

UNIVERSITÀ
DEGLI STUDI
DI PADOVA

UNIVERSITA' DEGLI STUDI DI PADOVA

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Interdepartmental Centre of Research and Service for Biology
and Regenerative Medicine**

**DOCTORAL RESEARCH SCHOOL OF REGENERATIVE BIOLOGY
AND MEDICINE
CURRICULUM IN TISSUE AND TRANSPLANT ENGINEERING**

XXII CYCLE

**Study and characterization of drug delivery systems
in regenerative medicine**

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Alla mia famiglia

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1. Symbols and Abbreviations

A Absorbance
AA amino acid
ACN acetonitrile
API Atmospheric Pressure Ionization
APS ammonium sulfate
ATP adenosine triphosphate
AUC Area Under the Curve
BMP Bone Morphogenic Protein
BSA Bovine Serum Albumins
° C Degree Celsius
C₀Initial concentration
CD Circular dichroism
Cl clearance
C_t concentration at time t
CURL Compartment for Uncoupling Ligand and Receptor
d density
D dose
Da Dalton
DAPI 4',6-diamidino-2-phenylindole
DCC *N,N'*-Dicyclohexylcarbodiimide
DMEM Dulbecco's Modified Eagle's Medium
DMF *N,N*-dimethylformamide
DMSO dimethylsulfoxide
DNA Deoxyribonucleic Acid (deoxyribonucleic acid)
DTT Dithiothreitol
ECM Extra Cellular Matrix
EDTA ethylenediaminetetraacetic acid
EPI epirubicin
EPR Enhanced Permeability and Retention Effect
ESI Electron Spray Ionization

FBS Fetal Bovine Serum
FDA Food and Drug Administration
FDMEM Folate-deficient Dulbecco's Modified Eagle's Medium
FITC fluorescein isothiocyanate
FR Folate Receptor
G-CSF Granulocyte Colony-Stimulating Factor
GH Growth Hormone
GPC Gel Permeation Chromatography
GPI glycosyl-phosphatidyl-inositol
HEPES 4 - (2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV Human Immunodeficiency Virus
HPMA poly-hydroxypropyl-methacrylamide
HUS hemolytic uremic syndrome
IC50 50% Inhibition Concentration
IFN Interferon
IMAC Metal Ion Affinity Chromathography
IPTG Isopropyl- β tiogalattopiranoside
J coupling constant
Kd Dissociation constant
ke elimination rate constant
LB Land of Luria Bertoni
LHRH Luteinizing Hormone-Releasing Hormone
LSB Laemmli Sample Buffer
MeCN Acetonitrile
Mn nominal molecular weight
mPEG- monometossi poly-(ethylene glycol)
mQ deionized water and filtered through Millipore filter ®
MSC Mesenchymal Stem Cells
MTT 3 - (4,5-Dimethylthiazole-2-yl) -2,5-diphenyl-tetrazolium bromide
M-VAC methotrexate, vinblastine, doxorubicin, cisplatin
Mw Molecular weight
NADH nicotinamide adenindinucleotide
NADPH nicotinamide adenindinucleotide phosphate

NBS Neonatal Bovine Serum
NHS *N*-Hydroxysuccinimide
NMR Nuclear Magnetic Resonance
NSCLC Non-Small Cell Lung Cancer Nitrilo triacetic acid,
NTA OC Osteocalcin
OD Optical Density
OP-1 Osteogenic Protein 1
OPN Osteopontin
PBS Phosphate Buffer Solution
PCR Polymerase Chain Reaction
PEG-poly (ethylene glycol)
pI Isoelectric point Acid,
PLGA poly (lactic-co-glycolic acid)
ppm parts per million
PTD Protein Trasduction Domain
RES reticuloendothelial system
RGD Argininina-Glycine-Aspartic
RI Refraction Index (refractive index)
Ribonucleic Acid RNA (ribonucleic acid)
RP-HPLC Reverse Phase-High Performance Liquid Chromatography
RPM Revolutions Per Minute
SCID Severe Combined Immuno Deficiency
SCLC Small Cell Lung Cancer
SDS sodium dodecyl sulphate
SMA styrene-maleic anhydride
SMANCS styrene-maleic anhydride-neocarcinostatina
t time $t_{1/2}$ half-life
TEA triethylamine
TEMED N, N, N', N'-Tetramethyl-ethylenediamine
TFA trifluoroacetic acid
TNBS acid 2,4,6-trinitro-benzenesulfonic
TNF Tumor Necrosis Factor
TOF Time of Flight Time of Flight

Tr retention time

TRIS tris-(hydroxymethyl)-aminomethane

UCBMSC Umbilical Cord Blood Mesenchymal Stem Cells

UTP uridine triphosphate

UV Ultraviolet

VD apparent volume of distribution

VEGF Vascular Endothelial Growth Factor

VPF Vascular Permeability Factor

α -MEM Minimum Essential Medium

\emptyset diameter

AMINOACID

Aspartic acid	Asp	D	Histidine	His	H
Glutamic acid	Glu	E	Leucine	Leu	L
Alanine	Ala	A	Lysine	Lys	K
Arginine	Arg	R	Methionine	Met	M
Asparagine	Asn	N	Proline	Pro	P
Cysteine	Cys	C	Serine	Ser	S
Phenylalanine	Phe	F	Tryptophan	Trp	W
Glycine	Gly	G	Tyrosine	Tyr	Y
Glutamine	Gln	Q	Threonine	Thr	T
Isoleucine	Ile	I	Valine	Val	V

2.Abstract

Drug delivery system (DDS) technology is particularly promising to improve the *in vivo* efficiency of active molecules. Moreover, it is possible to stabilize and prolong the half life at biologically active molecules, thus prolonging the *in vitro* activity. DDS's can be used either for the delivery of anticancer drug but also for the controlled release of growth factors essential for the tissue engineering. In this work DDS's have been investigate to develop new targeted anticancer drug or to control the release of growth factor for tissue engineering. In the first case polymer conjugates were chosen while in the second microspheres were used.

PEG conjugates

Anti-cancer drugs are very active molecules but they present limits that often prevent their success in chemotherapy. Common problems are a low half-life due to rapid kidney clearance, rapid inactivation by metabolic enzymes and low selectivity towards cancer cells and often a low solubility in water, these causing severe side effects.

