UNIVERSITÀ
DEGLI STUDI
DI PADOVA

UNIVERSITÀ DEGLI STUDI DI PADOVA

DEPARTMENT OF PHARMACEUTICAL AND PHARMACOLOGICAL SCIENCES

Doctoral School in Regeneration Biology and Medicine

Curriculum in Tissue and Transplant Engineering

Cycle XXV

DEVELOPMENT OF POLYMERIC DRUG DELIVERY SYSTEMS FOR BIOTECH PRODUCTS

SCHOOL DIRECTOR: Ch.mo Prof. Mariateresa Conconi

SUPERVISOR: Ch.mo Prof. Oddone Schiavon

PhD Student: Matteo Pasqualin

31 January 2013

Le cose non vanno mai come credi…

INDEX

ABBREVIATIONS AND SYMBOLS

°C	Celsius degrees
^{1}H-NMR	Nuclear magnetic resonance of the hydrogen
6-FAM	6-carboxyfluorescein
$A_{100\%lysis}$	absorbance value of the total hemoglobin released from RBCs
aa	amino acid/s
A_{blank}	absorbance value of the hemoglobin released from RBCs treated with PBS
Abs	absorbance
$Abs_{0.1\%}$	absorbance of a solution 1g/L or 1mg/mL
ACN	acetonitrile
AMD	age-related macular degeneration
A_{sample}	absorbance value of the hemoglobin released from RBCs in the sample
ATP	adenosine triphosphate
BCA	bicinchoninic acid
bp	base pair
CCM	cytidine 2';3'-cyclic monophosphate monosodium salt
CDI	1, 1'-carbonyldiimidazole
Da	Dalton
DDSs	drug delivery systems
dL	decilitre
DMSO	dimethylsulfoxide
DNA	desossiribonucleic acid
ds	double-stranded
EMA	European Medicines Agency
EMEA	European Agency for the Evaluation of Medicinal Products
EPO	erythropoietin
EtOH	ethanol
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
g	acceleration of gravity, equal to 9,80665 m/s^2
g	grams
GCSF	granulocyte colony-stimulating factor
h	hours
HA-ac	Hyaluronic acid derivatized with 4-aminobutyraldehyde diethyl acetal

HA-Na	Hyaluronic acid sodium salts
HA-N^{ter}-INS 1	HA-insulin -N^{ter} low loading
HA-N^{ter}-INS 2	HA-insulin -N^{ter} high loading
HA-N^{ter}-RNase A	HA-RNase A -N^{ter} selective conjugated
HA-N^{ter}-trypsin	HA-trypsin -N^{ter} selective conjugated
HCl	chloridic acid
HMWS	High Molecular Weight Substrate
kDa	kilo Dalton (10^3 Da, equal to ≈ 1,66053886 * 10^{-24} g)
L	liters
LMWS	Low Molecular Weight Substrate
m	meters
M	Molarity (mol/liter)
m	multiplet, multiplicity
mg	milli grams (10^{-3} grams)
min	minute
mL	milli liters (10^{-3} liters)
mM	milli Molar (10^{-3} moles/liters)
mmol	milli moli (10^{-3} moles)
mRNA	messenger RNA
MW	molecular weight
$NaBH_3CN$	sodium cyanoborohydride
NaCl	sodium cloride
nm	nano meters (10^{-9} meters)
nmol	nano moli (10^{-9} moles)
o.n.	over nigth
PAGE	polyacrylamide gel electrophoresis
PBS	Phosfate Buffered Solution
PDDs	Polymeric Drug Delivery System
PEG	polyethylene glycole
pH	- $\log_{10}$ $[H_3O^+]$
PK	pharmacokinetic
ppm	parts per million
RBCs	red blood cells
RES	reticuloendothelial system
rHA-RNase A	HA-RNase A random conjugated
rHA-trypsin	HA-trypsin random conjugated
RNA	ribonucleic acid
RNAi	RNA interference
RNase A	Ribonuclease A
RP-HPLC	Reverse Phase - High Performance Liquid Chromatography
rpm	revolution per minute

RT	room temperature
s	seconds
s	singlet, multiplicity
s.c.	sub cutaneous
siRNA	small interfering RNA
SEC-HPLC	Size Exlusion Chromatography - High Performance Liquid Chromatography
ss	single-stranded
t	time
t	triplet, multiplicity
TAME	N_α-*p*-Tosyl-L-arginine methyl ester hydrochloride
TBE	89 mM Tris base, 89 mM boric acid, 20 mM EDTA, pH 8.0
TEAA	triethylammonium acetate buffer
TFA	trifluoroacetic acid
t_R	retention time
TRIS	tris(hydroxymethyl)aminomethane
TRIS/acetate	tris(hydroxymethyl)aminomethane pH controlled with acetic acid
UV-Vis	ultraviolet-visible
v/v	volume per volume
VEGF	vascular endothelium growth factor
w/v	weight per volume
µg	micro grams (10^{-6} grams)
µL	micro liters (10^{-6} liters)
µm	micro meters (10^{-6} meters)
µmol	micro moles (10^{-6} moles)

Table I.I: Description of the oligonucleotides used in the project. Modifications, complementary, sequences and symbols.

	5' MODIFICATION	SEQUENCE	SYMBOL
a	Thiol	5'-ACTATTCCCGGGTAATGA-3'	HS-ACT-ATT-CCC-GGG-TAA-TGA
a_F	6-FAM	5'-ACTATTCCCGGGTAATGA-3'	FAM-ACT-ATT-CCC-GGG-TAA-TGA
a'	-	5'-AATTCATTACCCGGGAATAGT-3'	AAT-TCA-TTA-CCC-GGG-AAT-AGT
A	Thiol -	5'-ACTATTCCCGGGTAATGA-3' 3'-TGATAAGGGCCCATTACTTAA-5'	HS-ACT-ATT-CCC-GGG-TAA-TGA TGA-TAA-GGG-CCC-ATT-ACT-TAA
A_F	6-FAM -	5'-ACTATTCCCGGGTAATGA-3' 3'-TGATAAGGGCCCATTACTTAA-5'	FAM-ACT-ATT-CCC-GGG-TAA-TGA TGA-TAA-GGG-CCC-ATT-ACT-TAA
m	P	5'-ATTCCAAAGCTTCCCCCTA-3'	ATT-CCA-AAG-CTT-CCC-CCT-A
m'	6-FAM	5'-TAGGGGGAAGCTTTGG-3'	FAM-T-AGG-GGG-AAG-CTT-TGG
M_F	- 6-FAM	5'-ATTCCAAAGCTTCCCCCTA-3' 3'-GGTTTCGAAGGGGGAT-5'	ATT-CCA-AAG-CTT-CCC-CCT-A GGT-TTC-GAA-GGG-GGA-T-FAM
b	Thiol	5'-GGGTAATGA-3'	HS-GGG-TAA-TGA
b'	-	5'-AATTCATTACCC-3'	AAT-TCA-TTA-CCC
B	Thiol -	5'-GGGTAATGA-3' 3'-CCCATTACTTAA-5'	HS-GGG-TAA-TGA CCC-TTA-ACT-TAA
PEG-a	$mPEG_{20kDa}$-Mal	5'-ACTATTCCCGGGTAATGA-3'	ACT-ATT-CCC-GGG-TAA-TGA
PEG-SS-a	$mPEG_{20kDa}$-OPSS	5'-ACTATTCCCGGGTAATGA-3'	ACT-ATT-CCC-GGG-TAA-TGA
PEG-b	$mPEG_{20kDa}$-Mal	5'-GGGTAATGA-3'	GGG-TAA-TGA
PEG-A	$mPEG_{20kDa}$-Mal -	5'-ACTATTCCCGGGTAATGA-3' 3'-TGATAAGGGCCCATTACTTAA-5'	ACT-ATT-CCC-GGG-TAA-TGA TGA-TAA-GGG-CCC-ATT-ACT-TAA
PEG-SS-A	$mPEG_{20kDa}$-OPSS -	5'-ACTATTCCCGGGTAATGA-3' 3'-TGATAAGGGCCCATTACTTAA-5'	ACT-ATT-CCC-GGG-TAA-TGA TGA-TAA-GGG-CCC-ATT-ACT-TAA
PEG-B	$mPEG_{20kDa}$-Mal -	5'-GGGTAATGA-3' 3'-CCCATTACTTAA-5'	GGG-TAA-TGA CCC-TTA-ACT-TAA

1 ABSTRACT

Since the early 80's the forward steps in genetics and proteomics, have led a particular interest to biotech products, such as DNA and proteins. Although difficult, their large-scale production enabled the therapeutic use of this compounds. Proteins and DNA sequences can be very interesting therapeutic molecules owing to their high selectivity/affinity for the receptor or the specific site of action.

Unfortunately, some issues still limit their pharmaceutical use, such as the susceptibility to enzymatic degradation, rapid renal clearance and immunogenicity.

To overcome these limitations, many researchers are seeking solutions in the field of drug delivery systems (DDSs). In this respect, many systems have been developed and conjugation with PEG (polyethylene glycol) can be considered one of the leading approaches. PEGylation brings to the conjugated molecule great solubility and stability to proteolytic digestion, furthermore it reduces the tendency to aggregate and reduces the immunogenicity. Thanks to these advantages and the particular characteristics of PEG, to date, there are on the market 12 pegylated compounds: 9 are proteins, one peptide, one aptamer and a liposomal formulation, containing doxorubicin.

The improvements in the pharmacokinetic profile of these drugs, thanks to the use of drug delivery systems, can be also applied in the field of tissue engineering, where the same issues are of fundamental importance for the development of scaffolds for cells capable of releasing growth factors.

In the last years various polymers have been studied by many research groups to find an alternative to PEG, but its excellent biocompatibility and the know-how in its use has not brought any polymer to be truly competitive against PEG. Nevertheless, PEG presents some limits such as its non-biodegradability and in some case there are reports of antibodies against PEG. Therefore, there is an increased need for a PEG substitute.

In the first section of this work hyaluronic acid (HA) has been studied as a candidate polymer for bioconjugation of proteins (HAylation). HA, being biodegradable can compensate this limit of PEG. HA, is also present in humans and is metabolized by hyaluronidase. Moreover, HA has the advantage of a high loading compared to PEG, thanks to the presence of repetitive functional groups in each monomer.

This part of the work was focused on the study of HA conjugation (HAylation) to two model enzymes, trypsin and Ribonuclease A, and then to an interesting protein in pharmaceutical field, insulin.

In order to avoid cross-linking phenomena, only a fraction of all carboxyl groups of the polymer has been modified to aldehyde allowing the conjugation with the amino groups of the protein models. Furthermore, by modulating the

pH of reaction two protein-HA conjugates were obtained, selective *N*-terminal (pH 6) or random (pH 8), this taking advantage of the different pK_a values of the amino groups in the proteins.

The first products obtained with the enzymes Ribonuclease A and trypsin were tested verifying the residual activity compared to the native proteins. All conjugates, in particular those obtained by *N*-terminal selective conjugation, maintain a good activity on small substrates (30% decrease); only the HA-derived trypsin retains about 60% of residual activity against the substrate with a high weight molecular. Furthermore, enhanced stability over time was found for HA-trypsin respect to the free enzyme (45% on average) and also susceptibility to hyaluronidase was confirmed for both conjugates.

Polymer validation as potential protein carrier was then evaluated by preparing conjugates with bovine insulin, as an example of pharmacologically active protein. Two conjugates were synthesized by *N*-terminal selective conjugation starting from polymers with different degree of aldehyde derivatization, 4% and 21%, yielding products with a protein loading of 17% and 32% (w/w), respectively.

The therapeutic efficacy of the conjugates in comparison with insulin was tested in *Sprague Dawley* rats with induced diabetes. The conjugate with a lower protein loading was more effective and with a longer pharmacodynamic effect on the reduction on blood glucose level.

The second section of the work was focused on an innovative strategy of enzymatic PEGylation of oligonucleotides. Briefly, the method investigated on model oligonucleotides is composed of two steps: the first consists in the chemical conjugation of a short oligonucleotide to a PEG chain, the second step is the enzymatic-mediated conjuagation of the PEGylated oligonucleotide with a DNA sequence by the DNA T4 ligase.

To study the enzymatic PEGylation, 4 oligo sequences have been prepared as ligation model: two complementary pairs ending with sticky-ends in turn complementary (18-mer + 21-mer and 16-mer + 19-mer). The 18-mer has a thiol group in 5'-ending, in order to perform the coupling with PEG.

Applying some modifications to ligation classical protocols, excellent results were obtained: PEGylated portion completely ligate the other ds-DNA and no undesired products were found.

To further confirm the effective ligation, the ligated and PEGylated sequence was restricted with EcoRI. Indeed, the EcoRI recognized a sequence that is present only the ligated DNA. Complete restriction was found in absence and even in the presence of the polymer, further confirming the successes of ligation.

Furthermore it was investigated if a reduced number of bases coupled to PEG can still preserve the requirements for the ligase enzyme activity. Thus, the pair of the complementary sequences then coupled to PEG has been

reduced to half (9-mer + 12-mer). Even with a shorter PEGylated sequence a complete ligation was obtained.

In conclusion in this thesis it has been demonstrated that HA can be a valid alternative to PEG for protein conjugation.

In the field of oligonucleotide delivery an enzymatic approach of oligonucleotide conjugation can open new horizons that so far have not been completely explored.

1 RIASSUNTO

Dai primi anni 80 i passi avanti fatti nel campo della genetica e della proteomica, hanno portato ad un particolare interesse nei confronti dei prodotti biotecnologici, quali DNA e proteine. L'utilizzo terapeutico di queste entità, seppur non privo di difficoltà, è stato facilitato dalla loro produzione su larga scala. Proteine e sequenze oligonucleotidiche si sono rivelate interessanti come agenti terapeutici essendo molecole dotate d'elevatissima selettività/affinità per il recettore o il sito d'azione specifico.

L'impiego farmaceutico può evidenziare alcuni svantaggi che ne possono limitare l'utilizzo, come ad esempio la suscettibilità alla degradazione da parte di proteasi e DNasi, la rapida *clearance* renale e l'immunogenicità.

Per affrontare tali limiti, molti ricercatori hanno cercano soluzioni nel campo dei *drug delivery sistems* (DDSs). A tal proposito, sono stati sviluppati molti sistemi e la coniugazione al PEG (polietilen glicole) è risultata essere una delle più promettenti. La PEGhilazione, infatti, conferisce alle molecole coniugate maggiore solubilità e stabilità nei confronti della digestione proteolitica, una ridotta tendenza all'aggregazione ed una ridotta immunogenicità. Grazie a questi vantaggi ed alle particolari caratteristiche del PEG, ad oggi sono presenti nel mercato 12 composti PEGhilati: 9 sono proteine, un peptide, un aptamero ed una formulazione liposomiale (contenente doxorubicina).

Le migliorie apportate ai profili farmacocinetici di questi farmaci biotech grazie all'uso di DDSs possono essere anche impiegate nel campo dell'ingegneria tessutale, dove le medesime problematiche sono di basilare importanza per lo sviluppo di *scaffold* per cellule, in grado di rilasciare fattori di crescita.

Il polietilen glicole (PEG) è il polimero leader per la coniugazione di proteine. Negli ultimi anni diversi polimeri sono stati studiati per trovare una valida alternativa a questo polimero, ma la sua eccellente biocompatibilità e la conoscenza nel suo utilizzo non ha ancora portato nessun polimero ad essere realmente competitivo nei suoi confronti. Nonostante tutto, anche l'utilizzo del PEG presenta alcuni limiti, quali la non-biodegradabilità e la documentata presenza di anticorpi anti-PEG sviluppati in alcuni casi specifici. Per questo motivo si è alla ricerca di un polimero che possa validamente sostituire il PEG.

Nella prima parte di questo lavoro di tesi è stato studiato l'acido ialuronico (HA) per la bioconiugazione di proteine (*HAylation*). Essendo biodegradabile, l'HA può essere vantaggioso rispetto al PEG. L'HA è un polimero endogeno ed è metabolizzato dalle ialuronidasi, inoltre ha il vantaggio di poter raggiungere una capacità di *loading* elevate rispetto al PEG, grazie alla presenza di gruppi funzionali ripetitivi in ciascun monomero.

In questa parte del lavoro di tesi, la ricerca si è concentrata sullo studio della coniugazione dell'HA a due enzimi modello, Ribonuclease A e tripsina, e poi ad un interessante proteina per uso farmaceutico, l'insulina.

Per evitare fenomeni di *cross-linking*, solo una parte dei gruppi carbossilici del polimero è stata coniugata ad uno *spacer* aldeidico, consentendo la coniugazione con i gruppi amminici delle proteine. Inoltre, modulando il pH di reazione si sono potuti ottenere coniugati con legame selettivo all'*N*-terminale (pH 6) oppure *random* (pH 8), sfruttando la differente pK_a degli ammino gruppi nelle proteine.

I primi coniugati ottenuti con gli enzimi Ribonuclease A e tripsina sono stati studiati verificandone l'attività residua rispetto alle proteine native. Tutti i coniugati, in particolare quelli ottenuti per legame selettivo all'*N*-terminale, mantengono una buona attività su piccoli substrati (diminuzione del 30%); solo il derivato HA-tripsina mantiene circa il 60% di attività residua nei confronti del substrato ad alto peso molecolare. Inoltre, sempre per HA-tripsina, si è trovata una maggiore stabilità nel tempo rispetto l'enzima nativo (mediamente 45%) e si è confermata la suscettibilità di entrambe i coniugati nei confronti della ialuronidasi.

La valutazione del polimero come potenziale *carrier* per proteine è proseguita preparando dei coniugati con l'insulina bovina, come esempio di proteina farmacologicamente attiva. Sono stati sintetizzati due coniugati con modalità selettiva all'*N*-terminale a partire da polimeri con diverso grado di modifica con gruppi aldeidici, pari a 4 e 21% e si sono ottenuti prodotti con il 17 e 32% (p/p), rispettivamente, di *loading* proteico.

L'efficacia terapeutica dei coniugati in comparazione con l'insulina è stata testata su ratti *Sprague Dawley* con diabete indotto. Il coniugato con un minore *loading* proteico si è rivelato essere più efficace e con una riduzione dei livelli di glucosio nel sangue più prolungata.

Nella seconda parte di questo lavoro di tesi si è studiata un'innovativa strategia di PEGhilazione enzimatica di sequenze oligonucleotidiche al fine di sviluppare questo approccio per il *delivery* di oligonucleotidi. Il metodo è stato messo a punto con sequenze nucleotidiche modello e l'approccio è stato il seguente: una breve sequenza oligonucleotica viene legata chimicamente ad una catena di PEG. Poi, mediante l'azione catalitica della T4 DNA ligase la porzione di PEG-DNA viene coniugata ad un'altra sequenza oligonucleotidica.

Per lo studio di PEGhilazione enzimatica si è ideato un modello costituito da 4 sequenze oligonucleotidiche di riferimento: due coppie complementari terminanti con *sticky-ends* complementari a loro volta (18-*mer* + 21-*mer* e 16-*mer* + 19-*mer*). L'oligo di 18 nucleotidi portava in posizione 5' una funzione tiolica, che è stata impiegata per la coniugazione col polimero.

Dopo aver apportato alcune variazioni ai protocolli classici di ligazione si sono ottenuti ottimi risultati: completa ligazione del modello PEGhilato ed assenza di prodotti indesiderati.

Un'ulteriore conferma di ligazione del modello PEGhilato si è ottenuta tramite digestione con EcoRI. Infatti, solamente dopo la ligazione è possibile trovare nella sequenza oligonucleotidica il sito di restrizione dell'enzima. In presenza o in assenza di polimero la restrizione è avvenuta completamente.

Si è poi voluto indagare se una sequenza PEGhilata con un numero di basi ridotto potesse comunque mantenere i requisiti per essere substrato della T4 DNA ligase. Così, la coppia di sequenze complementari designata alla PEGhilazione è stata ridotta alla meta della sua lunghezza (9-*mer* + 12-*mer*). Anche con la sequenza PEGhilata così accorciata la ligazione è avvenuta completamente.

In conclusione questo lavoro di tesi ha dimostrato che l'HA può essere una promettente alternativa al più noto PEG per la modifica di proteine.

Nell'ambito del *delivery* di oligonucleotidi lo sviluppo di un approccio enzimatico di coniugazione può aprire nuovi orizzonti in questo settore il cui potenziale non è stato ancora esplorato.

2 INTRODUCTION

2.1 DRUG DELIVERY SYSTEMS

Drug delivery systems (DDSs) can be divided into a variety of subjects: micro- and nanoparticles, liposomes, matrixes or liquid gels, association with cyclodextrines and conjugation with natural or synthetic polymers. These systems can be seen as the first generation of "*nanomedicines*" (Duncan, 2006).

Drug delivery systems (DDSs) are particularly promising in improving the *in vivo* efficacy of active molecules. It is possible to prolong the *in vivo* half-life and protect the drug from enzymatic degradation, thus enhancing the *in vivo* bioactivity. DDSs can be used either for the delivery of classic low molecular weight pharmaceuticals with short PK profile (as anticancer drug) but also for biotechnologic products such as proteins and oligonucleotides.

The drug delivery in conjunction with the controlled drug release can reach unprecedented results in many therapeutic fields and can find application also in a field like tissue engineering.

2.1.1 Tissue Engineering

As a consequence of disease or trauma, the tissues and organs in our bodies may be unable to perform their anatomical and metabolic functions. Until recently the use of implants, and in severe cases transplants, is the only way to deal with these pathological conditions (Terada et al., 2000). In recent years, a new branch of regenerative medicine called tissue engineering is under development. This term, officially coined in 1988 by the National Science Foundation, indicates that this multidisciplinary field produces biological substitutes containing living and functional cells for regeneration, maintaining or improving performance of the tissue (Langer and Vacanti, 1993).

Tissue engineering represents the meeting point of different disciplines such as medicine, biology, engineering, and chemistry with the common purpose for obtaining or replacing organs or parts of organs in the human body. This will make possible to have a viable alternative to transplantation, as this can have many fundamental problems such as the chronic shortage of donors, the phenomena of organ rejection and the continued need to take immunosuppressive drugs (Terada et al., 2000). In general, a tissue engineering construct is formed by cellular component and a basic structure with function support. By production of extracellular matrix, the cells promote the interaction with the implantation site. A great advantage of this technique is that the cells can be donated from the patient himself; once selected they are grown *in vitro*

and subsequently replanted (Suh, 2000). The basic structure can be by an artificial component, polymer type, or from natural component that can ensure the support to the cell populations of interest.

