water bath until completely dissolved.
2. allow agar solution to cool down to 45°C and carefully pipette 3.5 ml into each Petri's dish. Allow to solidify.
3. carefully punch holes in the agar using the cork borer or gel punch so that you have a hole in the middle surrounded by 4-6 symmetrically disposed holes.
4. remove the plugs of agar from the wells using a Pasteur pipette attached to the vacuum line. The plugs have to be quickly and evenly removed.
5. pipette 20 μl of the respective antiserum in the proper dilution into the centre well and pipette the dilutions of antigen (in our case, protein (HSA) and modified protein (HES-HSA) in the surrounding wells.

4.2.3p MALDI-TOF

MALDI TOF instruments tend to be used for large biomolecules such as peptides and proteins. However, it is possible to use the technique for smaller inorganic molecules. One limitation is that the use of an large excess of matrix limits the lower limit of the scanning range. For most practical purposes it is not possible to get useful data below approximately 700 Daltons as this area tends to be predominately matrix ions.

The sample in solution, at a concentration of approximately 10 pmol/μl, is mixed with a solution of the matrix, at a typical concentration of 10 mg/ml, and 2 μl of the mixture is spotted onto a stainless steel plate. The solvent is allowed to evaporate at room temperature forming crystals of the matrix/sample mixture. The plate is introduced into the vacuum system of the instrument and allowed to pump down. A fast (4 ns) laser pulse irradiates the target spot producing a burst of ions, which are accelerated in an electric field and fly down to the detector.

The matrix absorbs the laser energy which causes it to desorb and ionize in a single step, along with the sample molecular ions. The laser light is focused on the 2 mm diameter target to a spot of approximately 150 -250 μm. The spot position is fixed on the ion optical axis, and the plate is moved around (under software control) to irradiate different parts of the target. The ions produced are accelerated up to 25 kV through a three stage source, which also focuses the ion beam and passed into the Time of Flight Mass Spectrometer. The matrix suppresser allows for the selective filtering out of lower mass ions to reduce the number of matrix ions that reach the detector, and is adjusted by the software.

4.2.3q Light-scattering detection [8]

Light scattering (LS) provides the absolute molecular weight (Mw) and size (radius of gyration $<r_g^2>$) of macromolecules in solution. The amount of light scattered is directly proportional to the product of the weight-average molar mass and the concentration of the macromolecule, i.e., LS ~ Mw·c.

Laser light scattering is a "non-invasive" technique that can be performed in either the batch or chromatography mode. In either instance the sample may be recovered at the end of the study. Unlike molecular weights that are estimated from gel size exclusion chromatography data alone, the Mw obtained from light scattering is *not* dependent upon either the Stokes radius of the protein (or other macromolecule) nor a calibration curve that depends upon running several standard proteins.

Since laser light scattering provides the weight average Mw for all molecules in solution it is generally more useful to utilize the chromatographic mode. Hence, if a weak dimer : tetramer interaction was being studied under conditions where there were equal weights of both species, the batch mode study would provide a Mw that would correspond to a trimer while the chromatography study would provide the Mw for both the (separated) dimer and tetramer as well as quantify the amounts of each species.

Although molecular weights are most widely determined by mass spectrometry, only light scattering and analytical centrifugation monitor the properties of macromolecules in solution and provide information about the oligomeric state of the protein. While a sedimentation equilibrium run may require 72 hours, a size exclusion chromatography/LS study can be completed in about one hour and a batch mode analysis can be completed in a few minutes.

The relationship linking he intensity of the scattered light, the scattering angle, and the molecular properties is simply

$$\frac{K^*c}{R(\theta)} = \frac{1}{M_w P(\theta)} + 2A_2c$$

where:

$R(\theta)$	is the excess intensity of scattered light at DAWN angle θ;
c	is the sample concentration;
M_w	is the weight-average molecular weight (molar mass);
A_2	is the second viral coefficient;
K*	is an optical parameter equal to $4\pi^2n^2(dn/dc)^2 / \lambda_0^4 N_A$
n	is the solvent refractive index and dn/dc in the refractive index increment
N_A	is Avogadro's number
λ_0	is the wavelength of the scattered light in vacuum.

The function $P(\theta)$ describes the scattered light's angular dependence. At low angles, the angular dependence of scattered light $P(\theta)$ depends only on the mean square radius $\langle r_g^2 \rangle$ independent of molecular conformation. A plot of $K^*c/R(\theta)$ vs. $\sin^2(\theta/2)$ yields a curve whose intercept give M_w and whose slope gives $\langle r_g^2 \rangle$.

4.2.4 Computational methods

4.2.4a Determination of the pK_a shift of the lysines in a model protein (HSA)

Model structure: The structure of HSA used for the calculations was the one published by SugioS., Kashima A. et al. [9], with PDB code 1AO6, and more precisely only the monomer A was considered.

Continuum Electrostatic calculations: On the above mentioned structure, CE calculations were made using Donald Bashford's software package MEAD (Macroscopic Electrostatics with Atomic Detail) [10]. The methodology considers the protein as a low dielectric medium immersed in a high dielectric solvent. The electric potential is determined by the Poisson-Boltzmann equation, which is solved by finite-difference methods. The details of the structure are incorporated into the placement of charges and dielectric boundaries. Moreover the calculation takes into account also the conformational flexibility of the side chains [11]. The calculation was limited to the lysyl ε-amino functions.

5 Results – *Protein drug conjugation*

5.1 Coupling strategies in HESylation

As already pointed out in the previous chapters, among the objectives of this work was the feasibility study of a selective coupling with polysaccharides, with the aim to develop a procedure where the polymer reacts by means of a single reactive group with the protein target. Besides the many hydroxyl groups, HES possesses this one unique reducing end per polymer chain whose different reactivity can be considered as a starting point for our coupling strategy.

We distinguished between two different approaches. In approach A the terminal aldehyde function is firstly oxidised to carboxylic acid by mild oxidation either in presence of halogens or with metal ions. The resulting carboxylic function allows a broader spectrum of coupling ways, all of them in general consisting of an activation step followed by the coupling reaction. Among the several activation methods suitable for the carboxylic function three of them were more deeply investigated: activation with carbodiimide (EDC – N-ethyl-N',N'-dimethylamino-3-propyl carbodiimide) in aqueous medium, active ester (p-nitrophenyl- and pentafluorophenyl-ester) in aqueous medium and activation with carbonyl diimidazole in DMF/water mixtures.

In the second coupling strategy (approach B) the aldehyde group may react as such with an amino function to yield a Schiff's base which will be reduced to a more stable secondary amino function.

At the beginning a two-step reaction was utilised, first building of the imine and second the reduction by mean of a usual reducing agent ($NaBH_4$).

As the Schiff's base formation is an equilibrium reaction, in order to shift it towards the product formation, also an *in situ* reduction was investigated with $NaBH_4$ as well as a pH sensitive reducing agent ($NaBH_3CN$).

5.2 Hydroxyethyl starch selective oxidation

As first reaction of the proposed reaction scheme (approach A), oxidation of different HES samples was carried out. According to the method described by Hashimoto et al.[1], iodine was chosen as a mild oxidation reagent, which should give a selective oxidation at the reducing end of the polysaccharide.

Table 5.1 lists the oxidation experiments performed for the optimisation of HES_{130} oxidation. Parameters varied in these experiments were mainly reaction time , solvent and molar ratios of reactants.

	HES (based on Mn)	***Iodine sol. 0.1 N***	***KOH sol. 0.1N***	***solvent***	***Reaction time***	***Yield***
I OXIDATION HES 130	1 g 2.4x10-5 mol	0.3 ml 3.0x10-5 mol	0.5 ml 5.0x10-5mol	water 4.0ml	4 h 25°C	30.1%
II OXIDATION HES 130	4 g 9.4x10-5 mol	1.0 ml 1.0x10-4 mol	1.5 ml 1.5x10-4mol	water 6.0ml	over night 25°C	24.8%
III OXIDATION HES 130	5 g 1.2x10-4 mol	1.2 ml 1.2x10-4 mol	1.5 ml 1.5x10-4mol	water 7.5ml	over night 25°C	24.3%
IV OXIDATION HES 130	5 g 1.2x10-4 mol	3.0 ml 3.0x10-4 mol	4.5 ml 4.5x10-4mol	water 7.5ml	over night 25°C	60.8%
V OXIDATION HES 130	5 g 1.2x10-4 mol	4.0 ml 4.0x10-4 mol	5.0 ml 5.0x10-4mol	water 7.5ml	over night 25°C	80.0%
VI OXIDATION HES 130	8 g 1.9x10-4 mol	7 ml 7.0x10-4 mol	11.5 ml 1.2x10-3mol	water 7.5ml	over night 25°C	88.4%
VII OXIDATION HES 130	10 g 2.4x10-4 mol	10 ml 1.0x10-3 mol	20 ml 2.0x10-3mol	water 7.5ml	over night 25°C	100%

Table 5.1: Summary of the experiments for the oxidation of HES_{130}.

More complete oxidation reactions were obtained by increasing the amount of iodine from 3.0 x 10^{-5} mol up to 1.0 x 10^{-3} mol. Acceptable yields were found for experiments V to VII, the latter showing nearly quantitative conversion at a molar excess of about 4-fold for I_2.

A similar picture was obtained in the case of HES 10 kD, as depicted in table 5.2. The amount of iodine solution was increased to reach almost 100% of oxidised material in experiment V (excess of I_2 about 5-fold). The oxidation degree was calculated by titration according to the Somogyi method (see Materials and methods).

	HES (based on Mn)	*Iodine sol. 0.1 N*	*KOH sol. 0.1N*	*Solvent*	*Reaction time*	*Yield*
I OXIDATION HES 10	5.0 g 1.4x10-3 mol	2.0 ml 2.0x10-4 mol	2.0 ml 2.0x10-4mol	Water 10.0ml	20 h 25°C	3.0%
II OXIDATION HES 10	5.0 g 1.4x10-3 mol	3.5 ml 3.5x10-4 mol	4.5 ml 4.5x10-4mol	Water 10.0ml	over night 25°C	5.3%
III OXIDATION HES 10	15.0 g 4.1x10-3 mol	21.0 ml 2.1x10-3 mol	31 ml 3.1x10-3mol	Water	over night 25°C	10.5%
IV OXIDATION HES 10	8.0 g 2.2x10-3 mol	83 ml 8.3x10-3 mol	180 ml 1.8x10-2mol	Water	over night 25°C	80.0%
V OXIDATION HES 10	7.0 g 1.9x10-3 mol	95 ml 9.5x10-3 mol	210 ml 2.1x10-2mol	Water	over night 25°C	100%

Table 5.2: Summary of the experiments for the oxidation of HES_{10}.

Later on, by changing the analytics for the determination of the oxidation degree from the Somogyi method to the more accurate BCA method (it uses a spectrometric evaluation instead of a manual titration, see Materials and methods), we observed that the material coming from the iodine/KOH oxidation had an actual lower oxidation degree than measured with the Somogyi method. The real oxidation degree was 70-75% instead of 100%.

Nevertheless theoretical yields could still be reached by repeating the oxidation process. It seemed likely that the oxidation efficacy would decrease by working in dilute solution, so it is necessary to eliminate the water and start again from a more concentrated solution. Moreover better results were obtained by using a 1 N KOH solution instead of the 0,1 N. In this way the final volume of the reaction was drastically reduced and the oxidation was more effective because the solution was more concentrated.

Another step in the optimisation procedure consisted in establishing the most convenient interval between two further KOH additions and the most appropriate volume of solution to be added. The addition of a 20µl drop every four minutes, in order to keep a constant concentration of the oxidant (IO^-) in solution served as a good compromise. This may be considered as a basis for automating the entire oxidation process in the future.

This oxidation strategy however needs long reaction times, therefore, in order to reduce the duration of this step, another method was introduced which allowed, by working at higher temperatures, to more easily obtain the desired oxidation degree. This approach uses the oxidising power of metal ions like Cu^{++} or Ag^+, an old reaction used to detect and quantify mono- and oligosaccharide which had not yet been used for synthesis of aldonic acids. The main reason was the difficult purification of the oxidised product from the reaction mixture. Working with high molecular weight polymers one may have two valid purification alternatives: ultrafiltration and dialysis. Selecting a membrane with an adequate molecular weight cut off, the low molecular weight by-products of the oxidation are easily eliminated. We chose dialysis as a preferred purification method.

This reaction leads to oxidation degrees normally higher than 95% (checked with the BCA method). In some cases it could happen that a second oxidation may be needed to reach the desired yield. Still the procedure is much faster than the iodine method.

Moreover by analysing with a light scattering detector both the products, oxidised with I_2 and with Cu^{++}, the oxidation with halogens seems to partially degrade the polymer. The figure 5.1 shows the differential molar mass of both products compared to the non-oxidised HES. The profile of the metal ions oxidised HES is much more similar to the original HES than the iodine oxidised product.

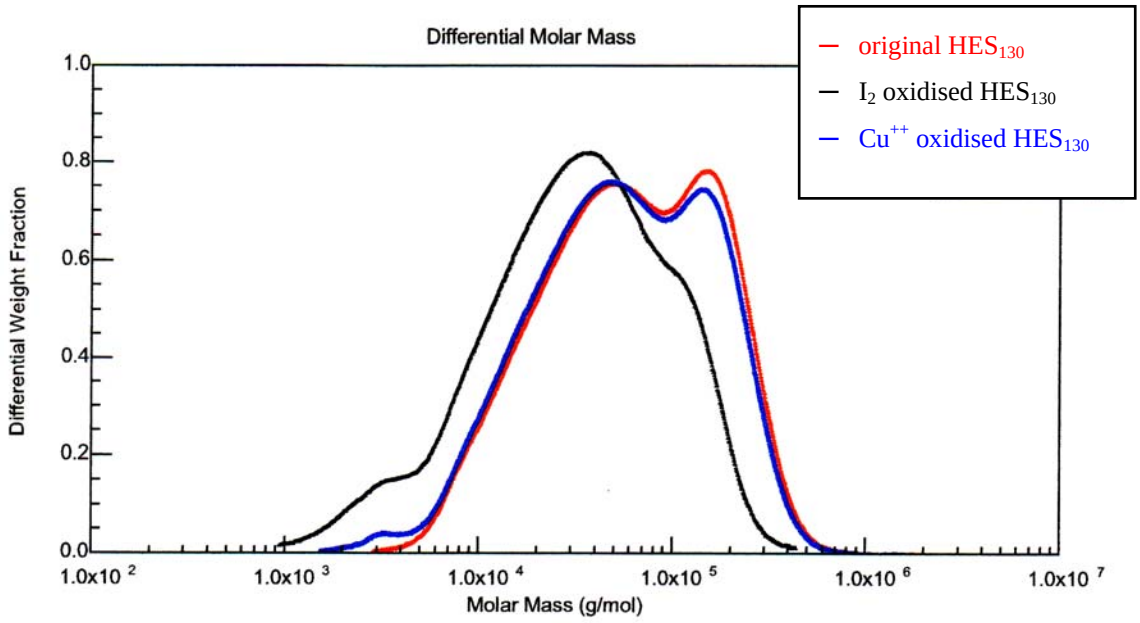


Figure 5.1: Differential molar mass distribution of two differently oxidised HES_{130} probes in comparison to the non-oxidised material.

This oxidation method has been successfully applied to HES of all different sizes.

5.3 Coupling of oxidised HES_{130} ($oxHES_{130}$) to a model protein (approach A)

In order to carry out a reproducible coupling strategy several trials were performed using human serum albumin (HSA) as easily available model protein. First of all, our objective was to obtain an evidence for HESylated protein, to demonstrate the feasibility of this coupling reaction. As second priority the coupling product should have only few modification points in order to avoid dramatic changes in the protein structure.

As the coupling product is supposed to be used as a drug to be injected directly into the blood stream, and is not supposed to be rapidly cleared another parameter has to be

considered: the final size of the conjugate and the molecular weight limit for glomerular filtration.

Therefore when a mono-HESylated protein is wanted, this single polymer chain has to be big enough to increase the molecular weight of the protein above a total molecular weight of 70kD which is actually the limit for glomerular filtration.

That is the reason why $oxHES_{130}$ was chosen for the first experiments. Having a Mw value of ~100kD, it ensures a considerable increase in Mw and might also prevent a rapid elimination after partial enzymatic hydrolysis of the carbohydrate portion by work of the α-amylase.

In case a multi-HESylated protein is desired as product also $oxHES_{10}$ and $oxHES_{25}$ were preferred. The logic behind this strategy may be the need either to have a better surface shielding towards high affinity antibodies or simply to avoid that a big portion of the polymer could be cleaved by means of a single enzymatic hydrolysis which takes place in the middle of the polysaccharide chain. In such case a the loss in molecular weight would lead to a rapid elimination of the modified protein.

oxHES possesses a unique carboxylic function corresponding to the former reducing end of the molecule. This group is available for being activated using the common COOH-activation methods, via carbodiimide, carbonyldiimidazole, active ester, mixed anhydride or halogenide of the acid. For our purposes the anhydride as well as the halogenide of the acid were excluded because they were considered to be too strong and non-selective. In fact, because of solubility problems the reactions were preferably performed in aqueous medium, and under these conditions the use of anhydrides and halogenides is no option since they would be hydrolysed by water. Moreover, since they are too strong, even in a non-aqueous solvent there would be higher probability for them to react with the –OH functions of the polymer (which are closer and in majority) than with the desired protein amino function, leading to cross-linkages of the polymer. Preferred activation strategies were then the milder ones, such as carbodiimide, CDI and active esters. In order to modulate the reactivity the reaction was also performed in a mixture DMF / Water in several different ratios.

The first idea was to carry out a coupling using a two-step reaction, with a pre-activation of the polymeric moiety. This should avoid the risk of undesirable protein activation which would yield protein-protein linkages. Since both polymer and protein are water-soluble we decided to perform the reaction in aqueous solution in order to keep the protein under physiologic conditions. As activator a water-soluble carbodiimide (EDC) was used. Table 5.3 lists the coupling reactions performed at the beginning with $oxHES_{130}$. Variations on the reaction conditions included the ratio of $oxHES_{130}$ to HSA, the presence of

HOBt as well as the pre-activation time and the total reaction time. Small amounts of dioxane were needed for completely dissolve HOBt.

In the third coupling reaction we obtained our best result using a direct approach without a preliminary activation of $oxHES_{130}$. We decided to mix all the reactants together and perform the coupling as a one-pot-reaction. GPC analysis of the reaction mixture showed a major broad peak at about 37 min and a minor peak at about 45 min, indicating material with a higher mol weight than HSA (figure 5.2). Only traces of unmodified HSA (retention time about 65 min) could be detected.

reaction	*HSA*	*ox-HES (based on Mn)*	*EDC*	*HOBt*	*solvent*	*activation time*	*reaction time*
I COUPLING ox-HES 130	300 mg 4.4x10-6mol	100 mg 2.4x10-6 mol	25 mg 1.6x10-4 mol	100 mg 7.7x10-4mol	H2O/dioxane 13 ml/2 ml	1.5 h 3-4°C	4 h 25°C
II COUPLING ox-HES 130	100 mg 1.5x10-6mol	300 mg 7.0x10-6 mol	15 mg 9.7x10-5 mol	100 mg 7.7x10-4mol	H2O/dioxane 10 ml/3 ml	1.5 h 3-4°C	over night 25°C
III COUPLING ox-HES 130	200 mg 3.0x10-6mol	3.8 g 8.9x10-5 mol	46.5 mg 3.0x10-4 mol	20 mg 1.5x10-4mol	H2O/dioxane 10 ml/3 ml	0 h	24 h 25°C
IV COUPLING ox-HES 130	100 mg 1.5x10-6mol	1.9 g 4.5x10-5 mol	25 mg 1.6x10-4 mol	20 mg 1.5x10-4mol	water	1.5 h 3-4°C	over night 25°C
V COUPLING ox-HES 130	200 mg 3.0x10-6mol	4.3 g 1.0x10-4 mol	100 mg 6.0x10-4 mol	0 mg	water	0 h	over night 25°C

Table 5.3: Summary of the experiments for the coupling of HSA to ox-HES 130 kD

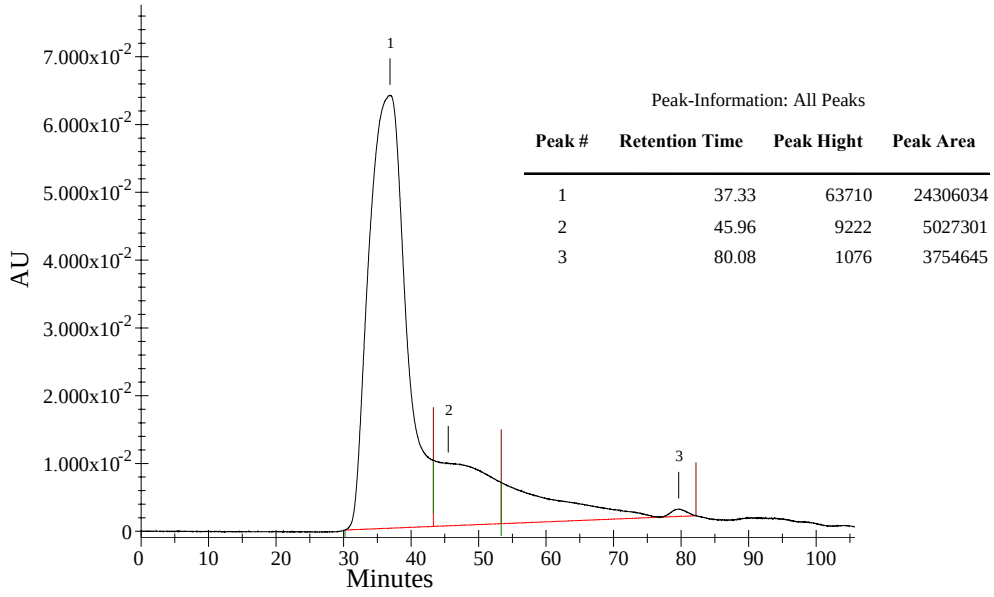


Figure 5.2: GP-FPLC chromatogram of the III coupling to HSA performed with $oxHES_{130}$. λ = 280 nm

Higher mol weight material was also detected by SDS-PAGE (fig. 5.3, lane 1). The result of the glycan detection (achieved with enzyme-conjugated anti-digoxigenin antibodies after western blotting and specific derivatisation of the polysaccharide with digoxigenin, see also Chapter 4) is shown in figure 5.3 (lane 1), where a significant staining indicates the presence of glycan in the coupling product, whereas no staining could be observed with creatinase used as negative control as well as in the case of unmodified HSA. Staining also occurred with transferrin included as positive control.

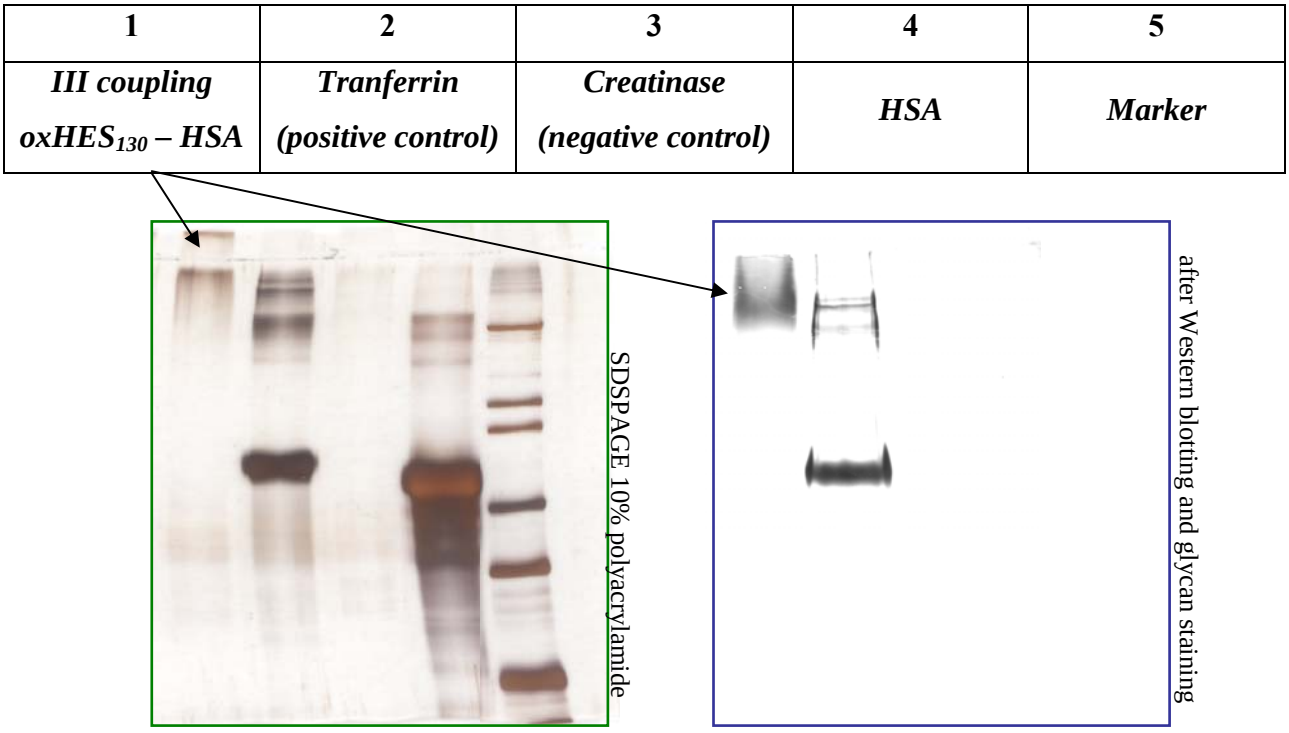


Figure 5.3: 10% acrylamide SDS-PAGE and glycan detection after Western blotting of the III coupling to HSA performed with $oxHES_{130}$.

In the fourth coupling reaction we decided to try whether the low yield obtained in the other coupling reactions (I and II) performed with a preliminary activation was due to the low oxidation degree or simply because of possible side reactions (coupling between different chains of $oxHES_{130}$ occurring in the pre-activation period).