To overcome this problems, polymeric conjugates were prepared by linking an anti-cancer drug to a polymer carrier. Polymeric conjugation improves the drug pharmacokinetic profiles by reducing drug clearance. Furthermore, a tumor targeting can be reached by two mechanisms: the first a passive accumulation into tumour tissue, known as the EPR (Enhanced Permeability and Retention) effect and the second an active targeting when a targeting molecule also is coupled to the conjugate. In this work an heterobifunctional poly-(ethylene glycol) was coupled to both epirubicin (EPI), an anti-cancer drug, and to folic acid (FOL) as targeting residue. The biological activity of the derivate FOL-PEG-EPI was studied in two different culture systems; the classic bi-dimensional (2D) system and the tri-dimensional (3D) system using Puramatrix hydrogel. The last should recreate an environment similar to the *in vivo* situation. The cytotoxicity activity studies were carried out on the following cell lines; HT-29, expressing a normal level of folic acid membrane receptor (FR), MCF-7, medium FR expression and KB-31cells over-expressing FR. The FOL-PEG-EPI cytotoxicity study, showed higher toxicity in KB-31 cells than MCF-7 and HT-29 in both 2D and 3D cell culture systems. Moreover, the use of the 3D culture system, displayed clearly that FOL-PEG-EPI had selective activity on cells over-expressing the folic acid receptor (KB-

31) compared to HT-29 cells where to obtain the IC_{50} was used a conjugate concentration 3 fold higher than the maximum one permitted in clinic. The uptake of conjugates and epirubicin were studied by flow cytometry, and confocal microscopy. In the first case the cytometry showed the fluorescence signal inside the cells for both FOL-PEG-EPI and epirubicin alone. Also, the confocal analysis have confirmed the internalization, displayed the epirubicin in the nuclei and the conjugate in perinuclear side.

Microspheres

Tissue engineering is based on various disciplines such as medicine, biology, engineering and chemistry fused to the common aim to obtain or replace organs, or parts of organs in the human body. In general, a construct of tissue engineering is formed by the cellular component and a basic structure with function of support. The cells need to be stimulate by proper growth factor to generate a functional tissue. When the solution of growth factors is injected into the site for regeneration, the biological effect is not always optimal, because the biologically active substances are spread away from the site of action very quickly, or because they cannot get to the targeting site. It is therefore essential to develop a technology that stabilizes the administration and the answer can be a DDS.

Therefore, the second part of this study was focused on tissue engineering of bone tissue. The research involved the use a the drug delivery system for the controlled release of TAT-OP1 protein, which stimulates osteogenic differentiation. Osteogenic protein-1 (OP-1 or BMP-7) is a member of Bone Morphogenic Proteins's family (BMPs). BMPs are a group of multi-functional growth factors belonging to the transforming growth factor β (TGF- β) superfamily. They are implicated in a variety of functions such as the formation of cartilage and bone, and the development of non-osteogenic tissues. BMPs are secreted as a precursor approximately four times longer than the mature form and share a C-terminal distinctive pattern (..C...CXGXC...CC...CXCX..) containing seven cysteines which are the active region of the proteins. In this study we used a recombinant fusion protein called TAT-OP1 which includes a TAT sequence, an Arg rich peptide, derived from a HIV protein, which allows the internalization. The construct TAT-OP1 has 162 amino acids starting with an N-terminal 6His-tag followed by the TAT sequence, a peptidase specific cleavage site (spanning 6 AA) and the C-terminal OP-1 domain (126 AA) containing the cysteines motif. When this type of

bioactive molecules is injected directly on the action site, it undergo a rapid inactivation and dilution effect, therefore this is the limit for *in vivo* use. To avoid this problem, the protein was encapsulated in polylactidecoglycolide (PLGA) microspheres. The bioactive molecules released from microspheres can be easily modulated by setting the formulation parameters and production technique. The spray drying technique was used to obtain the TAT-OP1 microspheres. The microspheres release of the TAT-OP1 over a period of 7 days was 98% and the encapsulation efficiency was 35%. The size measurement by SEM was 0.2-2 μm . The biological activity study on TAT-OP1 microspheres was conducted using pre-osteoblast MC3T3-E1 cell line at two different concentrations, 200nM and 27 nM. After the 7 and 14 days of treatment the cultures showed matrix mineralization and the assay testing for the alkaline phosphatase was positive. Also the presence of characteristic osteogenic markers, such as osteopontin and osteocalcin, was verified by immunofluorescence. These positive results led us to evaluate the biological activity of TAT-OP1 microspheres in a tri-dimensional culture system on cells isolated from umbilical cord blood (UCBMSC). The 3D model was made by using synthetic PuramatrixTM Hydrogel which is able to mimic the natural microenvironment.

Following encapsulation of the TAT-OP1 microspheres or the free TAT-OP1 into Puramatrix HydrogelTM, the cellular response to TAT-OP1 stimulation was evaluated using transmission electron microscopy (TEM) analysis to detect the production of bone-matrix. After 27 days of stimulation with TAT-OP1 loaded microspheres(200 nM), partially aggregated microfibrils were observed around the cells. Calcification deposit and hydroxyapatite crystals were detected only in the cultures treated with TAT-OP1 PLGA microspheres (200nM) controlled release system. Therefore the controlled release of TAT-OP1 from PLGA microspheres was verified to increase the stimulation effectiveness. Future investigations will be directed to further confirm the suitability of this approach to improve the *in vitro* osteogenic differentiation and the biological activity of TAT-OP1 for an eventual clinical application in the field of bone tissue engineering.

2.Riassunto

I sistemi di *drug delivery* (DDSs) rappresentano una tecnologia particolarmente promettente per migliorare l'efficacia *in vivo* e *in vitro* di molecole biologicamente attive con l'obiettivo di circoscriverne l'effetto su una determinata tipologia di cellule, migliorarne l'efficacia, prolungarne il periodo di emivita e ridurre la tossicità di una terapia.

In questo lavoro sono stati studiati due modelli di Drug Delivery: il primo riguarda lo sviluppo di nuovi farmaci antitumorali selettivi mediante un coniugato polimerico, mentre il secondo modello, che trova applicazione nell'ambito dell'ingegneria tissutale, riguarda il rilascio controllato di fattori di crescita mediante microsfele.