Therefore, the aims of tissue engineering are to design organs and prostheses, and to assess the interaction between biomaterial and cellular component facilitating the rapid and efficient regeneration of the original tissue. Moreover, the support must be biocompatible and biodegradable. To optimize the adhesion and cell growth, adhesion peptides are derived from the sequence RGD (arginine, glycine, aspartic acid), or the proteoglycan KSRS type (lysine, serine, arginine, serine). Another way to allow cell proliferation is to add sequences to the culture medium that belong to active growth factors specific for the type of cells used.

When the tissue around the defect has no inherent potential to regenerate, the tissue regeneration cannot always be expected by providing the scaffolding technology and space mentioned above. In this case, it would be better to combine the technology with cells and/or a growth factor, which has the potential to accelerate tissue regeneration. It is possible to use cells that were proliferated *in vitro* by the method described above.

To overcome the problem of the *in vivo* instability of biological substances such as protein and genes that are used to induce tissue regeneration, it is vital to use technology to develop the administration form. Indeed, when a solution of the growth factor is injected into the site requiring regeneration, the biological effect cannot be always predicted. This is because the growth factor is rapidly diffused away from the injection site. To enhance the *in vivo* efficacy of the growth factor, the drug delivery system (DDS) is promising. It is possible that when used in combination with an appropriate DDS technology, the growth factor enhances the *in vivo* proliferation and differentiation of key cells that promotes tissue regeneration. For example, the controlled release of a growth factor at the site of action over an extended time period is performed by incorporating the factor into an appropriate carrier. It is also possible that the growth factor is protected against proteolysis, as it is incorporated in the release carrier for prolonged retention of the activity *in vivo*. DDS technology can be also useful for half-life prolongation, absorption improvement and targeting, applicable in tissue engineering using protein and genes.

2.1.2 Biotech drugs

Biotechnology (or “biotech”) is defined as the use of living systems and organisms to develop or make useful products, or “any technological application that uses biological systems, living organisms or derivatives

thereof, to make or modify products or processes for specific use" (Convention on Biological Diversity of UN, 1992).

Biotech products are mainly represented by proteins, produced thanks to the recombinant DNA technology. Their applications involve health, feeding and agriculture.

The advent of recombinant DNA technology has occurred around the 70's and has led to a rapid development of the use of biotech in therapy, in particular for proteins. Often named simply "biological", biotech drugs are already widespread in fact, the clinical application of cytokines and other biological response modifiers such as thrombolytic agents, adhesion molecules, peptide fragments of agonists and antagonists of growth factors and their receptors and antibodies. They are commonly required for genetic or chronic diseases, where a key role protein is missing, mis-functional or lacking.

Very close to this kind of products there are also therapeutic oligonucleotides. They are not really biotech; in fact they are usually produced by synthetic systems. Although the synthetic production, they represent an originally natural component of the cell, then they frequently are comprised or closely related to "biotech product". They are prevalently indicated for tumour and genetic/rare disorders. It is possible mainly describe two classes for therapeutic application: short and long sequences. Short oligomers are possibly administered as "classical drugs" and they usually act blocking proteins translation (antisense technology and triple helix) or function (aptamers and ribozymes). Long sequences are used for gene therapy, the introduction of the correct gene sequence in a patient's genetic code.

This biotech drugs share common difficulties for widespread use in therapy. Firstly, they are fast cleared from the body by the action of reticuloendothelial system (RES), kidney excretion or spleen/liver capture. One of the best approaches to overcome these inconveniences is the conjugation to polymers, an approach following described.

2.2 POLYMER THERAPEUTICS

The term "polymer therapeutics" (Duncan et al., 1996; Duncan 1997) describes the family of compounds of drug delivery systems that include a water-soluble polymer as a common central component (core component) and a drug as active ingredient. The term includes polymeric drugs, polymer-drug conjugates, polymer-protein conjugates, non-viral vectors and polymeric micelles that trap covalently bound drugs (Duncan 2006).

The compounds obtained with these technologies are "new chemical entities" or "pro-drugs", according to fact that the conjugate is already active or that the drug bound needs to be released in order to performed its action. The

pro-drug is an inactive compound that is able to release the active drug at the site of action where the drug release is triggered by specific conditions in the target site. The conjugation of drugs with polymers form the so-called "Polymeric pro-drugs" which are part of the category of "Polymeric Drug Delivery System (PDDs)" (Jayant and Tamara, 2006). The task of these systems is to improve drug distribution, bioavailability, targeting, PK and provide a controlled release of therapeutic agent over time.

2.2.1 Use of polymers for the controlled release of biological active molecules

The delivery of biologically active molecules, especially with various types of polymeric systems has several advantages:

- increased half life;
- less frequent administration;
- increased maximum tolerated dose;
- protection of the drug from inactivation (Hoes and Feijen, 1989; Pasut and Veronese, 2012).

Drug release from polymeric systems may be due principally to a physical or chemical mechanism:

- physically controlled-release: the drug is encapsulated in an insoluble polymer membrane or dispersed in a soluble polymer matrix. The drug release occurs by diffusion.

- chemically/enzymatically controlled release: the active molecule is covalently linked to the polymer by a spacer or a sensible linkage that allows the release of the drug only in a specific condition (for example acid pH) or thanks to a specific enzyme cleavage (Cavallaro et al., 2001).

The bioactive molecule field has had great developments in bioconjugation, which provides the formation of a covalent bond between a polymer and an active molecule, creating a new chemical entity that has typical characteristics (Cavallaro et al., 2001).

2.2.2 Polymeric conjugated

The use of a soluble macromolecular carrier for the delivery of bioactive molecules can improve the pharmacokinetic characteristics preserving activity and bioavailability. The only characteristics modified are the half-life, its distribution in various body compartments and its elimination.

The use of carrier systems based on synthetic water-soluble polymers, defined as polymeric drug carriers, was proposed for the first time in 1975 by Helmut Ringsdorf (Ringsdorf, 1975). He suggested to covalently link a drug to a water-soluble polymer, through a linker sensitive to enzymatic or hydrolytic cleavage at the target site (Figure 2.1).

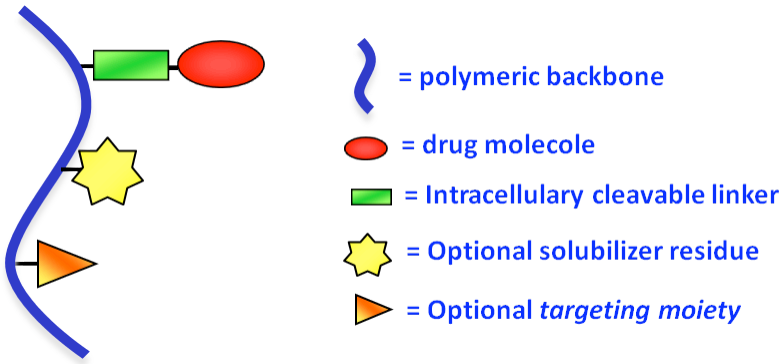


Figure 2.1: Model of polymeric drug carrier suggested by Ringsdorf.

The ideal conjugates should be composed as follow:

- an inert water-soluble polymeric carrier (polymer backbone);
- a linker between biodegradable polymer and active molecules (intracellulary cleavable linker) that is cleaved only at the site of action, releasing the active biomolecules;
- a targeting ligand that can be linked to the polymer structure, promoting the selective action of the drug towards the target cells while minimizing non-specific interactions;
- functional additive groups, which influence the solubility of the conjugate.

2.2.3 Characteristics of polymeric carrier

As mentioned earlier (§ 2.2.2) (Ringsdorf, 1975; Putnam and Kopecek, 1995), Ringsdorf polymeric carrier model must have certain characteristics. In this regards so far several polymers for drug delivery of natural and synthetic

origin have been studied. This resulted in the identification of requirements for an ideal carrier:

- the polymer must be biocompatible and not induce significant toxicity or immunogenicity;
- must be biodegradable by hydrolytic or enzymatic activity or have a molecular weight below the kidney excretion threshold;
- posses a low polydispersity;
- the polymer must be found in large-scale, low cost and made by simple economic processes;
- must be hydrophilic to ensure its solubility in body fluids and to increase the solubility of the bound drug;
- must possess the functional groups that allow binding of the drug and the residue targeting with simple chemical reactions, not involving toxicity or immunogenicity;
- the linkage between polymer and drug must be sufficiently stable in the bloodstream and easily hydrolysable in the target cells, in order to have a controlled release of the drug;
- the conjugate must present a sufficient carrying capacity, to ensure a proper amount of drug at the site of action;
- industrial production of the conjugate must be reproducible, economic and its analytical characterization must be complete and validated;
- the characteristics of the conjugate must be sufficient for an appropriate formulation with an high stability and easy administration.

2.2.4 Examples of polymer therapeutics

Different types of polymers are used as drug carriers in therapy, both of natural origin (dextran, dextrin, polysialic acid (PSA), hyaluronic acid (HA), human serum albumin, chitosan and DNA) and synthetic (N-(2-hydroxypropyl) methacrylamide copolymer (HPMA), poly-(styrene-co-maleic anhydride) (SMA), poly-(vinylpyrrolidone) (PVP), poly-(ethylene glycol) (PEG), poly-(N-acryloyl) morpholine (PACM), poly-(ethyleneimine) (PEI), poly-(divinyl ether-co-maleic anhydride) (DIVEMA)), hydroxy-ethyl-starch and polyoxazoline (Putnam and Kopecek, 1995; Maeda et al., 1992; Gregoriadis et al., 2005; Besheer et al., 2009; Ferguson et al., 2009; Mero et al., 2008, 2012; D'Este et al., 2010; Saravanakumar et al., 2010; Viegas et al., 2011).

Synthetic polymers are usually preferred to natural ones because they can be better tailored following specific characteristics, as controlled molecular weight, chemical composition, functionalization with active ingredients, targeting residues, biodegradable linkers and solubilizing groups. They are

usually less immunogenic than natural polymers especially because natural ones are usually extracted from bacteria or other animal species.

Polymers of natural origin, such as polysaccharides and poly-amino acids, are generally biodegradable, because they are hydrolyzed by catabolic mechanisms. The biodegradability is one of the main advantages of natural polymers, also present in some synthetic polymers, because it prevents any phenomenon of accumulation.

2.2.5 Bioactive molecules release

The bioactive molecules bound to macromolecular synthetic carrier can be release as following:

- passive hydrolysis
- pH-dependent hydrolysis
- enzymatic hydrolysis

The step of hydrolysis mainly involves those bonds that are sensitive to hydrolytic action, such as esters, and partially amides, carbonates and urethanes. The percentage of the released drug will depend on the stability of the bonds. This will be greater for the ester groups because they are very sensitive to hydrolysis, and decrease progressively with carbonates, urethanes and amides.

The acidic environment of lysosomes (pH of about 4.5-5.5) can be exploited to obtain pH dependent hydrolysis by linking of the bioactive molecules to the polymer through a linker sensitive to this environment (Shen and Ryser, 1981; Cavallaro et al., 2001). Enzymatic hydrolysis may occur in conjugates that present a polypeptide or another spacer, between the active molecule and carrier, sensitive to the action of one of the many enzymes present in the lysosomal level, such as phosphatases, esterases, glycosidases and peptidases (Soyez et al., 1996; Duncan et al., 1991). In the literature there are examples of conjugates designed to be sensitive to cathepsins, cysteine-dependent peptidase present in large quantities and with high activity at lysosomal level.

2.2.6 Problems and advantages of bioconjugation

Bioconjugation certainly represents an encouraging strategy for the administration of drugs that are characterized by low pharmacokinetic and pharmacodynamic profiles. Nevertheless, many issues must be resolved or refined to obtain an ideal bioconjugation, especially regarding the polymers:

- the chemical activation of the polymer used;
- a site specific conjugation, in order to prevent the inactivation or the activity reduction of the drug;
- development of analytical methods for a fast conjugate characterization (polymer and active residue);
- the achievement of low polydispersity polymers;
- the polymer approval for pharmaceutical use by the responsible authorities (FDA, EMEA).

Despite these limitations, bioconjugation improves the drug pharmacokinetic-pharmacodynamic properties, providing mainly the following positive effects:

- masking of antigenic/immunogenic sites of the drug, especially for protein;
- reduced renal excretion, due to the increased hydrodynamic volume;
- increased plasma half-life (thanks to the previous points);
- increased solubility of the drug in biological fluids;
- delivery of the drug to the target tissue (and accumulation) thanks to specific targeting residues;
- less frequent administration and lower doses than the free drug;
- increased patient compliance and consequent improvement in life quality.

2.3 HYALURONIC ACID (HA)

2.3.1 Chemical-physical characterization and functions

HA is a unique natural glycosaminoglycan, having a relatively simple linear structure of repeated non-sulfated disaccharide units composed of D-glucuronic acid and *N*-acetyl-D-glucosamine, joined together with alternating linkages, β-(1→4) and β-(1→3) (Almond, 2007) (Figure 2.2). In addition, intramolecular hydrogen bonds stabilize the structure.

It was initially obtained from rooster combs, while currently is mainly obtained by bacterial fermentation.

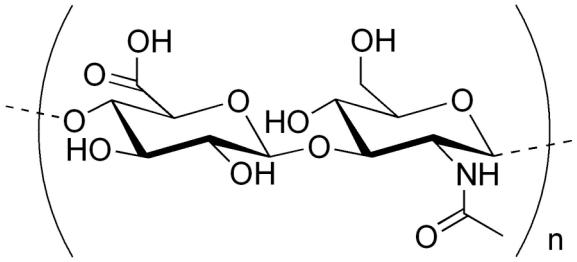


Figure 2.2: Chemical structure of hyaluronan.

At physiological pH, the carboxyl groups of the glucuronic acid residues are ionized, conferring to the polymer high solubility in water. In fact, it complexes many water molecules, strongly hydrating itself and forming solutions with high viscosity.

Hyaluronic acid is a biological molecule with very important roles in the body, essential for cells growth, mobility and differentiation. It is a major component of the connective tissue in which it maintains the degree of hydration, firmness, plasticity and viscosity of the amorphous matrix and gives the skin strength and shape retention. It is the main component of the vitreous humor and cartilages, where it has a key structural role in the organization of the cartilage extracellular matrix. The size of the molecule and its high hydration help the preservation of the shape of tissues. Furthermore, HA has other functions, like cementing and anti-shock substance, preventing the damage of cells after physical stress and lubricant in synovial fluid.

In addition, it plays as a physical filter to prevent the spread of bacteria, infectious agents, and viruses.

2.3.2 HA uses and applications

Hyaluronic acid is widely used in health care. The main uses are for surgical, ophthalmic and in the field of cosmetic dermatology (Balazs, 1983; Laurent and Frasent, 1992; Laurent, 1998; Ohri et al., 2004; Nakamura, 2006). Among the several therapeutic applications, the polymer is currently in clinical use in arthritis as a viscosupplement for the joints (Balazs end Denlinger, 1993). Furthermore, it is widely used in topical formulations for wound healing and in several injectable products for cosmetic applications (Muzzarelli et al., 2012; Palumbo et al., 2012; Laurent and Fraser, 1992; Balazs and Laurent, 1998). Consequently, its clinical safety is well established. It is also used to reduce pain and promote the treatment of inflammation and ulcers, like ulcers of the mouth and stomatitis, mainly caused by chemotherapy and radiotherapy.

In addition to these applications, the potentialities of HA have been investigated also in drug delivery as carrier of anti-tumoral and anti-inflammatory drugs (Banzato et al., 2008; Pitarresi et al., 2010; Saravanakumar et al., 2010; Homma et al., 2009). In the last two cases, the presence of several carboxylic groups is an advantage for the delivery of small drugs because it ensures a high loading. In contrast, as aforementioned, an approach of site selective conjugation is needed for protein delivery to avoid cross-linking.

Some other examples are hydrogels containing erythropoietin (Laurent, 1998), modified release formulations of human growth hormone (Motokawa, 2005), paclitaxel and doxorubicin conjugation (Luo et al., 2000; Luo et al., 2002). In the last years also our lab studied the potential value of this polymer in the conjugation of biologically active molecules (HAylation) (D'Este et al., 2010; Mero et al., 2013).

2.4 POLY(ETHYLENE GLYCOL) (PEG)

2.4.1 Chemical-physical characterization

Poly(ethylene glycol) (PEG) (Bailey and Koleske, 1976) is a synthetic amphiphilic polymer constituted by repeating oxyethylene units with molecular weight of 44 Da, and has the following structure:

$$HO[CH_2CH_2O]_nH \quad \text{or} \quad CH_3O[CH_2CH_2O]_nH$$

Figure 2.3: Structure of poly(ethylene glycol).

Its synthesis is schematically shown in Figure 2.4 and it involves three steps:

I. Initiation: it begins with the nucleophile attack of OH^- to ethylene oxide cyclic opening the epoxy ring and the formation of oxygen reactive alcohol;
II. Propagation: the reaction propagation continues with the addition of other ethylene oxide molecules;
III. Termination: the reaction is stopped by the action of a terminator.

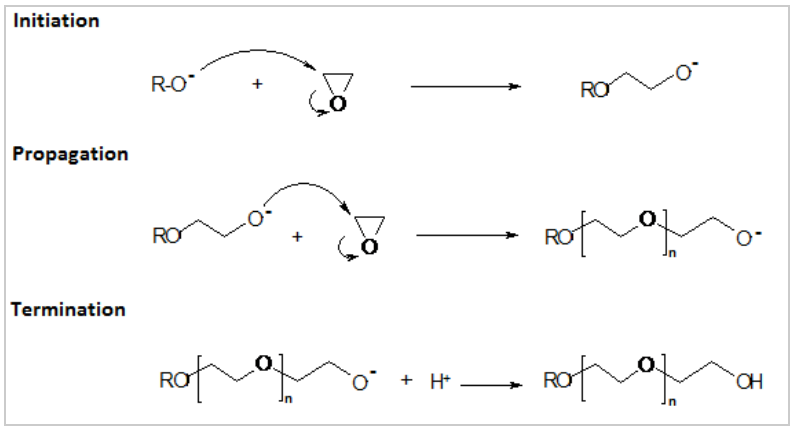


Figure 2.4: Synthesis of poly(ethylene glycol).

PEG is a polymer suitable for bioconjugation thanks to many characteristics: it is biocompatible, is not toxic and not immunogenic (Dreborg and Akerblom, 1990). It is also not charged and has only one or two functionals for drugs coupling. It is soluble in both aqueous and organic solutions (Powell, 1980; Mutter and Bayer, 1979). Being a non-biodegradable polymer, it must be used at molecular weights below 40 kDa to avoid body accumulation (Yamaoka et al., 1994). It is also readily commercially available and is not too expensive for a wide range of molecular weights and structures. All these features have allowed its widespread in the biomedical field, also thanks to its Food and Drug Administration (FDA) approval for human use.

Depending on the initiator used, different PEGylating agents can be synthesized. The mono-methoxy PEG (mPEG) mono-functional: is the leading polymer for protein conjugation.

2.4.2 PEG uses and applications

Polyethylene glycol is used in advanced pharmaceutical technology to conjugates principally proteins and chemical drugs. The bioconjugates present improved chemical and physical characteristics, such as:

- increased plasma half life;
- reduced renal excretion;
- reduced hydrolytic and enzymatic degradation;
- reduced uptake by the reticuloendothelial system (Zalipsky, 1995);
- increased solubility in water (Delgado et al., 1992);
- reduced immunogenicity and antigenicity (Dreborg and Akerblom 1990; Bhadra et al., 2002; Davis, 1992).

PEGylation is a technique that started in the seventies with particularly interest on proteins. It began from random amino PEGylations of model proteins (Abuchowski et al., 1977; Abuchowski et al., 1997) and presently accounts several PEGylated conjugates in clinical practice, some of which are blockbuster products (Maggon, 2007; Pasut and Veronese, 2007) (Table 2.1 and Table 2.2).

PEGylation technique has been widely used also for the delivery of anticancer drugs to specifically target the drug to the tumour tissue, preventing the distribution to other areas and avoiding side effects.

Examples of PEG conjugates with anticancer drugs include camptothecin, Ara-C, methotrexate and other taxanes. In all cases the aim is to target the antineoplastic agent in the tumour tissue, thus reducing systemic toxicity and also to increase the water solubility, improving overall the pharmacodynamic and pharmacokinetic profiles (Veronese et. al., 2004).

PEG is also used for the precipitation of proteins and nucleic acids, for the synthesis of peptides and oligonucleotides in liquid and solid phase and for the enzymatic catalysis in organic solvents. It is also used in cosmetic and pharma industry as excipient.

Recently, it is also widely used by pharmaceutical industry for the preparation of drug delivery systems (DDSs) such as liposomes, nanoparticles, nano-and microspheres, dendrimers and hydrogels (Bhadra et al., 2002).

2.5 PROTEIN THERAPEUTICS

2.5.1 Protein drugs

Since the advent of recombinant DNA technology, it was possible to produce proteins easily and in large amount.

In nature, proteins have the most dynamic and diverse roles compared to any other macromolecules. In the body, they catalyse biochemical reactions,

transduce signals (receptors), provide intracellular and extracellular scaffolding support, and transport molecules within a cell or from one organ to another.

They are estimated to be tens of thousands, each one with possibly different role. Mutation or disregulation of the translation can directly cause a disease. So, a disease caused by the lack of a protein can be treated with the administration of the protein itself. Viewed from the perspective of therapeutics, in fact, these represent a tremendous opportunity.

Presently, the Food and Drug Administration (FDA) has approved more than 130 different proteins or peptides for clinical use, and many more are in development (Leader et al., 2008).

Currently, protein drugs are very diversified in terms of production, function and origins: some are recombinant human proteins (for instance, insulin, growth hormone and erythropoietin), others are monoclonal antibodies (for instance, Remicade® (infliximab), Rituxan® (rituximab) and Erbitux® (cetuximab)) and still others are viral or bacterial proteins used as vaccines to elicit a specific immune response.

Nature did not evolve proteins for manufacture them *ex vivo*. For this reason, many human proteins produced in recombinant form are difficult to manufacture and some cannot be expressed at all in microbial cell culture.

Furthermore, in human body the serum half-life and tissue distribution of endogenously expressed proteins is carefully controlled *in vivo* to optimize their biological activity. Recombinant proteins therefore tend to be rapidly cleared and thus require frequent injections (thus, the growing interest in extending the serum half-life by, for example, polyethylene glycol conjugation or DDSs).