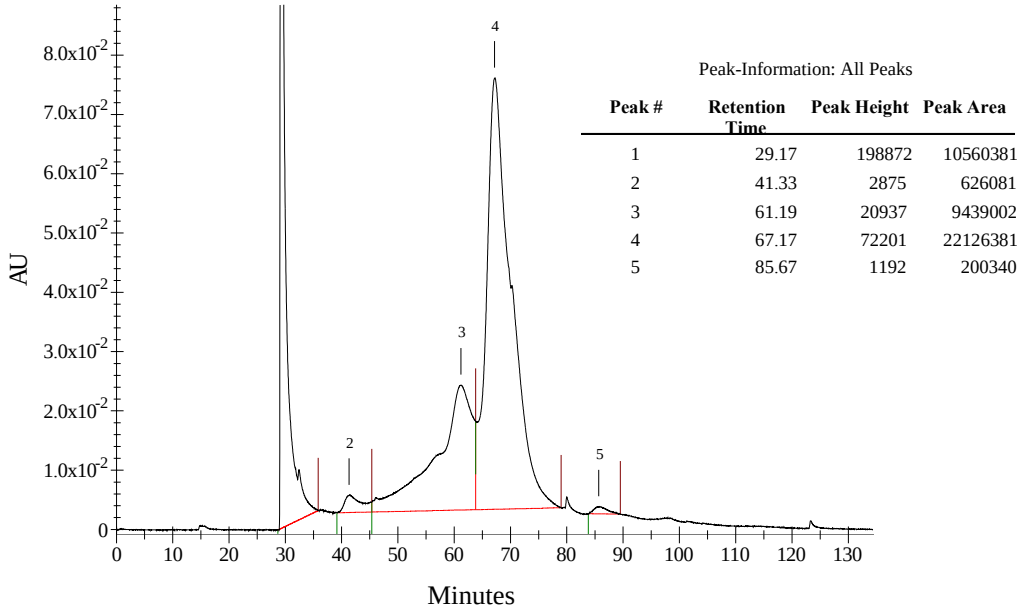


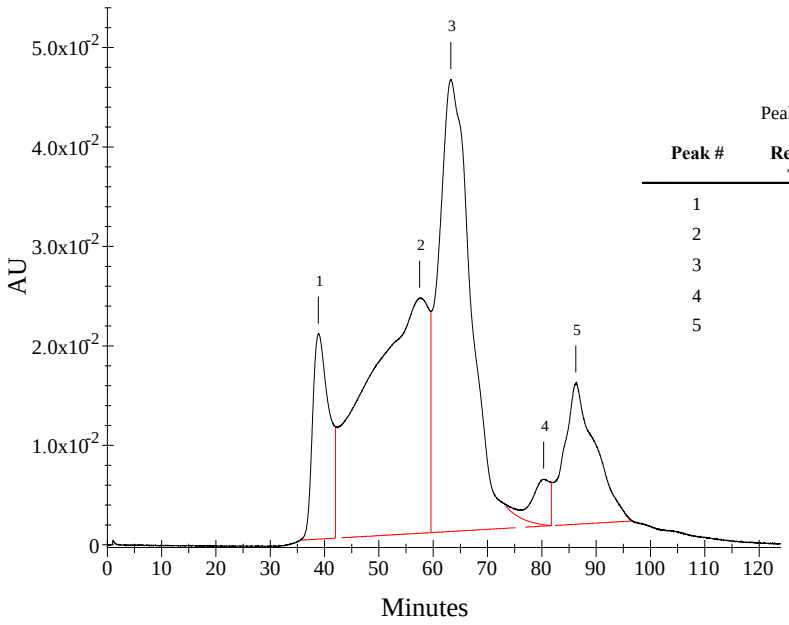
Figure 5.4: GP-FPLC chromatogram of the IV coupling to HSA performed with oxHES$_{130}$ (final sample). λ = 280 nm

The GPC chromatogram of the reaction mixture at the end of the reaction showed a product at 29 min besides a major peak of non-reacted HSA (fig. 5.4).

This result suggests that pre-activation of the oxHES$_{130}$ is not only unnecessary but may moreover result in lower yields possibly due to side reactions of the pre-activated oxHES$_{130}$.

In the fifth experiment again done without pre-activation, the triazole HOBt was omitted to see which effect that may have on the reaction. One of the advantages of this approach is that dioxane is not needed as co-solvent. The coupling product seemed to be more homogeneous than in the third coupling reaction, this is most probably due to the addition of the carbodiimide in small aliquots and not in one portion as had been done in the third experiment.

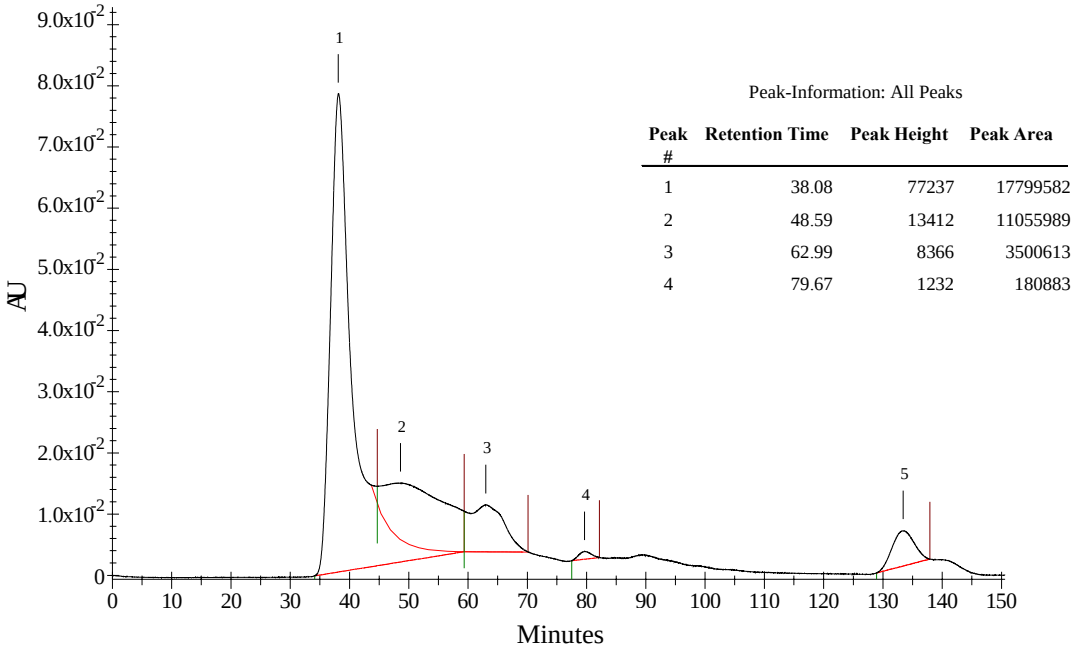
The results are shown in the GPC runs depicted in figures 5.5 and 5.6. After 2h reaction time the major peak in the chromatogram still was HSA (63 min), but reaction products could already be detected in this early phase (peaks 1 and 2 respectively). The major product after completion of the reaction (fig. 5.6) appeared at 38 min (peak 1) with additional material in a broad peak between 45 and 55 min. and non-reacted HSA (peak 3, at 63 min).



Peak-Information: All Peaks

Peak #	Retention Time	Peak Height	Peak Area
1	38.83	20565	4383312
2	57.5	23582	17836917
3	63.25	45350	19064726
4	80.33	4558	973144
5	86.25	14165	5567196

Figure 5.5: GP-FPLC chromatogram of the V coupling to HSA performed with $oxHES_{130}$. (first sample after 2h reaction run). λ = 280 nm



Peak-Information: All Peaks

Peak #	Retention Time	Peak Height	Peak Area
1	38.08	77237	17799582
2	48.59	13412	11055989
3	62.99	8366	3500613
4	79.67	1232	180883

Figure 5.6: GP-FPLC chromatogram of the V coupling to HSA performed with $oxHES_{130}$. (final sample after 24h reaction run). λ = 280 nm

The coupling product was also detected in SDS-PAGE (figure 5.7, lanes 2 and 5 respectively) and in Western blots with the glycan detection kit (data not shown).

1	2	3	4	5	6
Marker	***V coupling oxHES$_{130}$ – HSA (2x)***	***III coupling oxHES$_{130}$ – HSA (2x)***	***HSA***	***V coupling oxHES$_{130}$ – HSA***	***III coupling oxHES$_{130}$ – HSA***

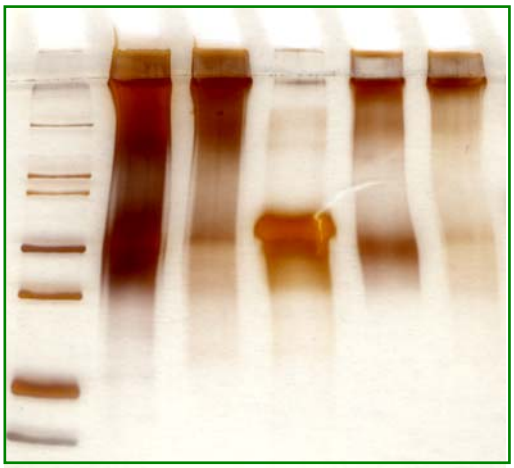


Figure 5.7: 7.5% acrylamide SDS-PAGE of the coupling reactions III and V, performed with oxHES$_{130}$ and HSA, with silver staining (in lanes 2 and 3 the samples loaded were two-fold concentrated).

Taking the results of these experiments together, it seems clear that the best coupling results were obtained without pre-activation of the oxHES$_{130}$. An explanation for this phenomenon may be found in the activation mechanism of EDC in water. As depicted in figure 5.8, unlike the common carbodiimide activations in organic solvents, the EDC activated carboxylic group does not react directly with an amino function (or it happens very slowly). The preferred reaction is the attack on another carboxylic function to form a symmetric anhydride which then reacts with the desired nucleophile. Of course, since during the pre-activation period the only nucleophile present in solution is water, the anhydride may be rapidly hydrolysed giving back the free carboxylic acids.

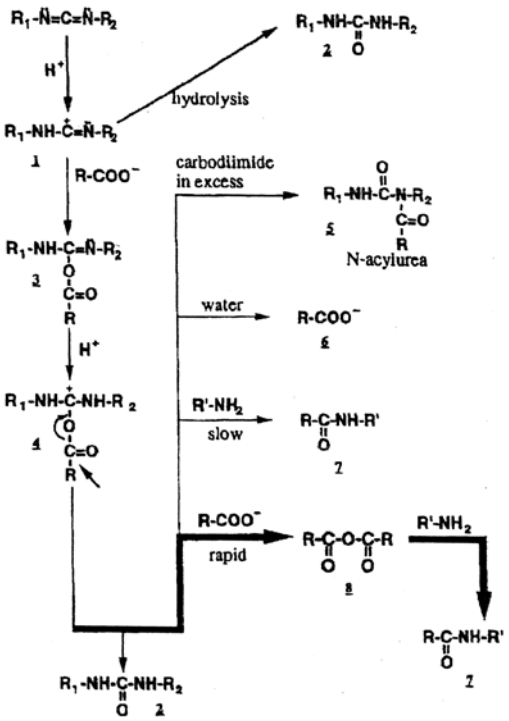


Figure 5.8: Reaction mechanism of carbodiimide in aqueous medium.

Therefore the procedure, as outlined above, results in a "burning cycle" which consumes EDC. That also explains the very low yield observed in the experiments performed with a pre-activation step.

The coupling III and V although successful, have to be considered as first test reactions, and therefore require optimisation. In fact, looking at the reaction conditions one may find at least three parameters which still can influence and improve the yield. First of all the ratio oxHES to HSA. Both coupling III and V were performed with a ~30 times excess of polysaccharide. This excess, although not extremely high, may be reduced during further optimisation procedure.

The activator EDC was also used in a large excess to overcome the loss due to the above discussed "burning cycles". Nevertheless it could be of interest to establish the ratio which will give the best results.

Another point worth to be clarified concerned the use of HOBt. The coupling V (without HOBt) already showed comparable results as coupling III (in presence of the triazole). As general rule, of course, it will be always preferred to work without HOBt to simplify the purification step and to avoid the use of organic solvents that might disturb the protein.

5.4 Optimisation of the reaction conditions (approach A)

Several experiments were performed to determine the dependence on pH and temperature. In fact no difference was found by changing pH in the range 4-10. Relevant degradation has been observed while working at extreme pH values as well as by increasing the temperature over 40°C.

The amount of $oxHES_{130}$ needed to achieve a quantitative modification of the protein was also object of investigation. Finally the minimum excess needed was found to be 2:1 on a molar basis.

Concerning EDC the improvement was not only focused on the ratio to the other reactants. All the experiments described in chapter 5.3 showed, at the end of the reaction, the presence of some precipitate. This was probably due to unspecific protein-protein cross-linking. Trying to improve the selectivity of the reaction, some experiments were carried out where, instead of a single addition, the activator was firstly dissolved in water and the aqueous solution was then slowly dropped in with the help of a dropping funnel. The

precipitate had almost disappeared but the yield of the coupling was also drastically decreased (figure 5.9).

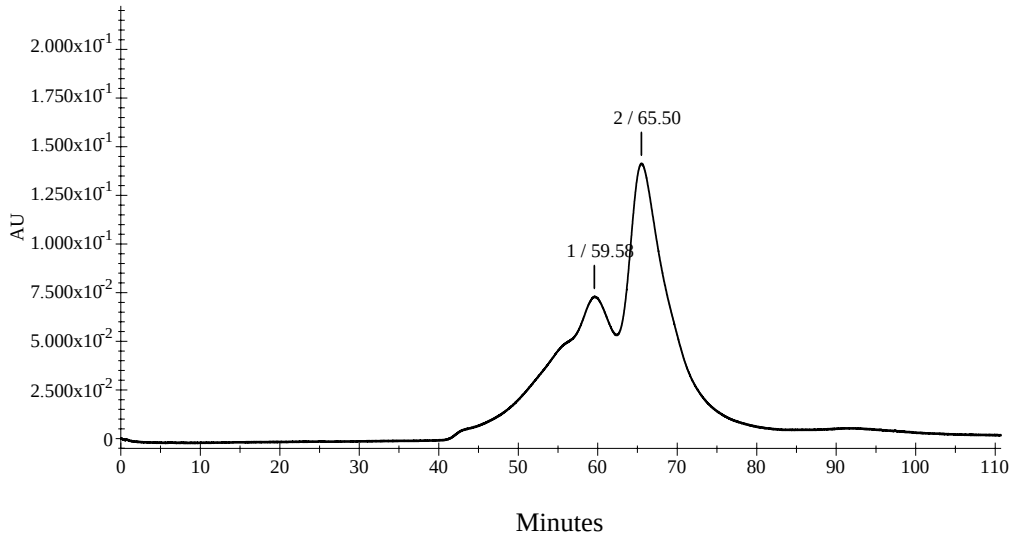


Figure 5.9: GP-FPLC chromatogram of final sample of a reaction performed using the same ratio EDC:oxHES$_{130}$:HSA used in the V coupling but with a slow addition of the activator. λ = 280 nm

A logic explanation for this phenomenon is that the concentration of EDC reached in the reaction is not sufficient. The more diluted the EDC solution is the higher is the probability for the activated form to react with water and consume the activator.

In order to avoid this problem and increase the yield, the following experiments were performed with repeated additions of EDC as aqueous solution always with the dropping funnel.

Finally we found a good compromise between addition period and frequency, the total reaction time, and the dilution factor; the optimised version (see Materials and methods) uses a 2 times excess of polysaccharide, a 800 times excess of activator inserted slowly, in three portions, to yield a final concentration of 4 mg of HSA per ml.

In figure 5.10 the analyses of the coupling product from the optimised version, confirm the almost complete HESylation of the protein.

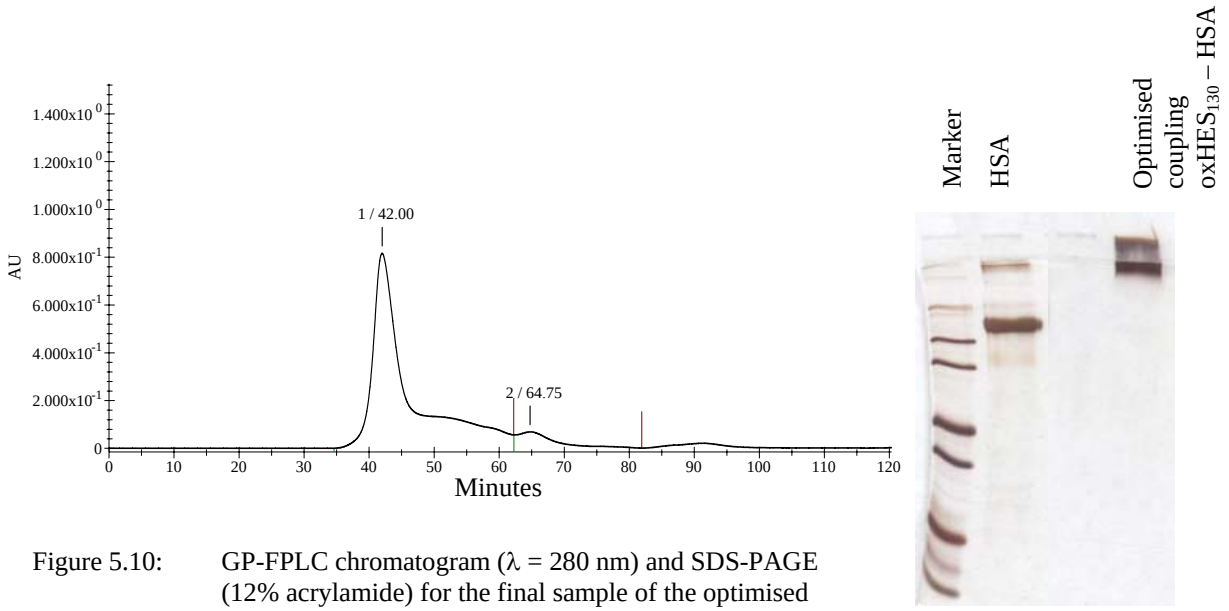


Figure 5.10: GP-FPLC chromatogram (λ = 280 nm) and SDS-PAGE (12% acrylamide) for the final sample of the optimised coupling reaction.

5.5 Optimisation of the conditions for coupling oxidised HES_{10} ($oxHES_{10}$) to a model protein (approach A)

Using all the experience gained by coupling $oxHES_{130}$ to HSA, the same reaction has been performed also with $oxHES_{10}$ in order to yield a multiple substituted product. This could be of use in case a single substitution does not give a product with the desired characteristics of molecular weight, antigenicity and body residence time.

After the first investigations aimed to define the most convenient ratios among the reagents the optimisation was carried out in a similar way as described for the $oxHES_{130}$. The polymer excess was reduced from 2700 times to only 20 times, while the optimal activator excess was found to be of 200 fold. As for the 130 kD species, the addition of the activator with the help of a dropping funnel was found to be adequate. In the figure 5.11 three chromatograms show the formation of the coupling product with the reaction time. The first and the second sample were taken two hours after the first and the second activator addition respectively. The third one was taken at the end of the run.

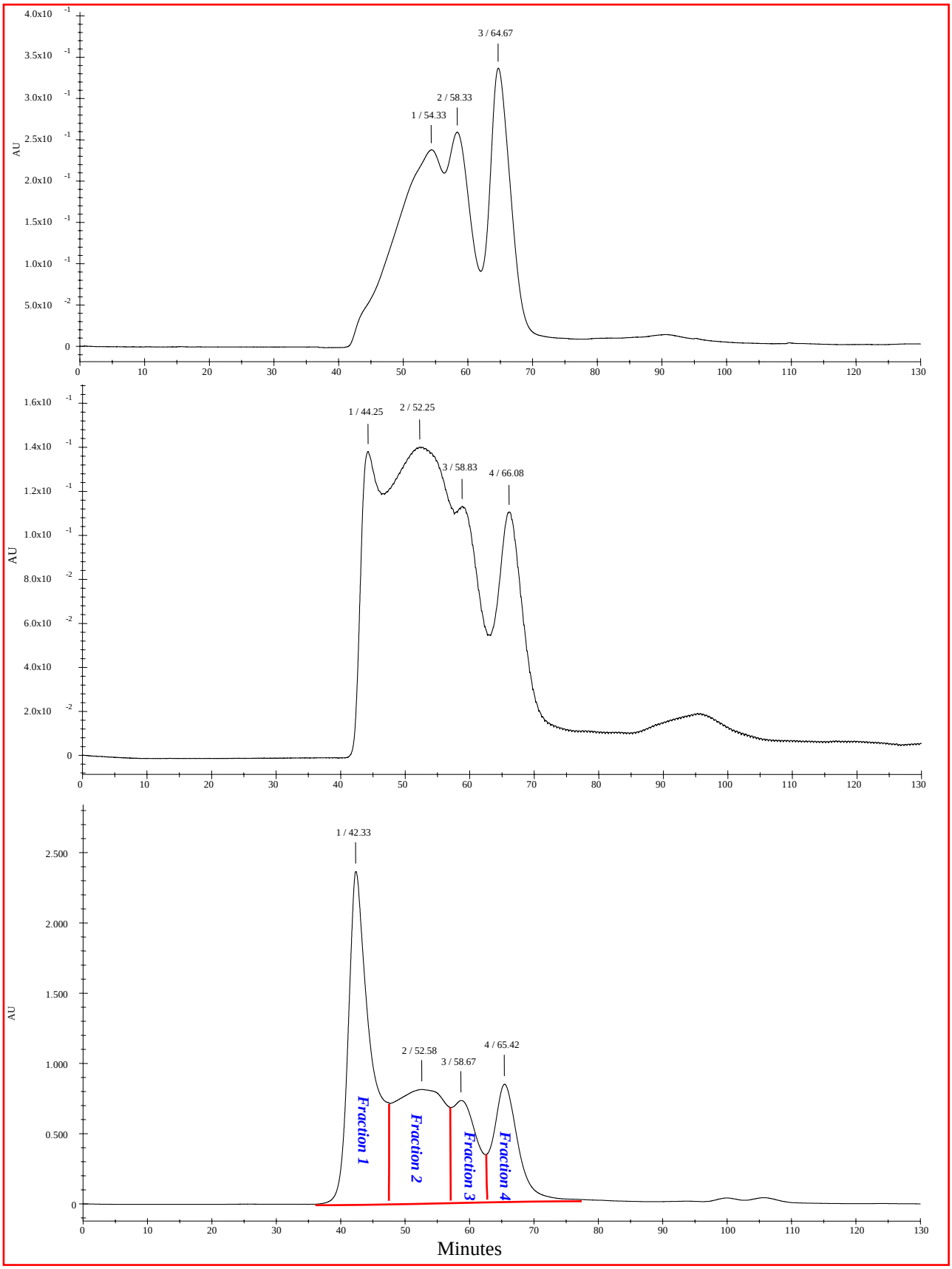


Figure 5.11: The three GP-FPLC chromatograms presented are respectively taken after 2h, 8h and 24h showing the kinetic of the coupling reaction optimised for $oxHES_{10}$. λ = 280 nm

The chromatograms clearly demonstrate the formation of three new peaks with higher molecular weight than HSA. The peak of HSA (~ 65 min) is still present, but appears only as minor peak. The yield can be calculated comparing the peak areas and was found to be around 85%.

1	2	3	4	5
Marker	HSA	Optimised reaction	Transferrin	Creatinase

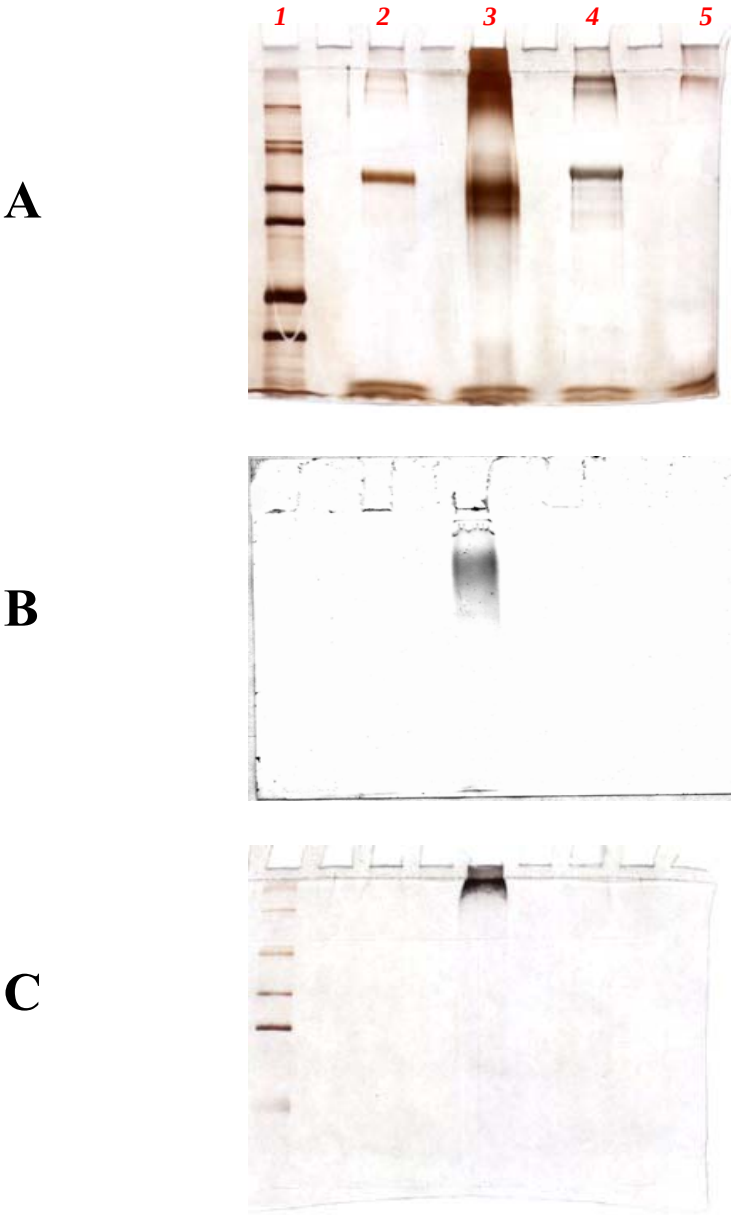


Figure 5.12: The three pictures are the result of the analysis of the coupling product (from the optimised reaction) by 10% acrylamide SDS-PAGE (**A**) followed by Western blotting and glycan detection (**B**). In the third picture (**C**) is presented the staining of the gel after the blotting which shows the proteins that were not completely transferred.

The electrophoresis (fig. 5.12) confirmed the result of the GP-chromatogram concerning the presence of products with higher molecular weight than HSA and moreover

gave the information that the increase in size could be attribute to the polysaccharide chains anchored to the protein. Moreover it has to be observed that both the retention time of the coupling product in the GP analysis and its profile on the SDS-PAGE gel are quite similar to those already recorded for the conjugate obtained with $oxHES_{130}$. Therefore, considering that HES_{10} is supposed to be at least ten times smaller in size than HES_{130}, this may represent an evidence of multiple conjugation.

Furthermore the GP analysis reveals the presence of at least three different conjugation products (see fig. 5.11). In order to investigate the nature of these peaks, in a semi-preparative GPC run, 100 mg of this product have been fractionated isolating the 4 different peaks which have then been analysed by SDS-PAGE and after blotting on nitrocellulose filter paper with the glycan detection kit.

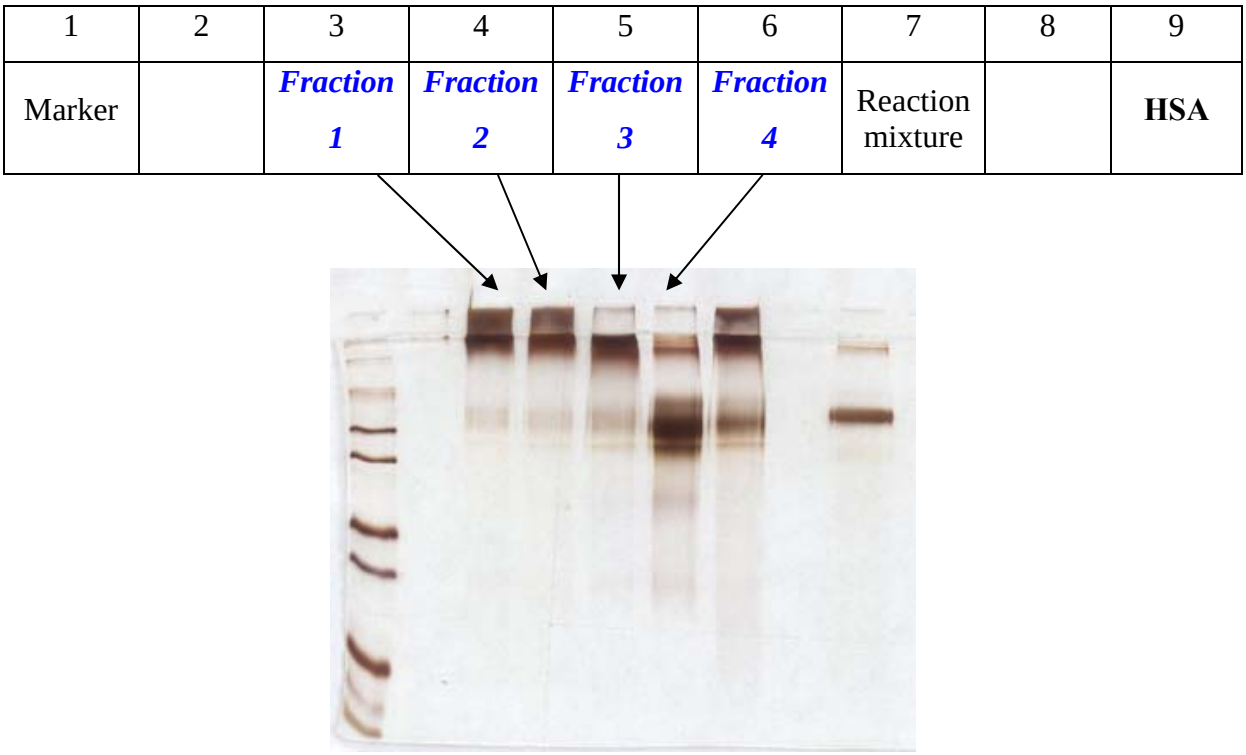


Figure 5.13: 15% acrylamide SDS-PAGE of the $oxHES_{10}$-coupled HSA fractionated on GP-FPLC column (optimised reaction, see also figure 5.10).