PEG coniugato

I problemi più comuni riguardanti i farmaci anti-tumorali possono essere dovuti ad un tempo di emivita basso a causa di *clearance* renale rapida, all'inattivazione rapida da parte di enzimi, alla scarsa selettività cellulare e spesso ad una scarsa solubilità in ambiente fisiologico, oltre a gravi effetti collaterali.

Per cercare di ovviare, almeno in parte, a questi problemi, è stato preparato un coniugato polimerico direzionato al quale è stato legato un farmaco anti-cancro. Il coniugato migliora il profilo farmacocinetico del farmaco riducendo la *clearance*. Il "selective tumor targeting" può essere attivo o passivo. Il primo riguarda ligandi di recettori associati al tumore, che raggiungono il bersaglio sfruttando l'affinità ligando-recettore. Il secondo sistema può essere ottenuto sfruttando il

cosiddetto effetto EPR (*enhanced permeability and retention effect*) grazie al quale molecole ad alto

peso molecolare raggiungono e si accumulano nell'ambiente peritumorale.

In questo lavoro è stato utilizzato un poli-(etilenglicole) eterobifunzionale legato ad epirubicina (EPI), un farmaco anti-cancro, e ad acido folico (FOL), come residuo di *targeting*.

L'attività biologica del derivato FOL-PEG-EPI è stata studiata in due diversi sistemi di coltura, il classico sistema bi-dimensionale ed il sistema tri-dimensionale utilizzando Puramatrix hydrogelTM. Quest'ultimo dovrebbe ricreare un ambiente simile a quello *in vivo*.

Gli studi di attività citotossica sono stati effettuati sulle seguenti linee cellulari: HT-29, MCF-

7 e KB-31 che presentano una diversa espressione del recettore di membrana per l'acido folico (rispettivamente normale espressione, medio-alta, alta). Lo studio di citotossicità su FOL-PEG-EPI ha mostrato maggiore tossicità su cellule KB-31, con sovra-espressione del recettore per l'acido folico, rispetto alle cellule MCF-7 e HT-29, sia in colture 2D che 3D. Inoltre, l'utilizzo del sistema di coltura tri-dimensionale ha dimostrato che FOL-PEG-EPI possiede attività selettiva sulle cellule KB-31, rispetto alle cellule HT-29 dove per ottenere l'IC₅₀ è stata utilizzata una concentrazione di coniugato 3 volte più alta della massima utilizzabile in clinica. L'*up-take* cellulare dei coniugati ed epirubicina sono stati studiati mediante citofluorimetria e microscopia confocale. Nel primo caso, la citofluorimetria ha mostrato la presenza del segnale di fluorescenza all'interno delle cellule sia per FOL-PEG-EPI che per epirubicina. L'analisi di microscopia confocale ha confermato l'internalizzazione, localizzando in zona nucleare il farmaco libero ed in zona perinucleare il coniugato.

Microsfere

L'ingegneria dei tessuti è un campo interdisciplinare che applica i principi dell'ingegneria e delle scienze della vita allo sviluppo di sostituti biologici per ristabilire, mantenere o migliorare la funzione di tessuti e organi danneggiati. In questa ricerca si fondono discipline di biologia cellulare, ingegneria, scienza dei materiali e chirurgia allo scopo di costruire, mediante la combinazione di cellule, materiali ("*scaffold*") e fattori di crescita, nuovi tessuti funzionali.

I fattori di crescita possono essere impiegati per riprodurre le condizioni fisiologiche che consentono alle cellule di crescere, moltiplicarsi e differenziarsi nei diversi tipi di tessuti, ma la loro somministrazione rimane ancora una sfida tecnologica a causa della loro breve emivita nonché della loro difficoltà nel raggiungere il sito di *targeting*.

La seconda parte di questo studio ha riguardato lo sviluppo di un sistema di Drug Delivery applicato all'ingegneria tissutale del tessuto osseo. La ricerca ha coinvolto l'utilizzo di un sistema di veicolazione di farmaci per il rilascio controllato della proteina TAT-OP1, che stimola la differenziazione osteogenica. *Osteogenic protein-1* (OP-1 o BMP-7) è un membro della famiglia delle proteine morfogeniche dell'osso (bone morphogenic proteins, BMPs). Le BMP vengono riconosciute come fattori di crescita osteoinduttivi, ovvero promotori della formazione di nuovo tessuto osseo e appartengono alla superfamiglia del TGF- β . Le BMP

sono secreti come precursori circa quattro volte più lunghi rispetto alla forma matura e possiedono una porzione C-terminale distintiva (pattern .. C ... CXGXC ... CC ... CXCX ..) contenente sette cisteine che costituiscono la regione attiva di queste proteine.

In questo studio è stata utilizzata una proteina ricombinante di fusione chiamata TAT-OP1 che comprende una sequenza TAT, un peptide ricco di arginina derivante dall'HIV e che permette l'internalizzazione. Il costrutto TAT-OP1, di 162 aminoacidi, comprende: una porzione N-terminale 6 His-tag seguito dalla sequenza TAT, un sito di *cleavage* peptidasi-specifico (*spanning* 6 AA) e il C-terminale con il dominio OP-1 (126 AA) contenente il motivo di cisteine. Quando questo tipo di molecola bioattiva viene iniettato direttamente nel sito di azione, viene sottoposta ad inattivazione e rapida diluizione; questo ne limita l'uso *in vivo*.

Per ovviare al problema sono state impiegate microsfere di poli-lattideglicolide (PLGA) per permettere un rilascio controllato di TAT-OP1 con l'obiettivo di mantenere un livello adeguato della proteina per tempi prolungati, migliorandone l'efficienza.

Il rilascio delle molecole bioattive può essere facilmente modulato settando i parametri nella formulazione e nella tecnica di produzione. La tecnica dello *spray drying* è stata utilizzata per ottenere le microsfere con TAT-OP1. Il rilascio dalle microsfere con TAT-OP1 è stato studiato in un periodo di 7 giorni e l'efficienza di incapsulamento era risultata del 35%. Le dimensioni al microscopio a scansione elettronica (SEM) risultavano comprese tra 0,2-2 µm. Lo studio dell'attività biologica su microsfere con TAT- OP1, è stato condotto utilizzando pre-osteoblasti MC3T3-E1 a due diverse concentrazioni, 200 e 27 nM.