2.5.2 Protein conjugation

For the conjugation of proteins, monofunctional or polyfunctional polymers have been used (i.e. having one or more reactive functions).

Especially in the past, dextran has been wudely used, because it's a polyfunctional polymer, uncharged and water-soluble. Many proteins were conjugated to dextran, with huge interest: streptokinase, plasmin, asparaginase and carboxypeptidase G. Despite the results obtained with these conjugates, dextran did not have great success in the derivatization of proteins, because, being a multifunctional product, it can give rise to a heterogeneous mixture of products and cross-linking problems.

Another polyfunctional polymer is the copolymer styrene-maleic anhydride (SMA), used in the conjugation of neocarcinostatina and superoxide dismutase. Homologous polymer is made up of divinyl ether and maleic anhydride (DIVEMA).

The polyfunctional polymers possess advantages in the enzymatic biocatalysis in organic solvent. In this case, the complex pattern that is formed by the coupling reaction between polymer and enzymes confer upon them a greater stability towards denaturation.

The cross-linking issue has been overcome by using polymers with a single reactive group. For this purpose, one of the more interesting has been polyethylene glycol (PEG), which, thanks to its monofunctional group prevents the formation of cross-links. Thanks to its characteristics and the possibility of obtaining homogeneous protein conjugates, the PEG is the most widely used polymer in bioconjugation (Veronese and Morpurgo, 1999). Up today 9 proteins and a peptide derivatives are commercially available (Table 2.1).

Table 2.1: PEGylated proteins on the market.

Trade name	Conjugate name	Active substance	Indication	Approval year
Omontys®	PEGinesatide	Functional analogue of EPO	Anaemia due to chronic kidney disease (CKD) in adult patients on dialysis	*2012*
Pegloticase®	Krystexxa	Uricase	Refractory chronic gout	*2010*
Cimzia®	Certolizumab pegol	Anti-TNF Fab'	Crohn's disease and rheumatoid arthritis	*2008*
Mircera®	PEG-EPO	EPO	Anaemia, causing symptoms in adults with chronic kidney disease	*2007 (Europe)*
Somavert®	Pegvisomant	hGH antagonist	Acromegaly	*2002*
Neulasta®	Pegfilgrastim	GCSF	Neutropenia	*2002*
PEGASYS®	Peginterferon-α2a	Interferon-α2a	Chronic hepatitis C, hepatitis B	*2001*
PegIntron®	Peginterferon-α2b	Interferon-α2b	Chronic hepatitis C	*2000*
Oncaspar®	Pegaspargase	Asparaginase	Leukemia	*1994*
Adagen®	Pegademase	Adenosine Deaminase (bovine)	Severe combined immunodeficiency disease (SCID) with a deficiency of adenosine deaminase.	*1990*

2.6 OLIGONUCLEOTIDE THERAPEUTICS

2.6.1 Oligonucleotides as drugs

Whole genes or short sequences of oligonucleotides have acquired relevance in the pharmaceutical field, mostly because of their selectivity of action and because in some cases they are not only a treatment but a definitive cure. They are generally chemically synthesized, especially relatively short sequences, but it is also possible to extract an entire genome from cells.

Genes are predominantly used for the treatment of genetic diseases, replacing the "wrong sequence" with the "right" and functional one. Short oligonucleotides are mostly involved in tumor or monogenic disease treatment. Substantially, they act inactivating or inhibiting a protein translation and production, usefull when the protein plays a key role in the pathological condition.

According to these actions, single stranded oligonucleotides could be principally subdivided in 6 different strategies, explained below.

Antisense technology

Antisense refers to opposing the normal order ("sense") of the code in DNA. Treatment consists in the administration of an RNA sequence, complementary to the sequence of mRNA coding for a protein. The formation of the double-stranded RNA will prevent the translation of the protein by the ribosome. Furthermore the double-strand will be recognized by the enzyme RNase H that degrades it (Figure 2.5).

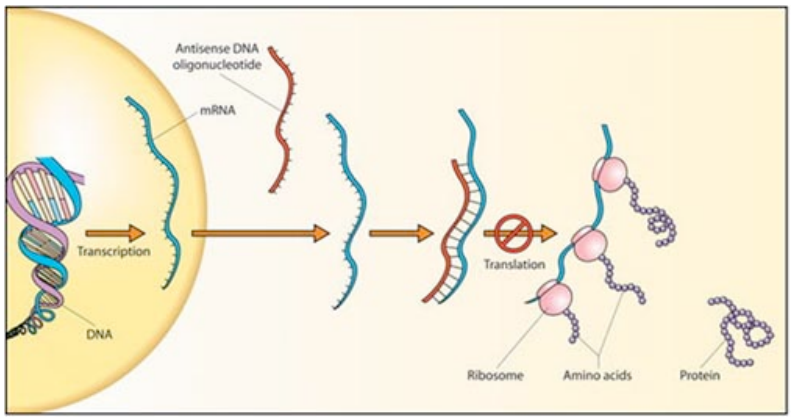


Figure 2.5: Antisense technology principle.

This strategy can be used to block expression of a gene that could be deleterious to the organism because overexpressed or malfunctioning.

It is possible to treat, for example, the overexpression of oncogenes, of cytokines that lead to an inflammatory state and angiotensinogen. OBLIMERSEN® recognizes mRNA coding for bcl-2 (antineoplastic overexpressed protooncogene in numerous types of cancer), and stimulates RNase-H which degrades the RNA restoring the balance between the protein pro- and anti-apoptotic (Cheson, 2007).

To enhance the delivery of RNA, liposomes and polymeric carriers have been exploited (Merdan et al., 2002; Ruponen et al., 2003).

Triple-helixes

Some nucleotides are able to complex with DNA in triple-helix forming regions that block the transcription and the translation that lead to the protein production; RNA polymerase is not able anymore to bind the DNA and transcribed it in mRNA. In this case the target is the gene.

Aptamers

It has been found that some nucleotide sequences (single or double strand) have the capacity to interact with high affinity to specific proteins, blocking their activity. They were called aptamers (from the Latin *aptus* - fit, and Greek *meros* - region). The best-known example is pegaptanib (Macugen®), product currently in the market (Table 3.2), used for the age-related macular degeneration of the retina. It is an oligonucleotide that binds Vascular Endothelial Growth Factor (VEGF). To improve the pharmacokinetic profile changes have been made in position 2' and 3' sugar. Moreover, in 5' has been linked by a chain of 40 kDa PEG (§ 2.6.2, Figure 2.6).

Ribozyme

Ribozymes are RNA sequences with catalytic activity able to cleave covalent bonds. They can be exploited to degrade the mRNA coding for defective or overexpressed proteins.

siRNA

Small interfering RNA (siRNA) is also known as short interfering RNA or silencing RNA. It represents a class of double-stranded RNA molecules, usually 20-25 base pairs in length. siRNA plays many roles, but its most notable is in the RNA interference (RNAi) pathway, where it inhibits the

expression of specific genes with complementary nucleotide sequence. siRNA also acts in RNAi-related pathways, for example, as an antiviral mechanism or in shaping the chromatin structure of a genome. The complexity of these pathways is only now being elucidated.

This mechanism was first discovered in plant, with an important role in post-transcriptional gene silencing (PTGS) (Hamilton and Baulcombe, 1999). Only at the beginning of this century it was proved that siRNAs could induce RNAi in mammalian cells (Elbashir et al., 2001). This discovery led to a great interest in harnessing RNAi for biomedical research and drug development.

DECOY

The RNA decoy (commonly known linc-MD1) acts as a protector of the mRNA. The decoy RNA protects mRNA from molecules that would stop its functions.

RNA decoys are exogenous molecules that have a sequence matching the natural RNA target of an RNA binding protein. Usually the RNA binding protein binds to its natural target, but when the RNA decoy is introduced into the cell then it binds some of the RNA binding protein. If enough of the decoy is in the cell, the RNA binding protein is mostly attached to the decoy and some of its natural RNA target is left unbound. This essentially "flips a switch" (makes a radical change) in the cell, as the process affected by the RNA binding protein-RNA interaction goes from its bound state to its unbound state.

The use of DNA or RNA as therapeutic agents, however, shows not negligible troubles, for example:

- the presence of enzymes with nuclease activity (non-specific, exo- and endonuclease), so the nucleotide sequence is degraded;
- being polyanions, they can not pass through the membranes because of the negative charges and the non-internalisation into the cell prevents its translocation to the nucleus;
- difficulty in targeting the nucleus.

Therefore, it was tried to implement changes in order to limit these disadvantages. Initial steps were done increasing the lipophilicity and resistance to nucleases. Studies on carriers have been suggested as strategies for the transport of these therapeutics to the nucleus. These systems were able to protect and improve the transport to the site of action (Merdan et al., 2002; Ruponen et al., 2003).

2.6.2 Oligonucleotide-polymer conjugation

Oligonucleotides are relatively new molecules in the pharmaceutical field. As mentioned in the previous paragraph, interest in them is growing up in the last decade.

Similar to proteins, they are very sensitive substance and only small changes on natural environment can destroy or inactivate them. For example, especially for RNA handling, sterilized material is needed; otherwise RNase enzymes could easily digest all material.

Conjugation to polymers also in this case can bring the advantages already mentioned for the therapeutic use of proteins, such as clearance prolongation and reduced enzyme degradation.

Studies have been conducted for example for LNA (Locked Nucleic Acid) antisense oligo. After PEGylation, hybridization is still optimal, circulation time and stability are prolonged. Thanks to these improvements lower doses are needed respect to the unmodified LNA for the same tumor growth inhibition (Bandaru et al., Enzon Pharmaceuticals, inc).

Other examples are present in literature, especially by Zhao, most of which declaring the effectiveness of PEGylation in oligos stability enhancement (Zhao et al., 2005; Zhao et al., 2007).

However, the best-known oligo is Macugen®, a PEG conjugated-oligonuclotide. The active molecule is Pegaptanib and is marketed by Pfizer (FDA N021756, 2004; EMA/671614/2010; EMEA/H/C/000620) (Table 2.2).

Table 2.2: PEGylated oligo on the market.

Trade name	Conjugate name	Active substance	Indication	Approval year
Macugen®	Pegaptanib	anti-VEGF aptamer	Neovascular (wet) age-related macular degeneration (AMD)	*2004*

Pegaptanib (Figure 2.6) is a PEGylated modified oligonucleotide that binds with high specificity and affinity to extracellular Vascular Endothelial Growth Factor ($VEGF_{165}$) inhibiting its activity (Ng et al., 2006; EMEA WC500026218).

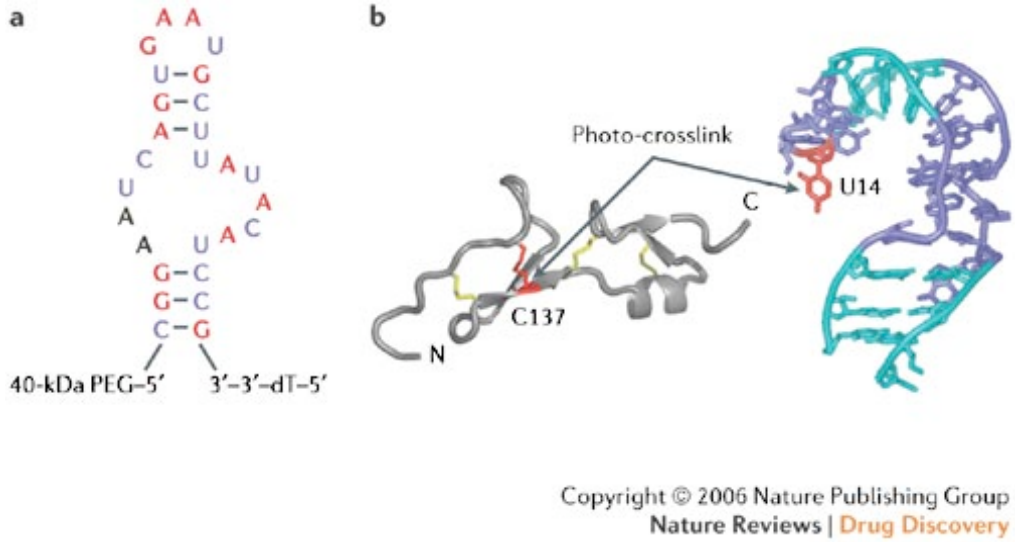


Figure 2.6: Pegaptanib structure and target binding. a) Sequence and predicted secondary structure of pegaptanib. 2'-O-methylated purines are shown in red, 2'-fluorine-modified pyrimidines are shown in blue and unmodified ribonucleotides are shown in black. The site of attachment of a 40-kDa polyethylene glycol moiety is shown. **b)** Interaction between the 55-amino-acid heparin-binding domain of vascular endothelial growth factor $(VEGF)_{165}$ and pegaptanib (Ng et al., 2006).

3 MATERIALS AND METHODS

3.1 MATERIALS

Hyaluronic acid sodium salts (HA, 200 kDa) were from Fidia Farmaceutici s.p.a. (Abano Terme, Italy). Ribonuclease A, trypsin, bovine insulin, casein and testicular hyaluronidase, methanesulfonic acid (CH_3SO_3H), 1,1'-carbonyldiimidazole (CDI), 4-aminobutyraldehyde diethyl acetal, sodium cyanoborohydride ($NaBH_3CN$), cytidine 2';3'-cyclic monophosphate monosodium salt (CCM), N_α-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME), triethylamine, ethanolamine, trifluoroacetic acid, copper (II) sulfate pentahydrate 4% (w/v), bicinchonicic acid solution, streptozotocin, D_2O, acetonitrile, ethanol, acetone, glacial acetic acid, hydrochloridric acid (HCl), dimethylsulfoxide (DMSO), adenosine triphosphate (ATP), ethanol (EtOH), triethylammonium acetate buffer (TEAA), triethylaamine (TEA) and salts of analytical grade were from Sigma-Aldrich Co (Taufkirchen, Germany).

All oligonucleotides were synthesized by Metabion International AG (Martinsried, Germany) and Diatech Pharmacogenetics (Jesi, Italy) and stored at -20°C in 10 mM Tris-HCl, 1 mM EDTA pH 8.0 (TE). The sequences and modifications are: **a**, 5'-Thio-ACT ATT CCC GGG TAA TGA-3'; $\mathbf{a_F}$, 5'-FAM- ACT ATT CCC GGG TAA TGA -3'; **b**, 5'-Thio-GGG TAA TGA-3'; **a'**, 5'-AAT TCA TTA CCC GGG AAT AGT-3'; **b'**, 5'-AAT TCA TTA CCC-3'; **m**, 5'-Pho-ATT CCA AAG CTT CCC CCT A-3'; **m'**, 5'-FAM-T AGG GGG AAG CTT TGG-3' (see Table I.I). PEG Maleimide 20 kDa ($mPEG_{20kDa}$-Mal) and ortho-pyridine disulfide PEG 20 kDa ($mPEG_{20kDa}$-OPSS) were from Shearwater Polymers, Inc. (Dallas, Texas, USA). Acrylamide:Bis-acrylamide 19:1 40% solution was from Merk (Darmstadt, Germany). T4 Polynucleotide Kinase (T4 PNK) and Reaction Buffer A were from Fermentas by Thermo Scientific (Waltham, MA, USA), T4 DNA Ligase, EcoRI 10U/mL, REact® 3 and Sybr Green II from Invitrogen (Life Technologies Ltd, Paisley, UK).

3.2 ANALITICAL METHODS

Spectrophotometer analysis of volumes larger than 0.5 mL was performed with a Lambda 25 UV-Vis Perkin-Elmer instrument (Northwolk, CT, USA) and for less than 5 μL with a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). ^{1}H-NMR spectroscopy was performed with Bruker 300 MHz spectrometer and NMR data were processed using the program Topspin (Bruker GmbH, Karlsruhe, Germany). Shimadzu analytical HPLC system was used with Zorbax GF-250 (Agilent Technologies, Palo Alto,

CA; 4.6 × 250 mm or 9.4 × 250 mm 4 μm) size exclusion chromatography (SEC) columns for analytical characterization and purification of HA-conjugates or with C18 Jupiter (Phenomenex, Torrance, CA, USA; 5 mm, 300 Å, 250 x 4.60 mm) reverse phase column in association with a TC-50 temperature controller (Eppendorf, Hamburg, Germany) for characterization and purification of PEG-oligo conjugates. Centrifugation was carried out with Hettich Zentrifugen mod. MIKRO 200 for small volume and Centrikon T-42K, Kontron Company, for large volume solution. The freeze-drying was performed with freeze-Hetossic Heto Lab Equipment. The dialysis Amicon® Ultra-15 membranes have been provided by Millipore (Billerica, MA, U.S.A.) and the dialysis tubing by Del Chimica Scientific Glassware (Naples, Italy). DNA acrylamide gels electrophoresis were run with a mini VE vertical electrophoresis system (10 x 10.5) by Amersham Biosciences (part of GE Healthcare, Glattbrugg, Switzerland). Imaging analysis were performed thanks to a Geliance 600 Imaging System in association with GeneSnap software (PerkinElmer, Waltham, Massachusetts, USA). Concentration of small volumes was performed with a Savant Speed Vac Concentrator (Thermo Scientific, Waltham, MA, USA). Aqueous and organic solutions were evaporated with Rotavapor mod. R II BÜCHI (Switzerland). Glucose level determination was tested by OneTouch® II assay kit (LifeScan Inc, Johnson & Johnson, Milpitas, U.S.A.).

3.3 SYNTHESIS AND CARACTHERIZATION OF HA-PROTEINS

In order to conjugate the polymer to NH_2-protein groups, the carboxilic residues of the glucuronic acid units were partially derivatizated with an acetal ending spacer (4-aminobutyraldehyde diethyl acetal). It will be then hydrolyzed into an aldehyde group to couples to proteins forming a reversible Shiff base. A secondary amine linkage will be formed after treating with a strong reducing agent ($NaBH_3CN$) (see Figure 3.1).

This method of conjugation also allowed modulating the polymer coupling in a random or selective way. In mild basic condition, the NH_2 group of lysines ($pK_a \geq 9.3$) on the protein surface are prevalently in the deprotonated form, so available for coupling (random approach). Instead, in slightly acid condition, only the *N*-terminal proteins residual are predominantly in deprotonated state (thanks to a lower pK_a, 7.6-8) generating a selective conjugation.

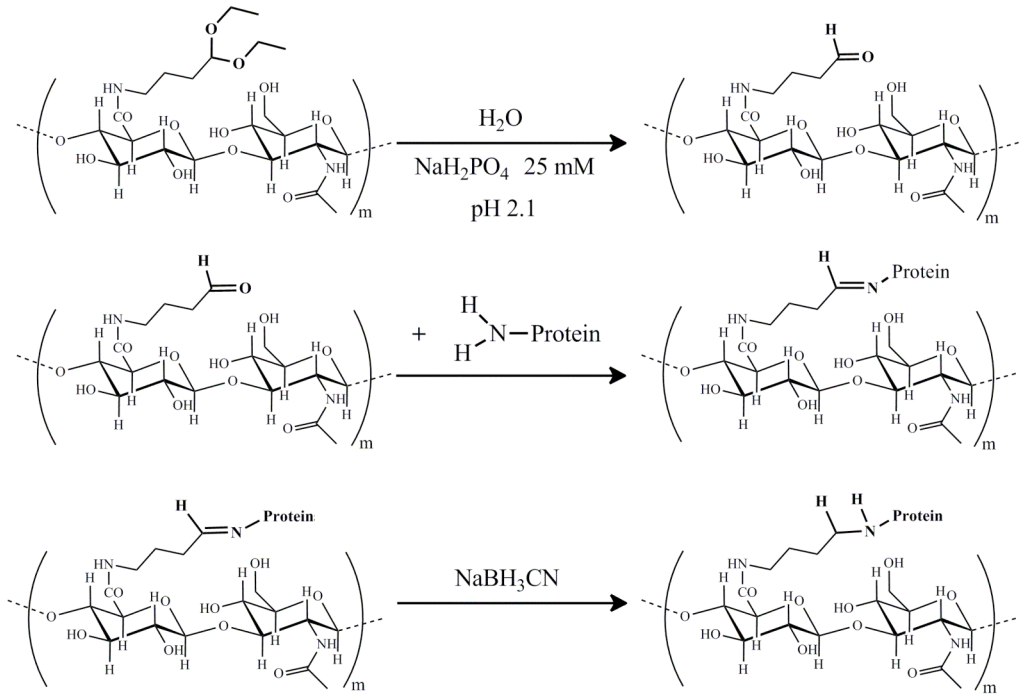


Figure 3.1: General scheme of the bioconjugation strategy of proteins.

3.3.1 Synthesis and purification of HA-acetal

HA sodium salt (100 mg, 0.25 mmol) was dissolved in 10 mL of DMSO with CH_3SO_3H (81 µL, 1.25 mmol). After complete dissolution, about 1-2 h, 1,1'-carbonyldiimidazole (203 mg, 1.25 mmol) was added, followed after 1 h by the addition of 4-aminobutyraldehyde diethyl acetal (0.25 mmol) (Figure 3.2). Triethylamine was used to raise the pH to 8.0. The reaction mixture was stirred at 40°C for 12 h and then 1 mL of NaCl saturated solution was added drop-wise, till the organic solution became opalescent. After 30 min of mixing, the polymer was precipitated slowly by adding 20 mL of chilly ethanol. The precipitate was kept at –20°C for 1 hour for a better sedimentation and than washed with ethanol/H_2O solutions at an increasing percentage of ethanol (70:30, 80:20, 90:10, v/v) to remove the excess of water soluble salts and reagents (filtering the powder after each step). A better purification of the product from unreacted amine was obtained dissolving the dried polymer in water and extensively dialysing against demineralized water before lyophilization. The derivatization degree was determined by ^{1}H-NMR, solubilizing about 4 mg of the powder in 0.75 mL D_2O.

HA-acetal signal in D_2O was observed at 1.1 ppm (*t*, -$(OCH_2$-**C<u>H</u>$_3)_2$**, acetal moiety), 1.85 ppm (*s*, 3H, -NHCO-**C<u>H</u>$_3$**, HA) and 3.2-3.8 ppm (*m*, GlcA, GlcNAc methylene H, methane-H, HA).