The gel electrophoretic analysis (fig. 5.13) demonstrated that the presence of free HSA is limited to very small traces in both the first and second peak, while it becomes more relevant in peak three and four. An opposite trend is to observe for the coupling product which partially remains in the stacking gel and does not even get into the running gel. The separation of this high molecular weight material was not possible with our GPC-method because of the low exclusion limit of the column used (Superose 12). As the exclusion limit lies around 400 kD, all molecules larger than 400 kD have been eluted together. Nevertheless

this value has not to be considered as the real size of the conjugate. In fact HES increases the hydrodynamic volume of the molecule much more than expected from its molecular weight (see also chapter 2). As a consequence of that, the conjugate “behaves” like a molecule much bigger than it really is, and on the GPC column (calibrated with standard proteins) gives results not comparable with the non-HESylated protein standard used for the calibration of the column.

5.6 Coupling of oxidised HES_{25} ($oxHES_{25}$) to a model protein (approach A) and optimisation for yielding a more homogeneous product

The results described for $oxHES_{130}$ and for $oxHES_{10}$ served already to show the feasibility of a covalent coupling of the polymer to HSA. The analyses by both GPC and SDS-PAGE clearly confirm the evidence of a conjugate. In spite of that, the coupling product seemed to be a mixture of differently coupled molecules.

The conjugate with $oxHES_{130}$ appears to be homogeneous in the GPC analysis, but this is most probably due to the fact that the product has a size very close to the exclusion limit of the column. Indeed, the electrophoresis shows at least two different product fractions present, one staying in the stacking gel and the other entering the running gel (see figs. 5.10 and 5.13).

The conjugation with $oxHES_{10}$, according to the GPC analysis of the optimised reaction, yielded at least three different products, considering that the main peak eluted at the exclusion limit and could also be a mixture of several different species.

In optimising the reaction conditions with $oxHES_{25}$ more consideration was given to the homogeneity of the product, with the aim to yield a mono-substituted protein. The choice of $oxHES_{25}$ was dictated by the need of a polymer chain to be not too big (like $oxHES_{130}$) which might exceed the exclusion limit of the GPC-column, but also not too small (like $oxHES_{10}$) because otherwise in case of a 1:1 coupling the resolution of the GPC method is not high enough to discriminate between the conjugate and the starting protein: $oxHES_{25}$ was supposed to fit for this purpose.

The first idea was to prepare some active esters of the polymer which could be selective enough to discriminate between differently reactive nucleophiles on the protein surface and at the same time sufficiently stable in aqueous medium. The coupling reaction was performed with p-nitrophenyl and pentafluorophenyl activated $oxHES_{25}$ (*pnPh*-HES_{25} and *pfPh*-HES_{25} respectively, see also Material and methods). This approach was abandoned because of very low yield in the activation step (~ 3%). Since it was not possible to discriminate between oxHES-active ester and oxHES-carboxyl acid (the two molecules have too similar chemico-physical characteristics), very large amounts of polymer had to be used and consequently large amounts of water were needed in order to perform the reaction. A typical result is shown in figure 5.14 and do not show a satisfactory yield, although some evidence of coupling was observed.

1	2	3	4	5
---	***Marker***	***pnPh-HES$_{25}$-HSA final coupling***	***HSA***	---

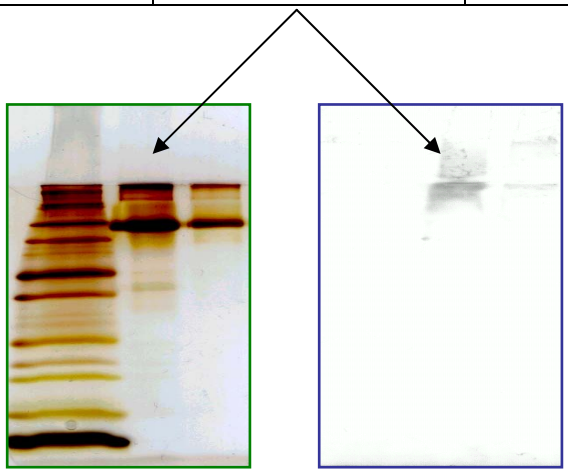


Figure 5.14: 15% acrylamide SDS-PAGE and glycan staining after Western botting of the optimised coupling of oxHES$_{25}$ to HSA using the active ester pre-activation.

In lane three there is some coupling product (confirmed also by selective glycan detection) but the main signal still is the non-reacted HSA.

A further try was performed using a stronger activator but in a mixture of water/DMF in order to somehow mitigate the reactivity of the active complex and of the nucleophiles. It was surprising to find that DMF not always disturbs the protein structure as long as a minimal quantity of water is present (at least 10-20%). In some cases, proteins treated with high concentrations of DMF for different reaction times (from 1h to 24h) could be recovered after dialysis without any loss in solubility and without significant changes in their fluorescence spectra. This allowed us to separately activate the polymeric moiety in anhydrous DMF (avoiding many possible side reactions, see chapter 6) and then add the activated polymer (actHES$_{25}$) to the protein solution (in a DMF/water mixture).

Under these conditions there are several parameters to adjust which could turn out to be relevant for the quality of the final conjugate, mainly ratio of oxHES$_{25}$ to HSA, excess of activator, the pre-activation time, the dropping period and the temperature.

Moreover the reaction time is also important. In general with this kind of approach the reaction time is shorter than with the previous one, on one hand, due to the higher reactivity of the activator (working in a solution poor of water activators like DCC or CDI are suitable),

and on the other hand to avoid a long contact with organic solvent for the protein which could result in loss of functionality.

The reaction was performed with a pre-activation step of the polymer in anhydrous DMF at 0°C followed by slow dropping into the protein solution. This procedure, after the usual optimisations, yielded a much more homogeneous conjugate as the analytical results clearly show. In figure 5.15 a chromatogram overlay is presented where the starting protein and the conjugate are directly compared. The difference in molecular weight is really small, nevertheless detectable. Using the calibration curve of the GPC-column (obtained with standard proteins) one can estimate the difference in size resulting after the coupling which was calculated to be about 106 kD.

In figure 5.16 the results of an SDS-PAGE analysis of the conjugate are presented. The coupling product signal is close to the HSA signal, although slightly shifted on the high molecular weight range. By comparison with the standard proteins of the marker a molecular weight of ~ 90-95 kD can be calculated. The discrepancy between the two analytical methods is explained by considering the hydrodynamic properties of HES (see also chapter 2). Having a hydration volume much bigger than a protein having the same size, HES confers also to the conjugate a larger hydrodynamic volume. But since the analytical method GPC has been calibrated with proteins the conjugate appear with the molecular weight of a protein having its volume.

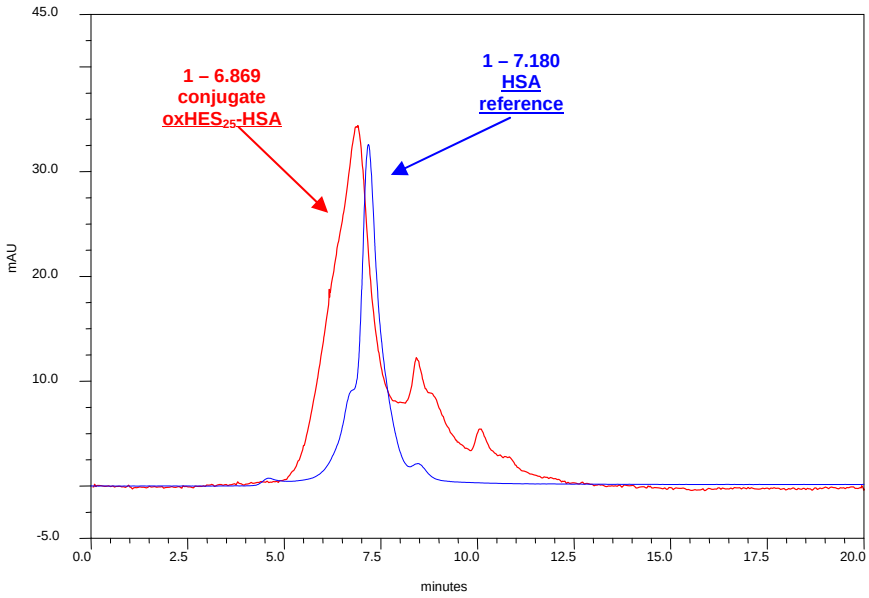


Figure 5.15: GP-HPLC chromatogram overlay of the coupling product obtained with actHES$_{25}$ (red) compared to the reference HSA (blue). λ = 280 nm

On the gel, the signal of the coupling product is broadly distributed around an average value. This is a typical proof for being a polymer conjugate. In fact, during the conjugation the protein looses its high characterisation level because of the polydispersity of the polymer.

1	2	3	4	5
Marker	***HSA***	***actHES$_{25}$-HSA conjugate (4x)***	**---**	***actHES$_{25}$-HSA conjugate (1x)***

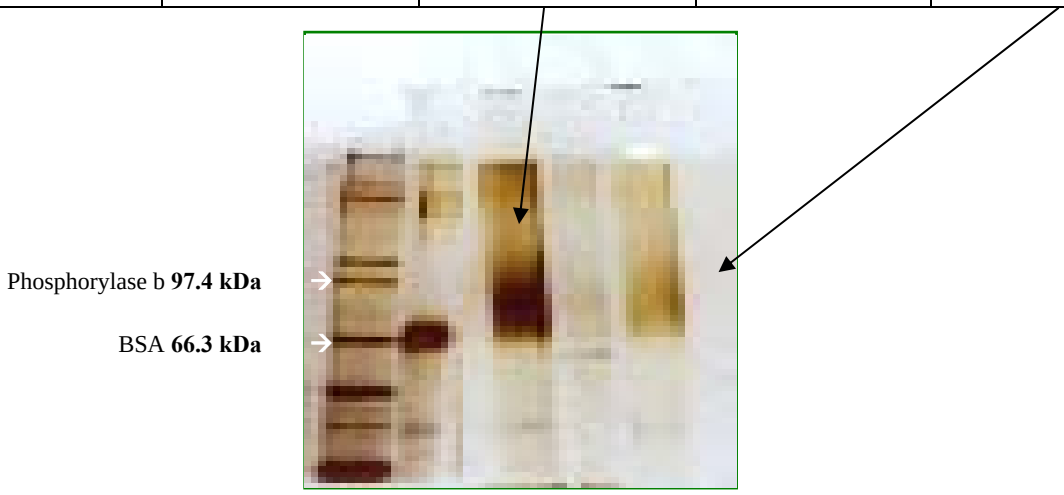


Figure 5.16: 7.5% acrylamide SDS-PAGE of the optimised coupling reaction between actHES$_{25}$ and HSA. The same sample has been loaded twice in two different concentrations.

In the chromatogram one can also observe that the peak of the conjugate, compared to the HSA peak, appears a bit smaller but broader. Also material with a higher molecular weight can be detected which may be the result of a multi-HESylated protein. Nevertheless the most significant product of the reaction remains to be the mono-HESylated HSA.

Figure 5.17 displays the fluorescence spectrum of this conjugate in comparison to the spectrum of the original HSA.

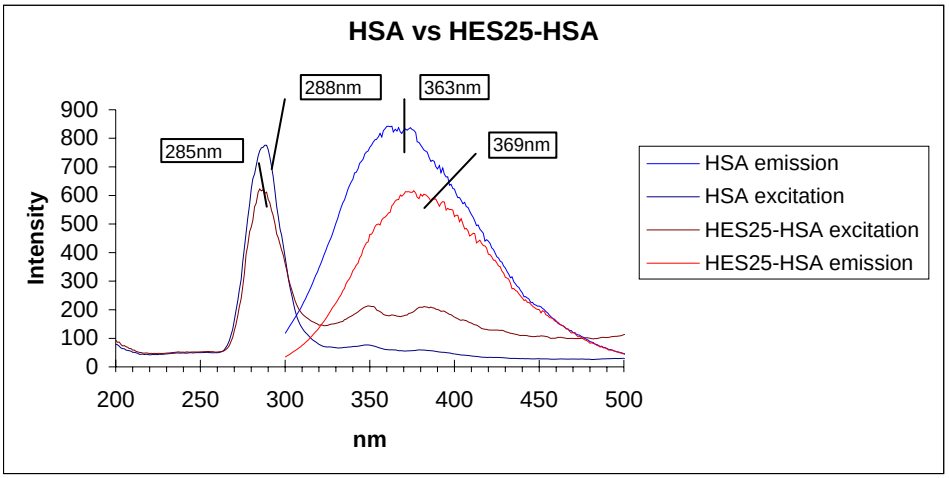


Figure 5.17: Comparison of fluorescence spectra between HSA and the coupling product with actHES$_{25}$. Emission spectra as well as excitation spectra are presented.

The spectra of the conjugate are shifted by about 6 nm from the normal values, considering that the solubility of the protein did not change probably the polymer coupling may not have disturbed the protein's tertiary structure too much.

Another parameter which could still improve this result was the reaction time. The reaction normally ran for 24 hours, the first 5 hours on ice at 0°C and thereafter for 19 hours at room temperature. Since the final product still presented some products of multiple conjugation, it was decided to analyse the kinetic of the reaction to find out which was the optimal time to stop it. Samples were taken at 0', 15', 30', 45', 60', 75' (end of dropping), 3h (end of cooling), 5h and overnight. All samples, loaded on a 7.5% acrylamide SDS-PAGE gel, are presented in figure 5.18. Also a chromatogram overlay of the four most relevant samples is shown.

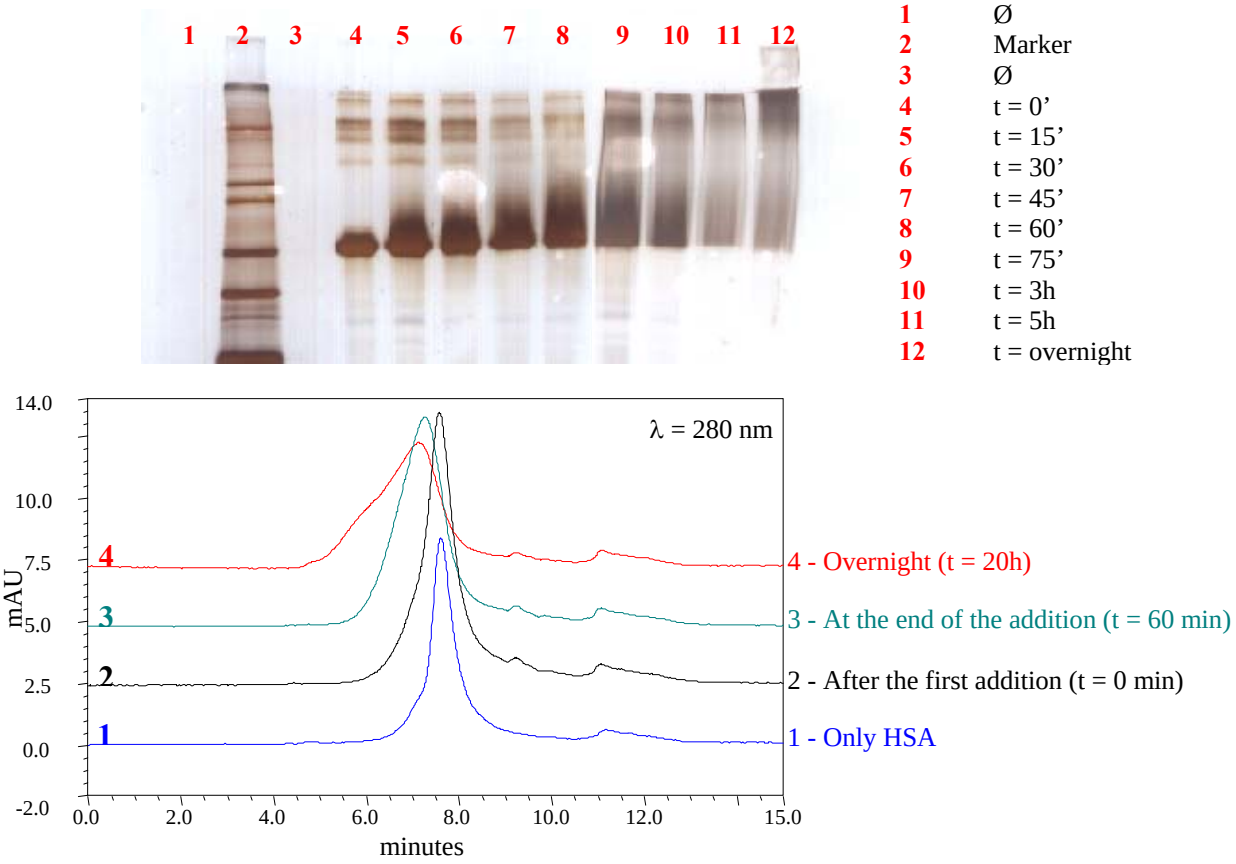


Figure 5.18: Kinetics of the coupling of actHES$_{25}$ to HSA analysed by SDS-PAGE (7.5% acrylamide) and by GP-HPLC. In the gel all samples have been loaded while the chromatogram overlay only shows four samples taken at different reaction times.

The kinetics shows that already after one hour reaction the main product in the solution is not HSA anymore. The main peak elutes already earlier implicating an increased molecular weight compared to the HSA. The peak moreover has become a bit broader than before. This

effect becomes more and more relevant during the reaction run. Looking at the chromatogram after 20 h (n° 4) it is clear that with a longer reaction time, the peak not only elutes earlier but, most important, becomes wider.

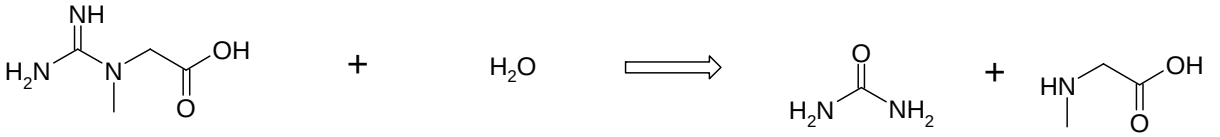
This result indicates that the reaction is quite fast in the initial phase (also considering that the whole process was performed at 0°C for the first hours), then it still proceeds but somehow more slowly. Moreover the initial phase seems to produce a homogeneous increase of the molecular weight for all molecules at the same time.

As an explanation for such a behaviour one can imagine that accessibility problems to the protein surface may subsist after the anchoring of the first HES chain. If the first polymer molecule does not find particular obstacles to get to its nucleophilic target, the next molecules do not have available the whole protein surface, being partially hindered by the previously attached polymer chain.

Nevertheless, even if more slowly, the conjugation goes on with time, causing the undesirable effect of worsening the homogeneity of the coupling product, as indicated by the broader peak in the overnight chromatogram. In the light of this kinetic behaviour, it seems to be a good compromise to stop the reaction one hour after the end of the addition of activated polymer.

5.7 Coupling of oxidised HES_{25} ($oxHES_{25}$) to creatinase (approach A) and optimisation of the activity yield

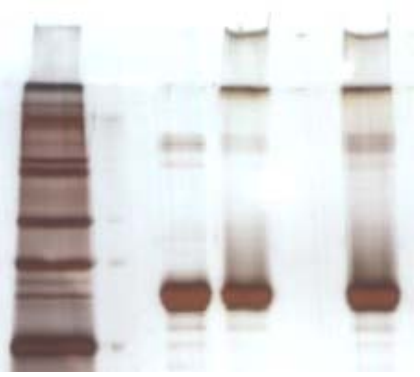
As model enzyme, creatine amidinohydrolase (creatinase), has been chosen because it is a non-glycosylated protein (the detection of glycosylated protein is used as an evidence for the conjugation) and because of the possibility to easily detect its enzymatic activity. Creatinase is a dimeric protein, with a molecular weight of 92 kD, which catalyses in vivo the reaction of deamination of creatine to produce sarcosine and urea.



creatine *urea* *sarcosine*

The coupling was carried out under the same conditions optimised for HSA and actHES$_{25}$. Analytic tests also confirmed the success of the conjugation (see fig. 5.19 for the GP-HPLC chromatogram and the SDS-PAGE gel).

1	2	3	4	5	6
Marker	**-**	***CRE***	***actHES$_{25}$-CRE (1x)***	**-**	***actHES$_{25}$-CRE (2x)***



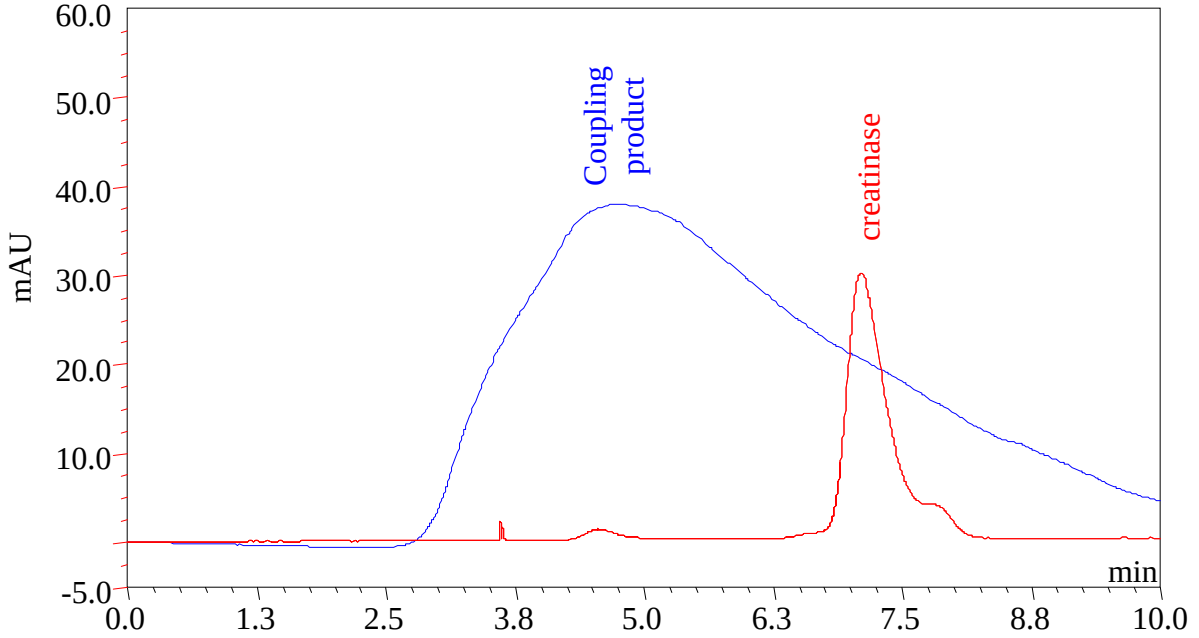


Figure 5.19: GP-HPLC chromatogram (λ = 280 nm) and 10% acrylamide SDS-PAGE gel of the coupling of actHES$_{25}$ to creatinase. In lane 6 a double concentration of the coupling product has been loaded.

The resulting product was unfortunately only partially soluble in water. This fact indicated that some irreversible modification had taken place during the reaction. Therefore a kinetic control of the reaction was carried out to more precisely determine the stopping time. Results of this experiments are shown in the figure 5.20.

1	2	3	4	5	6
Marker	***CRE***	***t=0'***	***t=5'***	***t=15'***	***t=30'***

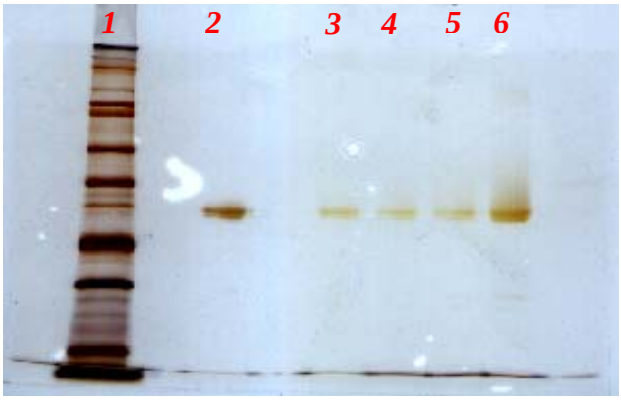


Figure 5.20: Kinetics of the coupling reaction of actHES$_{25}$ to creatinase.

The gel shows coupling product to be present after 30 minutes. In spite of repeating the coupling with a 1 h incubation instead of the 24 h, there was always a fraction of the product which was soluble in water anymore. Both soluble and insoluble fractions, dissolved in an adequate buffer, were analysed with the fluorimeter in comparison with the original enzyme, in order to get an idea about how much the polymer anchoring had influenced the protein structure.

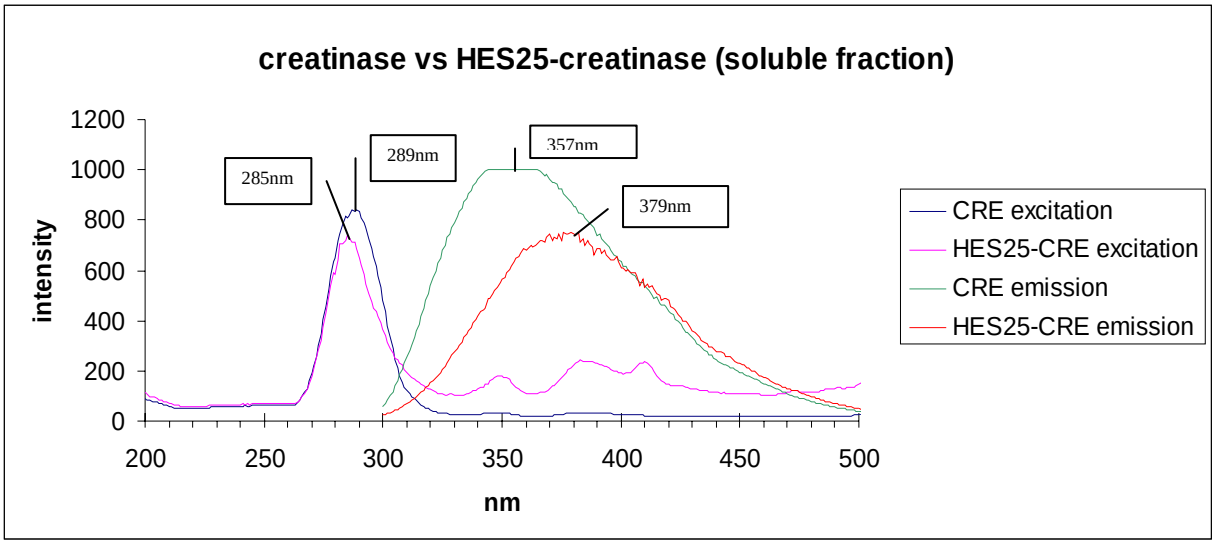


Figure 5.21: Fluorescence spectra of actHES$_{25}$-creatinase (HES25-CRE), only the soluble fraction after the coupling, compared with the original enzyme (CRE).