Dopo 7 e 14 giorni di trattamento, le cellule mostravano presenza di mineralizzazione della matrice, test per la fosfatasi alcalina positivo e presenza di caratteristici marcatori osteogenici, quali osteopontina e osteocalcina. Questi risultati positivi ci hanno portato a valutare l'attività biologica della TAT- OP1 in microsfere in un sistema tri-dimensionale utilizzando cellule staminali mesenchimali isolate dal sangue del cordone ombelicale (UCBMSC). Il modello 3D è stata ottenuto utilizzando la matrice sintetica Puramatrix hydrogelTM, che è in grado di simulare il microambiente fisiologico. A seguito dell'incapsulazione di TAT-OP1 libera o di microsfere con TAT-OP1 in Puramatrix hydrogelTM, la risposta cellulare alla stimolazione di TAT-OP1 è stata valutata grazie all'analisi di microscopia elettronica a trasmissione (TEM) per rilevare la produzione di matrice ossea. Dopo 27 giorni di stimolazione con TAT-OP1 (200 nM), si osservava la presenza di microfibrille parzialmente aggregate attorno alle cellule. Depositi di calcio e cristalli di idrossiapatite sono stati rilevati solo in culture trattate con

microsfere a rilascio controllato di TAT-OP1 (200nM). Pertanto, il rilascio controllato di TAT-OP1 da microsfere di PLGA sembra aumentare l'efficacia di stimolazione. Future indagini saranno dirette a confermare ulteriormente la capacità del presente approccio nel migliorare lo studio di differenziamento osteogenico *in vitro* e l'attività biologica della TAT-OP1 per un eventuale applicazione clinica nel campo dell'ingegneria tissutale dell'osso.

3.Introduction

3.1 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 [1]. 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 [2].

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 it 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 [1]. 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 [3]. 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 (Figure 1)

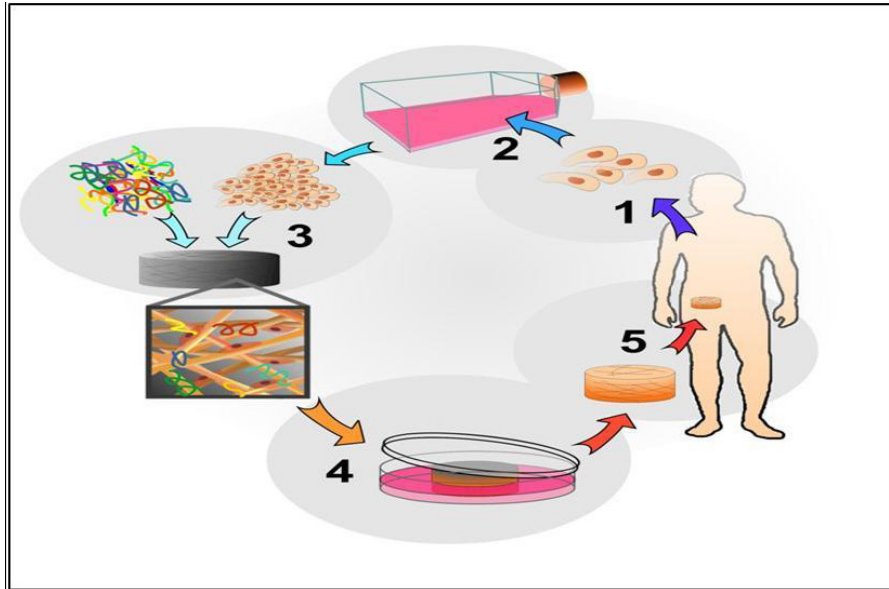


Fig. 1. Presentation outline: 1 removal of cells from the patient / donor, and 2 expansion in *in vitro* construction of the construct 3 cells / matrix / growth factors, 4 matrix in cell culture, 5 replanting of the construct in the patient.

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 which 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 which promotes tissue regeneration.

For example, the controlled release of the 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*. Other than the controlled release method, this DDS technology for half-time prolongation, absorption improvement and targeting are also applicable in tissue engineering using protein and genes.

3.1.1 Tissue regeneration based on DDS technologies of tissue engineering.

If the tissue to be repaired has a high potential for regeneration, it is conceivable that any potential, immature cells infiltrated into the scaffold implanted from the surrounding healthy tissue will result in the regeneration of new tissue. However, additional means are required if the regeneration potential of the tissue is very low, for example if there is a low concentration of cells and growth factors needed for new tissue generation. In practice, the possible methods are to supply appropriate growth factors, key cells or combinations of these to the site needing regeneration to promote cell-based tissue regeneration. Much research regarding tissue regeneration using various growth factors has been reported, and the importance of the design of the administration form to allow the factor to efficiently exert the biological activity for *in vivo* tissue regeneration has been demonstrated. However, in spite of this necessity, little research has been performed on the systematic DDS research of growth factors to induce tissue regeneration from the viewpoint of material sciences. In place of the growth factor protein itself, recently the application of a gene that encodes the growth factor has been increasingly noted to promote tissue regeneration [4]. A DDS technology or methodology will assist the system development of non-viral carriers which will permit the bioactive molecule delivery at the high level of efficiency as the viral system.

3.1.2 Use of growth factors in tissue engineering

To obtain a satisfactory result in tissue engineering is essential to create an environment within the extracellular matrix that is similar to the cells *in vivo* to allow the cells to proliferate and differentiate efficiently [5]. It is necessary to provide adhesion and

growth proteins which are in contact with the cells in their natural environment [6]. Growth factors can be used to promote and prevent the cellular proliferation, differentiation, migration and adherence. In nature, a single factor can be produced by several cell types and may have different actions based on the target cell against which it is to act [7]. The observed effect depends largely on the concentration of the protein in question, therefore *in vivo* there are complicated and delicate adjustment of synthesis; due to this the half-life of these factors can be very brief for example only a few minutes [8]. Therefore, it is known that these proteins play a crucial role in development and tissue repair; therefore they are essential in tissue engineering [9]. Among the most studied and widely used growth factors are; PDGF (platelet derived growth factor), HGF (hepatocyte growth factor), FGF (fibroblast growth factor), NGF (nerve growth factor) that stimulates the cells migration and the neurites extension, EGF (epidermal growth factor) a potent stimulator of cell proliferation, the family of TGF- β (transforming growth factor) that also belong to the BMP (bone morphogenetic proteins) that play an important role in the development of various tissues and organs [10]. There are different strategies to achieve these growth factors for example it is possible, using purified cells from an extract, perform cell therapy to stimulate the production *in vivo*, or use the recombinant DNA technique [11].