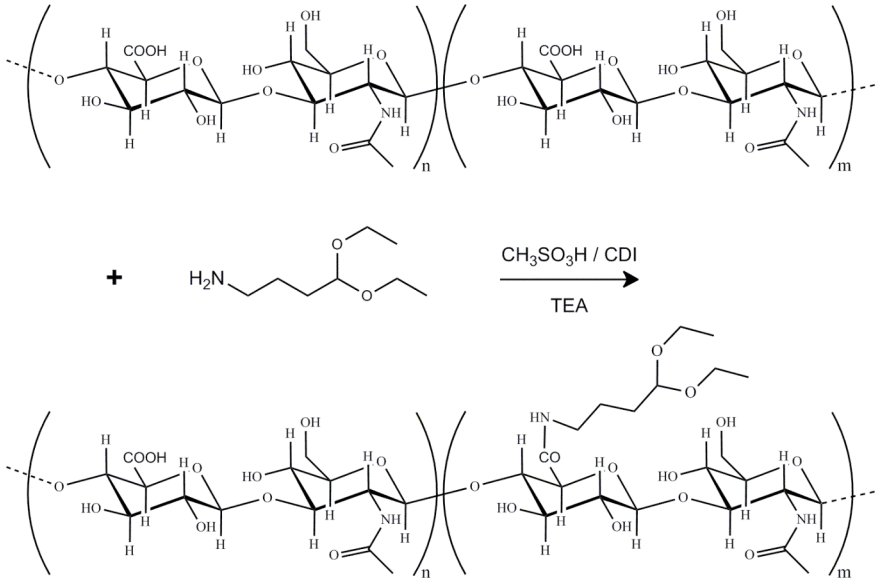


Figure 3.2: Scheme of HA-acetal synthesis. Reaction was conducted in DMSO.

3.3.2 Synthesis of random HA-RNase A and HA-trypsin (rHA-RNase A and rHA-trypsin) conjugates

According to the method of bioconjugation to NH_2 proteins' residues, we initially synthesized some protein models HA-conjugated. We choose two enzymes, RNase A and trypsin, because of the easy way to investigate the residual activity of the polymer-linked protein. First of all we obtained the random conjugates.

RNase A and trypsin conjugations were performed using the same procedure, as an example the reaction of rHA-RNase A is here reported. HA-acetal (11 mg, 1.09 μmol; degree of acetal modification 4% mol) was dissolved in 3 mL of 25 mM H_3PO_4 pH 2.1 and stirred for 1 h at 60°C. After cooling to room temperature, the pH value of the solution was raised to 8.0 with 0.1 M NaOH. RNase A (3 mg, 0.22 μmol), previously dissolved in 1 mL of 0.1 M Na_2HPO_4 buffer pH 8.0, was added. The reaction mixture was diluted to a final protein concentration of 0.5 mg/mL with 0.1 M Na_2HPO_4 buffer pH 8.0. After 1 h, $NaBH_3CN$ (0.69 mg, 10.95 μmol), dissolved in 10 μL of 0.1 M Na_2HPO_4 buffer pH 8.0, was added and the reaction was stirred at room temperature for 48 h. To saturate the still free and reactive aldehydic gruops, 5 equivalent of glycine, with respect to each equivalent of aldehyde in the starting HA, were added to the reaction mixture.

The reactions were analyzed by SEC with an analytical Zorbax GF-250 column (250 × 4.6 mm; 5 μm) eluted with 20 mM Na_2HPO_4, 130 mM NaCl pH 7.0, at a flow rate of 0.3 mL/min. The UV-Vis detector was settled at 280 nm. The purification was carried out with a semi-preparative GF-250 (250 × 9.4

mm) eluted with the same buffer used above at the flow rate of 1 mL/min. The purified conjugate was concentrated to 10 mL and the purity was confirmed by analytical SEC. The products were extensively dialyzed against demineralized water and lyophilized. The total protein content was determined by the average of the results obtained by bicinchonicic acid solution colorimetric assay (see section 3.3.5) (Smith et al., 1985) and $Abs_{0.1\%}$ at 280 nm.

rHA-trypsin reaction mixture was prepared by using 10.72 mg (1.07 μmol) of HA-acetal, 5 mg of trypsin (0.21 μmol) and 0.67 mg of $NaBH_3CN$ (10.73 μmol). The reaction was carried out at 4°C to prevent the autolysis of the enzyme.

3.3.3 Synthesis of *N*-terminal HA-RNase A and HA-trypsin (HA-N^{ter}-RNase A and HA-N^{ter}-trypsin) conjugates

In order to selectively conjugate HA-acetal to the model enzymes at the *N*-terminal amino group, the conjugation was carried out as above reported except for the pH of the solution of the coupling reaction mixture that was 6.0 instead of 8.0. The reaction was then stopped and purified as reported above.

3.3.4 Synthesis of *N*-terminal HA-insulin conjugates with different grade of loading: HA-N^{ter}-INS 1 and HA-N^{ter}-INS 2

After the results obtained by the HA-enzymes, only the selective bioconjugation was following used. Bovine insulin was chosen for coupling to HA as an example of protein of strong pharmaceutical interest. This time the focus of the observation was the protein loading content, so two conjugates were prepared.

Insulin (INS) was coupled to two HA-acetal derivatives with different degrees of activation, 4% and 21% mol, this yielding two conjugates: HA-N^{ter}-INS 1 and HA-N^{ter}-INS 2, respectively.

For the preparation of HA-N^{ter}-INS 1, after the hydrolysis of HA-acetal (8.81 mg, 0.87 μmol; degree of acetal modification 4% mol) to aldehyde as above reported, the pH value of the solution was raised to 6.0 and bovine INS (1 mg, 0.17 μmol), dissolved in 0.35 mL of DMSO, was added. After 1 h, $NaBH_3CN$ (0.11 mg, 174 μmol) was added.

For the preparation of HA-N^{ter}-INS 2, 1.66 mg of HA-acetal (0.87 μmol; degree of acetal modification 21% mol), 1 mg of bovine INS (0.17 μmol) and 0.11 mg of $NaBH_3CN$ (174 μmol) were used. Purification and protein content were carried out as reported for the preparation of HA-enzymes.

3.3.5 Protein concentration quantification with BCA colorimetric method

The amount of each protein conjugate is determined by reading the absorbance of the samples at the wavelength of 562 nm, using the method of the BCA assay (bicinchoninic acid). It is measured by the colorimetric reaction due to the reduction of Cu^{2+} to Cu^{1+} in an alkaline medium (the biuret reaction), by nitrogen peptide of the protein. Two molecules of BCA chelate a cation cuprous turning the solution color from blue-green to purple. The intensity of staining is almost linear with respect to protein concentration in a range of 20-2000 μg/mL.

This method was applied because the presence of the polymer should interfere in the determination of absorbance at 280 nm, usually used to calculate the protein concentration.

Samples and protein standards (0.2-1 mg/mL) were prepared with a final volume of 25 μL. The colorimetric reaction starts after adding to each sample twenty volumes (equal to 0.5 mL) of Working Reagent (50 parts of "reagent A" and 1 part of "reagent B", respectively containing bicinchonicic acid solution and copper (II) sulfate pentahydrate 4% (w/v)). After an incubation of 30 min at 37°C the absorbance at 562 nm is evaluated.

Samples concentrations are obtained from the calibration curve build with the standards absorbance values (Abs).

3.3.6 Determination of enzymatic activity

HA-enzymes conjugates were characterized on the residual activity respect the free protein. Specific substrate were chose: synthetic or protein origin and low or high molecular weight (LMWS or HMWS). Two substrates were used for the evaluation of trypsin activity: i) casein, a high molecular weight substrate for the determination of proteolytic activity and ii) N_{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME·HCl), a low molecular weight substrate for the determination of esterase activity.

3.3.6.1 Enzymatic assay for RNase A: hydrolysis of cytidine 2';3'-cyclic monophosphate (LMWS)

Free or HA conjugated RNase A samples (100 μL at 0.1 mg/mL protein concentration) were added to 1 mL of 0.15 mg/mL of CCM solution (0.46 mM) dissolved in 0.1 M Tris/acetate buffer pH 7.0. The hydrolysis of the substrate was evaluated by measuring the increase of absorbance at 247 nm for

5 min. The enzymatic activity was obtained by software elaboration end the final result was an averaged between three experiments.

3.3.6.2 Enzymatic assay for trypsin: proteolysis of casein (HMWS)

The hydrolysis rate of casein (MW 26000 Da) was evaluated by determining the absorbance of the digested peptides in solution after acid protein precipitation, according to Zwilling and Neurath (1981).

Briefly, in 2 mL test tubes, 500 µL of 0.1 M Tris/HCl pH 8.0 and 400 µL of 1% casein solution (dissolved in the same buffer) were added. To this solution 100 µL of free or HA conjugated trypsin samples (40 to 100 µg/mL protein concentration) were added. After 15 min at 30°C, 500 µL of a 5% trichloroacetic acid solution were added. The precipitate was pelleted by centrifugation at 5000 × *g* for 5 min. The absorbance of the supernatant was determined at 280 nm. The enzymatic activity was averaged between three experiments.

3.3.6.4 Enzymatic assay for trypsin: esterase hydrolysis of Nα-p-tosyl-L-arginine methyl ester (LMWS)

N_{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME·HCl), was used as low molecular weight substrate for the determination of esterase activity of trypsin. In this case, the method proposed by Hummel (1959) was used.

Breafly, in 2 mL test tubes, 500 µL of 40 mM Tris, 10 mM $CaCl_2$, pH 8.1 and 500 µL of 1.04 mM TAME·HCl solution, in the same buffer, were added. The hydrolysis of the substrate was evaluated by monitoring the absorbance at 247 nm over 5 min after the addition of 50 µL of free or HA conjugated trypsin samples (0.1 mg/mL protein concentration). The enzymatic activity was obtained by software elaboration end the final result was an averaged between three experiments.

3.3.7 Thermal stability of HA-conjugates

For the determination of thermal stability, RNase A, trypsin, HA-N^{ter}-RNase A and HA-N^{ter}-trypsin were separately incubated for 24 h at 37°C in PBS solution. At time 0, 6 h and 24 h of incubation, the necessary volumes of each solution were withdrawn to measure the residual enzymatic activity as above reported.

3.3.8 Digestion of HA-N^{ter}-RNase A and HA-N^{ter}-trypsin conjugates by hyaluronidase

Hyaluronidase from bovine testes was added to a solution of HA-protein conjugate at 0.1 mg/mL (protein concentration) in 0.1 M Na_2HPO_4 buffer pH 7 at the enzyme/HA molar ratio of 1/25 (w/w). The solution was incubated for 24 h at 37°C. At predetermined time points, a sample of 20 µL was analyzed by SEC under the same conditions reported above.

3.3.9 Erythrocyte compatibility study

One milliliter of freshly collected heparinized rat blood was centrifuged at 4000 × *g* for 10 min. The precipitated erythrocytes were washed three times with PBS buffer. The red blood cells (RBCs) were resuspended in PBS buffer and diluted to have an optical density in the 0.6–0.7 range at 670 nm. This stock erythrocyte suspension was freshly prepared and used within 48 h. For the compatibility experiment, 300 µL of the stock suspension were added to 300 µL of PBS containing HA-N^{ter}-INS 1 or HA-N^{ter}-INS 2 at protein concentrations ranging from 0.5 to 3 mg/mL. The samples were incubated at 37°C for 60 min under constant shaking. After centrifugation at 4000 × *g* for 10 min, the released hemoglobin was determined by measuring the absorbance at 414 nm. The experiment was performed also with HA sodium salts, using a polymer concentration range comparable to the HA content in the experiment with HA-N^{ter}-INS conjugates. Complete hemolysis, used as a positive control, was achieved by using a 1% v/v solution of Triton X-100 in water, a surfactant known to lyse RBCs. The RBC-PBS solution was used as the negative control. The percentage RBC lyses was calculated according the following formula:

$$\% \text{ lysis} = [(A_{sample} - A_{blank}) / (A_{100\%lysis} - A_{blank})] \times 100$$

where A_{sample} is the absorbance value of the hemoglobin released from the RBCs treated with the polymer or the conjugate solution; A_{blank} is the absorbance value of the hemoglobin released from the RBCs treated with PBS buffer; and $A_{100\%lysis}$ is the absorbance value of the hemoglobin released from RBCs treated with 1% Triton X-100.

3.3.10 Ethics statement

All animal procedures were approved by the Ethic Committee of the University of Padua and the Italian Health Ministry, and all animals received

care according to the DLGS 116/92 and in compliance with the "Guide for the Care and Use of Laboratory Animals".

3.3.11 Hypoglycemic potency of HA-N^{ter}-INS 1 and HA-N^{ter}-INS 2 in rats

Type I diabetes was induced in rats with streptozotocin (Junod et al., 1967; Akbarzadeh et al., 2007). Fifteen adult *Sprague-Dawley* male rats (250-300 grams, 75-90 days old) received a dose of 60 mg/kg of streptozotocin in 10 mM citrate, 0.15 M NaCl, pH 4.5 by intraperitoneal injection (i.p.). Diabetes was induced in 12 animals within 3 days, owing to beta cells destruction.

Animals were randomly divided in four groups and injected subcutaneously with insulin, HA-N^{ter}-INS 1, HA-N^{ter}-INS 2 (0.135 units per rats) or PBS. At predetermined interval time points glucose levels (mg/dL) in blood samples were measured using a OneTouch® II assay kit.

3.4 PREPARATION OF PEGYLATED OLIGOS BY ENZYMATIC PEGYLATION

In this section it was investigated the capacity of a DNA specific enzyme to act on a sequence when is linked to a polymer chain, especially for DNA ligase and exploit this method as a new enzymatic mediated PEGylation for oligomers.

3.4.1 Phosphorilation of a'

Chemically synthesized nucleic acids, if not opportunely modified, are not phosphorylated at their ends. Phosphorylation of 5'-ends is strictly required for enzymatic ligation of nucleic acids, as in our case ligases need a 5'-phosphate and a 3'-OH to link two oligonucleotides to form the phosphodiester bond.

3.4.1.1 5'-OH a' phosphorilation

The **m** oligonucleotide, was already 5'-P modified (see Table I.I), whereas **a'** was enzymatically phosphorylated using T4 PNK that catalyzes the transfer of the γ-phosphate from ATP to the 5'-OH group of oligonucleotides (forward reaction).

20U of T4 PNK were added to **a'** (1 nmol) in the presence of Reaction Buffer A (50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, pH 7.6) and ATP (10 nmol), in a total volume of 50 μL, at 37°C for 1 hour. Posphorilated **a'** was than recovered by precipitation.

3.4.1.2 Oligos precipitation

To obtain clean DNA after enzymatic reactions and reduction, unwanted protein residues and buffers were removed by precipitation.

Briefly, DNA solutions were added of 1/10 of the volume of 3 M sodium acetate pH 5.2 and of 3 volumes of cold 100% EtOH. The solution was then placed at –80°C for 15 min and centrifuged at 14000 rpm for 20 minutes at 4°C. EtOH was removed and the DNA pellet rinsed once with 70% EtOH. The washed pellets were centrifuged again for 10 min at 14000 rpm at 4°C. The EtOH was definitively removed and the pellets dried in a speed vacuum. After drying, pellets were re-suspended in 9/10 of the original volume.

3.4.2 Conjugation of PEG to DNA oligos

PEGylation was performed in two different fashions, obtaining a stable or a reducible linkage.

3.4.2.1 Synthesis of PEG-a and PEG-b

To synthesize **PEG-a** and **PEG-b**, 2 nmol of **a** and **b** respectively were mixed to a mild excess of $mPEG_{20kDa}$-Mal (molar ratio of oligo to PEG is 1:1.2) in 0.1 M H_2NaPO_4, 2 mM EDTA, pH 7.0 for 30 minutes at room temperature. The conjugation reaction was monitored by RP-HPLC and than the pegylated oligo was purified.

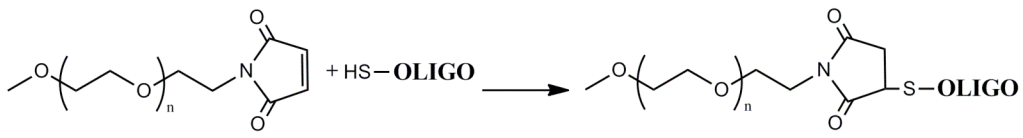


Figure 3.3: Scheme of mPEG-oligo synthesis.

Analysis and purification of PEG-oligos were performed with a C18 Jupiter (Phenomenex, USA; 5 μm, 300 Å, 250 x 4.60 mm), eluting at 1 mL/min with a gradient (reported below). Eluent A was 0.1 M triethylammonium acetate buffer (TEAA), pH 7.0 and ACN was the eluent B (50°C).

time (min)	0	25	27	29
% ACN	10	50	90	10

Conjugates were collected, dried and re-suspended in MilliQ water. The accurate contents of purified conjugates were evaluated by the individual molar extinction coefficients after UV-vis spectra analysis, using a nanodrop spectrophotometer.

3.4.2.2 Synthesis of PEG-ss-a

PEG-ss-a was prepared in the same condition used for **PEG-a** and **PEG-b**, excepting for the used polymer, $mPEG_{20kDa}$-OPSS, and the oligo polymer molar ratio (1:5). Purification was carried out after 24 h.

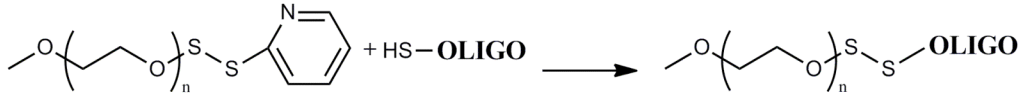


Figure 3.4: Scheme of mPEG-ss-oligo synthesis.

3.4.3 Annealing

Prior to ligation, the two ds DNA portions were formed. Many methods can be used to anneal complementary strands of nucleic acids. In each case, the goal is to denature the complementary strands to remove any secondary structure and then allow the strands to hybridize. Two factors that influence the efficiency of oligonucleotide hybridization are salt concentration and the rate of temperature decrease. Annealing occurs most efficiently when the temperature is slowly decreased after denaturation, especially when the oligonucleotides have high GC content or form hairpin structures.

3.4.3.1 "Classic" annealing

In the "classic" annealing procedure equal amounts of the top and bottom strand oligos to generate the ds portions of oligos were annealed (for example $\mathbf{a_F}$ and **a'** for $\mathbf{A_F}$, **m** and **m'** for $\mathbf{M_F}$).

We perform the annealing at a final oligo concentration of 50 μM, denaturing at 95°C for 5 min and the samples were slowly cool to room temperature. 0.25 nmol of each oligo portion ($\mathbf{A_F}$ and/or $\mathbf{M_F}$) were combined before the ligase reaction.

3.4.3.2 Annealing + "slow step"

This kind of annealing consists in the same procedure explained for "classic" annealing with an adding time of 5 h at RT in which the annealed portions were already mixed up before the ligation. A prolonged time of incubation favours the correct overlap of the sticky-ends.

3.4.3.3 "Mix it all" annealing

In "mix it all" procedure annealing the four single-stranded oligos (each ones in a final concentration of 25 μM) were mixed together and proceeded to anneal before the ligation. Also in this case annealing was done by denaturing at 95°C for 5 min and slowly cooling to room temperature.

3.4.4 $\mathbf{A_FM_F}$ ligation

According with the three annealing procedure previously describe (§ 3.4.3) ligation was performed as reported in Table 3.1.

Table 3.1: Different types of matching and pre-ligation procedure.

	PORTION I	PORTION II	PROCEDURE
i	$\mathbf{A_F}$	$\mathbf{M_F}$	"classic" annealing
ii	$\mathbf{A_F}$	$\mathbf{M_F}$	annealing + "slow step"
iii	$\mathbf{A_F}$	$\mathbf{M_F}$	"mix it all" annealing
iv	$\mathbf{A_F}$	$\mathbf{A_F}$	annealing
v	$\mathbf{M_F}$	$\mathbf{M_F}$	annealing

If not already mixed together, after the annealing procedure 0.25 nmol of each oligo portion ($\mathbf{A_F}$ and/or $\mathbf{M_F}$) were combined and than ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% w/v PEG_{8kDa}) and 4U of T4 DNA ligase were added, resulting in 6.25 mM ligated ds-DNA concentration ($\mathbf{A_FM_F}$, or $\mathbf{A_F^2}$ or $\mathbf{M_F^2}$). The reaction were maintained at 16°C o.n. and controlled by gel-electrophoresis (§ 3.4.11).

3.4.5 PEG-AM$_F$ ligation

Briefly, after the annealing + "slow step" procedure (§ 3.4.3.2)., 0.25 nmol of **PEG-A** and $\mathbf{M_F}$ were added of ligase buffer and DNA ligase, resulting in 6.25 mM ligated ds-DNA concentration. The reactions were maintained at 16°C o.n. and controlled by gel-electrophoresis (§ 3.4.11).

3.4.6 PEG-AM$_F$ ligation optimization

We monitored **PEG-AM$_F$** formation (§ 3.4.5) during time (10 min, 20 min, 30 min, 1 h, 2 h, 3 h) at three different temperatures: 10°C, 16°C and 21°C. At each time a small volume was withdrawn and the enzymatic reaction stopped with 25 mM EDTA and freezing.

The reactions were resolved in a vertical DNA PAGE (§ 3.4.11) and by RP-HPLC. DNA and fluorescein-modified components were analyzed at 260 and 494 nm respectively at the same conditions explained at § 3.4.2. In this case RP-HPLC elution was done in standard condition (RT), limiting the partial destabilization of the double-stranded caused by the high pressure.

3.4.7 PEG-ss-AM$_F$ ligation

As already reported for **PEG-AM$_F$** (§ 3.4.5 and 3.4.6), improvements in annealing procedure, time and temperature were applied, resulting in a ligation

reaction conduct at 21°C for 1 h. All parameters were the same, except for T4 DNA ligase buffer was DTT-free, to prevent the reduction of the disulfide bond between polymer and oligo.

3.4.8 PEG-BM_F ligation

PEGylation and ligation were developed as described above for **PEG-AM_F** in § 3.4.5 and 3.4.6. Results were also evaluated by DNA PAGE (§ 3.4.11).

3.4.9 PEG-ss-AM_F reduction

After ligation, a part of **PEG-SS-AM_F** was reduced in 0.1 M DTT, pH 7.0 for 3 h, to form **AM_F**. DNA material was washed from the buffers and excess PEG by precipitation as described in § 3.4.1.2.

3.4.10 EcoRI restriction

The ligation results were confirmed by EcoRI restriction, in fact only the ligated oligos present the exact sequence recognized by the enzyme (GAATTC).