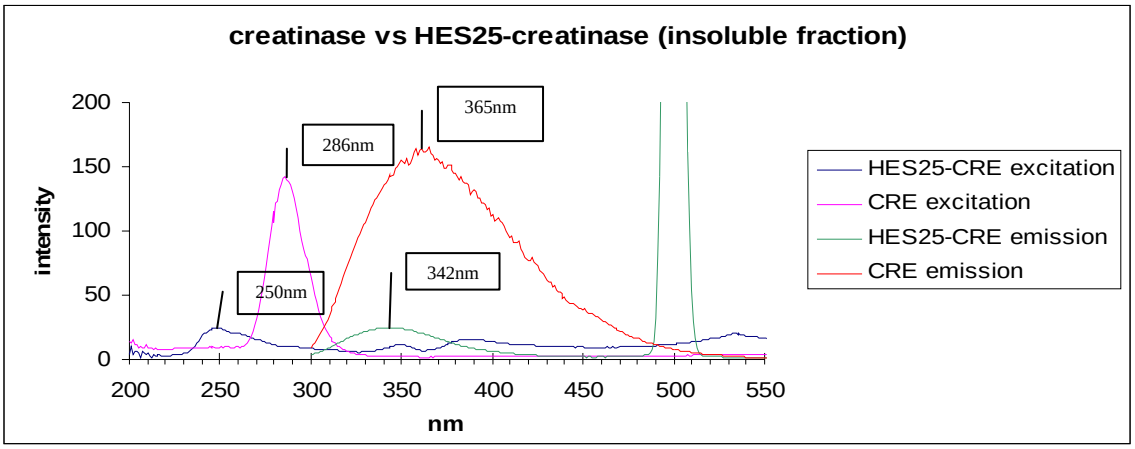


Figure 5.22: Fluorescence spectra of actHES$_{25}$-creatinase (HES25-CRE), only the insoluble fraction after the coupling, compared with the original enzyme (CRE). Both protein were dissolved using a 30mM phosphate buffer pH = 7.4 with 2.5mM of mercaptoethanol.

Figures 5.21 and 5.22 confirm the experimental data: the non-soluble product has a completely different fluorescence spectrum after the coupling, indicating that dramatic conformational changes have happened during the reaction. Also the soluble fraction shows marked differences in the spectrum.

The conjugation product was also checked for the functionality in order to find out to which extent the modification had affected the enzymatic activity.

Using a spectrophotometric test (see chapter 4) which detects the urea coming from the creatine cleavage, a kinetic study was performed using the same enzyme concentration for both original and HESylated creatinase. In the figure 5.23 the results are presented. No activity could be found in the polymer modified creatinase. Moreover by repeating the same experiment with a 10 fold more concentrated enzyme solution the result did not change, meaning the activity is completely lost.

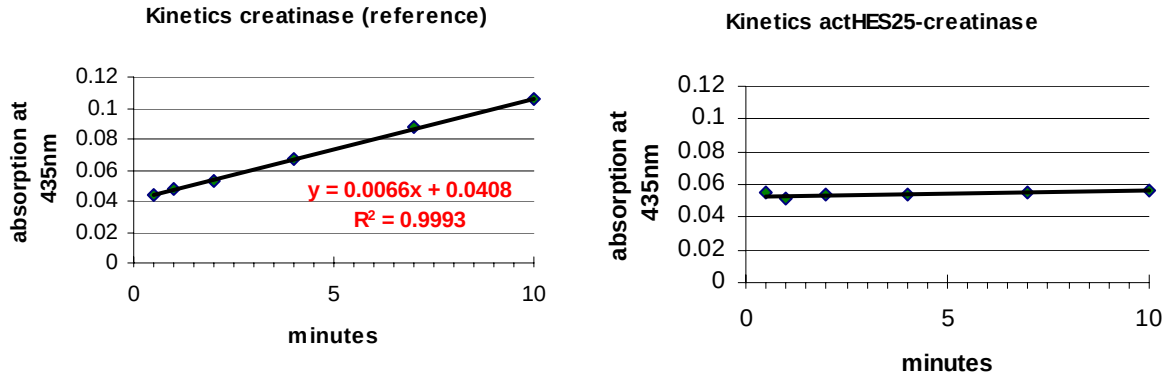


Figure 5.23: Enzymatic activity of creatinase measured monitoring the concentration of urea produced in the hydrolysis of creatine.

By further investigations with the unmodified enzyme (data not shown) it was found out that creatinase is very sensitive to DMF. Already after 5 minutes in contact with this solvent the enzyme has completely lost its catalytic function.

5.8 Coupling of oxidised HES_{25} ($oxHES_{25}$) to superoxide dismutase as functional protein (approach A) yielding a functional product

Since creatinase showed this kind of limitations, another enzyme was selected among the more stable ones. The choice was superoxide dismutase (SOD). SOD is a dimeric enzyme with a molecular weight of 32 kD, which has the capacity to eliminate the free radicals by catalysing the dismutation of superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen according to the following reaction:

$$2\ O_2^{\bullet -} + 2\ H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

This enzyme has also been chosen because of its pharmaceutical utility and the fact that it has already been coupled to PEG [1]. The PEGylated form is moreover easily purchasable from commercial suppliers.

The enzyme was coupled in the usual way by pre-activating the $oxHES_{25}$ with CDI in DMF. In figure 5.24 a time course of the coupling reaction is presented as analysed on 15% acrylamide SDS-PAGE gels with silver staining for proteins as well as for glycans. Furthermore this result has been confirmed by 10% acrylamide native PAGE (fig. 5.25) and glycan detection.

1	2	3	4	5	6	7	8	9
Marker	***SOD***	***t=0'***	***t=15'***	***t=30'***	***t=45'***	***t=60'***	***t=2 h***	***t=3 h***

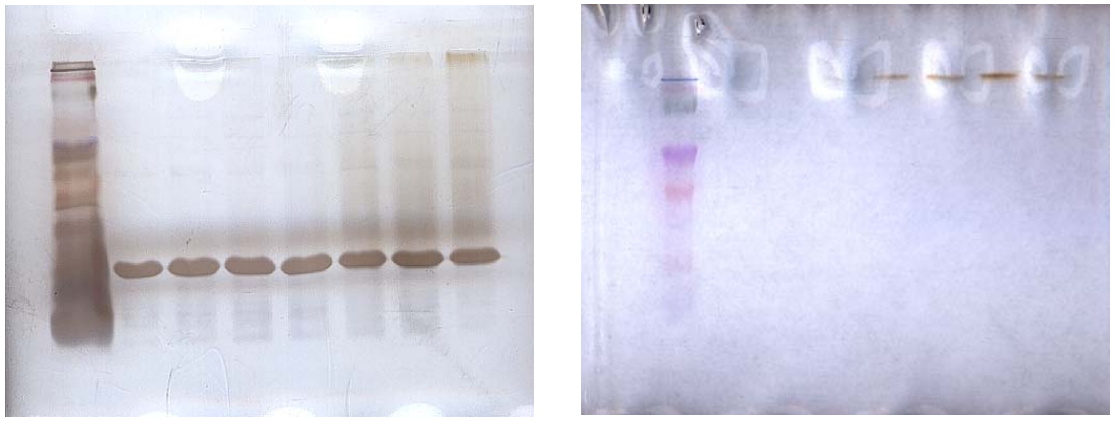


Figure 5.24: 15% acrylamide SDS-PAGE of the kinetics of the coupling reaction between $actHES_{25}$ and SOD. The gel on the right side has been selectively stained for glycans.

1	2	3	4	5
SOD	**-**	***actHES$_{25}$-SOD (1x)***	**-**	***actHES$_{25}$-SOD (2x)***

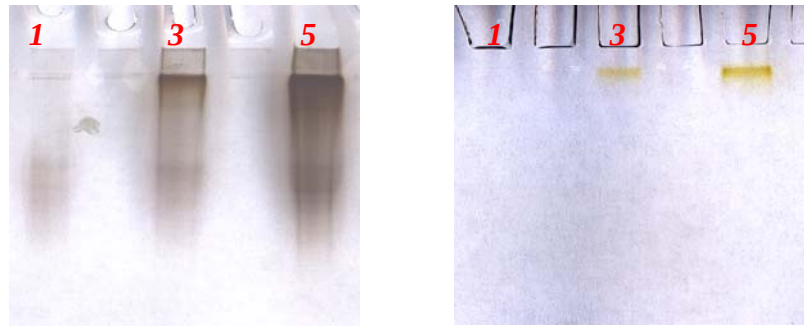


Figure 5.25: 10% acrylamide native-PAGE of the final product of the coupling reaction between actHES$_{25}$ and SOD. The gel on the right side has been selectively stained for glycans.

In the native gel the enzyme runs as a dimer while in the SDS-PAGE the two subunits run separately. On the native gel the amount of coupled enzyme seems to be higher than on the SDS-PAGE. It depends on the fact that modified enzyme (dimer) may contain an unmodified subunit which will be then separated under the denaturing conditions of the SDS-PAGE and appear together with the subunits coming from the non-reacted SOD.

These results provide clear evidence for the occurrence of coupling although still some non-reacted SOD could be detected.

In order to get rid of the non-reacted SOD as well as non-reacted HES a fractionation with ammonium sulphate was performed. After precipitation of the coupling product, four different fractions were obtained at different increasing salt concentration as summarised in the table below (samples not dialysed, containing still ammonium sulphate).

Fraction n°	% saturation of $(NH_4)_2SO_4$	protein content (BCA method)	saccharides content (phenol – sulphuric acid method)
1	45%	20 μg mg^{-1}	37 μg mg^{-1}
2	55%	29 μg mg^{-1}	135 μg mg^{-1}
3	87%	42 μg mg^{-1}	788 μg mg^{-1}
4	100%	11 μg mg^{-1}	417 μg mg^{-1}

The individual fractions have been then loaded on a native-PAGE gel obtaining the result showed in figure 5.26.

1	2	3	4	5	6	7
Frac. 1	**-**	***Frac. 2***	**-**	***Frac. 3***	**-**	***Frac. 4***

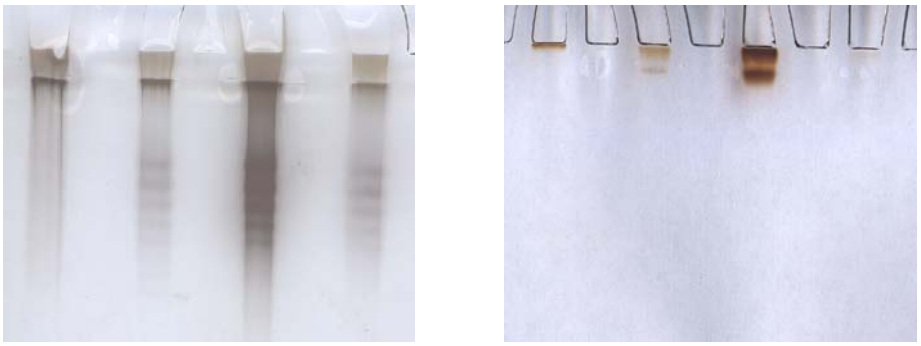


Figure 5.26: 10% acrylamide native-PAGE of the fractions resulting from the precipitation of the coupling product (oxHES$_{25}$-SOD) with ammonium sulphate. The gel on the right side has been selectively stained for glycans.

Surprisingly, a portion of the conjugate seems to precipitate at lower $(NH_4)_2SO_4$-concentration than the unmodified protein, which starts to be present only in the second fraction. This result is somehow unexpected because normally glycoproteins are known to precipitate at higher ammonium sulphate saturation than the non-glycosylated ones. An explanation could be the high molecular weight of such a conjugate which seems to be the largest present in the coupling mixture (compare the gel with glycan silver staining in fig. 5.26), and its large size may be the reason for such a phenomenon.

In fact, only in the first fraction pure coupling product could be detected, in the other fractions non-reacted SOD was present in all cases. As the limit for the HES$_{25}$ precipitation with ammonium sulphate lies around 85% of the saturation, one can assume that the first two fractions are free of non-reacted HES.

The coupling product of the first fraction has been analysed also by GPC and the chromatogram (showed in figure 5.27) confirms the purity of the conjugate (peak 1), almost free of non-coupled SOD (peak 2). The other peaks present in the lower molecular weight range are small impurities such as traces of ammonium sulphate or similar.

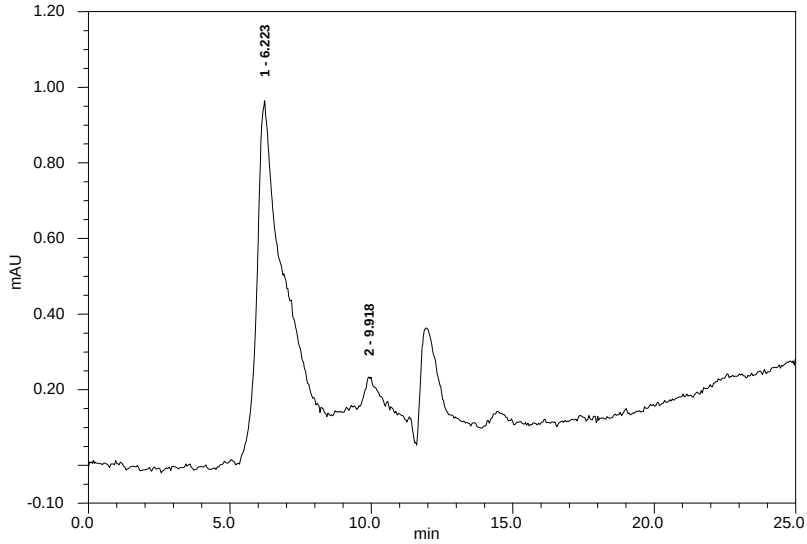


Figure 5.27: GP-HPLC chromatogram of the first fraction after $(NH_4)_2SO_4$-precipitation of the product of the coupling reaction between actHES_{25} and SOD. λ = 280 nm

The HESylated SOD has been finally compared with the original enzyme by fluorimetric analysis. The spectra, shown in figure 5.28, seem to ensure that no conformational changes did take place, indeed the two spectra are absolutely identical.

In the light of this result one may already assume that the enzyme present in the conjugate is active. In order to confirm this an activity test was performed on the HESylated SOD and as expected the activity found in the coupled product was exactly the 100% of the activity of the original enzyme.

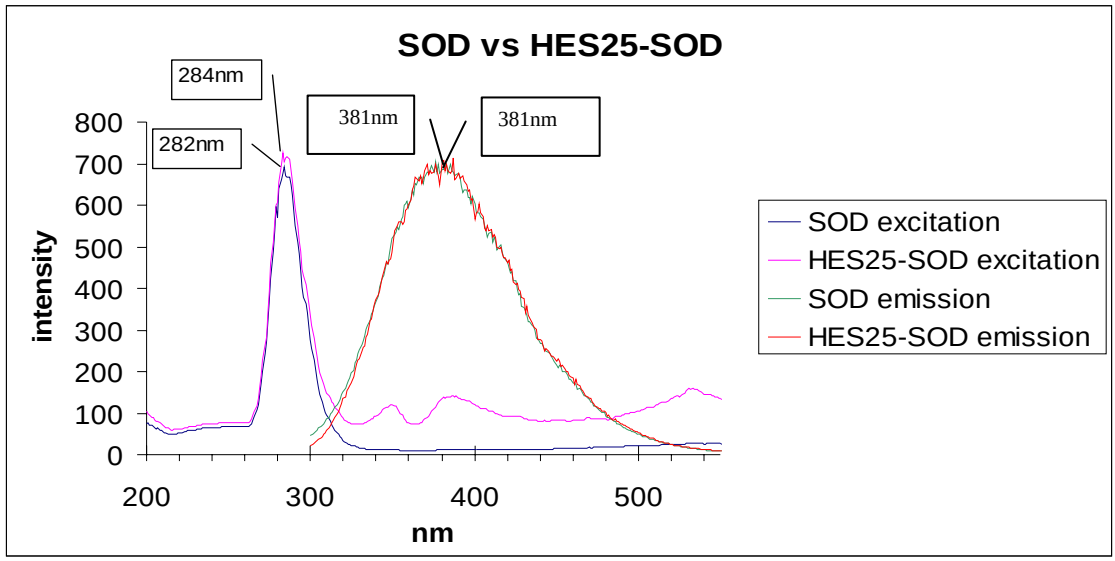


Figure 5.28: Fluorescence spectra of actHES_{25}-superoxide dismutase (HES25-SOD) compared with the original enzyme (SOD).

5.9 Coupling of HES_{130} to a model protein (approach B) and optimisation

As an alternative route to achieve coupling between HES and proteins the possibility of a direct reaction of the reducing end of the HES with amino groups of proteins to form a Schiff's base structure was evaluated (approach B). The reaction was driven into the desired direction by in situ reduction of the Schiff base to the respective amine with $NaBH_4$.

The experiments and corresponding reaction conditions of approach B with HES 130 kD are summarized in table 5.4.

reaction	*HSA*	*HES (based on Mn)*	*NaBH4*	*Na2HPO4 0.1 M*	*reaction time*
I COUPLING HES 130	50 mg 7.5x10-7 mol	500 mg 1.2x10-5 mol	500 mg 1.3x10-2 mol	0 ml	48 h 25°C
II COUPLING HES 130	100 mg 1.5x10-6 mol	1.0 g 2.4x10-5 mol	60 mg 1.6x10-3 mol	1 ml	20 h 25°C
III COUPLING HES 130	50 mg 7.5x10-7 mol	9.8 g 2.3x10-4 mol	285 mg 7.5x10-3 mol	1 ml	36 h 25°C

Table 5.4: Summary of the first experiments for the direct coupling of HSA with HES_{130}.

The third coupling reaction showed the best results. However as seen in the GPC chromatogram (fig. 5.29) the yields were in general lower than with approach A. The major peak in fig. 5.29 (peak 3, at 63 min) corresponded to unmodified HSA, whereas the two minor peaks were identified as coupling products by SDS-PAGE (fig. 5.30, left box, lane 4) and glycan detection western blotting (fig. 5.30, right box, lane 4). In the SDS gel the bands for the coupling product seemed to look similar to the higher mol weight bands in unmodified HSA (fig. 5.30, left box, lane 3), but did not show any signal in the Western blot. Also in control experiments we could show that these bands disappear under the reaction conditions, i.e. in the presence of $NaBH_4$.

This second approach seems to have problems due to the alkaline pH of the $NaBH_4$ solution and due to some bacterial contamination during the long reaction times. The high pH value might cause degradation of the protein in the reaction mixture. Phosphate buffer seems to reduce the hydrolysis problems. The contamination may be minimized by sterile filtration of the solutions used for the reaction (mainly protein, HES and phosphate buffer).

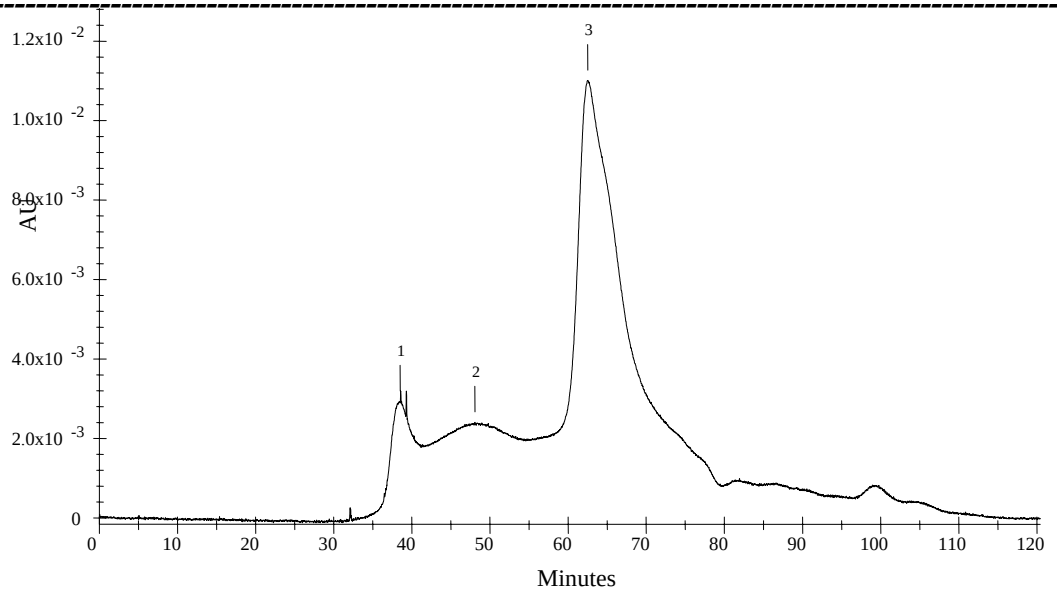


Figure 5.29: GP-FPLC chromatogram of the III coupling HES130 to HSA (approach B). λ = 280 nm

1	2	3	4	5
Transferrin	***Creatinase***	***HSA***	***III coupling HES_{130}-HSA***	***Marker***

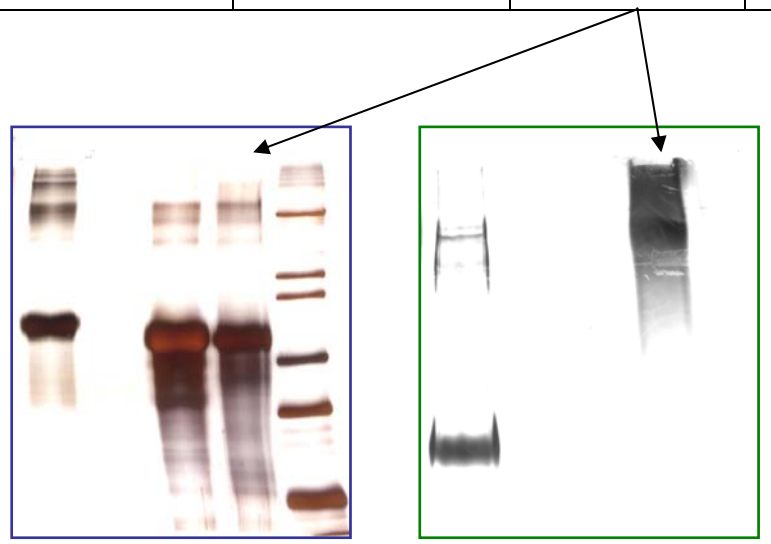


Figure 5.30: 10% acrylamide SDS-PAGE gel and Western botting with glycan staining of the III coupling reaction of HES to HSA using the approach B (via Schiff's base).

The low yield problem seems to be related to the rapid disappearance of aldehyde groups from the Schiff's base equilibrium. The reducing agent ($NaBH_4$) is not selective and seems to reduce aldehyde groups as well as the imide, but as the imide formation is extremely slow (due to the size of the molecules and to the small amount of reacting

groups per molecule) it finally results in a rapid reduction of all aldehyde groups to alcoholic functions.

In the optimisation experiments we focused principally on this topic. Among the several options we tried were the following:

- performing the reaction in a two-steps procedure;
- repetitive addition of HES to supply the one already been reduced;
- use of a more selective reducing agent.

Among these three options only the third was found to improve significantly the yield of the coupling. $NaBH_3CN$ was chosen as a more selective reducing agent in these experiments. This derivative is able to discriminate (depending on the pH environment) among aldehydes and imides. Under neutral or slightly acid pH conditions (pH 6-7), $NaBH_3CN$ has a rapid reduction rate for the imminium moieties while the reduction of aldehydes and ketones is negligible. This allows also to increase the reaction time significantly up to 6-7 days. In figure 5.31 a GP-HPLC chromatogram of the product of the optimised reaction (the final sample) is presented.

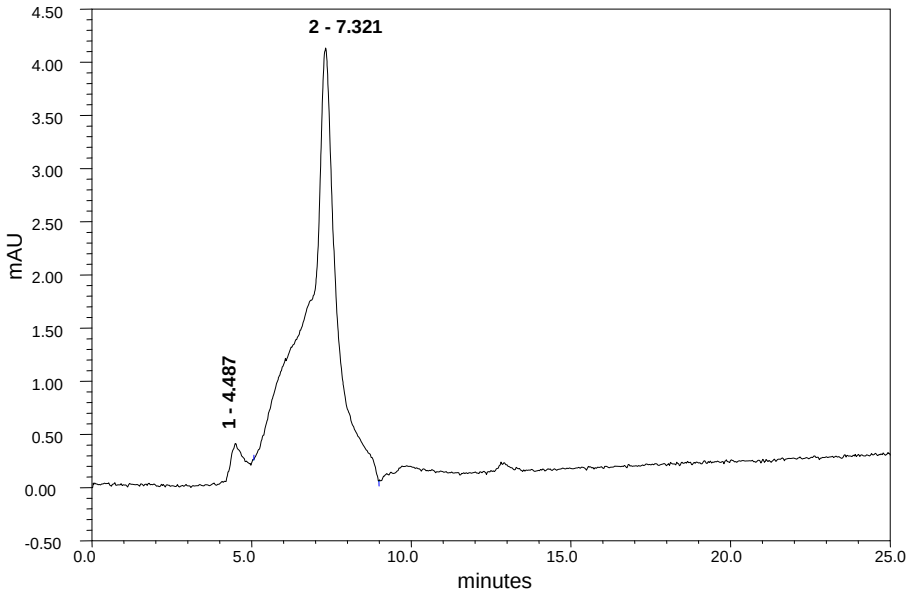


Figure 5.31: GP-HPLC chromatogram of the product of the optimised coupling of HES_{130} to HSA (using $NaBH_3CN$), final sample.

Although still a lot of non-reacted HSA is present the amount of the coupling product has become considerably larger, including not only peak n° 1 but also the material of the broad “shoulder” of peak n° 2 (HSA).

Nevertheless this strategy seems to have quite a lot of drawbacks, besides the low yield which still could be susceptible of improvement (possible variants in this respect include optimal pH conditions, use of immobilized cyanoborohydride, increase of reaction

temperature and prolongation of the reaction time). Mainly because of the very long reaction time, problems of stability in solution and contamination are arising. Therefore this approach is restricted only to proteins that are very stable in solution and, since this is not often the case, is considered, for the present work only as second choice.

5.10 Further characterisations of the conjugate product

The coupling product still possesses a low characterization level, mainly caused by the presence of the polymer which is polydisperse and strongly reduces the definition potential of many analytical methods. Nevertheless, there are still some investigations possible which can give us information about the molecular structure of the protein and the conformational variations occurring after anchoring the polysaccharide chains.

For this purpose MALDI-tof spectrometry was used. In figure 5.32 a typical MALDI spectrum for coupling product is shown.