3.1.3 Future direction of tissue engineering

Without using stem cells with high proliferation and differentiation potentials, it has only been possible to induce tissue regeneration using the controlled release system of biological active growth factors. Depending on the type, and site of target tissue or organ, it is necessary to make use of cells, their scaffold, the growth factor, the barrier membrane or their appropriate combinations. For the tissue engineering approach with growth factors, it is no doubt that the DDS technology or methodology is, and will be indispensable in the future. In terms of disease therapy based on the natural healing potential of patients, two forms of tissue engineering in the surgical and internal medicine disciplines should be carried out in the future. If a key growth factor is supplied to the target site at the right time, and over the appropriate period of time at the right concentration, the process of tissue regeneration could occur naturally. Once the right direction is given, it is possible that the intact biological system of the body will naturally start to function, resulting in automatic achievement of

tissue regeneration. There is no doubt that whenever growth factors and genes are used *in vivo*, their combination with DDS technology is essential. However, the present technology of controlled release does not always accurately regulate the amount and time length of growth factor release. Therefore, one practical approach is to release a growth factor necessary to increase the number of precursor, blastic or stem cells *in vivo*. It is practically impossible, however to control cell differentiation by the release technology of growth factors currently available, since the differentiation is normally regulated by the complicated network of growth factors in the restricted time, site, or concentration. Regenerative medical therapy is a new therapy based on the induction of tissue regeneration through cells and tissue engineering. To achieve regenerative medical therapy using tissue engineering technology and methodology, substantial collaborative research between material, pharmaceutical, biological and clinical scientists is needed. Even though superior stem cells can be obtained to use accompanied with development of basic biology and medicine of cells, it is impossible to apply the cells and the related scientific results to medical therapies for patients (regenerative medicinal therapy) unless an environment suitable for cell proliferation and differentiation is created and efficiently combined with those cells used. However, one of the main problems is the shortage of biomaterial research regarding tissue engineering such as scaffolding and DDS, aimed at tissue regeneration and the biological substitution of organ functions. Such researchers must have knowledge of medical, dental, biological and pharmacological disciplines, in addition to material sciences. It is vital to educate the researchers of interdisciplinary fields who have engineering background and can also understand basic biology and clinical medicine necessary for development of tissue engineering. One of the representative interdisciplinary research fields is DDS. The DDS technology is also applicable to create the non-viral vectors to prepare genetically engineered cells for regenerative medical therapy. Research and development of non-viral vectors with a high efficiency of gene transfection for stem cells are required. Tissue engineering technology is not only surgically used on damaged tissue and for regeneration induction treatment, but also applied using methodology of internal medicine to develop a new therapeutic method for chronic fibrosis diseases. As tissue engineering is still in its infancy, it will take a long time to become well established. However some research projects have already reached the stage of clinical applications. Increasing the significance of drug delivery in the future will help the progress of tissue engineering.

3.2 PEG Conjugates

3.2.1 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:

- Less frequent dosing
- Lower doses, and below the minimum toxic concentration
- Protection of the drug from inactivation resulting in prolonged half life [11]

The drug release from polymeric systems may be due to a physical or chemical mechanism:

- Physically controlled-release system: the drug can be found surrounded by an insoluble polymer membrane or dispersed in a in soluble polymer matrix; therefore there is a release by diffusion. In other cases the initially dry polymer can swell, implicating the osmotic process, especially in the membrane system;
- Chemically controlled release system: there is a link between the active molecule and biodegradable polymer, or the active molecule is dispersed in a matrix bioerodible [11].

Then there are delivery systems implanted *in situ*, where there is local release of the active substance from insoluble polymer.

The bioactive molecule field has had great developments in bioconjugation, which provides the formation of a covalent bond between a macromolecule of polymer and the active molecule, creating a new chemical entity that has typical characteristics [12].

3.2.2 Experimental model of conjugated polymeric

The use of a soluble macromolecular carrier for bioactive molecules delivery can alter the pharmacokinetic characteristics without altering the activity and bioavailability in the target site. The only characteristics modified are the half-life, its distribution in various body areas 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 [13]. He suggested combining the drug covalently-bonded with water-soluble polymer chains, subject to enzymatic or hydrolytic cleavage in the target site.

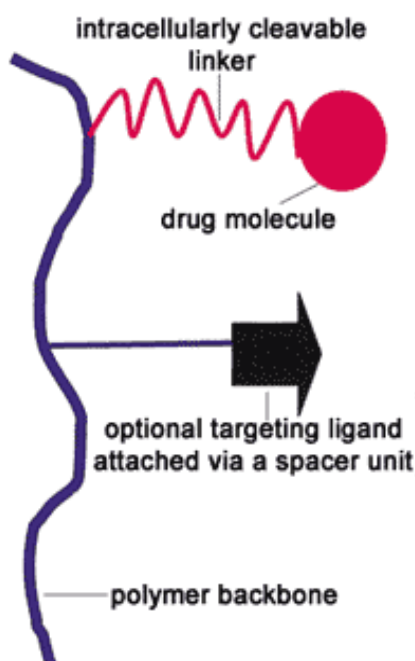


Fig 2: Model of polymeric carrier

This model suggested by Ringsdorf (summarized in Figure 2) must have particular characteristics and was made by:

- An inert water-soluble polymeric carrier (polymer backbone), with certain characteristics.
- A spacer interposed between biodegradable polymer and active molecules (intracellularly cleavable linker) capable of giving a bond that is broken only at the site of action, releasing the active biomolecules
- The bioactive molecule covalently linked to the linker
- Targeting ligand that may or may not be linked to the polymer structure, promoting the direction and specific uptake by target cells while minimizing nonspecific interactions
- Functional group additives, which influence the solubility of the conjugate

3.2.3 Characteristics of polymeric carrier

As mentioned earlier [13,14], the Ringsdorf polymer carrier model must have certain characteristics, and it has been tried in various polymers for drug delivery, both natural and synthetic. This has resulted in 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, and not have a molecular weight greater than 40kDa to aid elimination from the kidneys or liver to avoid accumulation
- Must have 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 link between the 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 the correct amount of drug at the site of action
- The conjugate must have a molecular weight that ensures the accumulation in the tumor tissue by EPR effect (Enhanced Permeability and Retention) and any excess drug should be removed quickly without reaching places where it can exert toxic 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

3.2.4 Bioactive molecules release

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

- Passive hydrolysis
- pH-dependent hydrolysis
- Enzymatic hydrolysis

The steps of hydrolysis mainly involve those bonds more sensitive to normal hydrolytic action, such as esters, amides, carbonates and urethanes. The percentage of the drug released will depend on the stability of the bonds themselves. 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 [11,14].