42 pmol of **AM_F** (obtained after reduction treatment of **PEG-SS-AM_F**), **A_FM_F** or **PEG-SS-AM_F** were mixed with 20U of EcoRI and REact® 3 buffer (5 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 10 mM NaCl). After 1h at 37°C samples and references were run in native DNA acrylamide gel electrophoresis (§ 3.4.11).

3.4.11 Native and denaturing polyacrylamide gel electrophoresis

Ligation and restriction product were resolved in native and/or denaturing (7 M urea) DNA polyacrylamide gel electrophoresis (PAGE) gels, prepared with 20% acrylamide in 1 X TBE buffer (89 mM Tris base, 89 mM boric acid, 20 mM EDTA, pH 8.0). Each well was fill with 10 μL of 2.5 μM oligo solution, previously added of loading buffer (containing glycerol as thickener, bromophenol blue and xylene cyanol as tracer).

Separations were run applying 150 V for 3h in a 1 X TBE bath. Gels were exposed to a fluorescent lamp to immortalized fluorescein signal, to Sybr Green II reagent to detect all the single-stranded sequences and, at the end, to ethidium bromide for double-stranded ones. Imagines were saved and analyzed with GeneSnap.

4 RESULTS

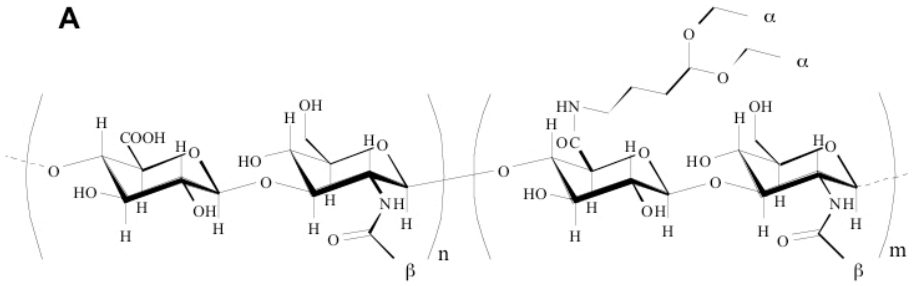
4.1 SYNTHESIS AND CHARACTERIZATION OF HA-PROTEINS

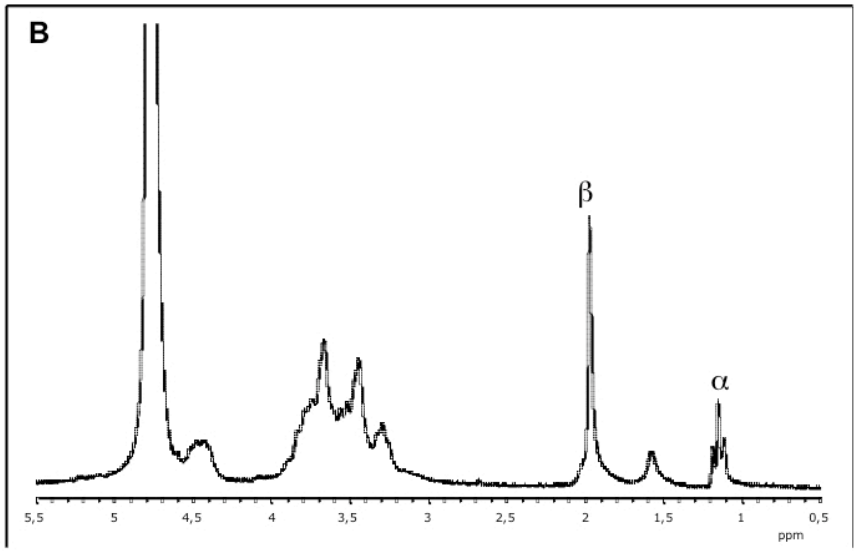
4.1.1 Synthesis and characterization of HA-acetal

In this study, the aldehyde groups were grafted on the HA backbone by exploiting a new strategy that allowed the preservation of HA backbone (oppositely to the widely used procedure of polysaccharide backbone oxidation with periodate). In this case, desired degree of aldehyde modification were achieved by coupling few HA carboxylic groups with 4-aminobutyraldehyde diethyl acetal. It will be then hydrolyzed into an aldehyde group useful for proteins coupling. Within this strategy a Shiff base will be first formed, then reduced to secondary amine treating with a reducing agent, $NaBH_3CN$ (see Figure 3.1).

A proper acetal derivatization was obtained carrying out the reaction in DMSO using the conditions reported in materials and methods. Even if the sodium salt of HA is only partially soluble in this solvent, the addition of CH_3SO_3H allowed a complete dissolution of the polymer within 1-2 hours.

HA-acetal was prepared at two different degrees of derivatization. In both cases the recovery were around 80%. The exact degree of modification was calculated by ^{1}H-NMR spectroscopy. The comparison of the integral value of the acetal group peak (1.10 ppm) with the integral value of HA acetyl group peak (1.85 ppm) demonstrated that the two polymer batches were activated at 4 and 21% mol (Figure 4.1).





δ_H (ppm)	Multiplicity	Assignment
4.65	*s*	2 H, H_2O
3.2-3.8	*m*	GlcA, GlcNAc methylene **H**, methane-**H**
1.85	*s*	3 H, -NHCO**CH_3**
1.10	*t*	6 H, -CH(OCH_2**CH_3**)$_2$

Figure 4.1: Structure and characterization of HA-acetal derivative. A) Chemical structure of HA-acetal derivative. **B)** ^{1}H-NMR spectroscopy of an HA-acetal derivative in D_2O: 1.10 ppm, t, (6H, -CH(OCH_2-C$\underline{H}_3$)$_2$, 4-aminobutyraldehyde diethyl acetal), 1.85 ppm, s, (3H, -NHCO-C$\underline{H}_3$, HA), 3.2-3.8 ppm, m (GlcA, GlcNAc methylene H, methane-H, HA).

4.1.2 Synthesis and characterization of HA-enzyme conjugates

Acetal groups of the HA derivative were hydrolyzed to aldehyde groups by a mild acid treatment. The hyaluronan-aldehyde derivative was conjugated to model enzymes at two pH values, 8.0 for random coupling at the level of amino group of lysines and 6.0 for selective protein *N*-terminus coupling. Random conjugation was performed for an indirect evaluation of the steric hindrance and the flexibility of HA on the protein surface.

The conjugation reactions were monitored by SEC. In Figure 4.2 (panel A), it is reported as an example the time course of the conjugation of HA-N^{ter}-trypsin. The disappearance over the time of the peak of free trypsin (t_R= 10.5 min) corresponded to the appearance of a new peak (t_R= 6.13 min), in agreement with the formation of HA-N^{ter}-trypsin conjugate that has a higher hydrodynamic volume.

All the purified conjugate were investigated by SEC to ensure the elimination of unreacted enzyme (example for HA-N^{ter}-trypsin in Figure 4.2, panel B). The purification was considered satisfactory when the peak of the starting protein (t_R around 10 min) had disappeared or was lower than 1%. Protein contents in the conjugates, shown in Tables 4.1 and 4.2, were averaged from very similar data obtained by bicinchonicic acid solution assay and absorbance at 280 nm on the basis of protein molar extinction. It was verified that HA did not affected both determinations.

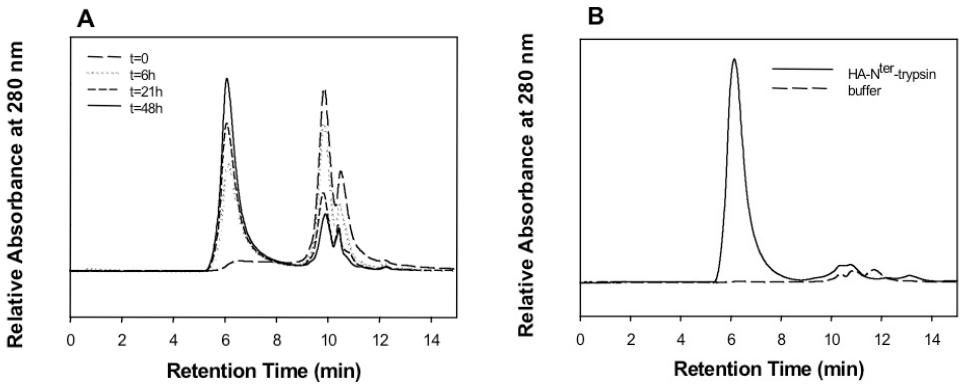


Figure 4.2: Example of conjugation of HA-acetal to trypsin. A) Conjugation reaction of HA-N^{ter}-trypsin at times of 0, 6, 21 and 48 h. **B)** Elution profiles of HA-N^{ter}-trypsin conjugate after purification. The low peak eluting at 10-12 min is not referred to free trypsin but it is due to the Tris buffer used to dissolve the sample as shown in the elution profile of buffer used as control. The analyses were carried out with size exclusion chromatography accordingly to the conditions reported in Materials and Methods section.

4.1.2.1 Measurements of HA-RNase A activity

The determination of RNase A activity in the corresponding conjugates, with the low molecular weight substrate, CCM, displayed a high enzymatic activity retention (Table 4.1). As expected, rHA-RNase A had a lower activity with respect to HA-N^{ter}-RNase A. This difference is probably due to the polymer chains that are a steric obstacle for the substrate recognition with the active site, an effect becoming more evident with the increase of the number of HA chains coupled per protein unit.

Table 4.1: Properties of HA-RNase A conjugates.

Sample	Protein content (% w/w)	Free Protein (%)	Yield of reaction (% w/w)	Activity (%)
RNase A	100		-	100
rHA-RNase A	21.4	< LOD	33.5	70.3 ± 3.05
HA-N^{ter}-RNase A	21.2	< LOD	48.5	82.9 ± 2.74

LOD = 0.01mg/mL

4.1.2.2 Measurements of HA-RNase A thermal stability

The thermal stability investigation, evaluated by monitoring the residual enzymatic activity over time at 37°C, showed that both RNase A and HA-N^{ter}-RNase A retained well the enzymatic activity by losing only 10% of the initial activity after 24 h of incubation (Figure 4.3).

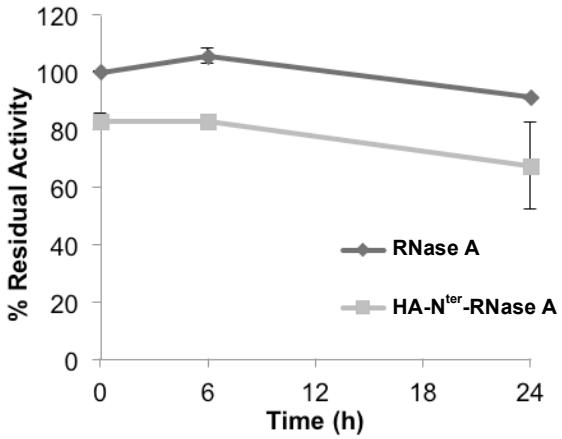


Figure 4.3: RNase A and HA-N^{ter}-RNase A thermal stability. Residual activity against CCM after 0, 6 and 24 h of incubation in Tris/acetate buffer pH 7.0. Results are presented as mean ± s.e.m. (n=3).

4.1.2.3 Measurements of HA-trypsin activity

The residual activities of rHA-trypsin and HA-N^{ter}-trypsin have been evaluated on low and high molecular weight substrates, TAME·HCl and casein, respectively. As expected, also in these cases the two conjugates

showed slightly different enzymatic activities on the basis of the method of coupling. The residual proteolytic activity on casein of rHA-trypsin and HA-N^{ter}-trypsin were reduced at 53% and 61%, while both conjugates displayed a better retention of esterolytic activity, 72% and 86%, respectively (Table 4.2). These results indicate that the polymer does not completely hinder the approach of even large substrates to the catalytic site, probably thanks to its backbone flexibility and hydrophilicity.

Table 4.2: Properties of HA-trypsin conjugates

Sample	Protein content (% w/w)	Free Protein (%)	Yield of reaction (% w/w)	Activity (%)
trypsin	100		-	100 (LMWS)[a] 100 (HMWS)[b]
rHA-trypsin	8.9	<LOD	21.4	72.0 ± 2.74 (LMWS)[a] 53.0 ± 6.46 (HMWS)[b]
HA-N^{ter}-trypsin	14.8	<LOD	41.4	85.6 ± 4.34 (LMWS)[a] 61.3 ± 6.10 (HMWS)[b]

LOD = 0.01mg/mL
[a]**LMWS:** activity evaluated against Low Molecular Weight Substrate
[b]**HMWS:** activity evaluated against High Molecular Weight Substrate

4.1.2.4 Measurements of HA-trypsin thermal stability

In the case of HA-N^{ter}-trypsin and trypsin, the residual activity over the time during incubation at 37°C decreased faster than for RNase A. Trypsin was quickly inactivated, showing 20% of residual esterolytic activity and 10% of proteolytic activity after just 6 h. The decrease of enzymatic activity for the conjugate over the time was slower than for free trypsin, as shown by the results after 6 h of incubation. In particular, the esterolytic and proteolytic activities were 59% and 34%, respectively, of that of trypsin at time zero (Figure 4.4, A and B). This demonstrates HAylation markedly increased enzyme stability reducing the typical autolysis process, an effect seen also after trypsin conjugation with other polymers (Gaertner and Puigserver, 1992).

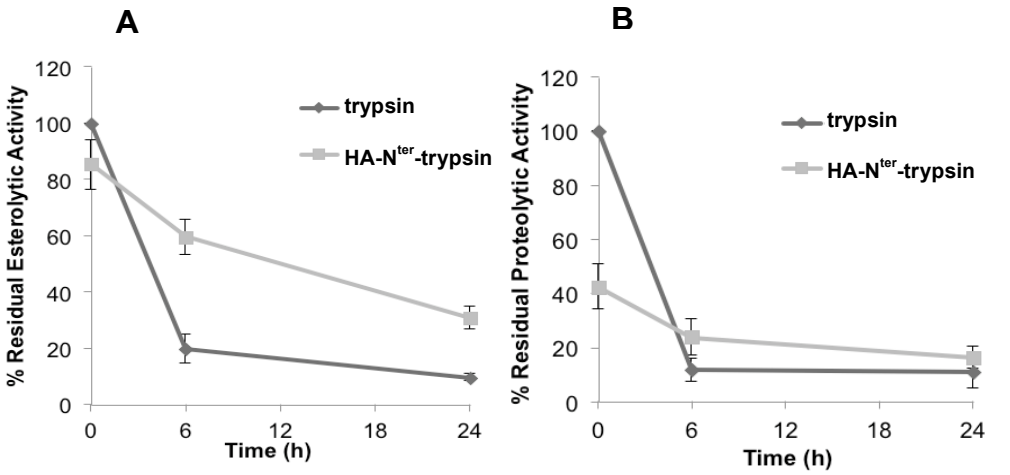


Figure 4.4: Trypsin and HA-N^{ter}-trypsin thermal stability. A) Residual activity against TAME (esterolysis) and **B)** casein (proteolysis) after 0, 6 and 24 h of incubation in Tris buffer. Results are presented as mean ± s.e.m. (*n*=3-5).

4.1.2.3 Treatment of HA-N^{ter}-RNase A and HA-N^{ter}-trypsin by hyaluronidase

HA is a biodegradable polymer and this represents a relevant advantage over the most used polymer in this field, PEG, which is not biodegradable (Pasut et al., 2008). It was therefore important to verify if hyaluronidase, the enzyme involved in HA degradation *in vivo*, was still able to degrade HA after its conjugation to a protein The SEC analysis showed that the conjugates' molecular weight was reduced in a time depended manner. In fact, the conjugates were eluted as a main peak at 6 min. After incubation with hyaluronidase, these peaks disappeared and new peaks with higher retention times, and consequently smaller molecular weights appeared over time (Figure 4.5).

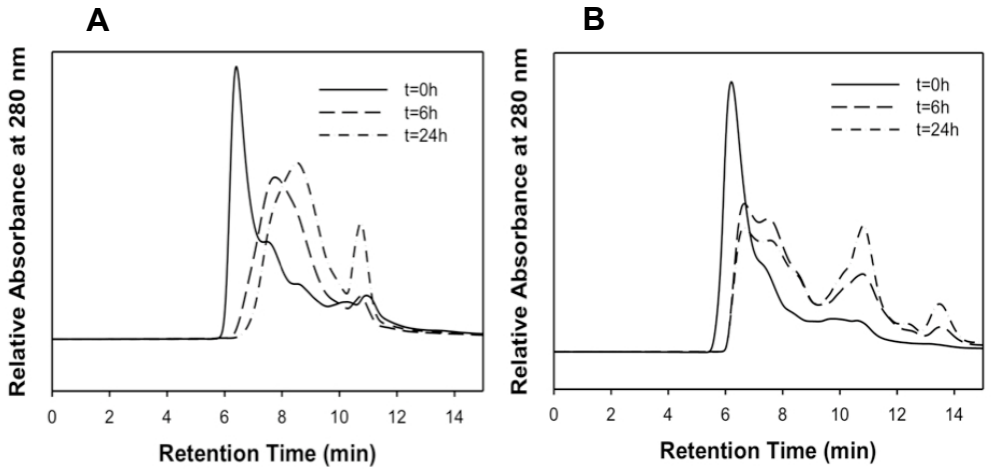


Figure 4.5: HA-enzymes degradation by hyaluronidase. A) HA-N^{ter}-RNase A, **B) HA-N^{ter}-trypsin** (t=0, 6 and 24h)**.** The SEC elution conditions are reported in Materials and Methods section.

4.1.3 Synthesis and characterization of HA-Insulin (HA-INS) conjugates

After testing and optimizing the conjugation of HA to model enzymes, the HA-acetal derivative was used to prepare a potential therapeutic conjugate with insulin. In this case two HA-acetal batches, differing in the degree of acetal substitution (4 and 21%), were used to also investigate the effect of protein loading.

The SEC elution profiles of purified products showed a main peak corresponding to the conjugates without the presence of detectable amounts of free insulin (Figure 4.6). As reported in Table 4.3, the percentages of aldehyde groups in the HA backbone correlates, although not proportionally, with the insulin loading in the final conjugates, 17.2% and 32% (w/w) for HA-N^{ter}-INS 1 and HA-N^{ter}-INS 2, respectively, thus suggesting a steric hindrance between the polypeptide chains.

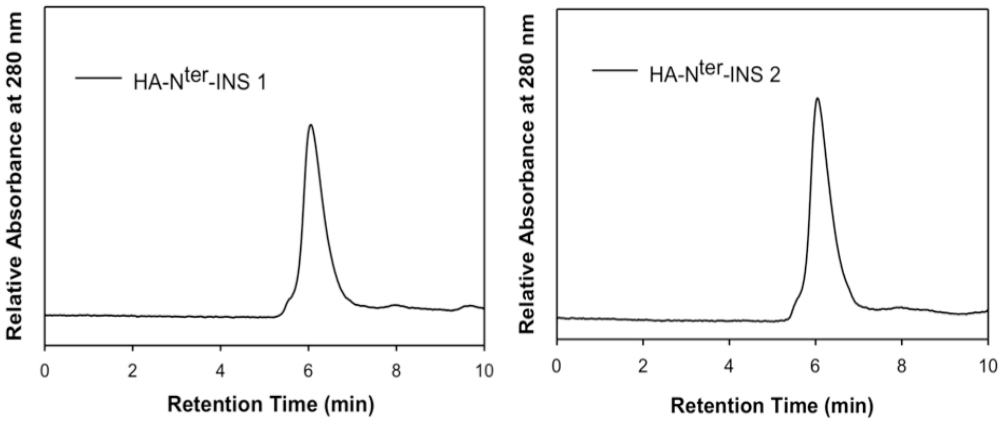


Figure 4.6: Elution profiles of purified HA-INS conjugates in size exclusion chromatography. The elution conditions are reported in Materials and Methods section.

Table 4.3: Properties of HA-INS conjugates

Sample	Protein content (% w/w)	Free protein (%)	Yield of reaction (% w/w)
HA-N^{ter}-INS 1	17.2	<LOD	9.6
HA-N^{ter}-INS 2	32.0	<LOD	18.0

LOD = 0.01mg/mL

4.1.3.1 Erythrocyte compatibility study

Before testing the activity of HA-INS conjugates *in vivo*, the effect of the polymer and HA-INS conjugates on the integrity of the erythrocyte membrane was investigated to evaluate the blood compatibility of the compounds.

The percent of cell lysis, as reported in Figure 4.7, showed that both conjugates were non-hemolytic at the concentration used for the *in vivo* study.

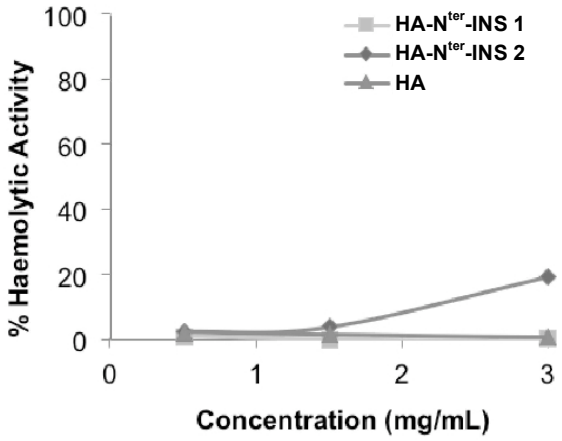


Figure 4.1: Haemolytic effect of HA and HA-INS conjugates on mouse red blood cells. Results are presented as % of hemoglobin release produced by the different compounds ± S.D. (n = 3). Due to similar values, some symbols overlap.

A partial lysis was only shown by HA-N^{ter}-INS 2 (< 20% lysis) but it was for a concentration 100 times higher than that used with the animals.

The starting HA and the insulin conjugate prepared with the HA-acetal at 4% activation degree showed negligible hemolytic activities.

4.1.3.2 In vivo evaluation of HA-INS activity

In order to study *in vivo* the residual activity of HA-INS conjugates, their glucose lowering effect was tested in rats. Type I diabetes was induced with streptozotocin in *Sprague-Dawley* rats, showing after the treatment an average basal blood glucose titer of > 200 mg/dL compared to 60-70 mg/dL in normal rats.

To each diabetic rat group was subcutaneously administered a single bolus of HA-N^{ter}-INS 1, HA-N^{ter}-INS 2, insulin or PBS (control). The glucose blood level for all groups was monitored over time and up to 24 h. The insulin equiv. dose was 0.135 IU per animal to avoid more severe hypoglycemic events, observed at higher doses.