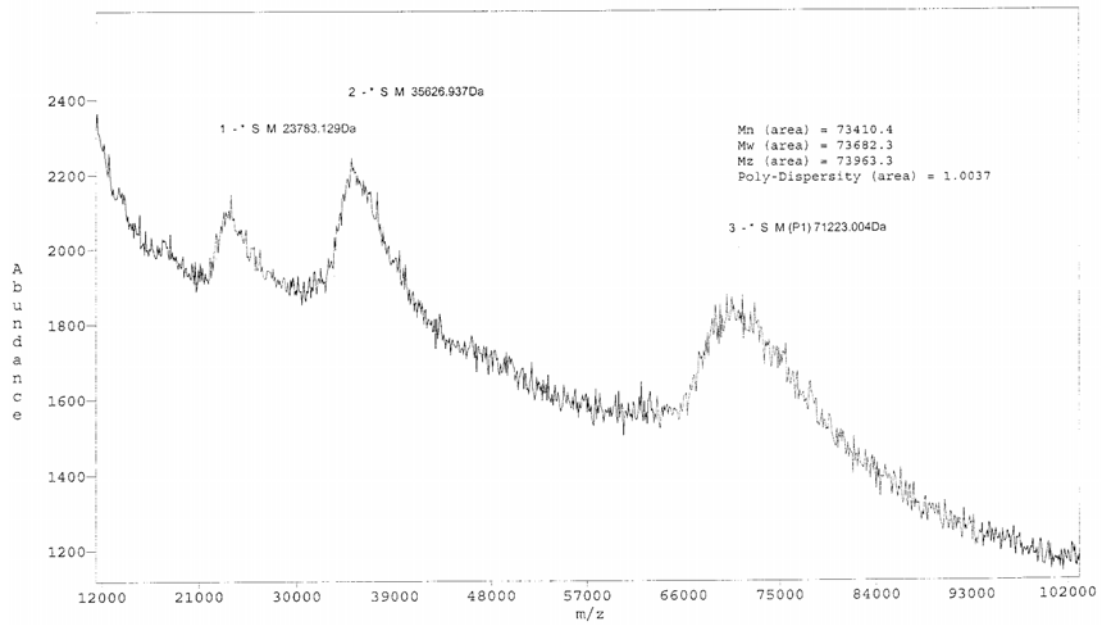


Figure 5.32: MALDI spectrum of the optimised coupling product of HES_{10} to HSA (using approach A).

In this MALDI spectrum the analysis of the conjugate between $oxHES_{10}$ and HSA is presented. The peaks are, as expected, not well defined. The first and the second peak are respectively the 3 times and 2 times charged coupling product, while the third peak represents the single charged.

In the figure 5.33 a spectrum overlay is presented where the conjugate and the original protein are compared.

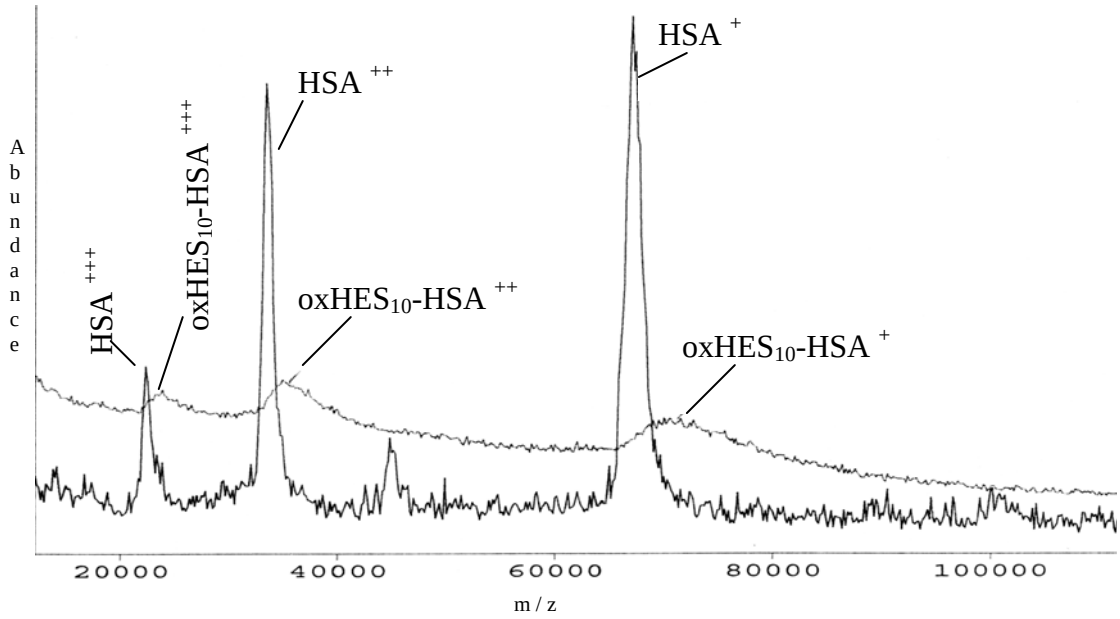


Figure 5.33: MALDI spectra overlay of the conjugate oxHES$_{10}$-HSA and the original protein.

In this spectrum an overlay between the conjugate and the unmodified protein is shown, which highlights the difference in molecular weight as well as the low definition level of the conjugate in comparison with a homogeneous entity like the pure protein.

A further confirmation of the quantity of polysaccharide attached to the protein surface is given by a saccharide colorimetric test (see chapter 4) to be performed after isolation of the protein fraction from the non-reacted HES (e.g. by $(NH_4)_2SO_4$ precipitation or isolation of a GPC peak). This analysis can quantify the amount of polymer, and estimating the protein concentration (e.g., by BCA assay) it is possible to calculate the average weight of polymer per protein molecule. Dividing this value by the average molecular weight of the polymer chains one can obtain the average number of HES chains anchored.

Another important question arising after the coupling is surely the identification of the coupling sites. In collaboration with Dr. Czodrowski (University of Marburg), by means of a molecular modelling software (MEAD – Macroscopic Electrostatics with Atomic Detail, see chapter 4) it was possible to limit the number of all possible targets (we only considered lysines) according to reactivity and accessibility of the side chains.

The reactivity was evaluated comparing the pK_a value of each lysyl ε-amino function in the protein to the normal value (calculated theoretically in solution and in the absence of ions or other amino acids).

The table 5.5 summarises the calculated pK_a values. The lysines whose pK_a value did not differ from the normal one (10.4), have been omitted.

Lysine n°	Calculated pK_a value	Difference
199	6.75	- 3.65
190	7.71	- 2.69
414	8.45	- 1.95
436	9.47	- 0.93
195	9.49	- 0.91
536	9.49	- 0.91

Table 5.5: List of the lysines present in HSA which show a pK_a shift (6 out of a total of 57 lysines). These values have been calculated using a molecular modelling software (MEAD – Macroscopic Electrostatic Atomic Detail, see chapter 4).

The lower the pK_a value is, the higher is the probability to find the amino function in the non-protoned form, meaning able to take part in the coupling reaction.

Of course what is missing in this sort of prediction are the steric parameters. HES, being a branched polymer, might react only with a small number of all lysines exposed on the protein surface because of hindrance problems.

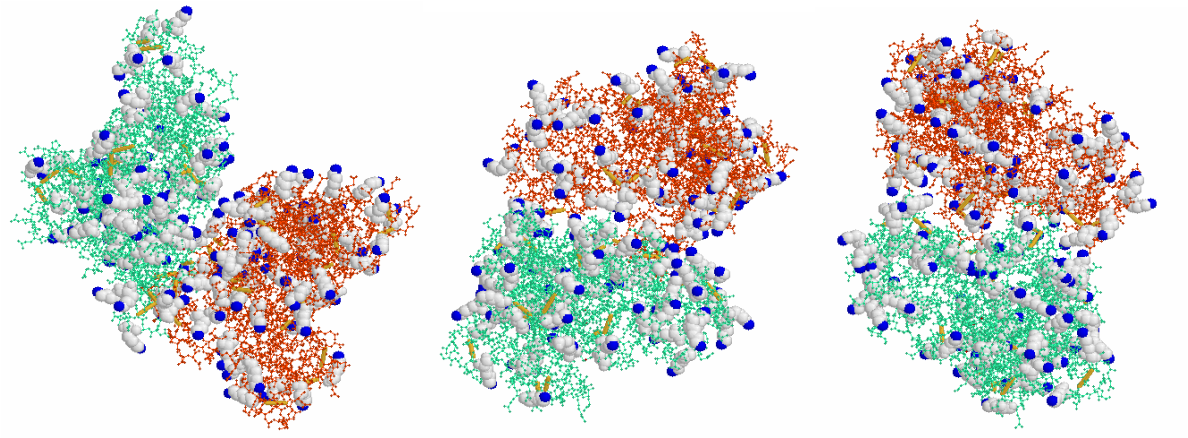


Figure 5.34: Three-dimensional views of a dimeric structure of HSA. ε-amino function of lysine residues are in evidence.

In the figure 5.34 a HSA dimer is shown in three different perspectives. Each subunit (green or red) contains 57 lysine residues which have been represented in white. The ε-amino function is represented in blue.

Not every lysine residue is reachable, only some of them might get close enough to the reactive end group of the polymer. The elimination of the lysines which are not exposed on the surface does not really help since they are normally very few. In the case of HSA for example, only one lysine (Lys 106) does not seem to be accessible from the solvent.

6 Discussion - *Protein drug conjugation*

6.1 HES–Protein conjugates

Polymer conjugation is of increasing interest in pharmaceutical chemistry for delivering drug of simple structure or complex compounds such as peptides, enzymes and oligonucleotides. However the most active research field is for certain that of polypeptides and protein conjugation, for the two following reasons: first of all because a greater number of therapeutically interesting compounds can now be produced by genetic engineering in large quantity and, secondly, because these products are difficult to administer to patient for several inherent drawbacks. Proteins are in fact easily digested by many endo- and exo-peptidases present in blood or other body fluids and tissues; most of them are immunogenic to some extent and finally, they are often rapidly excreted by glomerular filtration. Covalent polymer conjugation at protein surface was demonstrated to reduce or eliminate these problems, since the bound polymer may behave like a shield hindering the approach of proteolytic enzymes, antibodies, or antigen processing cells. Furthermore, the increase of molecular weight of the conjugate allows overcoming the kidney elimination threshold.

Hydroxyethyl starch possesses several properties which can be considered of some utility in the protein conjugation field: it is a highly hydrophilic, highly branched polymer, it is easily available even as very high molecular weight compound (up to 450 kDa), it has well known degradation patterns, presents a known dependence of its degradation rate on its structural parameters like degree of modification and position of substitution, it is derived from a natural occurring source, and it is inert towards the immune system (see chapter 2).

With the present work a coupling chemistry was established which allows to handle this polymer as a mono-functional entity yielding cleaner and more predictable conjugation products. Our results show that most of the limits that lead to the minor role of polysaccharides in the bioconjugation field could be overcome and even may allow to replace PEG as a standard polymer in the coupling reactions.

6.2 Feasibility of HES–protein conjugation

The experiments performed in this thesis were aimed firstly to demonstrate the feasibility of HES-protein conjugation and to control the coupling condition to yield the desired conjugate. The use of HSA (human serum albumin) as a model protein in the first phase provided a first assessment of the reaction conditions needed in order to obtain mono- or multi-substituted protein.

Of course every protein, being a different entity, deserves a separate optimisation, nevertheless the experience gained with this model protein facilitates this step and represents a more than valid starting point.

There were two main coupling strategies selected:

- activation of a single carboxyl function yielded on the HES chain by selective oxidation of the terminal aldehyde group (called approach A);

- Schiff's base formation between an amino function of the protein and the terminal aldehyde group of the HES chain followed by *in situ* reduction to yield a secondary amine (called approach B).

6.2.1 Mono- or multi-HESylated protein

The HESylation technology is very versatile. As different proteins do not share the same properties and problems concerning their pharmacokinetics (stability or elimination rate), the HESylation has to be aimed to the specific protein target. Parameters like molecular weight and molecular structure, stability towards peptidases, stability in solution, intrinsic antigenicity and of course desired therapeutic effect have to be accurately balanced before planning the synthetic strategy.

The knowledge of the molecular structure is needed in order to select the right size of the HES chains to be anchored, as well as the degree of substitution of the material (see chapter 2). In this respect, also the therapeutic effect, particularly the desired duration of the therapeutic effect, may be a determinant. In fact, knowing the pharmacokinetic behaviour and the degradation rate of HES (which is mainly achieved by means of an α-amylase), is possible to predict how long the

conjugate approximately will stay in the bloodstream before being eliminated by glomerular filtration.

Assuming 70 kD as the general limit for the glomerular filtration in the kidney, the mass of the protein has to be increased at least above this value. The more the molecular weight of the conjugate overcomes this size limit, the longer the conjugate is supposed to stay in circulation in the blood. Furthermore the degree of substitution of the HES is another determinant parameter. In general one may affirm that the higher the substitution degree is, the slower the amylase digestion happens and the longer the conjugate remains in the bloodstream. Of course these rules have to be considered very general, because a lot of other factors may and do influence the residence time in the bloodstream, nevertheless they represent a valid starting point.

Once decided how large has to be the rise in molecular weight, two alternative ways have to be evaluated: reach the desired size with a single substitution by means of a big chain or using a multiple substitution with several small polymer chains.

Each way presents advantages and drawbacks, as summarised in the table below.

	Advantages	***Drawbacks***
Mono substitution	- minor disturbance of the protein structure; - higher probability to keep the functionality of the protein.	- shielding effect not complete; - possibility of a big loss in MW by hydrolysis which may take place in the middle of the polysaccharide chain.
Multi substitution	- more complete shielding of the protein surface; - more regular degradation rate.	- major trouble for the tertiary structure; - higher probability of partially (or completely) loosing the protein activity.

As it can be seen, there is no way to predict in advance which approach may be preferred for a given protein. Each case has to be separately considered in the light of these general rules considering its reactivity versus specific antibodies or its intrinsic capacity to evoke an immune response in the host.

Special attention has to be given to those proteins constituted by several subunits. Also in this case one may distinguish between two general cases: the single subunit is active by itself or the activity is present only in association with the other subunits.

In the first case, a multi-HESylation may be preferred treating the single subunit as a independent entity to be modified. It often happens that after multi-HESylation (mainly when the reaction took place under denaturing conditions) the subunits cannot get in the original structure

anymore and remain separated; so a single substitution involving only one monomer would leave the other monomers unmodified, meaning with the same characteristics as those of the original protein (e.g. antigenicity). Moreover the modified subunit will have a smaller size, compared to the compound we wanted to obtain, which was supposed to keep intact the quaternary structure after the coupling. It is evident that in these cases the size and the number of polymer chains to conjugate have to be related to the single monomer rather than on the naturally occurring protein.

A different strategy is needed when the protein is only active in its assembled form. In this case it could still be convenient to try a mono-HESylation (under mild, physiologic conditions) which is supposed to disturb not so much the quaternary structure and may prevent the separation of the single monomers.

Once the more adequate approach for the protein of interest is decided, the chemical way to perform the conjugation has to be chosen. Along this research work, four coupling chemistries have been proposed, three of them being performed in aqueous medium and the other one in DMF / water mixtures. The stability of the proteins, the sensitivity to the organic solvents, are the main variables conditioning the choice.

6.2.2 Coupling in water with oxHES and EDC

This coupling chemistry represented our first attempt to obtain evidence of conjugation between proteins and oxidised HES. EDC is a strong activator commonly used in aqueous solvents. In our experiments it turned out to be very effective in coupling the polymer to the protein although also some drawbacks were noticed.

EDC gives the best yields when used in a single-step reaction, meaning without a polymer pre-activation step. Nevertheless in our case, the pre-activation of the polymer is desirable because it drastically reduces the number of possible coupling combinations. In fact, the proteins own carboxylic functions could possibly be activated as well as the corresponding function on the polysaccharide and then react with a nucleophile. Considering only this aspect, in order to perform a cleaner reaction, one would choose a reaction scheme including a pre-activation step.

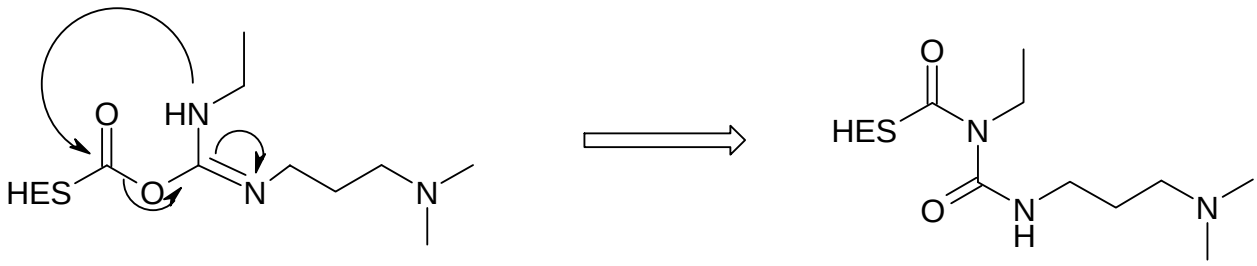
On the other hand, one has to consider that the carboxylic group, once activated, is very reactive and in aqueous medium would react with the most abundant nucleophilic species present: the water. Actually, the mechanism is a bit more complex, and includes a first step where the active carboxyl group reacts with another carboxyl group to yield a symmetric anhydride, and then the

anhydride reacts with the nucleophile. As during the pre-activation step the protein molecules are not yet in the solution, the only nuclephile present turns out to be the water. Therefore the symmetric anhydride is promptly hydrolysed yielding the two starting carboxylic acids and consuming a EDC molecule. As net effect this process “burns” the activator without yielding a considerable amount of reactive intermediate.

In this respect the attempt to include HOBt in the reaction mixture has to be taken into account. HOBt was supposed to react with the active intermediate more rapidly than water because of its negative charge. The active ester resulting from this reaction has much milder activity than the anhydride, although not extremely stable. In our case the coupling performed in presence of the triazole did not improve the yield and the selectivity of the previous reactions.

That is basically the reason why a one-pot-reaction was finally chosen. Even in this case the cycles consuming EDC cannot be avoided but were bypassed supplying a large excess of the activator.

This procedure can also lead to further problems. Indeed, when a big excess of carbodiimide is used, a part of the carboxylic functions tends to react internally with one of the nitrogen atoms present in the activator yielding an N-acylurea (see scheme below). This by-product is very stable and will not react with any nucleophile.



Active oxHES **N-acylurea**

Therefore there seem to be some intrinsic limits in this method really difficult to overcome. There is still room for optimisation by changing mainly pH and temperature in order to modulate the reactivity, but the above mentioned side reaction will always occur to some extend, therefore an excess of HES may be applied.

6.2.3 Coupling in water with oxHES-active esters

An elegant solution for performing the coupling in aqueous solution was offered by some active esters of carboxylic acid which are moderately stable in water, without loosing reactivity. The already mentioned HOBt derivative, cannot be considered to be such an ester because it is not very stable and already the isolation as an intermediate would present some problems. This kind of esters could serve also to discriminate among targets of different nucleophilicity, only the ones possessing a certain reactivity would take part in the coupling.

As preferred active ester p-nitrophenol- and pentafluorophenol-ester (a bit more reactive) were chosen which are also easy to detect by UV-visible light and fluorescence respectively. The active ester formation was carried out in a polar non-protic organic solvent (DMF, DMSO or N-methylpyrrolidone) using DCC as activator. Unexpectedly the yield of this reaction turned out to be very low (around 5%) and there is no technique available to separate the activated HES and the non activated one, because globally the physico-chemical characteristics of the polymer did not change that much by inserting an aromatic ring at one end of the chain.

As practical consequence, assuming a 5% activation of the polymer, we would have to use a 20:1 excess of polymeric material already to match a 1:1 stoichiometry between ester activated-polymer and protein. Since the stoichiometry used reached sometimes ratios of up to 30-50 to 1, the large amount of polysaccharide needed would cause either a dramatic increase in the viscosity of the reaction mixture or an increase in the water volume needed.

This strategy was not used because of this problem but theoretically remains the most advantageous one, the only one which combines an acceptable reactivity with a high selectivity; so it would be still worth to carry out investigations in this direction and to try to overcome the limits.

6.2.4 Coupling in water with HES via Schiff's base

Another possibility in water is given by the so-called approach B. In this case HES does not need any previous treatment and has to be used as such. The terminal aldehyde group of the polymer reacts with high selectivity with amino functions to yield a imminium ion quite unstable that needs to be reduced to a more stable secondary amine. As summarised in the figure 6.1, the overall reaction is the sum of several equilibrium steps.

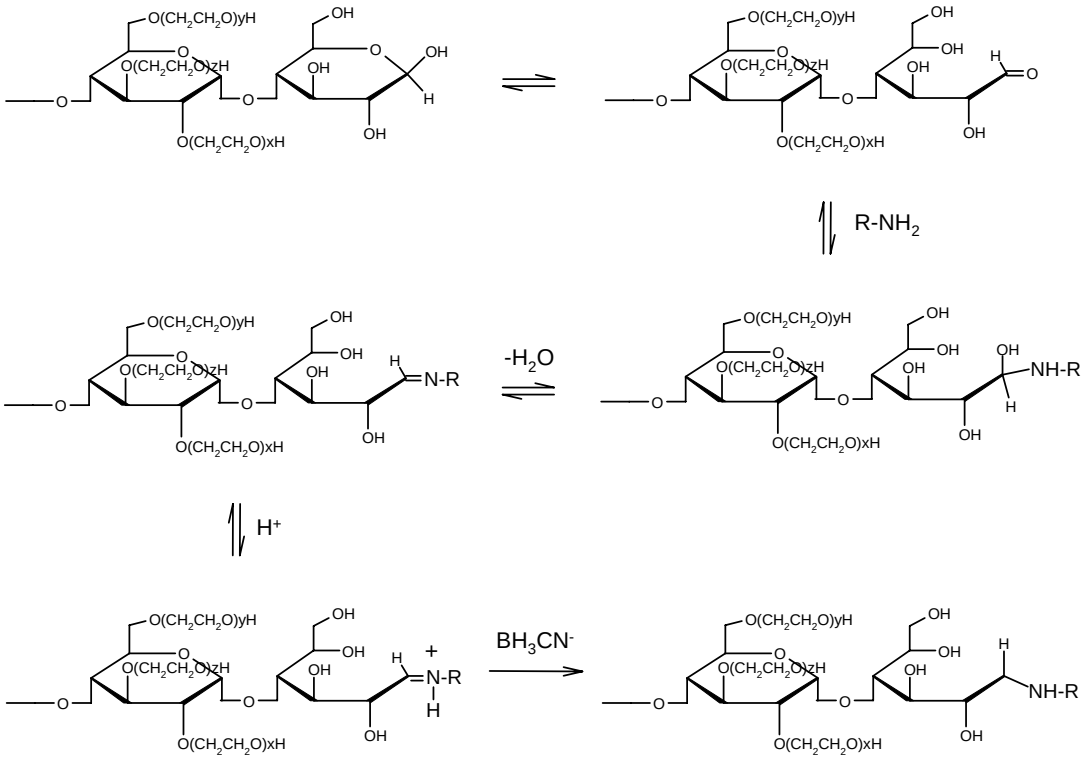


Figure 6.1: Reaction scheme of the coupling strategy via Schiff's base (Approach B)

With our reagents this equilibrium is not really favourable for product formation because of the big size of the molecules and of the few aldehyde present in solution. Indeed the probability for the two reagents to meet each other in the right conformation seems to be quite low.

The reduction is a very delicate step. The reduction step can be used to pull the equilibrium towards product formation, because the imminium ion, once reduced, does not participate anymore in the equilibrium. Therefore it was decided to perform the reaction in the presence of the reducing agent.

At the beginning a strong reducing agent was used ($NaBH_4$) and the reaction gave only very low yields. In fact this agent was able to reduce not only the few imminium intermediates but also the other aldehyde groups that had not yet reacted. Finally the reaction did not proceed because no more reactive groups were present.

Therefore as reduction agent $NaBH_3CN$ was chosen because it has a reducing power sensitive to the pH. At low pH values it is really strong and unselective, but at neutral or slightly basic pH values has a negligible reduction rate versus aldehydes while it remains still reactive enough to reduce the imminium ion to secondary amine.

Nevertheless there are still some limits in this approach, mainly related to the low reactivity of the aldehyde terminal function. In order to obtain acceptable yields the reaction has to run for

several days (normally 5-6). Of course not every protein can spend such long time in solution without showing degradation phenomena or loss of activity. Moreover due to the long reaction time and the nature of the reagents, it was necessary to work in absolutely sterile conditions in order to avoid bacterial contamination.

This method finally represents a valid alternative for those proteins (or peptides) with enhanced resistance in solution, and is not concerned by the problem of selectivity always yielding a conjugate without cross-linkages.

Another objection against this strategy is related to the marked toxicity of the $NaBH_3CN$. As the conjugate is supposed to be used as injectable drug, even the smallest traces of $NaBH_3CN$ need to be eliminated. For this work it has been achieved by a long dialysis against water (several days in solution for the protein) but also ultrafiltration would fit. An elegant solution could also be the use of immobilised $NaBH_3CN$ commercially available on the market.

6.2.5 Coupling in DMF / water mixtures with oxHES and CDI

The coupling to a model protein was also achieved in the presence of organic solvents. Since non-polar side chains are more soluble in organic solvents than in water, hydrophobic interactions are weakened by organic solvents and that could compromise the stability of the protein's tertiary structure. On the other hand, since the stability and formation of peptide hydrogen bonds are enhanced in a low-permittivity environment, certain organic solvents may actually strengthen or promote formation of peptide hydrogen bonds. Organic solvents, such as acetone and alcohol, also can cause denaturation of proteins and their effects are reduced by low temperatures.

The net effect of an organic solvent on protein structure, therefore, usually depends on the magnitude of its effect on various polar and non-polar interactions. At low concentration, some organic solvents can stabilize several enzymes against thermal denaturation. At high concentrations, however, all organic solvents cause denaturation of proteins because of their solubilising effect on non-polar side chains.

Normally, in order to keep intact the folding of the protein and its tertiary structure conditions very close to the physiological ones are desired. Nevertheless the use of DMF in presence of a certain percent of water (10-20%) was found to be tolerable without loosing the capacity of the protein to assume its original folding after eliminating the organic solvent in the case of HSA and SOD (superoxide dismutase).

DMF was chosen because it possesses peculiar properties being non-protic but polar at the same time, so able to form hydrogen bonds with the amino acids side chains exposed on the protein surface. Other organic solvents, such as methanol, ethanol, propan-2-ol and acetone, are generally used to rapidly precipitate proteins from a diluted aqueous solution. These act by reducing the dielectric of the medium and consequently reducing the solubility of proteins by favouring protein-protein rather than protein-solvent interactions. Organic solvents are not widely used on a large scale also because of the tendency of proteins to undergo rapid denaturation by these solvents if the temperature is allowed to rise much above 0°C.

In order to avoid these drawbacks we decided to work in a DMF / water mixture at 0°C for a short reaction time.

The need of working in a poorly aqueous medium was originally dictated by a pure chemical problem concerning the so-called approach A. In fact, as already described before, in presence of water, the activated polymer has a strong tendency to react with water preferably than with the desired nucleophiles.

Moreover working in the presence of DMF it is possible to pre-activate the polymer separately avoiding a direct contact of the protein with the activator which might lead to an even less desirable side reaction: protein-protein cross-linking.

The opportunity to use DMF as solvent has finally to be evaluated considering the specific protein. If for the HSA an evident deviation from the natural structure could not be observed in the conjugate, for some other proteins unfortunately this method is not suitable. They are mainly enzymes with a prosthetic group (e.g., heme in horse radish peroxidase) or simply too sensitive to denaturation by organic solvents. This is the case for creatinase, although the coupling reaction occurs almost with theoretical yield, the conjugate product completely lost its activity. A possible explanation can also be that, by means of the conjugation with HES, the protein may be locked in a wrong conformational state (caused by the organic solvent) and is not able anymore to adopt the original folding even in the physiological buffer.

Other proteins like SOD seem to be able to restore the natural conformation after switching the solvent from the DMF / water mixture to a physiological buffer. The fluorescence spectra of the coupled SOD and the natural one are in fact identical.

This approach is from the chemical point of view the most efficient one because in most of the cases it results in complete HESylation of the protein. Nevertheless because of the non-physiological coupling condition its applicability is restricted only to stable structures. For sure it cannot be considered as a first choice in protein conjugation whereas for small peptides (which are

normally more flexible and can easily adopt the original folding) may represent the preferred strategy.