Last enzymatic hydrolysis may occur in conjugates that present a polypeptides spacer between the active molecule and carrier, sensitive to the action of many enzymes present in the lysosomal level, such as phosphatases, esterases, glycosidases and peptidases [16,17]. Different peptide spacers have been studied, especially for the peptide and protein conjugates, and according to the amino acid sequence chosen, there is an action of either enzyme. In the literature there are examples of conjugated polymers designed to be sensitive to cathepsins, cysteine peptidase-dependent present in large quantities and with high activity at lysosomal level.

3.2.5 Problems and advantages of bioconjugates

The bioconjugates is certainly an encouraging strategy to increase the effectiveness of injectable drugs because it allows us to obtain new chemical entities with specific chemical, physical and biological characteristics that positively affect the pharmacokinetics and pharmacodynamics of the therapeutic agent. There are issues to overcome before we obtain the ideal bioconjugate including the improvement of the chemical bond to obtain activation, conjugation that does not affect the stability of the polymer and the active site of the drug remains available. Development of analytical methods for the characterization of the conjugate and its components, the achievement of well defined polymers with low polydispersivity, the polymer have been already approved by the FDA.

Apart from these problems that are still being resolved, the bioconjugates certainly presents many advantages:

- Masking of antigenic sites of the drug, especially for protein drugs with resulting reduced uptake by the immune system
- Reduction of renal excretion, due to the high hydrodynamic volume
- Increased plasma half-life, linked to previous point
- Increased the solubility of normally low solubility drugs in biological fluids
- Specific direction of the drug in tumor tissue due to the presence of targeting residues

- Greater retention of the conjugate with subsequent release of drug in tumor tissue by EPR effect.
- Possibility of less frequent, and lower doses
- Reduced toxicity in other tissues and organs, as a result of the previous point
- Increased patient compliance and with improvement of quality of life
- New internalization mechanism for the bioactive molecules into cells

The main advantage is the direction of the drug only to the target site, exploiting the characteristics of the tumor tissue and the bond polymer-drug [18].

3.2.6 EPR Effect

To target only the macromolecular conjugates in tumor tissue, thus avoiding damage to healthy tissue, we can link to the conjugate a targeting residue, so it is recognized by specific receptors on the surface of cancer cells. However, while cancer drugs with low molecular weight were distributed equally in healthy and diseased tissue, macromolecular drugs as well as natural macromolecules such as albumin, could only passively accumulate in cancer tissue; this phenomenon was observed for the first time by H.Maeda, has been called Enhanced Permeability and Retention, or more commonly EPR effect [19].

The EPR effect is due to two main phenomena:

- Increased vascular permeability (Enhanced Permeability) compared with normal tissue, due to irregular blood vessel architecture [20], which is discontinuous endothelium in tumor tissue; extensive production of vascular mediators that facilitate the extravasation, including bradykinin, prostaglandins, NO, peroxynitrite, VEGF / VPF (vascular endothelial growth factor / vascular permeability factor) [21], active angiogenesis and high vascular density. All of these factors allow massive macromolecules and small particles extravasation, with molecular mass between 20 and 800kDa
- Decreased drainage of the lymphatic system in tumor tissue (Enhanced Retention) which reduces the clearance of macromolecules resulting in accumulation in the tissue itself [22]

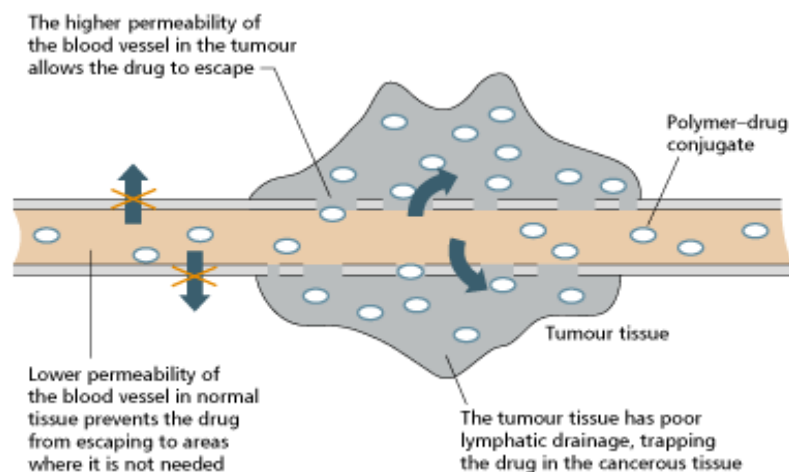


Figure 3 EPR Effect

By exploiting these tumor tissue characteristics can be selectively delivered on it the antineoplastic agents like bioconjugates, thus limiting the diffusion in normal tissues, because their blood vessels structure are less permeable than the macromolecular construct still on the blood stream.

3.2.7 Uptake endocytosis

Traditional chemotherapy uses drugs with low molecular weight, and is often accompanied by high toxicity as the molecules are spread rapidly throughout the body, entering by simple diffusion in cells. This occurs in the first minutes following intravenous administration as there is high percentage of the injected dose which leaves the circulatory system to be distributed ubiquitously around the body. This results in the need for an increased dose; in addition there is an inevitable damage to healthy tissue.

The bioconjugation technique can partially limit the damage because the accumulation in solid tumors due to the EPR effect mentioned earlier, is facilitated by the high molecular weight of the conjugate. This can limit the cellular uptake only at the endocytosis mechanism, predominantly in tumor cells [11].