The experimental results are shown in Figure 4.8. The lowering effect on glucose blood level of insulin was observed at 1 h post-injection, but the glucose titer returned already to the initial value after 2 h. Interestingly, HA-N^{ter}-INS 1 showed a slower but prolonged onset of the lowering effect, in fact the glucose titer reached the minimum concentration at 6 h and then progressively returned to the initial value. The prolonged activity of HA-N^{ter}-INS 1 can be due to a combination of the increased molecular weight of the conjugate and the prolonged release of conjugate fractions at a lower molecular weight from the site of injection, by the action of hyaluronidase.

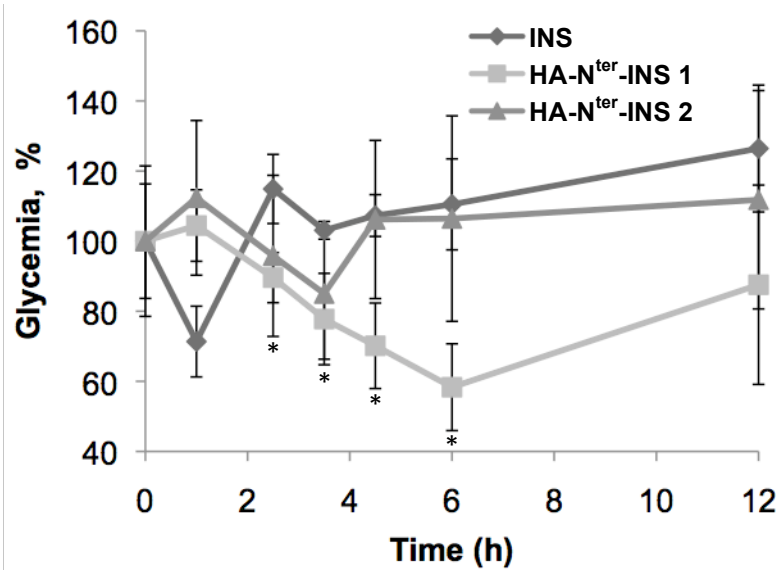


Figure 4.8: Blood glucose levels in streptozocin-induced diabetic *Sprague-Dawley* rats. Injection of bovine insulin, HA-N^{ter}-INS 1, HA- N^{ter}-INS 2. Results are presented as mean ± s.e.m. (*n*=3-5). Significance: * = $p < 0.05$ vs insulin.

Unexpectedly, HA-N^{ter}-INS 2 showed no significant effect on glucose titer. Probably the higher insulin loading of HA-N^{ter}-INS 2 led to a steric entanglement affecting the receptor/protein recognition.

4.2 PREPARATION OF PEGYLATED OLIGOS BY ENZYMATIC PEGYLATION

In this section of the work an innovative enzyme-mediated oligo PEGylation was studied. The method is composed of two steps. The first is a chemical PEGylation of a short oligomer; the second is the enzymatic ligation of the oligo-PEG with another oligo sequences, catalyzed by T4 DNA ligase.

In order to investigate the feasibility of this enzyme-mediated oligo PEGylation, a model of four DNA sequences was studied. A simple scheme is shown in Figure 4.9.

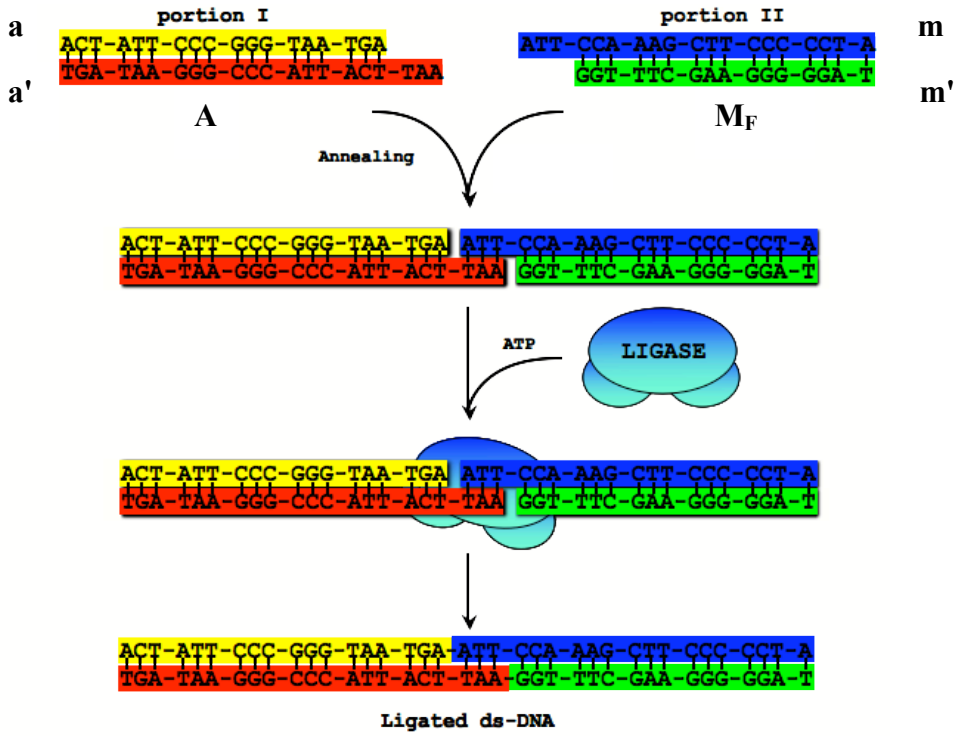


Figure 4.9: Scheme of the model ligase reaction.

4.2.1 Synthesis of PEGylated oligos

The two 5'-Thiol modified oligos, **a** and **b**, were conjugated to $mPEG_{20kDa}$-Mal exploiting the Michael addition.

The yield of conjugation in RP-HLPC analysis was about 93%. To remove unreacted PEG and oligo, the conjugates were purified by RP-HPLC (example in Figure 4.10), obtaining **PEG-a** and **PEG-b** for each conjugation reactions.

PEG-ss-a was synthesized by coupling the thiol of **a** with $mPEG_{20kDa}$-OPSS, thus forming a disulfide bridge. Also in this case the yield of conjugation was comparable with the other conjugates and RP-HPLC was used for purification.

All the conjugates presented a recovery of about 80%, as determined spectrophotometrically.

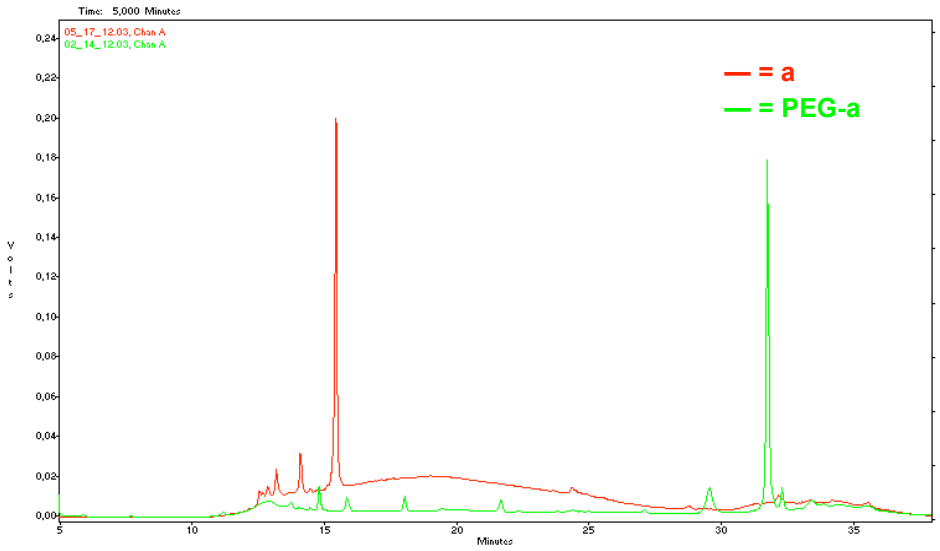


Figure 4.10: Example of oligo and PEG-oligo RP-HPLC elution. Red line: **a**, green line: purified **PEG-a**. C18 Jupiter (5 µm, 300 Å, 250 x 4.60 mm), flow 1 mL/min, eluting with a non-linear gradient (A: 0.1 M TEAA, pH 7.0; B: ACN. 5 min 5% B, 20 min 40% B, 25 min 40% B, 30 min 70% B, 50°C).

4.2.2 Ligation whit T4 DNA ligase

4.2.2.1 A_FM_F ligation and protocol optimization

Before to perform the ligation with the PEGylated oligo, the enzymatic reaction was optimized with the not-pegylated oligos. Three different methods of annealing were tested to promote the maximum results of ligation and to avoid undesired products.

After the annealing of the two double-stranded portions, an intermediate passage named "slow step" was added (see § 3.4.3.2). The two portions were mixed and kept at RT for 5 h. This time promoted the perfect pairing of the short sticky-ends of the components (AAT for $\mathbf{A_F}$ and TTA for $\mathbf{M_F}$) that in not optimized conditions could also combine in a wrong way, leading to the formation of $\mathbf{A_F^2}$/$\mathbf{M_F^2}$. Figure 4.11 shows that under the selected conditions a single ligated product is obtained (lane 4). Instead, traces of unwanted products were found with the other two procedures (simple annealing in lane 3, "mix it

all" annealing in lane 5). In fact, the formation of $\mathbf{A_F}^2$ and $\mathbf{M_F}^2$ in spite of the central missing base coupling in the overhang portions (A:A for $\mathbf{A_F}^2$ and T:T for $\mathbf{M_F}^2$) (Figure 4.11, lane 6 and 7) are reported.

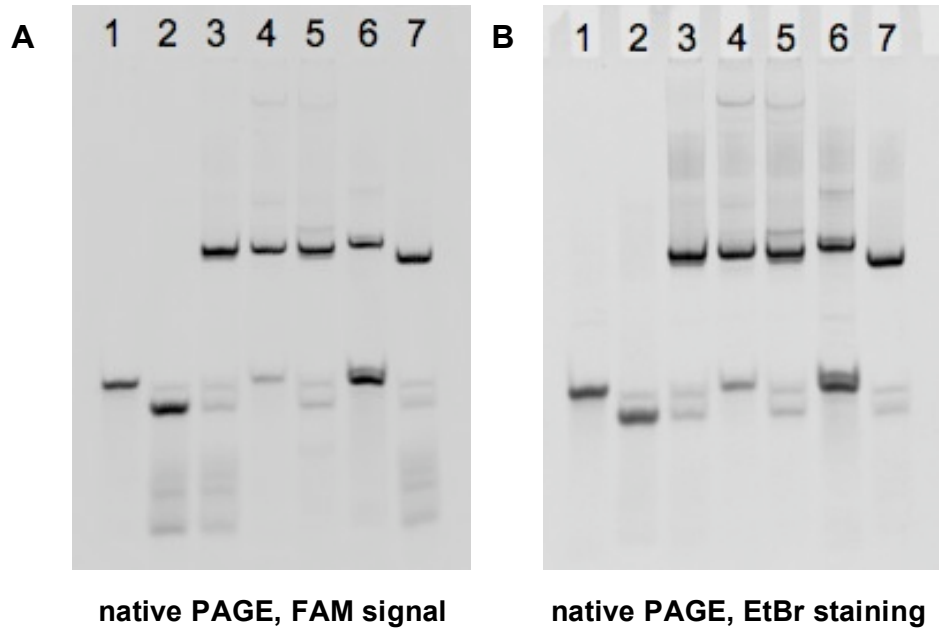


Figure 4.11: Different procedure of annealing pre-ligation of $\mathbf{A_FM_F}$ and $\mathbf{A_F}^2$ and $\mathbf{M_F}^2$ formation proving. A) FAM signal; **B)** EtBr staining signal. Lane: 1, $\mathbf{A_F}$, 2, $\mathbf{M_F}$, 3, annealing procedure pre-ligation of $\mathbf{A_FM_F}$, 4, annealing + "slow step" procedure pre-ligation of $\mathbf{A_FM_F}$, 5, "mix it all" annealing procedure pre-ligation of $\mathbf{A_FM_F}$, 6 and 7, $\mathbf{A_F}^2$ and $\mathbf{M_F}^2$ formation. Samples were applied on a 20% native PAGE containing 1X TBE buffer. The mobility of the ds oligos was detected using the Geliance 600 Imaging System.

4.2.2.2 PEG-AM$_F$ ligation reaction

PEG-AM$_F$ was prepared following the modified protocol settled for $\mathbf{A_FM_F}$. To confirm **PEG-AM$_F$** formation in DNA PAGE two characteristics is required: the FAM signal and a low electrophoretic mobility, the last caused by the hydrodynamic volume increased after PEGylation.

In Figure 4.12 is shown the different electrophoretic mobility between the references and the PEGylated complex (lane 6); furthermore, high intensity of fluorescent signal is present at the base of the well, indicating that the ligation was successful. We also supposed that the polymer strongly stabilized the double-strand sequence because even in strong denaturing condition (panels C and D), the PEGylated complex was still in the double-stranded form and the mobility of the entire fluorescent ligate was very low.

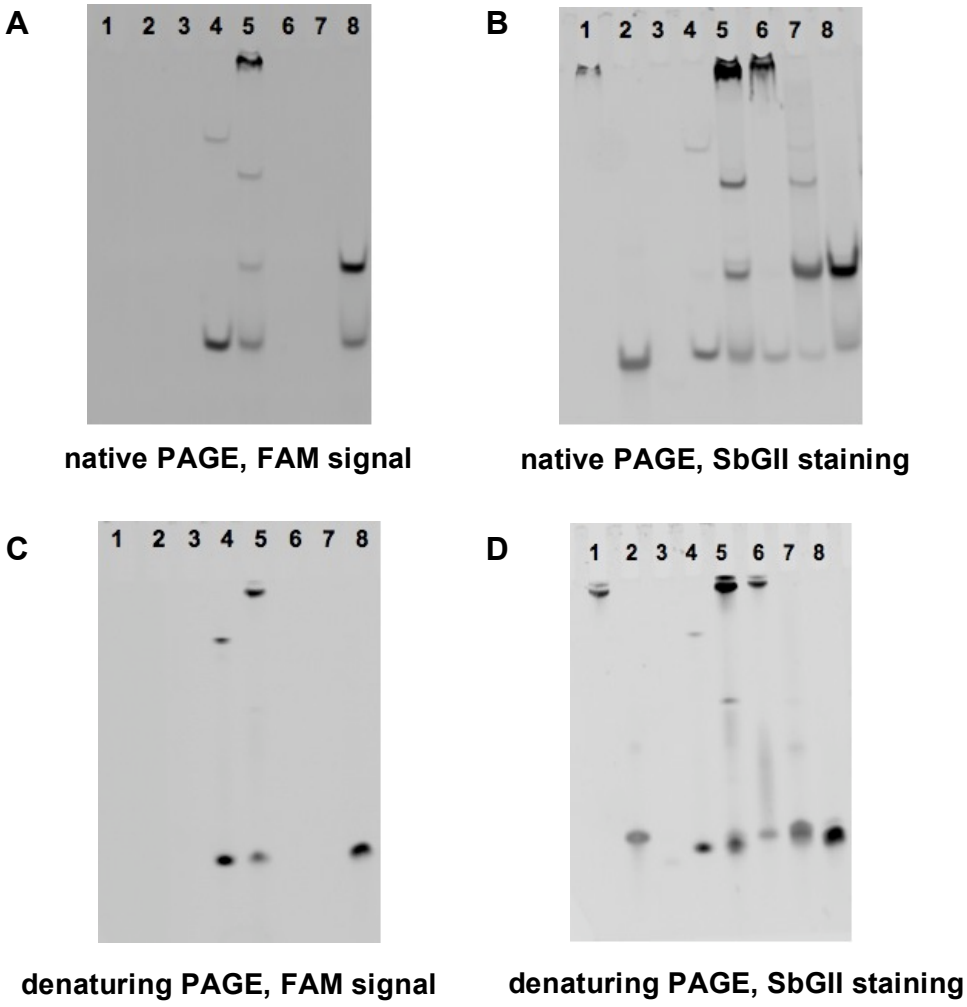


Figure 4.12: PEG-AM$_F$ Ligation. A) native PAGE, FAM signal; **B)** native PAGE, Sybr Green II® staining; **C)** denaturing PAGE, FAM signal; **D)** denaturing PAGE, Sybr Green II® staining. Lanes: 1, **a**, 2, **a'**, 3, **m**, 4, **m'**, 5, mix **PEG-AM$_F$** ligation, 6, **PEG-A**, 7, **A$_F$**, 8, **M$_F$**. Samples were applied on a 20% PAGE containing 1X TBE buffer. The mobility of the ds oligos was detected using the Geliance 600 Imaging System.

4.2.2.3 PEG-AM$_F$ ligation reaction time course

In order to increase the yield of ligation, **PEG-AM$_F$** formation was monitored during the time at three different temperatures. As expected, DNA PAGE (Figure 4.14) and RP-HPLC data (Figure 4.13 and Table 4.4) suggested qualitatively and quantitatively that at the higher temperature the reaction was faster. At 21°C the ligation was complete after about 1 h. After this time only unwanted products were formed (especially **M$_F^2$**).

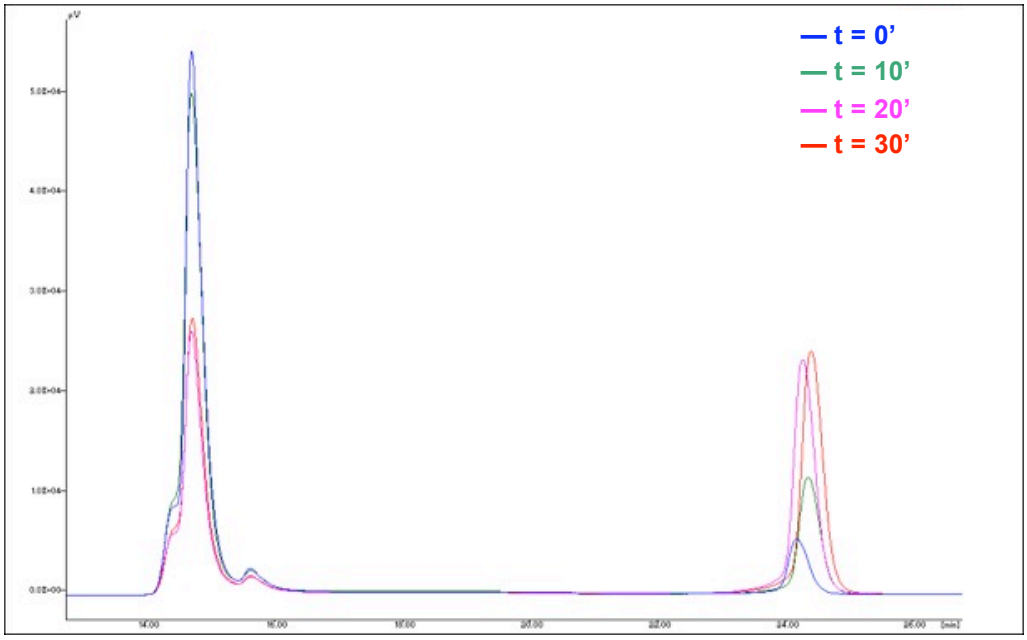
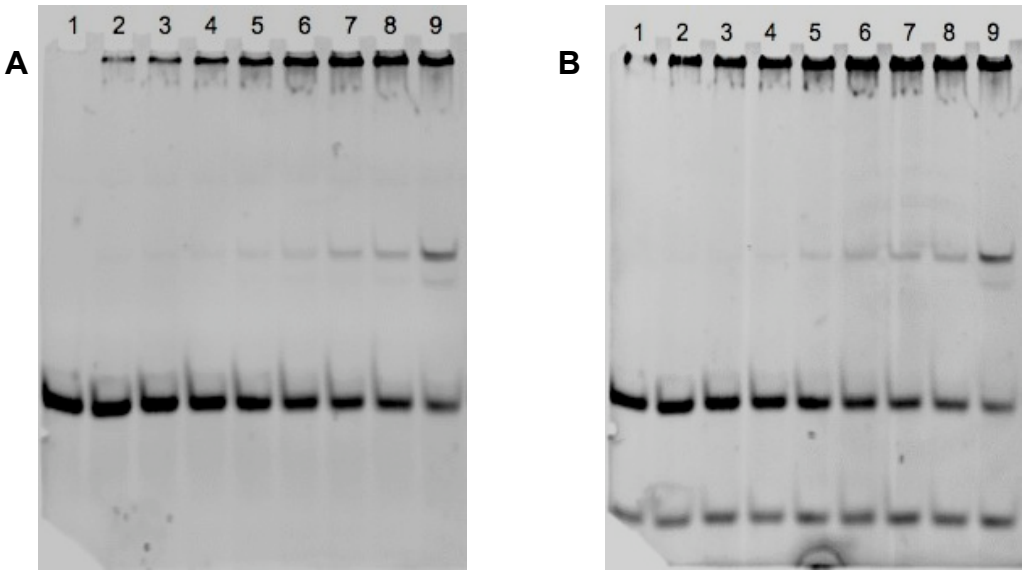


Figure 4.13: PEG-AM$_F$ formation. Monitoring by RP-HPLC. Example of elution profile timing samples of 21°C experiment. C18 Jupiter (5 μm, 300 Å, 250 x 4.60 mm), flow 1 mL/min, eluting with a non-linear gradient (A: 0.1 M TEAA, pH 7.0; B: ACN. 5 min 5% B, 20 min 40% B, 25 min 40% B, 30 min 70% B, 50°C). λ= 494 nm.

Table 4.4: PEG-AM$_F$ formation quantified by RP-HPLC. Data are calculated on the AUC of the **PEG-AM$_F$**. o.n. time of each ligation (10°, 16° and 21°C).