In this work no peptides have been included but some experiments have already been performed giving promising results.

6.3 Characterisation of the conjugates

The results obtained with HSA, creatinase and superoxide dismutase clearly show that the coupling occurs with both anchoring approaches, the first one (approach A) being the more effective. A multiple coupling on the same protein to a certain extent cannot be avoided if a theoretical yield is desired. In fact a moderate excess of the polymer is needed to complete the reaction and even with high molecular weight chains (HES_{130}) traces of poly-HESylated proteins (generally 2-4 polymer chains) have been detected besides the main mono-HESylated product (see chapter 5, figure 5.10).

But, even a mono-HESylated protein generally exists in a number of variants. Considering both components of the conjugate, it is clear that, while the protein is a unique entity, the polymer is not very well defined. The molecular weight of the polymer only represents an average value of all different species present. A measure of how different the several polymer chains are, is given by the value of polydispersity index (PDI).

$$\boldsymbol{PDI = Mw / Mn}$$

The polydispersity index is the ratio of the weight average molecular weight to the number average molecular weight (see also chapter 2). It indicates the distribution of individual molecular weights in a batch of polymers. The higher the polydispersity is, the worse is the quality of the material. In fact, working with polymers, it is not possible to exactly discriminate between chains of similar size. As a consequence, polymer-protein conjugates possess the same polydisperse variation in molecular weight, that can only be given as an average value.

Besides the polydispersity, there is another problem which can compromise the characterisation level of the coupling product. Even in the case of mono-substituted proteins, several different possible nucleophiles (i. e. amino acid residues to be modified) are present on the same target protein. This implicates a micro-heterogeneity in the HESylated protein even at a 1:1

stoichiometry, meaning a single HES chain coupled per protein molecule but at different sites on the protein surface. This phenomenon is difficult to detect because the conjugate has the same physico-chemical properties but has a very high probability to occur (similar data are reported in protein conjugation with PEG [1]).

Although micro-heterogeneity could seem to be a minor problem because it does not modify the main characteristics of the protein, some relevant side effects may be based on it. In the case of conjugated enzymes or, in general, functional proteins, the derivatives could show different activity profiles, depending on the anchoring site, which, as net effect, may turn out in a partial loss of the original activity. Obviously if the polymer binds near the active site cleft of an enzyme, or near the recognition site of a receptor or of an antibody, the functionality of the protein may result compromised. In some cases, in order to safeguard the activity of the protein, it is possible to perform the coupling reaction in presence of a substrate analogue or an inhibitor (in case of enzyme) or in presence of the target molecule, so that the functional part of the molecule is not available for the coupling. A further development of this approach, already used in PEG conjugation, that proved to be more efficient is to work in a heterogeneous state, i. e. in the presence of an active site inhibitor linked to an insoluble resin. Because of steric effects, the resin-inhibitor complex hinders the conjugation also at the active site surroundings [2].

In HES conjugation the risk of loss of activity is relatively reduced because of the nature of the polymer itself which is highly branched and bulky, so that the probability of HESylation is not the same for every nucleophile but is related to their accessibility on the protein surface. Due to this hindrance the amino acids of the active site of an enzyme are usually not available for HESylation. This consideration is not valid for those enzymes which process high molecular weight substrates, antibodies or receptors with high molecular weight ligands, because in this case the interactive surface must be wide enough to bind high molecular weight interactive partners. In those cases the approach to the reactive site may be even more hampered by a bulky polymer like HES than by a flexible polymer like PEG.

Another possible problem imputable to the micro-heterogeneity may be the different reactivity against antibodies. To have different anchoring sites means to have different shielded portions of the protein surface or, from an other point of view, different antigenic determinants exposed on the protein surface. Unless the polymeric portion is big enough to completely cover the protein surface independently from the coupling site, there could always be some epitopes not very well masked and therefore able to induce a immunogenic response. Considering the statistical distribution of the possible anchoring sites on a protein, it could be possible for all antigenic epitopes of the protein to be present in a conjugate sample although not all on the same molecule

but as the sum of all antigenic epitopes exposed on all different conjugate molecules. This phenomenon becomes even more important in case of allergenicity reaction which are known to be independent from the amount of allergen, so that very small quantities may evoke very strong allergic reactions.

6.3.1 Determination of the real molecular weight of the coupling product

Due to the polymer polydipersity, the determination of the conjugate mass can only be obtained as an average value. The presence of the polymer greatly reduces the resolution in any analytical method. Moreover the behaviour of the polymer in the analysis often does not reflect its real size but the size of a much bigger molecule. This is based on the structural properties of the polysaccharide, mainly its ramifications and hydration volume.

Typical for this behaviour is the case of the gel permeation chromatography. The technique separates molecules based upon their Stokes radius. Amylopectin, even being branched, is quite a compact molecule. The hydroxylethylation process serves to widen the amylopectin structure expanding the space between the different branches, resulting in a relevant increase of the hydrodynamic volume of the molecule (see also Chapter 2). The consequence on the coupling product is that it behaves in the analytic system like a larger molecule and, as the method has been calibrated with proteins (that are more compact than HES), the analysis gives a mass value which would be the mass of a protein having the same volume than the conjugate. The error is proportional to the size of the polymer chain attached but becomes more and more relevant as the size of polymer part exceeds the size of the protein part in the conjugate. It explains also the different results obtained with the different HES species.

In the GP-HPLC analysis of the coupling with oxHES_{25}, the mono-HESylated HSA had an apparent molecular weight of 106 kD. The same product, analysed by SDS-PAGE, showed a more realistic molecular weight distributed between 90 kD and 95 kD. In this case, although not being the major portion of the conjugate, the polymer caused already more than a 10% error in the determination of the real molecular weight by GPC. The situation becomes more complicated in case of multi-HESylation products, as happened in the coupling with HES_{10} and even more complicated when cross-linkage products are present.

Analysis by electrophoresis gave more acceptable results, concerning the determination of the molecular weight. The limit of this method was principally the difficulty to separate products

larger than 200 kD. As we have seen in the case of the coupling with HES_{130} the conjugates could only partially enter the running gel meaning they had a size bigger than 200 kD (molecular weight of myosin, the largest protein in the marker). The separation and characterisation of such conjugates seems to be very difficult to carry out because the single spot at the upper end of the running gel might contain more than one product (e.g. mono- and di-HESylated proteins), and may be also some protein-protein cross-linkage products. The glycan detection, that was usually performed in combination with the electrophoretic analysis, gave only an additional information regarding the presence of HES in correspondence of the protein signals in the silver stained gel but could not give any information about the content of HES in comparison with proteins.

Better chances to obtain a pattern of the individual conjugate species may be expected performing a MALDI mass analysis. Unfortunately this method cannot be considered quantitative, it only gives an idea about all species present in the sample but it does not give any information about the relative occurrence of the components in the sample. This limitation is due to the different rate of extraction from the matrix during the ionisation process. The product of coupling between HSA and $oxHES_{10}$ analysed by MALDI showed three wide peaks corresponding respectively to the mono-, di- and three-HESylated protein.

Another method to confirm the results coming from these analyses is a saccharide determination by phenol – sulphuric acid assay (see also chapter 4). This procedure, used to assay the total neutral sugar content in a sample in solution, does not require a prior hydrolysis of the saccharide chains and is very sensitive. It was performed after precipitation of the proteins and glycoproteins with ammonium sulphate in order to get rid of the non reacted HES which could disturb the results. This assay, matched with a BCA protein assay, can give an idea of the ratio of HES:protein in the conjugate. Unfortunately this method does not discriminate among cross-linked HES-protein conjugates and non cross-linked HES-protein conjugates, so the analysis of the complete reaction mixture will give as result only an average value. More information may be deduced performing the assay directly on individual fractions coming from the GP-HPLC.

The above mentioned analytical methods, matched together can give an approximate idea about the size of the final product.

The low definition level of the product was somehow expected; in fact in the protein conjugation field is quite normal to consider the properties as an average of several species present in the coupling product. In the same way as for the determination of other properties like: remaining activity, affinity for the physiological ligand, antigenicity, also the molecular weight will be defined as the average of the size of all different products present, which sometimes may include also cross-linkage products.

6.3.2 Identification of the coupling site

The precise sites of polymer coupling along the protein primary sequence are very difficult to identify. The experience already gained in this field using PEG as polymer provides information that the coupling takes place at almost all possible nucleophilic functions on the protein surface [3]. In this case the identification of the coupling sites would not reveal any significant information because in average all nucleophiles would be present in the PEGylated form as well as in the non-PEGylated form (depending on the stoichiometry of the reaction).

For HESylation the situation may look somehow better because of the low molar excess of polymer in the reaction mixture and the use of high molecular weight chains. As shown in the case of the coupling with $oxHES_{25}$ the mono-substituted product can be obtained as main product, so we can assume that most probably under the same conditions a similar product could be obtained also with other HES derivatives.

Additionally the steric hindrance of the HES compared to that of PEG may lead to better defined products. In fact, HES is a bulky branched polymer, so it does not show the same flexibility as a liner polymer like PEG. Therefore HES will not attack every amino acid target on the protein surface but will couple only to those which are most exposed.

In the light of this consideration, a peptide mapping of the conjugate product may reveal information of preferred coupling sites. With this analysis it is also possible to get an idea about the occurrence of a homogeneous coupling.

The pattern of the peptides resulting from the complete enzymatic cleavage of the conjugate, compared to the pattern coming from the original protein should show that a small number of peptides are formed in the case of the conjugated protein. It means that the cleavage occurs only at certain of all possible cleavage sites. The missing peptides indicate that the peptidase is not able to cut at all specific points in case of the HESylated protein. In other words the polymer has a shielding effect in the same areas and prevent the contact of the enzyme at the possible cleavage sites. Besides the missing peptides there must be also some new peptides (at least one) appearing which are supposed to be the HES-modified ones.

The amino acid sequence of the new peptides present could be then carried out in order to find out the modified amino acids.

6.3.3 Coupling product functionality prediction by mean of fluorescence spectrum

For those proteins which possess a biological activity, besides the coupling yield and the product homogeneity, the remaining functionality is checked as another very important parameter.

In case a functional assay is available to define the activity left in the conjugate, we have found that the comparison of the fluorescence spectra between native and HESylated protein, can be considered a reliable prediction of the extend of the activity change after the modification. In fact modifications in the tertiary structure are detectable as changes of the fluorescence spectrum of the protein.

Assuming that to the completely folded structure corresponds the 100% activity, we can affirm that the more the fluorescence spectrum of the conjugate differs from the original one the higher is the probability to find a loss of functionality.

This hypothesis was completely confirmed by the results obtained with HSA, creatinase and superoxide dismutase. In fact the fluorescence spectrum of HES-HSA was not very much different from the one of the native HSA and, in line with this observation, we could not observe any precipitation (another clear symptom of denaturation).

For creatinase we have observed and analysed two different fractions, a soluble one and an insoluble one. As expected from their fluorescence spectra both fractions did not show any activity left. Moreover, the soluble fraction had a fluorescence spectrum somewhat more similar to the original protein whereas the spectrum of the insoluble fraction clearly showed that some dramatic structural changes had happened.

Finally, for SOD we could not observe any change in the fluorescence spectrum and this result was also confirmed in the activity assay. Indeed the activity left in the conjugate was 100% of the original activity.

In conclusion this method could represent a first rapid screening alternative for evaluating the functionality of the coupling product.

6.3.4 Prevention of interactions between the conjugate and other macromolecules

Besides the possibility of identifying the coupling site, peptide mapping methods may also give an indication about the extent of interactions with other macromolecules. In fact, a low rate of cleavage could mean that the shielding properties of the polymer serve for protection purposes also in other fields.

For example, for the peptide mapping trypsin could be chosen, an endopeptidase which cuts at the carboxylic-side of arginine and lysine. Since also the anchoring of the polymer is supposed to take place at the lysine residues it may result extremely efficacious in protecting the protein from this enzyme. On the other hand also the size of the polymer used has to be taken into account.

Then if this polymer chain would be able to prevent the interaction with the proteolytic enzyme, there is a high probability for this conjugation to serve also in preventing the interaction with antibodies and in general with other macromolecules.

The interaction between antibodies and antigens is very important because the conjugate are thought to be used as therapeutics to be administered directly in the bloodstream (intravenously). One of the major reasons which limit the clinical use of proteins as drug is the induction of immunological responses. Therefore among the objectives of protein conjugation there is also the reduction of antigenicity and immunogenicity.

This effect as result of polymer strands has already been reported in the case of PEGylation[3], therefore there are very good chances to find the same effect also in the case of HESylation. Experiments aimed to demonstrate this property of the coupling product have not yet been carried out but are planned for the near future. For a first qualitative investigation it was sufficient to perform a double immunodiffusion experiment comparing the behaviour of both native and modified protein. In the experiment showed in figure 6.2 the native HSA was compared with the HESylated one at two different concentrations. Pure $oxHES_{130}$ was also loaded on the plate in order to detect possible interferences. The coupled HSA at the same concentration of the reference did not give any precipitation line. It was necessary to use a 10-times higher concentration to obtain a comparable result. Therefore we can conclude that the reactivity towards antibodies, although not completely abrogated, has been strongly reduced after the coupling reaction.

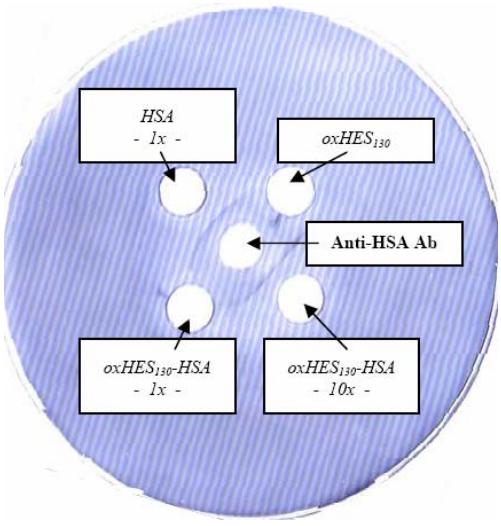


Figure 6.2: Immunodiffusion assay. HSA, and $oxHES_{130}$-HSA were loaded in an agar gel in presence of polyclonal anti-HSA antibodies.

But this ability of the HES-conjugate to remain apart from other macromolecules can turn out to be also a disadvantage. For those proteins whose functionality depends on their interaction with other proteins (e.g. receptors, antibodies, enzymes with macromolecular substrates, etc.), the coupling with a highly hindered polymer like HES can yield a completely non-functional derivative. This is one of the cases in which the prediction of the activity by means of fluorescence spectrum comparison would not help. Even in the case that the intrinsic activity is not lost (as one would estimate looking at the fluorescence spectrum) the conjugate can show zero functionality merely because of steric problems. In some cases the real functionality of the protein may be detected by means of small substrate analogues able to mimic the natural substrate without being affected by steric hindrance.

An elegant way to overcome this drawback is to perform the reaction in the presence of the macromolecular ligand in order to make sure that the area on the protein surface which has to recognise the ligand would not be involved in the coupling reaction. In a even more effective way, the ligand could be immobilised on a insoluble resin which would ensure a protection not only for the recognition site but also for its near surroundings.

6.4 A direct comparison between HESylation and PEGylation technologies

6.4.1 Problems with PEG modification

- Toxicity

Polyethylene glycol (PEG) is approved by the FDA for parenteral use, topical application, and as a constituent of suppositories, nasal sprays, foods, and cosmetics [4]. PEG is of relatively low toxicity when administered orally or parenterally [5] and only large quantities invoke severe adverse reactions. Severe toxic events attributed to PEG have been observed in two settings: using low-molecular-weight PEG after absorption of topically applied PEG and when PEG has been used to test intestinal absorption. Significant amount of topically applied LMW-PEG are absorbed and this has been implicated in the death of patients suffering from severe burn, who were treated with PEG-based antimicrobial creams [6]. These patients developed renal failure associated with metabolic acidosis, elevated anion and osmolality gaps, with a raised bound and free calcium levels but normal or decreased free calcium ions. Subsequent studies in rabbits confirmed the suggestion that it was the PEG content of the cream that was responsible for the metabolic abnormalities [7]. Mass spectrometric data confirm the presence of glycol metabolites (hydroxyglycollic acid and diglicollic acid homologues) in serum and urine of patients treated topically with PEG [8]. These carboxylic acids cause the metabolic acidosis. Both the mono- and diacids formed are potent chelators of calcium [9], causing calcium mobilisation to maintain the level of ionised calcium. The metabolic products of PEG, acids and aldehydes, are toxic to renal epithelial cells [10], thus exacerbating the metabolic abnormalities but reducing clearance of PEG and its metabolites. Whereas these studies yielded important information about the potential toxicity of PEG metabolites when present in higher concentration, such life-threatening toxicities are most likely only relevant for the chronic administration of large quantities of PEG-modified proteins or drugs. Experience with intravenously administered PEG-proteins revealed in general slight to moderate side reactions like itching and pruritus. Occasionally more critical adverse reactions like immediate hypersensitivity or pancreatitis (in some severe cases) were reported [11, 12]. In general higher mol weight PEG´s appear to have a lesser toxicity compared to the smaller, readily water soluble polymer chains [13]. Despite the more pronounced tendency to cause toxic reactions, the latter low molecular weight PEG chains are preferred for modification reactions, because of their better solubility in water.

- Immunogenicity

PEG on its own is not very immunogenic, nevertheless there are some cases of anti-PEG antibodies described in rabbits [14] and in humans [15]. Naturally occurring antibodies have been reported in 0.2% of the normal population. Following sensitisation with PEG-ragweed extract and PEG-honey-bee venom, anti-PEG antibodies were found in 50% of the patients after the first treatment [15]. As discussed above, mild to moderate hypersensitivity immediate-type reactions to PEG-modified drugs are quite common. Similar to their toxicological potential, smaller sized PEGs also increase the chance for such allergic reactions compared to larger chains [13].

6.4.2 Advantages of hydroxyethyl starch (HES)

- Properties as plasma substitute

In the last 10-15 years HES has been more and more used as plasma substitute in the current clinical practice. This long experience has shown the unique safety and biocompatibility of this material. In Germany HES is the most widely used hydrocolloid with the least side effects. The low disturbance of the coagulation [16] and its optimal rheological properties (low colloid osmotic pressure, low plasma and urine viscosity) [17] are responsible for the excellent acceptance of HES in the field of volume replacement and hemodilution.

Having used this polymer almost exclusively via direct infusion into the blood stream, substantial literature data on HES pharmacokinetics and on the possibility of interactions with plasma proteins and the immune system are available. No polymer even approved for parenteral use could claim a better definition of its in vivo behaviour and a longer experience in human practice [18-27].

- Toxicity of HES

Another point to be considered is the amount of HES which is normally used to restore the plasma volumes. These values are quite high (maximal approved daily dose ~ 1.2 g/kg). Compared to any normal drug administration this is at least two orders of magnitude higher. Even in concentrations 5 times the maximal daily dose, as used in toxicity tests, no toxic reaction could be

observed (LD_{50} was higher than 21.6 g/kg in acute toxicity test in rats). Also of relevance is the subchronic and subacute toxicity which could be interesting in the case of a chronic therapy with a HES-modified drug. The experimental results showed, as unique drawback, a reversible accumulation (deposit effect) strictly related to the molecular weight and to the degree of substitution of the polymer if used in high doses. Besides this reversible deposit effect this colloid causes no toxic, teratogenic, mutagenic or antigenic reactions, it is highly biocompatible and well tolerated parenterally as well as locally [28-32].

- HES metabolism

Another advantage relates to the HES degradation pathway [32]. Starch itself is a naturally occurring hydrophilic polymer. When starch is introduced into the bloodstream it is digested mainly by means of the enzyme α-amylase which cuts α(1–>4) glycosidic bonds in polysaccharides. The fragments (oligomers of glucose) of the digested product are rapidly cleared from the vascular compartment through glomerular filtration and/or metabolism.

The hydroxyethylation of the starch serves to slow down the rate of digestion/excretion of the polymer. No traces of HES were found in feces [33, 34]. In fact the residence time in the bloodstream is determined by the polymer size; as soon as the degradation process reduces the size below the glomerular filtration exclusion limit the polymer is cleared out. The hydrolytic activity of the α-amylase is inhibited by the presence of hydroxyethyl groups on sugar units, mainly because of steric hindrance [35, 36].

This property makes HES much more versatile than any other biocompatible polymer because by modifying its substitution parameters and its molecular weight it is possible to predict the half-life in the blood stream and to decide how long the polymer will remain in the body before being eliminated. As these parameters could be chosen within wide ranges, tailor-made solutions for individual pharmacokinetic profiles of drugs can be offered.

6.4.3 Practical considerations

Normally protein conjugation with polymers aims to increase the molecular weight up to at least the glomerular filtration exclusion limit (65-70kD). HES in that respect has two big advantages compared to PEG.

PEG solubility tends to decrease with longer polymer chains. Therefore 20kD-PEG seems to be limit in size for yielding water soluble coupling products. HES is available in much larger sizes than PEG (400kD is already on the market but even higher MW species can be produced), and even at such MW is still keeping its excellent water solubility. In conclusion a single point substitution may be sufficient in case of HES to achieve the desired molecular weight, whereas with PEG multiple substitution sites may be necessary. Regarding modification of proteins it seems obvious that multiple random substitutions increase the probability of impairing the protein functionality.

For instance superoxide dismutase (SOD) is an enzyme with a molecular weight of 65kDa which has been modified with PEG. To achieve a considerable Mw increase 12 chains of PEG-5000 were needed. The same mol weight can be obtained modifying SOD with one molecule of HES 60-70 kD resulting in a unique modification site and a much higher chance to keep intact the enzyme activity. Our own studies with HES-coupling to superoxide dismutase showed as expected a very high retention of the biological activity in the modified enzymes (> 95% of the original activity).

One might argue that having several modification sites the modified protein may be somehow better shielded from the immune systems recognition. But unlike PEG, which is a linear polymer, HES has a highly branched structure. This structure is extremely useful because it gives a good shielding effect not only at the substitution site (as a linear polymer is expected to do) but also in the surrounding area. Such an “umbrella-like” structure is more effective in protecting proteins from proteolysis, in hindering the approach of antibodies, and in reducing immunogenicity. The same mechanism limits also the number of HESylation sites on a given protein, allowing a conjugation to the more accessible amino acid residues only. As a result the coupling product is much better defined and exhihibits a smaller number of positional isomers [37].

6.5 Conclusions and perspectives

Many proteins possess very interesting therapeutic effects which still cannot be exploited because of administration problems (low stability, low solubility, high reactivity towards the immune system, rapid excretion and degradation). The HESylation technology intends to overcome these limitations by means of anchoring on the protein surface an inert, highly hydrophilic, natural based polymer: hydroxyethyl starch.

Our preliminary experiments already gave very promising results showing, besides the feasibility of a selective coupling with the polysaccharide, the possibility to yield still functional conjugates with increased molecular weight and lower reactivity towards antibodies.

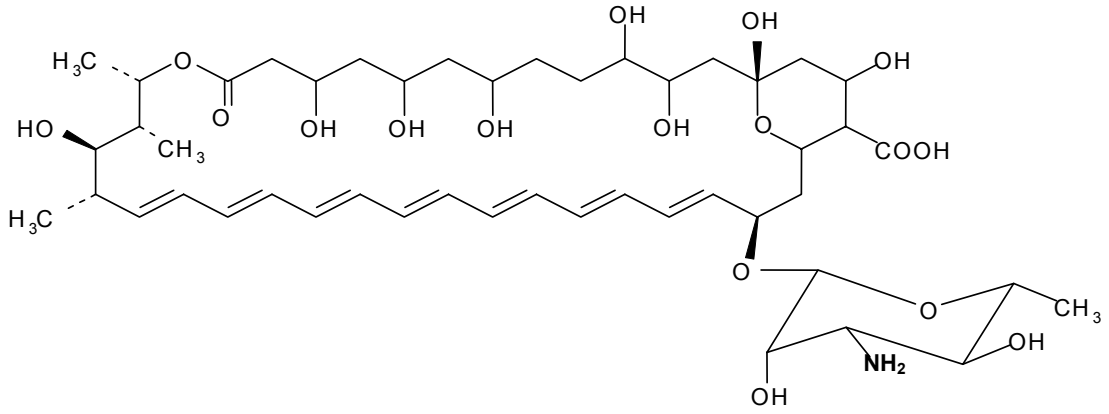
We intend to proceed in the near future by completing the picture with *in vivo* tests which will definitively assess the validity of our theories.

As the technique is of commercial interest, it has been the object of two patent applications.

7 Results – *Low molecular weight drugs*

7.1 Amphotericin B

Amphotericin B is a polyene antibiotic with a very enhanced anti-fungal activity. Its structure (see figure below) has a very lipophilic side which is also responsible for the activity, and a hydrophilic portion represented by the amino-sugar moiety (micosamine) and by a carboxylic function on the main ring.



This structure has very low water solubility, because of the two ionisable functions has some solubility only at extreme pH values.

As target function for the coupling the primary amino function was chosen.

7.1.1 Coupling with oxHES and an activator in water/DMSO mixtures

At the early beginning, based on the promising results obtained in coupling oxHES to human serum albumin, the reactions were performed in presence of EDC/HOBt as activators. Due to the extremely low water solubility of the drug the solvents used were mixtures of DMSO and water in different ratios. At the end of the reaction an aliquot of the reaction mixture was analysed with a GP-FPLC system using as eluent the same DMSO/water mixture used for the reaction. In table 7.1 are summarised these reactions.

	oxHES$_{130}$	*Amphotericin B*	*EDC*	*HOBt*	*Solvent*	*Time*
I. COUPLING	460 mg $1.1x10^{-5}$ mol	5 mg $5.4x10^{-6}$ mol	207 mg $1.1x10^{-3}$ mol	144 mg $1.1x10^{-3}$ mol	DMSO	20 h 25 °C
II. COUPLING	460 mg $1.1x10^{-5}$ mol	5 mg $5.4x10^{-6}$ mol	207 mg $1.1x10^{-3}$ mol	144 mg $1.1x10^{-3}$ mol	H_2O	24 h 50 °C
III. COUPLING	460 mg $1.1x10^{-5}$ mol	5 mg $5.4x10^{-6}$ mol	207 mg $1.1x10^{-3}$ mol	144 mg $1.1x10^{-3}$ mol	0.1 M Borate pH 11 DMSO (6:3)	24 h 50 °C
V. COUPLING	460 mg (as Na^+ salt) $1.1x10^{-5}$ mol	5 mg $5.4x10^{-6}$ mol	207 mg $1.1x10^{-3}$ mol	144 mg $1.1x10^{-3}$ mol	H_2O/DMSO (5:1)	24 h 50 °C

Table 7.1: Summary of the experiments with carbodiimide activation in water/DMSO mixtures.

The results of these experiments were unexpected. The GP-FPLC chromatograms showed only a very small amount of coupling product. The yields were never higher than 5%. Only in the V coupling reaction a first relevant signal for the coupling product could be detected. The oxHES was in this case not in the acid/lacton form but in the Na^+ salt form, so that it could better react with the activator. The yield was around 14%. In figure 7.1 the chromatogram of the V coupling reaction is shown.