The interaction with the plasma membrane, depending on the conjugates structure could have three mechanisms of pinocytosis, summarized in Figure 4:

- A fluid phase
- Adsorption
- Receptor-mediated [17]

In the **fluid phase** pinocytosis do not have any interaction with the plasma membrane and it is the conjugate concentration in the extracellular fluid that determines the speed and extent of the process.

Adsorption occurs after non-specific interactions with the membrane due to the presence of hydrophobic groups or positive charges on the surface of the conjugate, and it can increase the endocytosis.

Finally, **receptor-mediated** pinocytosis occurs when the macromolecules are linked with the residues complementary to the receptors or surface antigens: in this way the conjugate is recognized and endocytic only by the target cells.

Figure 4.3 Shows the internalization process of the drug-polymer conjugate (P-D) and the target drug-polymer conjugate ()-PD).

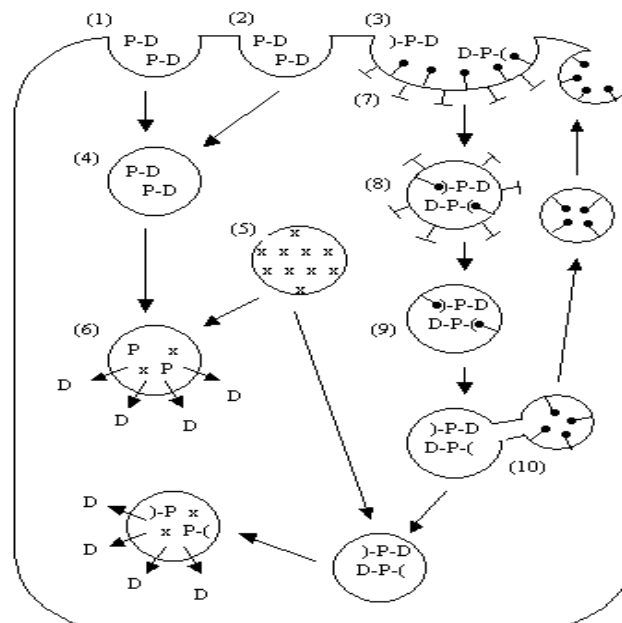


Figure 4: Uptake by endocytosis of conjugates polimerici

Fig 4 Showed all three mechanisms of pinocytosis: **a fluid phase** (1), **by adsorption** (2) and **receptor-mediated** (3). In the first 2 cases (1 and 2) the pinocytic capture occurs with invagination of the plasma membrane, which captures the macromolecules present in the extracellular fluid, or deposited on the membrane. The conjugates are enclosed in vesicles and delivered to the endosomal compartment which is a pH about 6 (4). Here we can check the hydrolysis of the links or spacers susceptible to acid pH, following the endosome merges with a primary lysosome (5) to give a secondary lysosome (6). In the lysosomal compartment the

macromolecules are exposed to more acidic environment (pH 4.5-5.5), and there are many enzymes capable of hydrolyzing specific bonds. The lysosomal membrane, such as plasmatic membrane, is impermeable to macromolecules and allows only products with a low molecular weight from enzyme degradation passage into the cytoplasm: so at this point the molecules of the active substance are released from the carrier and can diffuse into the cytoplasm giving their pharmacological action. In the latter case (3) i.e the receptor-mediated pinocytosis, the conjugate is linked with targeted residues bound to receptors and the cell surface antigens (•—), which are found inside clathrin-coated dimples (⌣) (7). It forms a primary endosome (8) which subsequently loses the clathrin coating and becomes CURL (Compartment for Uncoupling Receptor and Ligand) (10). Inside this organelle, the conjugates separate from the receptors which return to the cell surface. The endosome containing the conjugate then merges with lysosomes and as described in the mechanisms (1) and (2), gives rise to the secondary lysosome. The drug released from the carrier can now spread in the cytoplasm of treated cells.

3.2.8 Types of polymers used as drug carriers

Polymers currently used as drug carriers can be divided into

- Natural polymers
- Synthetic polymers

The polymers of natural origin are mainly polysaccharides and poliaminoacidi and have the advantage of being biodegradable and so easily removed from the body. However there are some drawbacks that should not be underestimated. The active molecule bond can sometimes reduce the sensitivity of the polymer to the degrading action of the enzymes, making it less biocompatible [23]. It can also give immunogenicity [24] and cause dangerous immune reactions. The polymers of natural origin which are often used include:

- DNA: easily internalized by cells through endocytosis and is hydrolyzed only in lysosomal and has been used as drug carriers since the early 70s [23,25]
- Dextrane: polysaccharide of bacterial origin, it is used primarily as a blood expander but it provide an easily immunogenicity [17,27]
- Albumin: this is an endogenous molecule with low toxicity and good stability, and when linked to drugs it increases plasma half-life and activity [28].

The synthetic polymers have low immunogenicity, which can be easily synthesized and derived; in addition they are not biodegradable but can be prepared with dimensions to facilitate the elimination by the kidneys. Among the most widely used ones are:

- Poly-lysine: polymer obtained by the bond between carboxyl groups and α -amino groups on the lysine amino acid, which has a number of free ϵ amino groups where the drug can bind to
- SMA copolymer of styrene and maleic anhydride to derive the widely used anticancer drug protein neocarzinostatina, giving the conjugate SMANCS [29]. By protecting the protein from degradation, it is rendered less immunogenic and is more cumbersome with a prolonged time residence within tumours. It also has a negative surface charge that permits the interaction with the plasma membrane, and endocytosis allowing the reduced renal excretion. This conjugate is used in the treatment of hepato-cellular carcinoma [29,30];
- HPMA (or poly-idrossipropylmetacrilamide): polymer of methacrylic acid containing peptide spacers and often activated with p-nitro-phenyl ester in order to allow the binding with the active molecule, which undergoes hydrolysis within the cell. As it is not biodegradable, it is used with a molecular weight below 45 kDa in order to be excreted by the kidneys without accumulation. The preparation of various drug-HPMA conjugates shown that the polymer: increases the water solubility of the linked drug, guarantee the stability by decreasing the toxicity, the specific link with targeting compounds specifically recognized the cells, this also allows administration of lower and less frequent, due to reduced renal excretion and decreased inactivation
- PEG: synthetic polymer, discussed the next paragraph.