	0 min	10 min	20 min	30 min	1 h	2 h	3 h	o.n.
10°C	0.29	7.87	29.66	29.57	73.71	94.14	95.26	100
16°C	7.75	18.31	52.32	68.13	78.21	82.42	82.82	100
21°C	17.26	35.49	88.36	86.77	96.23	98.78	99.06	100



native PAGE, FAM signal **native PAGE, EtBr staining**

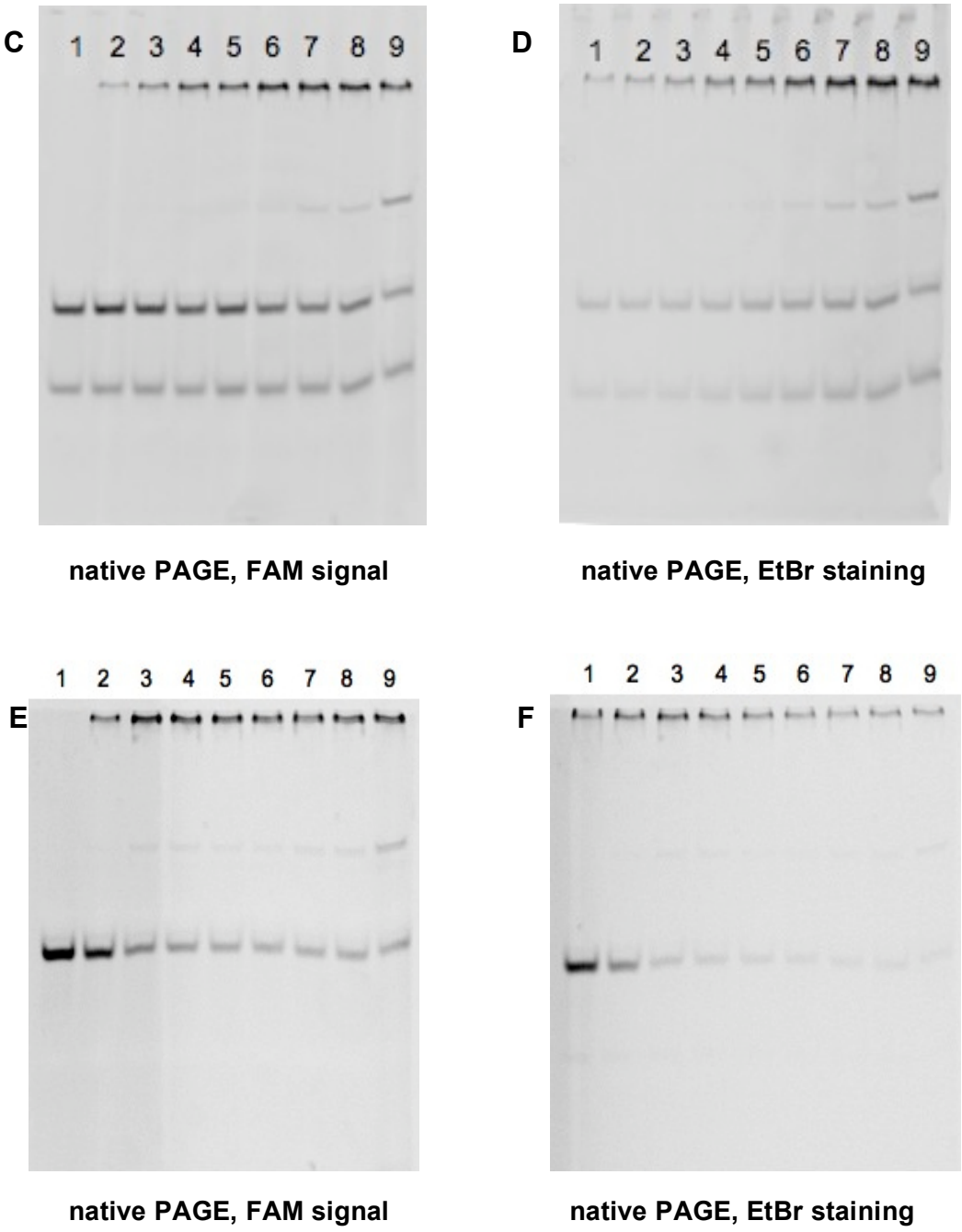


Figure 4.14: Time monitoring of PEG-AM$_F$ ligation at 21°C. Panels **A)**, **C)** and **E)**: FAM signal. Panels **B)**, **D)** and **F)**: Sybr Green II® staining. Lanes: 1, ref. **PEG-A+M$_F$**, 2, t=0, 3, t=10 min, 4, t=20 min, 5, t=30 min, 6, t=1h, 7, t=2h, 8, t=3h 9, t=o.n. Samples were applied on a 20% native PAGE containing 1X TBE buffer. The mobility of the ds oligos was detected using the Geliance 600 Imaging System.

4.2.3 EcoRI restriction

To further confirm the effective ligation of the pegylated complex, the restriction enzyme EcoRI was used. Indeed, the couples of oligos were designed to form the EcoRI consensus sequence (GAATTC) after ligation.

The reversible conjugate **PEG-SS-a** was prepared for this experiment with the aim to avoid potential steric entanglement by the PEG chain attached to the oligo. In fact after DTT treatment PEG is released from the DNA complex, which can then be treated with EcoRI. As shown in Figure 4.15, after reduction $\mathbf{AM_F}$ is still present as the double-stranded fluorescenated $\mathbf{A_FM_F}$ (lane 5 and 3). The enzyme EcoRI was active on $\mathbf{A_FM_F}$ and as expected on reduced **PEG-SS-$\mathbf{AM_F}$** ($\mathbf{AM_F}$) but interestingly the enzyme was able to recognize and clive the substrate also in the PEGylated form (**PEG-SS-$\mathbf{AM_F}$**) (lane 6, 8 and 7 respectively).

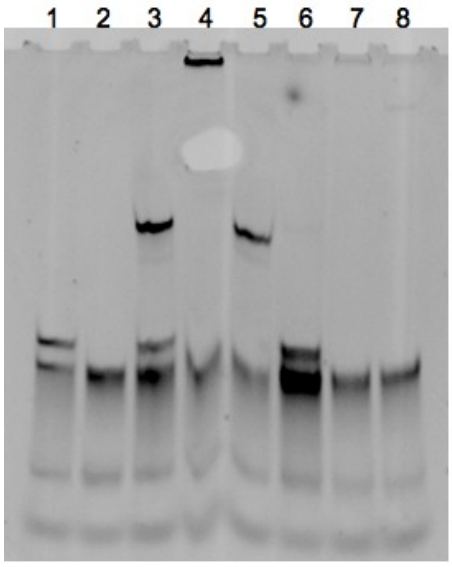


Figure 4.15: Ligated DNA EcoRI restriction. FAM signal. Lanes: 1, $\mathbf{A_F}$+$\mathbf{M_F}$, 2, **PEG-SS-A**+$\mathbf{M_F}$, 3, $\mathbf{A_FM_F}$ ligated, 4, **PEG-SS-$\mathbf{AM_F}$** ligated, 5, reduced **PEG-SS-$\mathbf{AM_F}$** ($\mathbf{AM_F}$), 6, restricted $\mathbf{A_FM_F}$, 7, restricted **PEG-SS-$\mathbf{AM_F}$**, 8, reduced and restricted **PEG-$\mathbf{AM_F}$**. Samples were applied on a 20% native PAGE containing 1X TBE buffer. The mobility of the ds oligos was detected using the Geliance 600 Imaging System.

4.2.4 PEG-$\mathbf{BM_F}$ ligation reaction

Once verified the capacity of DNA ligase to act on a PEGylated oligo complex, it has been important to verify if the enzyme could work also on PEGylated oligos with a reduced number of bases. The size of the oligos

coupled to PEG was reduced by half, resulting in the 9-mer **b** with the complementary 12-mer **b'**.

In Figure 4.16 it is shown that the fluorescent signal of **PEG-BM$_F$** in the lane 4, which is compared with the non-PEGylated form **BM$_F$**, has a very low electrophoretic mobility. This confirms that DNA ligase performed its action even when the distance between polymer site of coupling and the site of ligation is just 9 base pair.

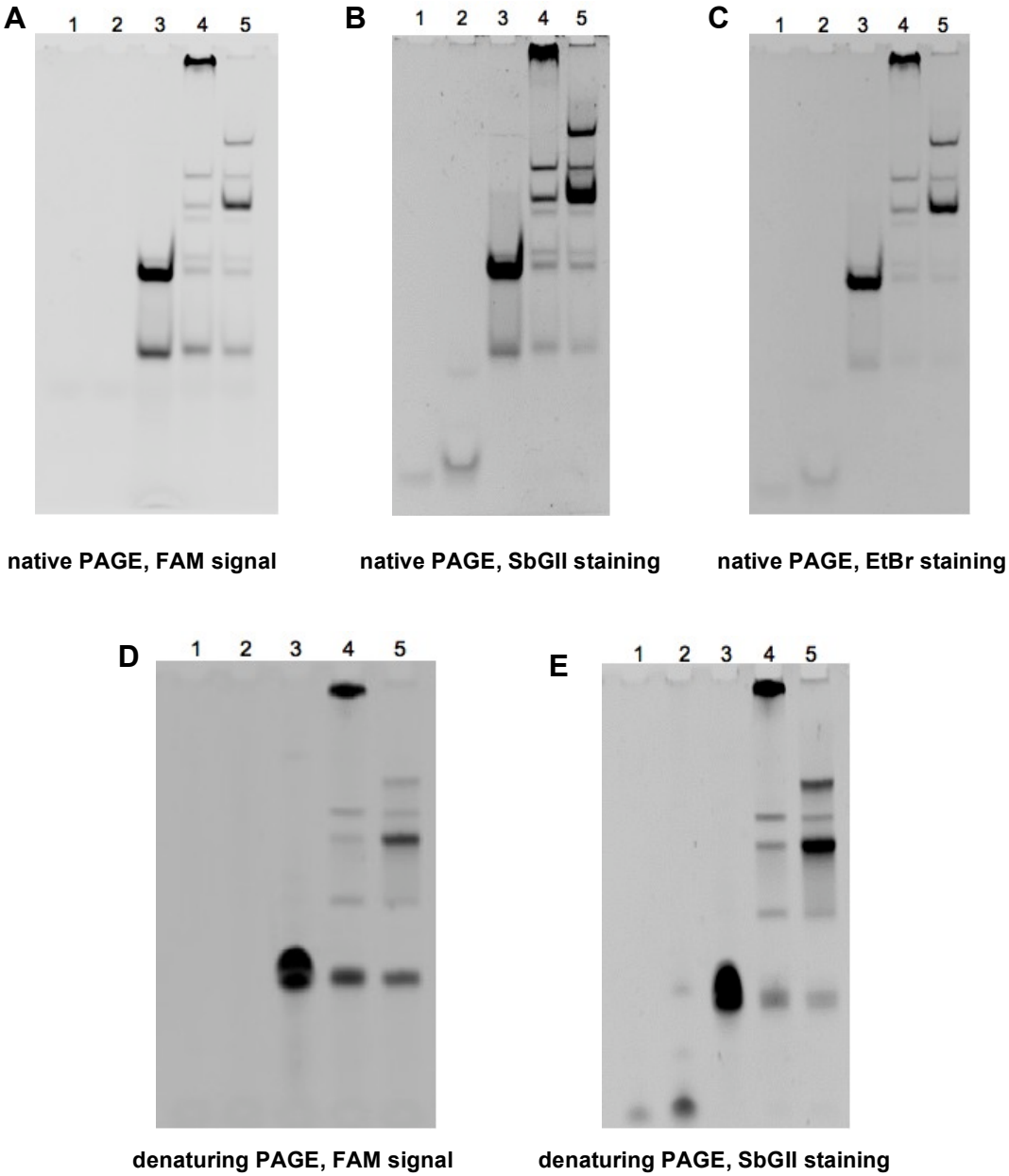


Figure 4.16: PEG-BM$_F$ Ligation. A) native PAGE, FAM signal, **B)** native PAGE, Sybr Green II® staining, **C)** native PAGE, EtBr staining, **D)** denaturing PAGE, FAM signal, **E)** denaturing PAGE, Sybr Green II® staining. Lanes: 1, **b**, 2, **b'**, 3, **M$_F$**, 4, mix **PEG-BM$_F$** ligation, 5, mix **BM$_F$** ligation. Samples were applied on a 20% PAGE containing 1X TBE buffer. The mobility of the ds oligos was detected using the Geliance 600 Imaging System.

5 DISCUSSION

Since the early 80's the forward steps in genetics and proteomics, have led a particular interest to biotech products, such as DNA and proteins. A better understanding of the molecular mechanism at the bases of pathologies and the large scale production of these molecules, help their success as therapeutics (Walsh, 2003).

The strengths of proteins and genes are their high selectivity/affinity and high efficacy. Nevertheless, their pharmaceutical use presents some disadvantages, which may limit their use, such as the susceptibility to degradation by enzymes, rapid renal clearance and immunogenicity.

Many researchers are seeking solutions in order to avoid these drawbacks, which include chemical modification or the use of drug delivery systems (DDSs). For proteins, some examples are genetic mutation, like amino acid substitutions and insertion of glycosilation sites, or formulation in DDSs. For oligos, chemical modifications of the backbone are the most used (for example at the level of ribose or phosphate group).

One of the most successful DDSs is the covalent attachment of polyethylene glycol (PEG), also named PEGylation. It has become one of the most exploited approaches for the delivery of therapeutic proteins (Harris and Chess, 2003; Pasut and Veronese, 2007) and oligomers (Zhao et al., 2005; Zhao et. al, 2007; Ng et al., 2006).

The advantages achieved by PEGylation can be summarized as follows: remarkable *in vivo* half-life prolongation (Pasut and Veronese, 2012), elimination or reduction of protein immunogenicity (Mero and Veronese, 2008) and increased stability (Veronese et al., 2007).

The effectiveness and potentials of this technique are demonstrated by the several conjugates already on the market, such as Neulasta®, Pegasys®, Mircera®, Cimzia® and others (see Table 2.1 and 2.2). On the other hand, PEG presents some weaknesses that might restrict its full exploitation; nevertheless it is still considered the polymer of choice.

A known limit is its non-biodegradability, which was easily overcome by using polymers with molecular weights below the kidney excretion threshold.

Furthermore, a recent concern comes from some studies reporting cases of development of specific anti-PEG antibodies. These were detected in the serum of patients treated with PEG-asparaginase (Armstrong et al., 2007) and PEG-uricase (Sherman et al., 2008). Up to now, these events occurred only when the polymer was coupled to large and highly immunogenic proteins. Although the frequency of such reports is still limited, there is an unmet need of developing other polymers than PEG, offering a valid alternative that can also overcome the patent constrains of PEGylation.

A forward step in protein conjugation might occur by the use of biodegradable polymers. Such polymers can prevent protein limitations such as aggregation and fast kidney clearance, problems also solved by using non-biodegradable polymers, but at the same time they can avoid the risks of polymer accumulation in the body. Furthermore, polymer degradation allows the recovery, at least in part, of protein activity that is usually reduced after polymer conjugation (Duncan et al., 2008; Hardwicke et al., 2008).

Biodegradable polysaccharides, such as dextran, alginate, inulin, hyaluronic acid (HA), have been widely investigated as protein carriers. In the first studies, the conjugation strategies were based on random couplings between the protein's amino groups and either the carboxylic groups of the polymer, if present, or aldehyde groups generated by periodate oxidation of the polymeric backbone (Torchilin et al., 1988). The main limitations of these approaches were undesired cross-linking, low homogeneity of conjugates and potential formation of soluble aggregates, which yielded difficulties in terms of characterization, batch-to-batch reproducibility and immunogenicity.

In this respect, the first section of this work was focused on the potential role of a new HA-aldehyde derivative as carrier of proteins

Hyaluronic acid is a natural polysaccharide largely present also in humans. HA has a relatively simple linear structure of repeating non-sulfated disaccharide units composed of D-glucuronic acid and *N*-acetyl-D-glucosamine (Almond, 2007).

HA possesses several of the desired properties of a polymer for protein conjugation, such as biodegradability, non-immunogenicity and non-toxicity. In addition to the applications already in clinical use, HA has been thoroughly studied as carrier of low molecular weight drugs (Banzato et al., 2008). For the delivery of proteins, a multi reactive polymer can easily yield heavy cross-linked conjugate mixtures with a high inhomogeneity. This limit prevents the direct use of HA for protein conjugation through activation of its carboxylic groups.

The development of new HA derivatives for site selective protein coupling can circumvent this constraint and allows the exploitation of HA in this field. Polymers with aldehyde functionalities are the best choice for selective *N*-terminal protein conjugation owing to their mild reactivity in comparison to other electrophile groups such as carboxy succinimidyl esters (Hu and Sebald, 2011).

Among all the different available chemical routes for introducing aldehyde groups into the HA backbone, in this study it was preferred a strategy that allowed the coupling of few HA carboxylic groups with 4-aminobutyraldehyde diethyl acetal. This approach offered several advantages. Firstly, the butyraldehyde group is less reactive than other aliphatic aldehydes, namely propyl or ethyl aldehydes (Hu and Sebald, 2011). 4-aminobutyraldehyde

diethyl acetal contains a protected aldehyde functionality that avoids the stability issue of aldehyde groups during long-term storage and it can be easily deprotected in the active form just before protein conjugation. Furthermore, the degree of derivatization can be well controlled and its determination in HA-acetal batches can be straightforwardly evaluated by ^{1}H-NMR, this offering a precise control over the reagents ratio in the following protein conjugation reaction.

The new HA derivative was selectively linked to model proteins by exploiting the *N*-terminal conjugation. This method is based on the different pK_a values between the *N*-terminal α amino group, about 7.6-8, and the ε amino group of lysines, about 10-10.2 (Wong, 1991). Another important advantage of the method is its suitability for almost all proteins.

The potentials of this HA-acetal for protein conjugation (HAylation) and delivery were assessed with two model enzymes and the therapeutic polypeptide insulin. In the first case, the enzymes trypsin and RNase A offered the opportunity to investigate the effect of HA coupling on their enzymatic activity. The conjugation was performed at two pH values, 8.0 for random coupling at the level of lysines and 6.0 for selective protein *N*-terminus linking. Random conjugation was performed for an indirect evaluation of the steric hindrance and the flexibility of HA on the protein surface. In fact, the polymer chains might represent a great steric obstacle for the substrate recognition with the active site, an effect becoming more evident with the increase of the number of HA chains coupled per protein unit. But, the backbone flexibility may reduce in part the steric hindrance effect.

Interestingly, HAylation determined only small reductions of enzymatic activities, this being always above 70% on small substrates for both trypsin and RNase A. As expected, the activity reduction was more significant when measured with large substrates (casein for trypsin) but still it is above 53% even for rHA-trypsin. These results indicate that the polymer does not completely hinder the approach of even large substrates to the catalytic site, likely thanks to its backbone flexibility and hydrophilicity. Anyway, *N*-terminal conjugates were more active compared to random ones.

The thermal stability studies showed that HAylation does not cause detrimental effects on protein structure over the time after incubation at 37°C in physiological buffer. In fact, the activity of HA-N^{ter}-RNase A was comparable to that of the native enzyme. In the case of trypsin, the polymer markedly increased enzyme stability because HAylation reduced the typical autolysis process, an effect seen also after conjugation with other polymers (Gaertner and Puigserver, 1992).

HA is a biodegradable polymers and this represents a relevant advantage over the most used polymer in this field, PEG, which is non biodegradable (Pasut et al., 2008). It was therefore important to verify if hyaluronidase, the

enzyme involved in HA degradation *in vivo*, was still able to degrade HA after its conjugation to a protein. Figure 4.5 shows that the HA moiety of conjugates is digested by hyaluronidase thus confirming the biodegradability of the polymer also after conjugation. This feature offers the possibility to prepare conjugates that *in vivo* are slowly digested into protein conjugates with short HA chains that should allow a recovery of the protein activity lost after HAylation.

After testing and optimizing the conjugation of HA to model enzymes, the HA-acetal derivative was used to prepare a potential therapeutic conjugate with insulin and the conjugates were studied *in vivo*.

In this case two HA-acetal batches, differing in the degree of acetal substitution (4 and 21%), were used to investigate also the effect of protein loading. The percentages of aldehyde groups in the HA backbone correlates, although not proportionally, with the insulin loading in the final conjugates, 17.2% and 32% (w/w) for HA-N^{ter}-INS 1 and HA-N^{ter}-INS 2, respectively, thus suggesting a steric hindrance between the polypeptide chains.

Unexpectedly, the *in vivo* study in diabetic rats showed that HA-N^{ter}-INS 1 had an enhanced hypoglycemic activity with respect to HA-N^{ter}-INS 2. In fact, in the case of HA-N^{ter}-INS 1 the lowering effect on blood glucose level was maintained for more than 6 h when, in comparison, insulin exhausted its effect after 1 h.

Conversely, HA-N^{ter}-INS 2 was even less effective than insulin. Probably the higher insulin loading of HA-N^{ter}-INS 2 led to a steric entanglement affecting the receptor/protein recognition. On the other hand, it is also possible that the greater loading, which yielded a bigger conjugates, hampered the diffusion from the site of *s.c.* injection to the blood stream, although the glucose level was monitored for up to 24 h.

Nevertheless, the prolonged and enhanced hypoglycemic effect of the HA-N^{ter}-INS 1 conjugate, compared to free insulin, revealed that HA conjugation is able to modify the PK profile of proteins by increasing their half-lives and behaving as a depot system. All these findings encourage the use of HAylation technology for the conjugation of proteins of pharmaceutical interest.

Summarizing, HAylation performed with this new HA-acetal derivative demonstrated great potentialities of development for protein delivery. The obtainment of a stable and easily characterizable HA derivative, suitable for site selective protein conjugation, was a prerequisite for the full exploitation of the biodegradability advantage of HA over the most known polymer in the field of protein conjugation, PEG (Mero, et al., 2013). The biodegradability of HA backbone, preserved after protein conjugation, should represent one of the main strength of HA because it allows the release of protein conjugates with short HA oligomers *in vivo*, thus allowing at least a partial recovery of protein activity. On other hand, it should be highlighted that HAylation might not be

suitable for immunogenic proteins because, for the same reason reported above, it would not offer a complete shielding of immunogenic sites over the time.

In conclusion, the results presented in this study warrant further broader and thoroughly investigations of HAylation. Other *in vivo* studies on HA conjugation with therapeutic proteins are presently under way in our lab for thoroughly investigating the *in vivo* behavior of several conjugates.

As mentioned above, closely to proteins, oligonucleotides are very promising molecules as future therapeutics. They are mainly involved in the treatment of tumour and genetic diseases.

Likewise to proteins, the therapeutic use of oligos has some limitations. First of all the short plasma clearance. Other oligos characteristic can also limit their administration, for example the strong and marked negative charge, the long sequences and the difficulty to reach the site of action (the nucleus). In order to overcome these restrictions, once again DDSs can be helpful, enhancing for example nucleus targeting and acting as a protecting-shield from circulating enzyme degradation (Merdan et al., 2002; Ruponen et al., 2003).

Particular cases of short oligomers are represented by aptamers and ribozymes. They consist mainly of RNA but their functions are strongly related to proteins. Instead to represent, as usual, a copy of DNA responsible for the synthesis of proteins, they act as an antibody and an enzyme, respectively.