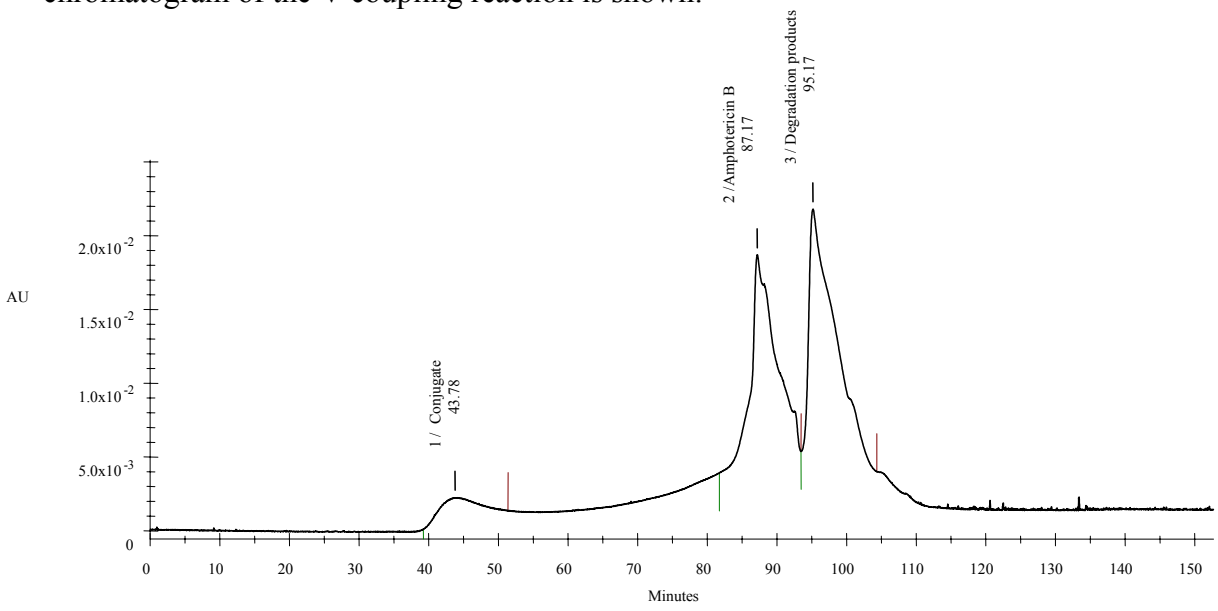


Figure 7.1: GP-FPLC-chromatogram of the V coupling reaction. Coupling of oxHES 130 kD with Amphotericin B in water/DMSO 5:1 (Eluent: water/DMSO 3:1; detection at 385 nm).

7.1.2 Coupling in water with HES oxidised with $NaIO_4$ (*"peroxHES"*)

According to a procedure described by Falk et al. [1] a new coupling strategy was followed. Falk described coupling of Amphotericin B to arabinogalactans previously oxidised with sodium periodate. This oxidation is, of course, unspecific and many aldehyde groups are formed per HES molecule. Although the substitution on C_2 and C_3 greatly reduces the number of possible oxidation sites on the HES chain, a slight molar excess of drug has been required. In table 7.2 two reactions are reported.

	oxHES$_{130}$ (NaIO$_4$)	***Amphotericin B***	***Solvent***	***Reaction time Temperature***
IVa. COUPLING	250 mg $0.6x10^{-5}$ mol	30 mg $3.2x10^{-5}$ mol	H_2O	48 h 25 °C
IVb. COUPLING	300 mg $0.7x10^{-5}$ mol	10 mg $1.1x10^{-5}$ mol	0.1 M Borate pH 11	70 h 25 °C

Table 7.2: Summary of the experiments with $NaIO_4$-oxidised HES_{130}.

After stopping the run, the solution was lyophilised. A sample of the solid material has been then analysed by GP–FPLC using pure DMSO as eluent. The coupling reaction IVb gave the best result with a yield about 35%. (Figure 7.2)

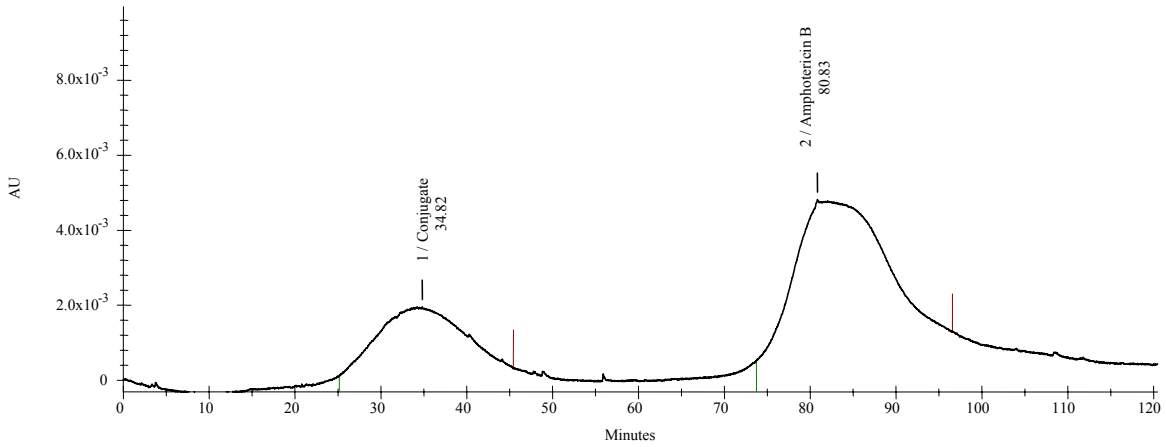


Figure 7.2: GC-FPLC chromatogram of the coupling IVb.Coupling of $NaIO_4$-oxidised HES_{130} with Amphotericin B in borate-buffer (eluent: DMSO; Detection at 385 nm).

7.1.3 Coupling with oxHES and an activator in dry DMSO

Since the experiments performed in aqueous mixtures of DMSO did not give the expected results, the solvent was changed to anhydrous DMSO because this solvent is able to dissolve both the oxHES as well as the Amphotericin B. In this way, having both reagent in a homogeneous phase, better yields could be obtained. Table 7.3 summarises these experiments.

	oxHES$_{130}$	***Amphotericin B***	***EDC/DCC***	***HOBt***	***Solvent***	***Time Temp.***
VI. COUPLING	460 mg $1.1x10^{-5}$ mol	5 mg $5.4x10^{-6}$ mol	207 mg E $1.1x10^{-3}$ mol	144 mg $1.1x10^{-3}$ mol	dry DMSO	24 h 25 °C
VII. COUPLING	264 mg (Na^+) $5.4x10^{-6}$ mol	5 mg $5.4x10^{-6}$ mol	128 mg D $6.2x10^{-4}$ mol	84 mg $6.2x10^{-4}$ mol	dry DMSO	24 h 25 °C
VIII. COUPLING	460 mg (Na^+) $1.1x10^{-5}$ mol	5 mg $5.4x10^{-6}$ mol	227 mg D $1.1x10^{-3}$ mol	144 mg $1.1x10^{-3}$ mol	dry DMSO	24 h 25 °C

Table 7.3: Summary of the experiments with carbodiimide activation in anhydrous DMSO.

Unfortunately also this new approach did not improve the reaction substantially. The VI reaction had a yield not higher than 5% so in the next two tries oxHES was used in the Na^+ salt form. This change resulted in an increased yield which did never become higher than 15% (VII coupling reaction).

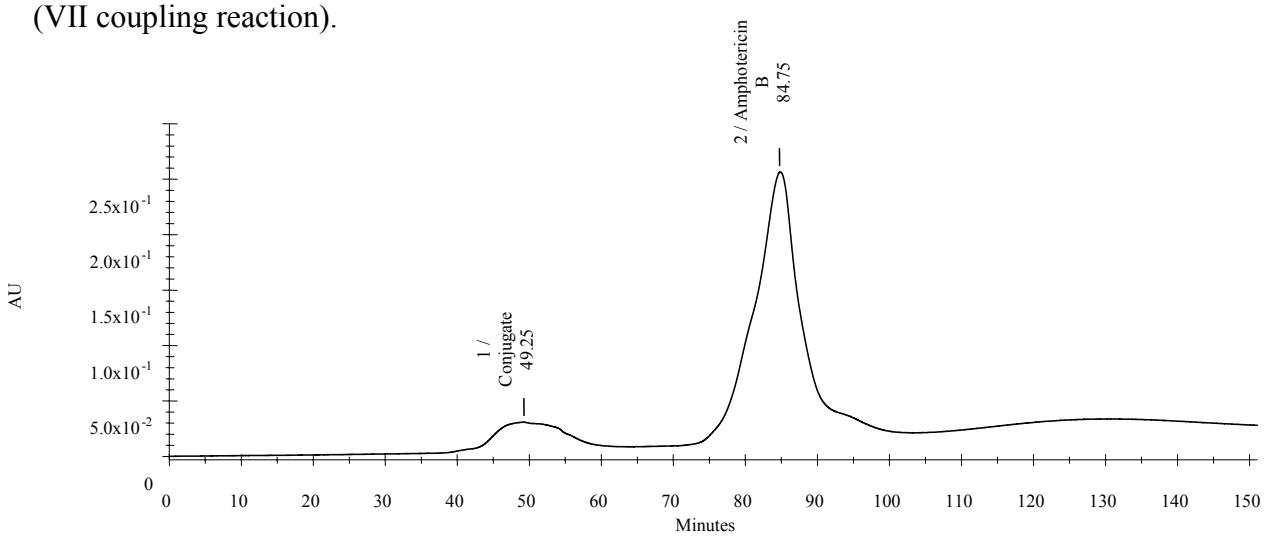


Figure 7.3: GP-FPLC chromatogram of the VIII coupling reaction. Coupling of oxHES130 as Na^+ salt with Amphotericin B in anhydrous DMSO (Eluent: DMSO; Detection at 385 nm).

7.1.4 Couplin with oxHES in dry DMSO without activator

Although the previously described approaches left room for further optimisations, the strategy has been changed once more. The reason is that the lacton form of oxHES may be considered as already a reactive form, and would we found the optimal coupling conditions, we could have a clean reaction without other reagents than the two parts of the conjugate.

The reaction had to be realised in strictly anhydrous conditions and by high temperature. Moreover the gained experience with Amphotericin B obliged us to work under inert atmosphere and in absence of light to reduce any degradation process. Of course in that respect the high temperature does not help. In the table 7.4 the most relevant experiments are resumed.

	$oxHES_{130}$	**Amphotericin B**	**Dry DMSO**	**Reaction time**	
I COUPLING	460.0 mg $1.1x10^{-5}$ mol	5.0 mg $5.4x10^{-6}$ mol	6.0 ml	90 h 40 °C	in dark under N_2
II COUPLING	690.0 mg $1.62x10^{-5}$ mol	5.0 mg $5.4x10^{-6}$ mol	6.0 ml	24 h 60 °C	in dark under N_2
III COUPLING	460.0 mg $1.1x10^{-5}$ mol	5.0 mg $5.4x10^{-6}$ mol	6.0 ml	24 h room temp.	in dark under N_2
IV COUPLING	1150.0 mg $2.7x10^{-5}$ mol	5.0 mg $5.4x10^{-6}$ mol	6.0 ml	24 h room temp.	in dark under N_2
V COUPLING	395.0 mg $9.2x10^{-6}$ mol	2.8mg $3.0x10^{-6}$ mol	4.0 ml	24 h 70 °C	in dark under N_2
VI COUPLING	395.0 mg $9.2x10^{-6}$ mol	2.8mg $3.0x10^{-6}$ mol	4.0 ml	24 h 70 °C	in dark under N_2
VII COUPLING	650.0 mg $1.52x10^{-5}$ mol	2.8mg $3.0x10^{-6}$ mol	4.0 ml	24 h 70 °C	in dark under N_2
VIII COUPLING	650.0 mg $1.52x10^{-5}$ mol	2.8mg $3.0x10^{-6}$ mol	4.0 ml	24 h 70 °C	in dark under N_2

Table 7.4: Summary of the experiments without carbodiimide activation in anhydrous DMSO.

Starting from the first try, the new strategy seemed more promising. The first reaction gave already a 25% yield as shown in figure 7.4. In this case a new column Superose 12 (Pharmacia) has been used, the resolution is significantly better.

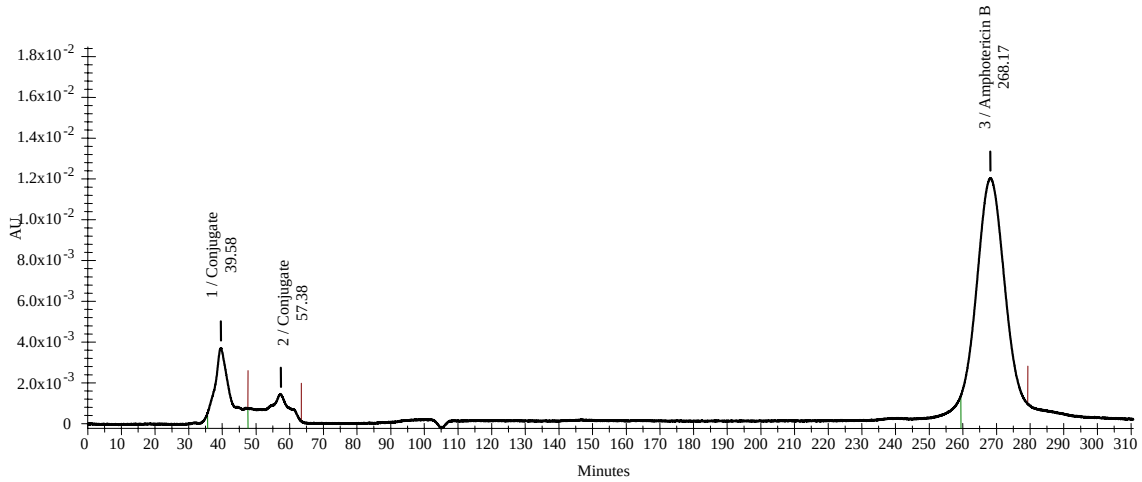


Figure 7.4: GP-FPLC chromatogram of the I coupling reaction. Coupling of oxHES$_{130}$ with Amphotericin B in anhydrous DMSO without any activator (eluent: water, detection at 385 nm).

The next experiments aimed at the optimization of the coupling conditions, mainly the temperature and the ratio between oxHES and the drug. Finally the best compromise found was a temperature of 70°C with an oxHES molar excess between 3:1 (V coupling) and 5:1 (VII coupling). The final yield was in both cases higher than 95% which allows us to eliminate a purification step from the production protocol. The reaction mixture was diluted with water and dialyzed in order to eliminate all DMSO present, and finally lyophilized. Figure 7.5 shows a chromatogram of the final product on GP–FPLC using water as eluent, because, as anticipated, the conjugate is perfectly soluble in water.

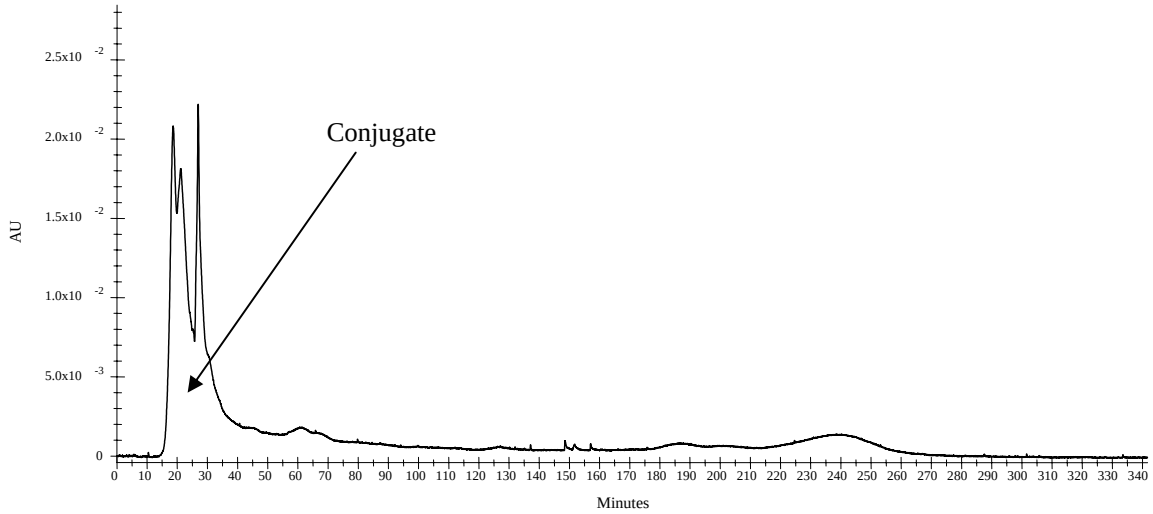


Figure 7.5: GP-FPLC chromatogram of the VII coupling reaction. Coupling of oxHES$_{130}$ with Amphotericin B in anhydrous DMSO without any activator. The final product has been dialysed against distilled water and lyophilised. (eluent: water, detection at 385 nm).

The three different peaks near the column dead volume can be explained remembering that HES is not a pure chemical entity but a polydisperse mixture of many different molecular weights around an average value.

7.1.5 Purity control by RP-HPLC

Finally to check the purity of the conjugate a different analytical method based on Reverse Phase Chromatography was established. Surprisingly the purified conjugate, analysed by RP–HPLC gave a completely different result compared to the GP–FPLC chromatogram. (see figures 7.6 and 7.7)

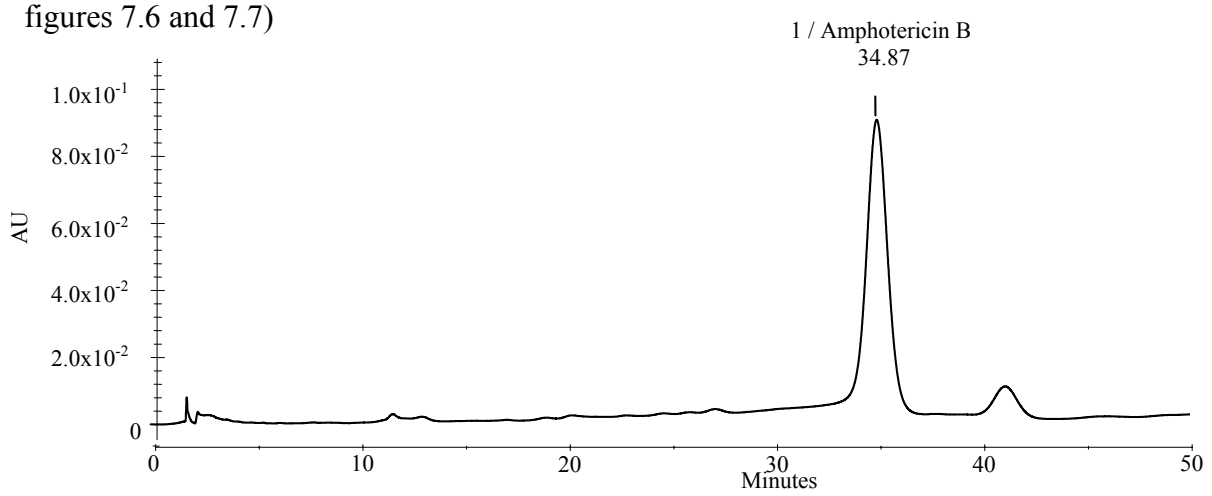


Figure 7.6: RP-HPLC reference chromatogram of Amphotericin B. (Eluent: 63% NH_4^+ CH_3COO^- 0.2 M : 37% CH_3CN, detection at 385 nm).

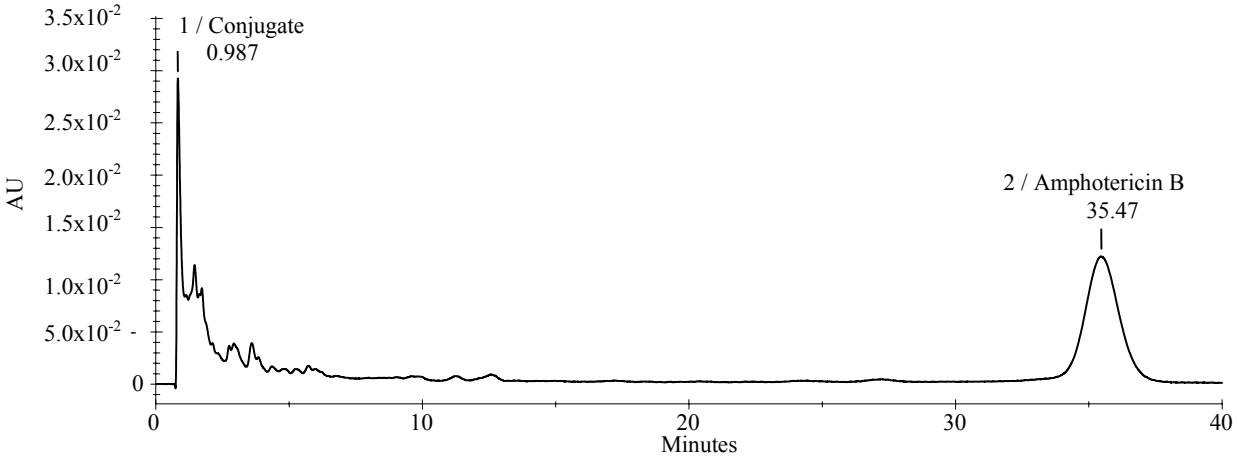


Figure 7.7: RP-HPLC chromatogram of the "purified" Amphotericin B conjugate. (Eluent: 63% NH_4^+ CH_3COO^- 0.2 M : 37% CH_3CN, detection at 385 nm).

The real amount of coupled Amphotericin B is much lower than supposed and there is still a lot of free, non-reacted drug.

The conjugate was then once more analysed with GP–FPLC to exclude that hydrolysis had occurred and the chromatogram confirmed that this was not the case. The other question arising was: why so much free Amphotericin B could not be removed in the dialysis step? One possible satisfactory answer could be the formation of macromolecular structures such as micelles, as proposed in figure 7.8.

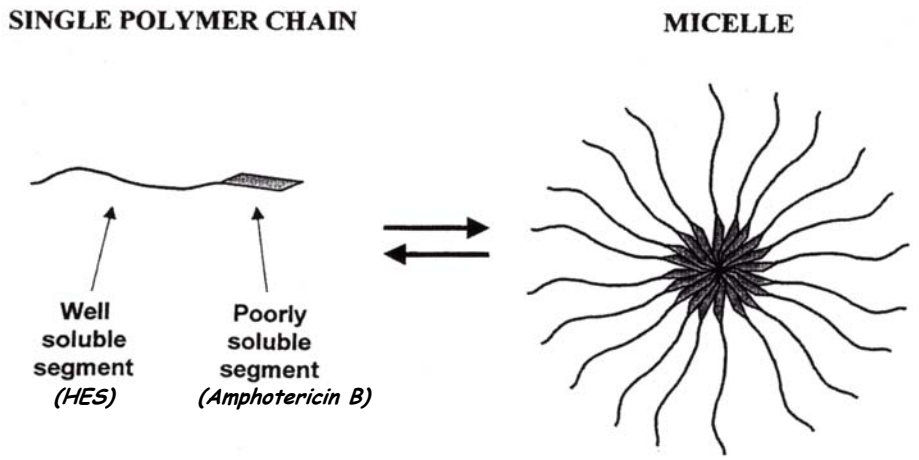


Figure 7.8: The general scheme of micelle formation from amphiphilic molecules.

In our case the poorly water soluble segment is represented by the drug, while the highly hydrophilic portion is represented by the polymer. These molecules may be able to organise themselves in micellar structures with a highly lipophilic core which also entraps non conjugated drug. This assumption finds further confirmation considering the natural attitude of Amphotericin B to form aggregates [2].

Another possible explanation could be a non-specific absorption phenomenon of the drug on the polymer surface. It is generally accepted that also polysaccharides tend to organize in minimum free energy structures exposing the hydrophilic side (hydroxyl functions) to the surface in contact with the aqueous solvent. This often results in the creation of small lipophilic domains where only the backbone of the polymer is present. These pockets, like those of the cyclodextrines, could then entrap and stabilize hydrophobic molecules (e.g. Amphotericin B) in aqueous medium.

Both these theories would explain also the different behaviour in the two analytical models. The GPC was performed in water and intrinsically this method uses mild separation conditions, these structures keep intact during the run. On the other hand RPC is based on the in-

teraction of the substance with a lipophilic matrix (C_{18} in our case), and moreover uses a considerable amount of organic solvent in the eluent, enough to break down the macromolecular structure and deliver its content (the free drug).

Since we made this observation, we used RP–HPLC as the standard analysis for Amphotericin B and restarted the optimisation. Also this time we reached very high yields, between 85% and > 95%. The old procedure, which used dialysis to get rid of the DMSO, was also changed to a precipitation procedure in acetone. This operation turned out to be moreover efficient in eliminating all non-reacted drug as well as degradation by products. The conjugate is now really pure as can be seen from the chromatogram shown below in figure 7.9.

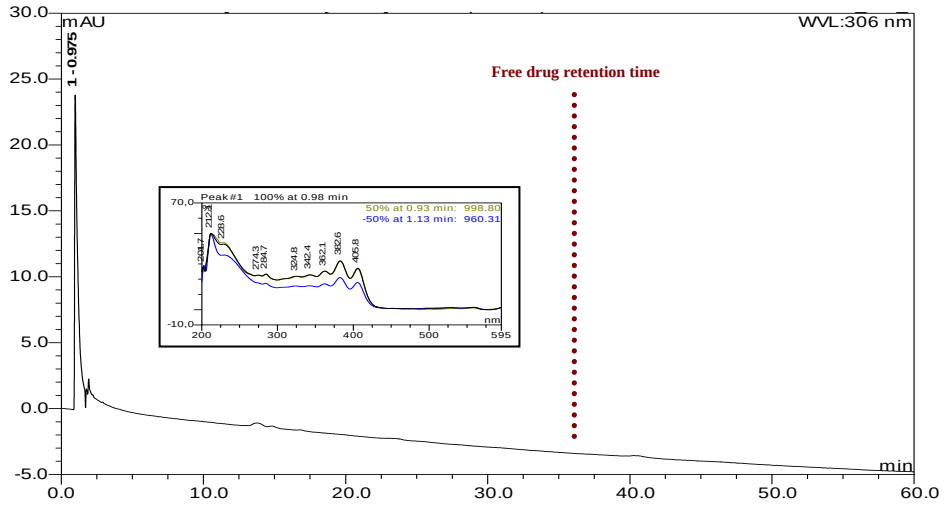


Figure 7.9: RP–HPLC chromatogram of the purified Amphotericin B conjugate (Eluent: 63% $NH_4^+CH_3COO^-$ 0.2M : 37% CH_3CN, detection at 385nm) and UV spectrum of the first peak.

7.2 Spectroscopic determination of the drug content in the conjugate

As the coupling site at the Amphotericin B did not involve the chromophore of the drug, we assumed the relationship between drug concentration and UV absorption to remain the same after the coupling. So firstly a calibration curve of Amphotericin B in DMSO was prepared (figure 7.10).

Then three different amounts of conjugate were dissolved in DMSO and analysed in the spectrometer. From the absorption of these samples at 414 nm an average drug content was calculated. The drug content calculated in this way always correlated to the yield derived from the respective peak area in the RP–HPLC chromatograms.

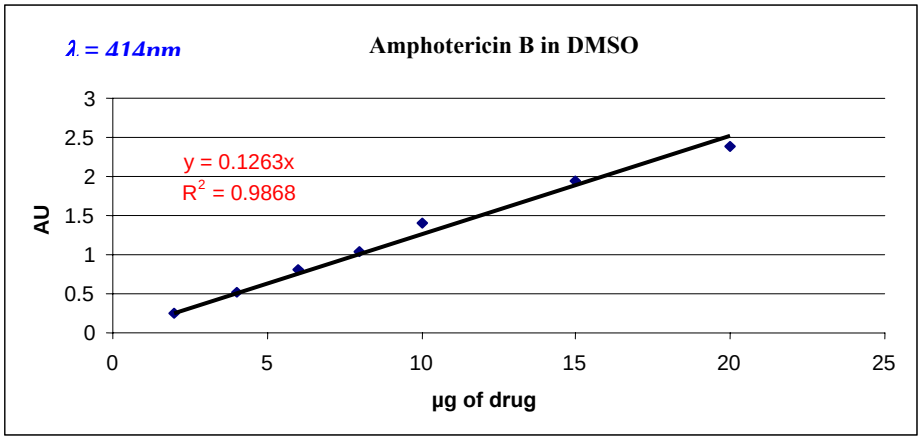


Figure 7.10: Amphotericin B calibration curve in DMSO.