The most known aptamer is MACUGEN® (pegaptanib), already in the market since the 2004, an anti-VEGF PEGylated aptamer (FDA P04-110; EMA/671614/2010; EMEA/H/C/000620), used in the treatment of new vascular age-related macular degeneration (Ng et al., 2006). In fact, as mentioned for PEG-proteins, PEGylation of oligomers has shown several advantages: prolonged half-life, low enzyme degradation, less administration frequency (Zhao et. al., 2005; Zhao et. al., 2007).

In the second section of this work it was investigated an innovative PEGylation strategy for oligo sequences, based on an enzymatic-mediated conjugation. Differently from many classical conjugation syntheses, the enzymatic ligation is conduct in mild condition, always maintaining a stable environment for the oligomers.

PEG is available with many functionalizations, but no function is useful for our purpose to enzymatically join the polymer to an oligo. To overcome this issue, a short DNA sequence was attached to the polymer. The DNA sequence was modified with an -SH reactive group in 5'-ending and thiol-reactive PEGs ($mPEG_{20kDa}$-Mal and $mPEG_{20kDa}$-OPSS) were used for the linkage.

The selected enzyme was T4 DNA ligase, which catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA. This enzyme is able to join blunt or cohesive

end termini as well as repair single stranded nicks in duplex DNA, RNA or DNA/RNA hybrids (Engler and Richardson, 1982).

To study the enzymatic PEGylation, a ligation model was appositely design, composed of four reference DNA sequences (Table I.I; **a** 18-mer, **a'** 21-mer, **m** 19-mer, and **m'** 16-mer). As required by the enzyme, the four oligos match in two complementary pairs (**A**, **a** + **a'**; $\mathbf{M_F}$, **m** + **m'**), in this case both ending with sticky-ends complementary in turn (AAT and TTA, respectively). In particular, **a** was modified at the 5'-termini with a thiol group for PEGylation.

As expected, **PEG-a** synthesis was easy and fast and purification by RP-HPLC gives good yield, always around 80%.

After verifying the correct functionality of ligase (on $\mathbf{A_FM_F}$), some modifications to classic protocols allowed to obtain excellent results with the PEGylated oligo: complete **PEG-AM$_F$** ligation and absence of undesired products.

Ligation products were resolved by DNA-PAGE in native and denaturing condition, showing for **PEG-AM$_F$** a characteristic fluorescent band at the base of the walls. This is explained by the very low electrophoretic mobility of the PEGylated sequences and the 6-FAM modified **m'**. In the denaturing migration, it was expected to find two different bands for **PEG-AM$_F$**: a non-fluorescent band for **PEG-am** and a fluorescent one for **a'm'**. In fact, generally denaturing condition separates a double-strand sequence in the two complementary homologues. Despite 7M urea condition, no difference with native electrophoretic migration were shown, proving a strong stabilization of the double-stranded sequence by PEGylation.

PEG-AM$_F$ ideal conditions were then defined monitoring the reaction during the time at different temperatures. Despite the short length and the consequently low melting temperature, the best results were obtained at 21°C for 1 hour.

To further confirm the effective ligation, the PEGylated and ligated sequence was restricted with EcoRI enzyme. Indeed, the EcoRI consensus sequence (GAATTC) was formed only after ligation. In particular, reversible conjugate **PEG-SS-a** was prepared for this experiment with the aim to avoid potential steric entanglement by the PEG chain attached to the oligo. After ligation, in a part of the mixture the polymer was removed by DTT treatment. As expected, EcoRI was active on the reference $\mathbf{A_FM_F}$ and the reduced **PEG-SS-AM$_F$** (**AM$_F$**), but interestingly the enzyme was able to recognize and cleave its specific substrate also in the PEGylated form (**PEG-SS-AM$_F$**). This evidence further confirms that a PEG chain attached to the reference model does not modify its susceptibility to enzymatic reaction.

Furthermore, it has been important to verify if the DNA ligase activity could be affected by PEGylated oligo with a reduced number of bases. Thus, the PEGylated portion **A** was been reduced by half into **B** (**b** 9-mer and **b'** 12-mer).

As described for **a**, also **b** was modified with a thiol group in 5'-ending position and exploited for polymer conjugation. Even with a so short PEGylated sequence the model was efficiently ligated, resulting in complete **PEG-BM$_F$**.

Summarizing, a model of enzymatic PEGylation for double-stranded DNA sequence was optimized, involving T4 DNA ligase enzyme. This model was well characterized and standardized, so potentially applied to every DNA sequence (but appositely modified for complementary sticky-ends). This method can be considered an innovative approach in PEGylation, because of the enzymatic mediated coupling and the potential reversibility of the process, with the possibility to remove the polymer if it is necessary (by chemical reduction of the disulfide bond or enzymatically). Moreover, it was proved that PEGylation strongly stabilized the ds-sequences even in strong denaturing condition.

Furthermore, a PEGylated 9-mer was chosen in order to better investigate if T4 DNA ligase could also works closely to the site of PEGylation, confirming that it was still active. At the same time, a short PEGylated oligo means a cost reduction.

This easy method, with the improved characteristics of PEGylated DNA sequences, offers new possibilities in the use of oligos in diagnostic and therapeutic field.

A future proposal for this approach is the substitution of DNA ligase with RNA ligase, in order to involve also single-strand oligo and enrich the applications.

6 REFERENCES

Abuchowski, A., McCoy, J. R., Palczuk, N. C., van Es, T., Davis, F. F. (1977). Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.* 252, 3582-3586.

Abuchowski, A., van Es, T., Palczuk, N. C., Davis, F. F. (1997). Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J. Biol. Chem.* 252, 3578-3581.

Akbarzadeh, A., Norouzian, D., Mehrabi, M. R., Jamshidi, S. H., Farhangi, A., Allah Verdi, A., Mofidian, S. M. A., Lame Rad, B. (2007). Induction of diabetes by streptozotocin. *Indian Journal of Clinical Biochemistry, 22*, 60–64.

Almond, A. (2007). Hyaluronan. *Cell. Mol. Life Sci. 64,* 1591–1596.

Armstrong, J. K., Hempel, G., Koling, S., Chan, L. S. (2007). Antibody against poly-(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer 110,* 103–111.

Bailey, J. F. E., Koleske, J. V. (1976). Poly(etylene-oxide), *Academic Press New York*.

Balazs, E. A. (1983). Sodium hyaluronate and viscosurgery. Healon (sodium hyaluronate). A guide to its use in ophthalmic surgery. *Miller D., and Stegmann R., Eds. Wiley*. New York.

Balazs, E. A., Denlinger J. L. (1993). Viscosupplementation: a new concept in the treatment of osteoarthritis. *J. Rheumatol. 20*, 3-9.

Balazs, E. A., Laurent, T. C. (1998). The chemistry, biology and medical applications of hyaluronan and its derivatives. In T.C. Laurent, (Ed.), *Round table discussion: New applications for hyaluronan* (pp 325–326). Portland Press, London.

Bandaru, R., Zhao, J., Richards, M., Wu, D., Xia, J., Zhao, H., Dai, Q., Zhang Y., Conover. C. Novel Releasable PEG-Conjugates of LNA Antisense Oligonucleotides as Potent mRNA Antagonists to Cancer Targets. *Enzon Pharmaceuticals, Inc.*, 20 Kingsbridge Road, Piscataway.

Banzato, A., Bobisse, S., Rondina, M., Renier, D., Bettella, F., Esposito, G., Quintieri, L., Meléndez-Alafort, L., Mazzi, U., Zanovello, P., Rosato, A. (2008). A paclitaxel-hyaluronan bioconjugate targeting ovarian cancer affords a potent in vivo therapeutic activity. *Clin Cancer Res. 14,* 3598–3606.

Besheer, A., Hertel, T. C., Kressler, J., Mäder, K., Pietzsch, M. (2009). Enzymatically catalyzed HES conjugation using microbial transglutaminase: Proof of feasibility. J. *Pharm. Sci. 98,* 4420–4428.

Bhadra, D., Bhadra, S., Jain, P., Jain, N. K. (2002). Pegnology: a review of PEG-ylated systems, *Pharmazie 57,* 5-29.

Cavallaro, G., Pitarresi, G., Licciardi, M., Giammona, G. (2001). Polymeric prodrug for release of an antitumoral agent by specific enzymes. *Bioconjugate Chem., 12(2)*, 143-151.

Cheson, B. D. (2007). Oblimersen for the treatment of patients with chronic lymphocytic leukemia. *Ther. Clin. Risk. Manag.* 3(5), 855-870.

Convention on Biological Diversity (1992), United Nations.

D'Este, M., Pasut, G., Renier, D., Rosato, A. (2010). Process for the synthesis of conjugates of glycosaminoglycanes (GAG) with biologically active molecules, polymeric conjugates and relative uses thereof. WO 2010/145821.

Davis, F. (1992). The origin of PEGnology, *Adv. Drug Deliv. Rev.* 54, 457-458.

Delgado, C., Francis, G.E., Fisher, D. (1992). The uses and properties of PEG-linked proteins, *Crit. Rev. Ther. Drug Carrier Syst. 9,* 249-304.

Dreborg, S., Akerblom, E. B. (1990). Immunotherapy with mono-methoxypolyethylene glycol modified allergens. *Crit. Rev. Ther. Drug Carrier Syst. 6(4)*, 315-365.

Duncan, R. (1997), Polymer Therapeutics for tumor specific delivery. *Chemistry & Industry, 7*, 262-264.

Duncan, R. (2006). Polymer conjugates as anticancer nanomedicines. *Nature Rev. Cancer 6,* 688-701.

Duncan, R., Dimitrijevic, S. and Evagorou, E. G., (1996). The role of polymer conjugates in the diagnosis and treatment of cancer. *STP Pharma Sciences, 6*, 237-263.

Duncan, R., Gilbert, H. R. P., Carbajo, R. J., Vicent, M. J. (2008). Polymer masked–unmasked protein therapy. 1. Bioresponsive dextrin–trypsin and –MSH conjugates designed for alpha-amylase activation. *Biomacromolecules, 9,* 1146–1154.

Duncan, R., Hume, I. C., Yardley, H. J., Flanagan, A., Ulbrich, K., Subr, U., Strohalm, J. (1991). Macromolecular prodrugs for use in target cancer chemotherapy: melphalan covalently coupled to N-(2-Hydroxypropyl) methacrylamide copolymers. *J. Controlled Release, 16*, 121-136.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature 411*(6836), 494-498.

EMA/671614/2010; EMEA/H/C/000620; EMEA WC500026218.

Engler, M. J. and Richardson, C. C. (1982). The Enzymes (Boyer, P. D., ed.) Vol. 5, p. 3, *Academic Press*, San Diego.

FDA N021756 (2004). Approves New Drug Treatment for Age-Related Macular Degeneration.

Ferguson, E. L., Duncan, R. (2009). Dextrin-phospholipase A2: synthesis and evaluation as a novel bioresponsive anticancer conjugate. *Biomacromolecules*, *10,* 1358-1364.

Gaertner, H. F., Puigserver, A. J. (1992). Increased activity and stability of poly(ethylene glycol)-modified trypsin. *Enzyme Microb. Technol. 14,* 150-155.

Gregoriadis, G., Jain, S., Papaioannou, I., Laing, P. (2005). Improving the therapeutic efficacy of peptides and proteins: A role for polysialic acids. *Int. J. Pharm. 300,* 125-130.

Hamilton, A. J., Baulcombe, D. C., (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science 286*(5441), 950-952.

Hardwicke, J., Ferguson, E. L., Moseley, R., Stephens, P., Thomas, D., Duncan, R. (2008) Dextrin–rhEGF conjugates as bioresponsive nanomedicines for wound repair. *J. Control. Release, 130,* 275-283.

Harris, J. M., Chess, R. B. (2003). Effect of PEGylation on pharmaceuticals. *Nat. Rev. Drug Discov. 2,* 214–221.

Hoes, C. J. T., Feijen, J. (1989). The application of drug-polymer conjugates in chemotherapy. *Drug Carrier Systems,* 9, 57-109.

Homma, A., Sato, H., Okamachi, A., Emura, T., Ishizawa, T., Kato, T., Matsuura, T., Sato, S., Tamura, T., Higuchi, Y., Watanabe, T., Kitamura, H., Asanuma, K., Yamazaki, T., Ikemi, M., Kitagawa, H., Morikawa, T., Ikeya, H., Maeda, K., Takahashi, K., Nohmi, K., Izutani, N., Kanda, M., Suzuki, R. (2009). Novel hyaluronic acid–methotrexate conjugates for osteoarthritis treatment. *Bioorg. Med. Chem. 17,* 4647-4656.

Hu, J., Sebald, W. (2011). N-terminal specificity of PEGylation of human bone morphogenetic protein-2 at acidic pH. *Int. J. Pharm. 413,* 140-146.

Hummel, B. C. (1959). A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. *Can. J. Biochem. Physiol. 37,* 1393-1399.

Jayant K., Tamara M., 2006. Polymer-drug conjugate: Progress in polymeric prodrug. *Prog. Polym. Sci. 31*, 359-397.

Junod, A., Lambert, A. E., Orci, L., Pictet, R., Gonet, A. E., Renold, A. E. (1967). Studies of the diabetogenic action of streptozotocin. *Proc. Soc. Exp. Biol. Med. 126,* 201–205.

Langer R., Vacanti J. P. (1993), Tissue engineering. *Science, 260*, 920-926.

Laurent T. C. (1998). The chemistry, biology and medical applications of hyaluronan and its derivatives. *Portland Press, Wenner-Gren intern.*, London.

Laurent, T. C., Fraser, J. R. E. (1992). Hyaluronan. *FASEB J. 6,* 2397–2404.

Leader, B., Baca, Q. J., Golan, E. D. (2008). Protein therapeutics: a summary and pharmacological classification. *Nature Reviews Drug Discovery 7*, 21-39.

Luo, Y., Bernshaw, N. J., Lu, Z. R., Kopecek, J., Prestwich, G. D. (2002). Targeted delivery of doxorubicin by HPMA copolymer-hyaluronan bioconjugates. *Pharm. Res. 19*, 396-402.

Luo, Y., Ziebell M. R., Prestwich G. D. (2000). A hyaluronic acid-taxol antitumor bioconjugate targeted to cancer cells. *Biomacromolecules, 1*, 208-218.

Maeda, H., Seymour, L. W., Miyamoto, Y. (1992). Conjugates of anticancer agents and polymers: advantage of macromolecular therapeutics in vivo. *Bioconjug. Chem. 3*, 351-362.

Maggon, K. (2007). R&D paradigm shift and billion-dollar biologics. S.C. Gad (Ed.), *Handbook of Pharmaceutical and Biotechnology*, Wiley, New York, 161-198.

Merdan, T., Kopecek, J., Kissel, T. (2002). Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Advanced Drug Delivery Reviews 54(5)*, 715-758.

Mero, A., Fang, Z., Pasut, G., Veronese, F. M., Viegas, T. X. (2012). Selective conjugation of poly(2-ethyl 2-oxazoline) to granulocyte colony stimulating factor. *J Control. Release, 159,* 353-361.

Mero, A., Pasqualin, M., Campisi, M., Renier, D., Pasut, G. (2013). Conjugation of hyaluronan to proteins. *Carbohydrate Polymers 92*, 2163-2170.

Mero, A., Pasut, G., Dalla Via, L., Fijten, M. W., Schubert, U. S., Hoogenboom, R., Veronese, F.M. (2008). Synthesis and characterization of poly(2-ethyl 2-oxazoline)-conjugates with proteins and drugs: suitable alternatives to PEG-conjugates? *J. Control. Release 125,* 87-95.

Mero, A., Veronese, F. M. (2008). The impact of PEGylation on biological therapies. *BioDrugs 22,* 315–329.

Motokawa K., Hahn S. K., Kim S. J., Hahn S. K., Kim M. J., Kim D. H., and Lee Y. P. (2005). Development of a novel sustained release formulation of recombinant human growth hormone using sodium hyaluronate microparticles. *J. Controlled Release 104*, 323-335.

Mutter, M., Bayer, E. (1979). The peptides. *Academic Press*, New York, 285-332.

Muzzarelli, R. A. A., Greco, F., Busilacchi, A., Sollazzo, V., Gigante, A. (2012). Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regeneration: a review. *Carbohydrate Polymers 89*, 723-739.

Nakamura, T., Kato, T., Togawa, H., Yasugi, K., Konishi, H., Sekimori, Y. (2006). Conjugate of water-soluble modified hyaluronic acid and GLP-1 analogue. W02006/095775.

Ng, E. W. M., Shima, D. T., Calias, P., Cunningham, E. T., Guyer, Jr. D. R., Adamis A. P. (2006). Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nature Reviews Drug Discovery 5*, 123-132.

Ohri, R., Hahn, S. K., Stayton, P. S., Hoffman, A. S., Giachelli, M. (2004). Hyaluronic acid grafting mitigates calcification of glutaraldhyde-fixed bovine pericardium. *J. Biomed. Mater. Res. 70A*, 159-165.

Palumbo, F. S., Pitarresi, G., Fiorica, C., Matricardi, P., Albanese, G., Giammona, G. (2012) In situ forming hydrogels of new amino hyaluronic acid/benzoyl-cysteine derivatives as potential scaffolds for cartilage regeneration. *Soft Matter 8*, 4918-4924.

Pasut, G., Mero, A., Caboi, F., Scaramuzza, S., Sollai, L., Veronese, F. M. (2008). A new PEG-beta-alanine active derivative for releasable protein conjugation. *Bioconjug. Chem. 19,* 2427-2431.

Pasut, G., Veronese, F. M. (2007). Polymer-drug conjugation, recent achievements and general strategies. *Prog. Polym. Sci. 32,* 933-961.

Pasut, G., Veronese, F. M. (2012). State of the art in PEGylation: The great versatility achieved after forty years of research. *J. Control. Release 161,* 461-472.

Pitarresi, G., Palumbo, F. S., Albanese, A., Fiorica, C., Picone, P., Giammona, G. (2010) Self assembled amphiphilic hyaluronic acid graft copolymers for targeted release of antitumoral drug. *Journal of Drug Targeting 18*, 264-276.

Powell, G. M. (1980). Polyethylene glycol, *Handbook of water soluble gums and resins* (R.L. Davidson, Ed.), pp 18-1-18-31, McGraw-Hill, New York.

Putnam, D., Kopecek, J. (1995). Polymer conjugates with anticancer activity. *Adv. Polym. Sci. 122*, 55-123.

Ringsdorf, H. (1975). Structure and properties of pharmacologically active polymers. *J. Polym. Sci. Polym. Symp. 51*, 135-153.

Ruponen, M., Honkakoski, P., Rönkkö, S., Pelkonen, J., Tammi, M., Urtti. A. (2003). Extracellular and intracellular barriers in non-viral gene delivery. *Journal of Controlled Release, 93(2)*, 213-217.

Saravanakumar, G., Choi, K. Y., Yoon, H. Y., Kim, K., Park, J. H., Kwon, I. C., Park, K. (2010). Hydrotropic hyaluronic acid conjugates: Synthesis, characterization, and implications as a carrier of paclitaxel. *Int. J. Pharm. 394,* 154-161.

Shen, W. C., Ryser, H. J. P. (1981). Cys-aconityl spacer between daunomycin and macromolecular carriers: a model of pH sensitive linkage releasing drug from a lysosomotropic conjugate. *Biochem. Biophys. Res. Commun.* 102, 1048-1054.

Sherman, M. R., Saifer, M. G., Perez-Ruiz, F. (2008). PEGuricase in the management of treatment-resistant gout and hyperuricemia. *Adv. Drug Delivery Rev. 6,* 59-68.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem. 150,* 76-85.

Soyez, H., Schacht, E., Vanderkerken, S. (1996). The crucial role of spacer groups in macromolecular prodrug design. *Adv. Drig Del. Rev 21*, 81-106.

Suh, H. (2000). Tissue restoration, tissue engineering and regenerative medicine. *Yonsei Med. J. 41(6)*, 681-684.

Terada, S., Sato, M., Sevy, A., Vacanti, J. P. (2000). Tissue engineering in the twenty-first century. *Yonsei Med. J. 41(6)*, 685-91.

Torchilin, V. P., Maksimenko, A. V., Mazaev, A. V. (1988). Immobilized enzymes for thrombolytic therapy. *Methods Enzymol. 137,* 552-566.

Veronese, F. M., Mero, A., Caboi, F., Sergi, M., Marongiu, C., Pasut, G. (2007). Site-specific pegylation of G-CSF by reversible denaturation. *Bioconjug. Chem. 18,* 1824-1830.

Veronese, F. M., Morpurgo, M., (1999). Bioconjugation in pharmaceutical chemistry. *Il Farmaco* 54, 497-516.

Veronese, F. M., Pasut, G., Guiotto, A. (2004). Protein, peptide and non-peptide drug PEGylation for therapeutic application, *Expert Opin. Therap. Patents* 14, 859-894.

Viegas, T. X., Bentley, M. D., Harris, J. M., Fang, Z., Yoon, K., Dizman, B., Weimer, R., Mero, A., Pasut, G., Veronese, F. M. (2011). Polyoxazoline: chemistry, properties, and applications in drug delivery. *Bioconjug. Chem. 22,* 976-986.

Walsh, G. (2003). Pharmaceutical biotechnology product approved whitin the European Union. *Eur. J. Pharm. Biopharm*. *55*, 3-10.

Wong, S. S. (1991). Reactive groups of proteins and their modifying agents. Chemistry of protein conjugation and cross-linking. *Wong, S. S. Ed.*, CRC, Boston.

Yamaoka,T., Tabata, Y., Ikada, Y. (1994). Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice, *J. Pharm. Sci. 83(4)*, 601-606.

Zalipsky, S. (1995). Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates, *Bioconjugate Chem. 6,* 150-165.

Zhao, H., Greenwald, R. B., Reddy, P., Xia, J., Peng, P. (2005). A new platform for oligonucleotide delivery utilizing the PEG prodrug approach. *Bioconjugate Chem*, 16(4), 758-766.

Zhao, H., Peng, P., Longley, C., Zhang, Y., Borowski, V., Mehlig, M., Reddy, P., Xia, J., Borchard, G., Lipman, J., Benimetskaya, L., Stein, C. A. (2007). Delivery of G3139 using releasable PEG-linkers: impact on pharmacokinetic profile and anti-tumor efficacy. *J. Control. Release*, *119*, 143-152.

Zwilling, R., Neurath, H. (1981). Invertebrate protease. *Methods Enzymol. 80,* 633-664.