Repetitive batches of conjugate were prepared and the drug content was found in the range of 2.0 to 5.0 µg of Amphotericin B / mg of conjugate, corresponding respectively to a yield of 40% to 60%, in some reactions even approaching 100%.

7.3 Water solubility of the HES_{130}–Amphotericin B conjugate

One batch of conjugate with $oxHES_{130}$ was used to determine the solubility in water. As expected the physico–chemical properties were dictated by the polymeric part. Hydroxyethyl starch is highly water soluble, the limit for the solubility depends on the viscosity of the solution. In fact, the viscosity increases together with the amount of starch dissolved, and the higher the viscosity is the slower becomes the dissolution process.

The same behaviour has been observed for the coupled Amphotericin B. After reaching a concentration of 700 mg/ml, without observing any precipitate or suspension, the solution became very viscous and the time required to get more conjugate in solution was getting longer and longer. The value assumed for the water solubility is therefore greater than 700 mg/ml.

The same measurement, repeated with batches having a different drug content, did not show any dependence from the drug content.

Amphotericin B itself is completely insoluble in water at neutral pH. It is possible to reach a concentration of 0.1 mg/ml by working at extreme pH values (2 or 11). Converting the value of 700 mg/ml in amount of drug effectively dissolved in water we got a maximum drug content up to 3.5 mg per ml of water. This corresponds to a 35-folds increase in water

solubility compared to the solubility at pH 2 of the free Amphotericin B and a more than 1000-fold increase compared to the solubility at physiological pH, respectively. It is clear that even in the case of a non-satisfactory coupling yield the possible reachable drug concentration in water is markedly higher than that of the free, non-coupled drug.

7.4 Antimycotic activity

After the chemical optimisation, the determination of the drug content and the solubility of the conjugate, the next logic step was to check the pharmacological activity of the coupling product. Amphotericin B is a highly potent antimycotic, first choice against *Candida albicans* infections. The test aimed to define the Minimal Inhibitory Concentration (MIC) of the coupling product and to compare it with the free drug.

The experiments showed the effectiveness of Amphotericin B after covalent coupling to HES (hydroxyethyl starch) by growth inhibition of the pathogen *Candida albicans* according to a standardised procedure (DIN 58940).

After dissolving the freeze-dried coupled product in PBS (phosphate buffered saline) the solution was filtered through a sterile non-pyrogenic filter and subsequently a MIC test was carried out. The MIC desired value, according to DIN 58940, lies between 0.125 and 1.0 µg/ml.

Placement of samples on the microtitration plate:

	H	**G**	**F**	**E**	**D**	**C**	**B**	**A**
[µg/ml]	10	5	2.5	1.25	0.625	0.31	0.156	0.078
1	antifungal effect							
2	no effect							
3	Amphotericin B, not filtered, in PBS (1%DMSO initial content)							
4	Amphotericin B, not filtered, in PBS (1%DMSO initial content)							
5	Conjugate-Amphotericin B, not filtered, in PBS							
6	Conjugate-Amphotericin B, not filtered, in PBS							
7	Conjugate-Amphotericin B, filtered, in PBS							
8	Conjugate-Amphotericin B, filtered, in PBS							

MIC 0.31 µg/ml

MIC 0.156 µg/ml

MIC 0.156 µg/ml

The MIC of uncoupled Amphotericin B was determined at 0.31 µg/mg. However, there is only marginal growth in wells B3 and B4 at a Amphotericin B concentration of 0.156 µg/mg. The MIC of coupled Amphotericin B dissolved in PBS lies at 0.156 µg/mg before and

after sterile filtration. Sterile filtration of the dissolved coupled product does not lead to increase of MIC, valid proves that the conjugate to be definitely dissolved and not suspended in water.

In fact the coupled Amphotericin B seems to preserve its anti-fungal potential after anchoring to the polymer. This fact together with the increased water solubility of our conjugate makes it an excellent and versatile antimycotic for parenteral administration, as well as for topic use.

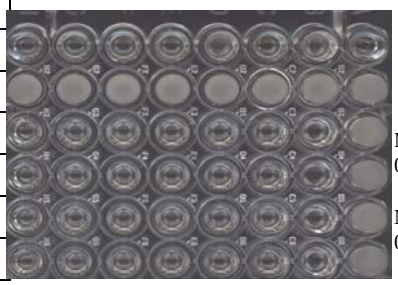
7.5 Conjugate stability in different storage forms

In order to prove its stability, the activity of coupled Amphotericin B has been tested systematically during 6 months. The conjugate, stored in dark at –18°C, proved to keep the same anti-fungal activity for the complete test period.

Reassured by such a good result, we tried a stability test under less favourable conditions. The conjugate was dissolved in water and, after sterile filtration, incubated at 25°C. An aliquot of this solution was analysed every ten days (over three months) to check the amount of the residual activity. The result was very promising:

Placement of samples on the microtitration plate:

	H	G	F	E	D	C	B	A
[µg/ml]	10	5	2.5	1.25	0.625	0.31	0.156	0.078
1	antiungal effect							
2	no effect							
3	Amphotericin B, not filtered, in PBS (1%DMSO initial content)							
4	Amphotericin B, not filtered, in PBS (1%DMSO initial content)							
5	Conjugate-Amphotericin B, sterile filtered, in PBS after 90 days							
6	Conjugate-Amphotericin B, sterile filtered, in PBS after 90 days							



After 90 days storage at 25°C the MIC of the coupled product as well as uncoupled Amphotericin B was kept at 0.156 µg/mg. This means the substance has not lost any activity after staying more then one month in solution and at room temperature. The only caution has been to store the solution under light exclusion.

This test is still running at the moment in order to determine the ultimate storage time.

7.6 Haemolytic effect of the conjugate compared to another water soluble Amphotericin B formulation

The haemolytic potential has been checked by incubation of erythrocytes with several concentrations of coupled Amphotericin B. The same experiment has been performed in parallel with an injectable formulation of the drug available on the market under the trade name Fungizone from Bristol Meyer Squibb. In this formulation the drug is complexed with deoxycholate to improve the water solubility. The diagram in figure 7.11 shows the results.

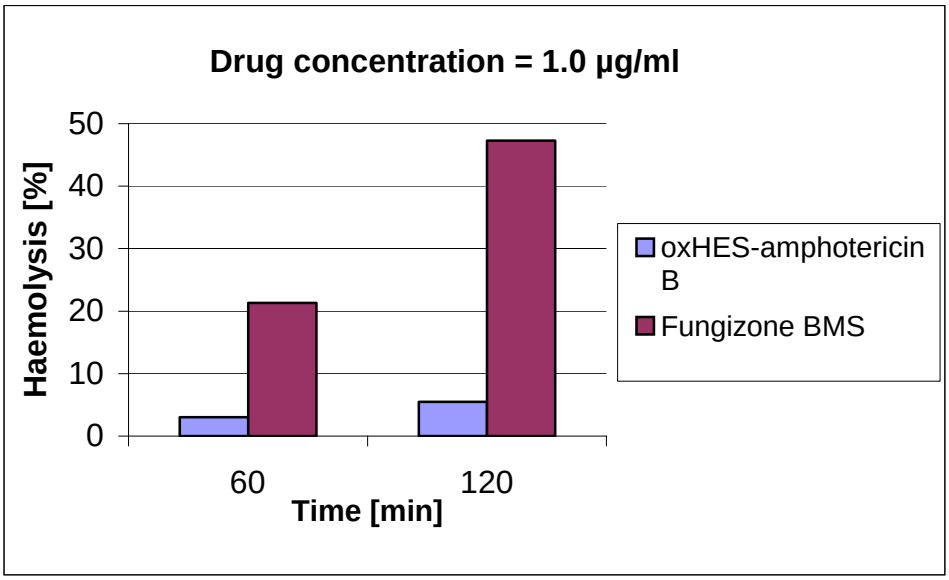


Figure 7.11: Diagram of the haemolytic activity measured for the two different Amphotericin B formulations at therapeutic concentration.

The three concentrations tested did not show any evidence for any difference in the high concentration range. Nevertheless at the lowest concentration a relevant difference can be observed. The deoxycholate salt compared to our conjugate has a 7 times higher haemolytic potential after one hour incubation which becomes even higher increasing the incubation time (about 10 times); in other terms the HES coupled Amphotericin B shows a lower affinity for human cells membranes compared to the injectable formulation.

8 Discussion – *Low molecular weight drug conjugation*

8.1 HES–Amphotericin B conjugate

Amphotericin B is a polyene macrocyclic membrane-active antifungal antibiotic produced by *Streptomyces nodosus* M4575, which for as long as 40 years has been a salvaging medical drug in the treatment of systemic fungal diseases [1]. All polyene antifungal agents form complexes with ergosterol (a membrane sterol present only in fungal cell membranes) and disrupt the fungal plasma membrane, resulting in increased permeability, leakage of the cytoplasmic contents and death of the fungal cell. The drug is very efficient, having predominantly a fungicidal effect, and is particularly administrated to immunosuppressed patients, including solid organ and bone marrow transplant recipients [2], acquired immunodeficiency syndrome subjects [3] and patients with solid tumours and haematological malignancies [4].

However the clinical application of Amphotericin B is hampered firstly by its negligible solubility in aqueous solutions and the lack of peroral drug formulation for systemic use, and secondly by increased occurrence of side effects, especially nephrotoxicity.

The current formulations available on the market are not satisfactory and the development of new, effective, parenteral antifungal drug delivery systems has gained great attention.

The marketed formulation Fungizone, forms a micellar dispersion after the addition of water to the lyophilised sodium deoxycholate–Amphotericin B mixture. Fungizone exhibits major clinical limitations: in systemic use, both sodium deoxycholate and Amphotericin B are not selective enough, and therefore, the therapeutic index of Fungizone is very narrow.

The recently appeared liposomal formulation (AmBisome) allows larger doses of the drug with much lower side effects and toxicity [5, 6, 7]. However, problems, such as the high cost of production and the need of a repeated i.v. infusions for successful treatment, prevent their widespread use. Moreover, the lipid based formulations share a common disadvantages with all particulate systems which are: physical and chemical instability and difficulty in the sterilisation.

Another approach to improve drug performance and reducing toxicity is the conjugation to a polymeric carrier. Conjugation of an insoluble drug to a highly hydrophilic, biodegradable polymer may increase the water solubility of the drug, drug circulation time,

and accumulation in the diseased tissue [8], resulting in an improved therapeutic effect and therapeutic index.

Hydroxyethyl starch (HES) is a highly branched polysaccharide with an extremely large water solubility. For the conjugation with Amphotericin B, HES_{130} (MW~130kD) has been used, but also HES_{10}, HES_{25}, HES_{70}, HES_{200}, and HES_{450} are available on the market, and almost every MW–species can be easily manufactured. The observed solubility of the conjugate was higher than 700 mg/ml corresponding to a drug concentration larger than 3.5 mg/ml. We chose HES_{130} for the coupling reaction because, due to its molecular weight, it is not rapidly excreted by glomerular filtration, ensuring a protracted efficacy. The table 8.1 shows the maximum drug concentration reachable with the most common HES species (assuming that the solubility of HES in water remain the same changing the molecular weight species).

HES	***Mn***	***Drug content per ml***
10	2.61	> 58.1mg
25	14.35	> 10.6mg
70	36.21	> 4.2mg
130	**43.29**	**> 3.5mg**
200	106.16	> 1.4mg
450	447.07	> 0.3mg

Table 8.1: Maximum achievable drug concentrations with the commonly used HES species. (Mn is the normalised mass, see chapter 2).

Such relevant improvement of the solubility can overcome the major drawbacks which normally characterise the Amphotericin B therapy, giving the opportunity to easily reach the therapeutic dose, avoid the long infusion time needed, be injected directly in aqueous solution, be ready for the administration without other solubility enhancers and to facilitate the sterilisation step.

Moreover, the pharmacokinetic behaviour of the conjugate will be governed by the HES chain, and this represents a big advantage since the pharmacokinetic of HES has been investigated for quite long time and has a known dependence on parameters like size, substitution degree and C_2 / C_6 ratio (see also chapter 2). Depending on the kind and the severity of the infections, different conjugates could be administered (or even mixtures of them) to keep the desired concentration in blood as long as required from the patient

conditions. Long lasting formulations could be prepared to avoid repeated administrations, providing the toxicity of the conjugate would be low enough to be tolerated by the patient.

8.1.1 Chemistry of the HES-Amphotericin B conjugation

Hydroxyethyl starch, besides the several hydroxyl groups, possesses on each strand a unique aldehyde function. In order to achieve a 1:1 stoichiometry in the coupling, the difference in reactivity between aldehyde and hydroxyl groups has been exploited. As target function on the drug the primary amino function has been chosen.

This aldehyde group has been firstly oxidised to the carboxylic group, in order to have a broader spectrum of possible coupling strategies in order to yield a stable amide bond. Generally these reaction require the carboxylic group to be previously activated (as halide, anhydride, active ester, etc) or an activator to be present in the reaction mixture to perform an *in situ* activation (carbodiimides, carbonyl diimidazole, etc).

In spite of all these methods being suitable for the coupling, a much more elegant alternative has been exploited. As shown in figure 8.1 the carboxylic function can be easily transformed into a lacton function by dehydration. The resulting lacton is already a reactive form of the carboxylic acid and is able to react selectively with amines to yield the desired amide.

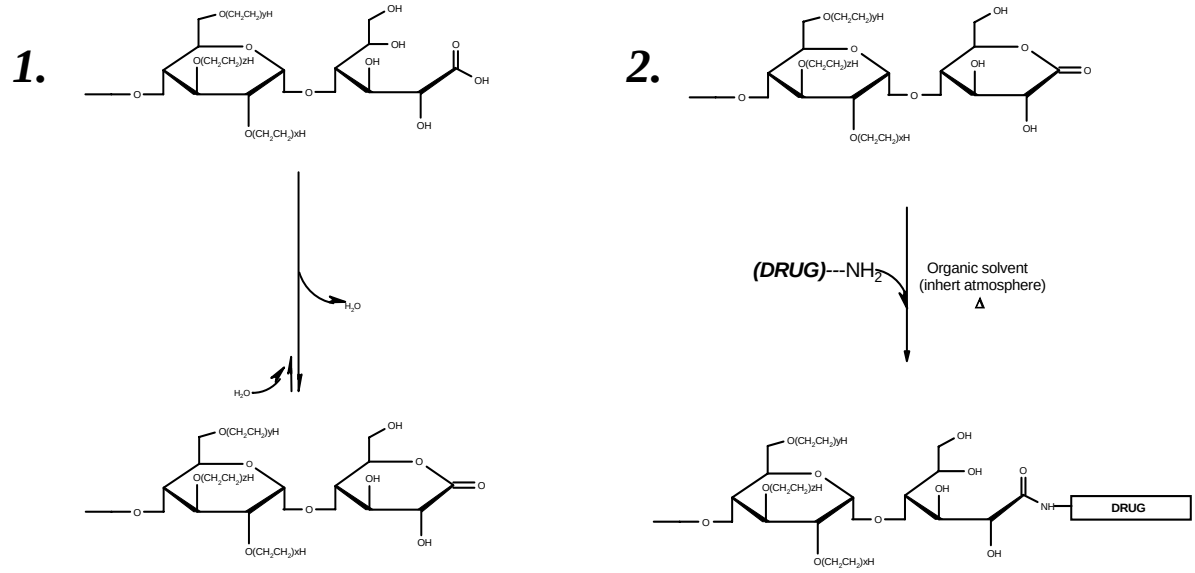


Figure 8.1: Scheme of the reaction used for the conjugation of amphotericin B with $oxHES_{130}$. Step 1: lactonisation; step 2: aminolysis of the lacton.

This procedure is much "cleaner" and more selective than the others mentioned above. To work in the absence of activator turns out to be an advantage not exclusively in terms of costs of the materials but mainly in terms of simplifying the purification step.

8.1.2 Activity of HES-Amphotericin B conjugate

HES_{130}–Amphotericin B has been tested in comparison with the free drug. The results show clearly (see chapter 7) that the coupled version keeps 100% of the original activity, indeed no difference could be observed between the HES conjugate and the free Amphotericin B.

This result may seem somehow unexpected. In fact the conjugate is not a classic pro-drug which would release the intact drug after undergoing enzymatic hydrolysis, the relatively stable amide bond will be hydrolysed only extremely slowly, so the conjugate has to be considered as a new chemical entity. Then the question is: why such bulky and hydrophilic polysaccharide does not change the drug affinity for the membrane sterols?

An explanation for this surprising result could be that the HES anchoring has taken place at a already hydrophilic side of the molecule, which did not change the local chemico-physical properties and leave the pharmacophoric part untouched [10]. (Figure 8.2)

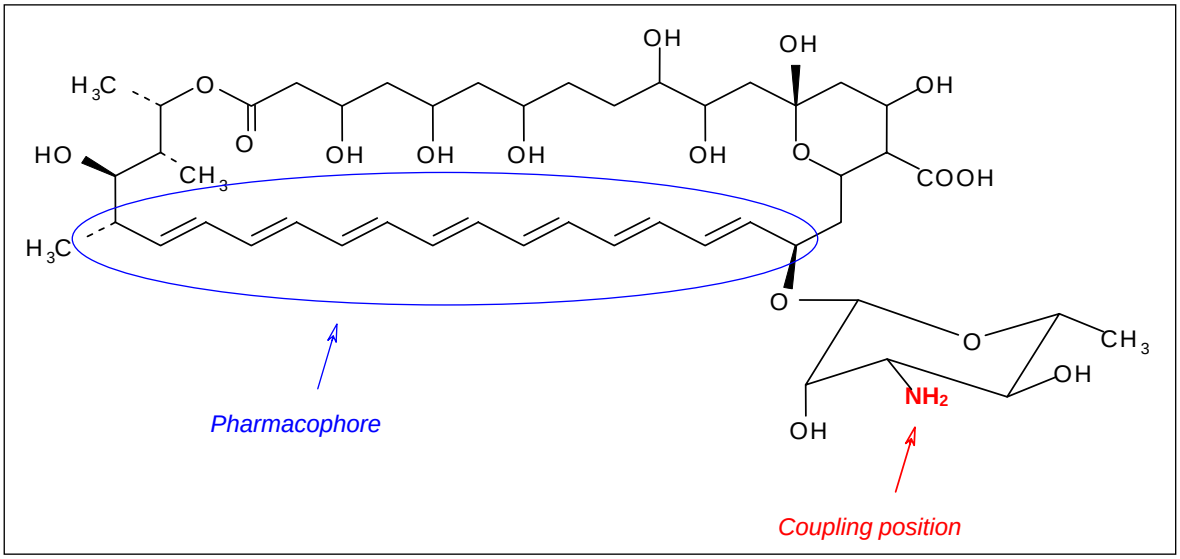


Figure 8.2: Amphotericin B chemical structure.

8.1.3 Stability of HES-Amphotericin B conjugate

The next question was the stability of the molecule. Amphotericin B is a very sensitive molecule *per se*: it is sensitive to light and oxygen exposure [11].

For a formulation, already available on market (Fungizone, Bristol Mayer Squibb) for i.v. administration, refrigeration and light protection are recommended. Nevertheless, intact vials are reported to be stable at room temperature for two weeks up to one month. The manufacturer indicates potency loss (5 to 10%) to occur in one month storage at room temperature [12].

Fungizone reconstituted with sterile water for injection without preservatives and stored in the dark is stable for 24 hours at room temperature and for one week under refrigeration (2 to 8°C).

The pH range for optimum clarity and stability is 6–7. At lower pH values the colloidal dispersion may become turbid and colloidal particles may coagulate.

So it is clear that a lot of caution has to be taken in handling such drug.

Stability test performed on our product gave very promising results. The solid product has been stored for more than 6 months at –20°C without any loss of activity. The solution in sterile water shows an unexpected stability even at room temperature (25°C) for at least 90 days (end of the investigation period).

The conjugate does not suffer any degradation or decrease in efficiency by changing pH and does not seem to need any other care than the light protection. In comparison with the two formulations available on market HES–Amphotericin B needs much less cautions and can be handled also by a not specialised person.

Moreover the sterilisation procedure becomes quite simple because sterile filtration can be performed without the risk to lose parts of the drug.

8.1.4 Selectivity of HES-Amphotericin B conjugate and toxicity

The antimycotic activity of Amphotericin B seems to depend on the affinity that this drug shows for cell membrane sterols [10]. The binding of the drug to the membrane sterols turns out in an increase of the cell membrane permeability which causes an abnormal concentration of the metabolites in the intracellular compartment and the consequent death of

the cell. Amphotericin B shows affinity for all eukaryotic cell membrane sterols, but particularly for ergosterol, the sterol present in fungal cell membranes. Nevertheless, the affinity of this drug to cholesterol (the main sterol in the mammalian cell membranes) is still relevant and seems to be the reason, together with its low water solubility, of the many side effects of Amphotericin B.

Due to its extremely low water solubility, in aqueous medium Amphotericin B has a strong tendency to aggregate in micelles. Recent studies [9] have shown that Amphotericin B should be in a micelle form in order to induce K^+ permeation in cholesterol–containing liposomes (model for mammalian cell membrane) and that any other form of the antibiotic (monomeric or self–associated) induces K^+ leakage from ergosterol–containing liposomes (model for fungal cell membrane). As Amphotericin B has a very low critical micellar concentration (CMC) its intrinsic selectivity in the monomeric form will be masked by the action of micelles leading to an indiscriminate attack towards every kind of membrane sterols, and therefore to toxic effects.

Following this hypothesis, the most selective form of Amphotericin B would be a solution in which the polyene is completely present as monomer. In its monomeric state, Amphotericin B has a characteristic absorption band at 409 nm; upon aggregation, however, its apparent molar absorptivity decreases in magnitude [9]. In figure 8.3 two spectra are shown of a HES_{130}–Amphotericin B solution and Amphotericin B–deoxycholate (Fungizone), respectively, at the same concentration.

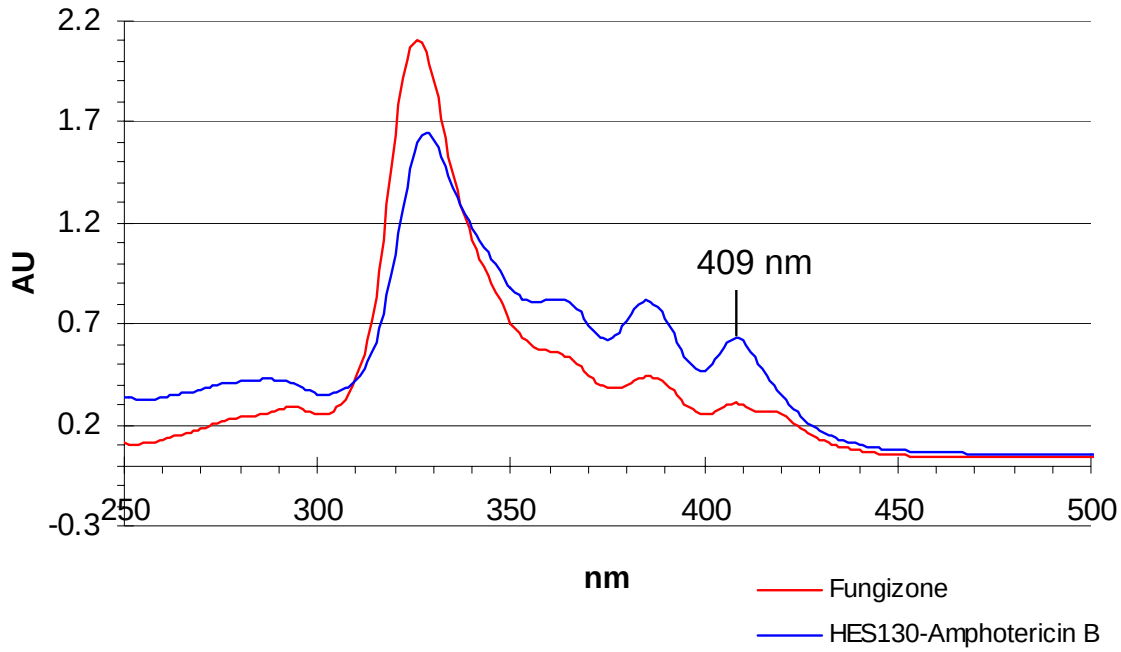


Figure 8.3: Spectra of 20 µg/ml solutions of Fungizone and HES_{130}–Amphotericin B.

It is evident that the peak at 409 nm is much higher in the case of our conjugate, indicating the presence of the drug still as monomer. On the other hand the Bristol Mayer Squibb formulation gave a spectrum which indicates the CMC to have already been reached.

The spectral data are also confirmed by the haemolysis test. This test is the most rapid way to show the affinity of the drug for human membrane sterols. The binding of Amphotericin B to the cholesterol of the erythrocyte cell membranes causes a disruption of the cell membrane with consequent leakage of haemoglobin in the solution. The content of haemoglobin, estimated spectrometrically, gives a measure of the affinity for the mammalian cell membrane sterols. In therapeutic concentrations the haemolysis rate caused by HES–Amphotericin B is about 10 times lower than the one resulting after incubation with Fungizone (see chapter 7).

Both results confirm the assumption that by HES conjugation, it is possible to significantly increase the CMC of the drug, thus avoiding a non-selective rupture of biological membranes at high concentrations of the agent. Since the loss of selectivity and the poor water solubility appear to be the main reasons of the Amphotericin B toxicity, the HES conjugate seems to have all properties of an ideal candidate in antifungal therapy; it offers the same potency as the free drug with drastically reduced side effects, and maintains high selectivity even at much higher concentrations.

8.1.5 Conclusions and perspectives

Amphotericin B is a highly effective antimycotic with a low therapeutic index (ratio between Effective Dose in 50% of the cases and Lethal Dose in 50% of the cases). Almost 50 years after its discovery [10], although severe side reactions are reported, it remains the most effective chemotherapeutic agent against systemic mycoses.

The intrinsic toxicity of the molecule, due to the strong tendency to aggregate in solution, only allows a rather low maximal tolerated dose (1 mg/kg of body weight per day) to be administered, in a concentration of 0.1 mg/ml, by slow intravenous infusion over approximately 2 to 6 hours.

The hydroxyethyl starch–Amphotericin B conjugate, besides keeping intact the whole antimycotic potential of the drug even in the coupled form, has the big advantage to stabilise the molecule in its monomeric state avoiding self–aggregation. Since most probably the

micelle form is responsible of the unselective attack of Amphotericin B against mammalian cells, the effect of the coupling is, in fact, a general reduction of undesired effects obtained by preventing the aggregation in solution at therapeutic concentration as well as keeping a high water solubility.

This new molecule allows for the antifungal activity to be separated from its haemolytic activity, and the magnitude of this separation will increase as the critical micelle concentration (CMC) of the conjugate increases. In other words, even higher concentration could be reached without having relevant aggregation and therefore side effects. This is a promising achievement especially for those cases where the maximal tolerated dose is still ineffective (i.e. leishmanial infections, especially when the immune status of the patient is compromised and therefore larger drug doses are required in order to kill parasites in unusual locations) [12].

If compared with the current marketed formulations, HES_{130}–Amphotericin B has moreover many other advantages which include enhanced stability (even in solution at room temperature), easiness in preparing and handling the formulation, easy sterilisation and well known pharmacokinetics.

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