3.3 PEG

3.3.1 General aspect

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

HO [CH₂CH₂O] n or CH₃O [CH₂CH₂O] nH



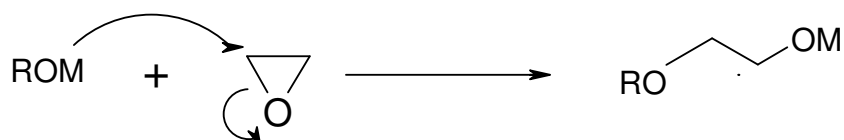
Fig 5: Structure of poly-(ethylene glycol)

Its synthesis is shown schematically in Fig 5.

This involves three stages:

- This begins with the nucleophile attack to ethylene oxide cyclic opening the epoxy ring and the formation of oxygen reactive alcohol;
- Follows the propagation of the reaction by the addition of other epoxidic molecules;
- Finally, the cessation of the reaction by the action of a terminator.

Initiation



Propagation



Termination

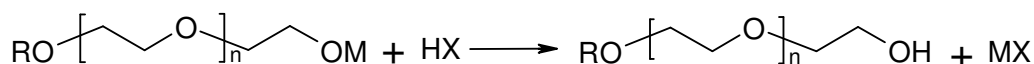
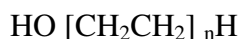


Fig 6: Synthesis of poly-ethylene glycol.

The PEG is a polymer suitable for the bioconjugates, as its characteristics include being biocompatible, it is neither toxic, allergenic nor immunogenic [31], is not charged and has only one functional group giving a clean chemistry. It is soluble in both aqueous and organic solutions [32,33] it is not biodegradable except for molecular weights below 40 kDa, it is easily eliminated by the kidney or liver function and does not accumulate [34]. It is also readily commercially available and is of low cost in a wide range of molecular weights and structures. All these features would allow its wide use in the biomedical field. Finally it was

approved by the Food and Drug Administration (FDA) as a constituent of foods, cosmetics and pharmaceutical preparations, injections, topical, rectal and nasal applications. The type of polymer that is formed depends on the initiative used:

- In aqueous solution of ethylene oxide reacts with a hydroxyl giving a bifunctional PEG-diol:



- In the presence of a methoxy anion in DMF gives the monometossi PEG (mPEG) monofunctional: $\text{CH}_3\text{O} [\text{CH}_2\text{CH}_2\text{O}]_n\text{H}$

- Using an initiator bi-or polyfunctional branched polymers are obtained.

3.3.2 Main methods for bioconjugates activation

Due to the fact the alcohol function of the PEG is less reactive under the conditions needed for the bioconjugates, the polymer must be first functionalized with different agents according to the group which will be bound to it:

- The amino acid bond will use used PEG-epoxide, aldehyde-PEG or PEG-succinimidyl; with PEG-thiol groups iodacetamide;

- PEG-COOH with the hydroxyl groups activated with N-idrossisuccinimide or p-nitrofenilcloroformiato.: These also react with amines [35]. lysine ϵ and α - polypeptide [36,36,37,38]. The binding of PEG to proteins and peptides, exploited different functional groups of amino acids, among them are the most reactive cysteine thiol groups and amino groups in the. The carboxyl groups of aspartic acid and glutamic acid and C-terminal groups how a low reactivity aqueous solution, the guanidine group of arginine is very basic and is present in protonated form at pH values acceptable for the stability of the protein. However the alcoholic functions of serine and threonine, the imidazole ring of histidine and the phenolic group of tyrosine are certainly less reactive but can be used in special conditions [39]. The number of polymer chains linked to it is variable and depends on the amino acid sequence and the reaction conditions. In addition to the mPEG usually used for conjugation with drugs to avoid the crosslinks leading to non-homogeneous products, there is also a PEG branched marketed. Here two PEG chains are linked to a lysine to obtain a compound with two chains and a single functional group activity, giving the characteristic "umbrella effect" [16]. Proteins are among the most reactive

• mono-functional reagents: Harri [40] has

described the main methods for the activation of PEG for the subsequent protein derivation. This usually occurs by linking the activated PEG-amino lysine groups $\text{CH}_3\text{O} [\text{CH}_2\text{CH}_2\text{O}]_n\text{H}$ $\text{mPEGCH}_3\text{O} [\text{CH}_2\text{CH}_2\text{O}]_n\text{-R}^*$ $\text{mPEG activated CH}_3\text{O} [\text{CH}_2\text{CH}_2\text{O}]_n\text{-R-NH-Prot. PEG-Protein}$

The methods used to activate the mPEG are:

- Using 2,4,6-trichloro-s-triazine (cyanuric chloride): the resulting derivative diclorotriazinico can react with nucleophilic functional groups of the protein (amino, hydroxyl, sulfhydryl). This reagents lack of selectivity can often lead to loss of biological activity in different peptides;
- Use of 1.1-carbonildiimidazolo activating agent to give a carbamate [41]: the PEG obtained is therefore less reagent;
- Conversion of the terminal hydroxyl sulfuric ester [42];
- Introduction of a carboxyl group sull'ossidrilic of PEG and subsequent conversion into foreign penthouse with NHS and DCCl.
- bifunctional reagents: the use of bifunctional reagents can bind 2 molecules whose conjugation can be prevented for steric reasons; the 2 terminal hydroxyl groups of PEG chain can be activated by the same or different reactive groups. The PEG Eterobifunctional derivatives are widely used as agents for macromolecular cross-linking reactions or as "spacers" between 2 different molecules [43,44,45,46]. In the literature there are: synthesis of biologically active conjugates apoenzima-cofactor [47], preparation of polymer supports for solid phase peptide synthesis [48] and targeting systems for active molecules [44]. Among the PEG eterobifunctional commercially available, there is a derivate with the ester N-idrossisuccinimidico on one side, and on the other either a group Vinylsulphonic or ammine group protected by t-Boc or Fmoc group to prevent the reaction between the active ester and the amino group present in the same PEG. These offer significant opportunities for cross-linking reactions and conjugation. The t-Boc and Fmoc groups are also easily removed with trifluoroacetic acid (TFA) and piperidine [49] respectively. There are several methods to synthesize PEG eterobifunzionali [50]:
- Limit the molar equivalents of each reagent;
- Polymerized ethylene oxide in the presence of an anion that eventually becomes end-group [51,52,53].

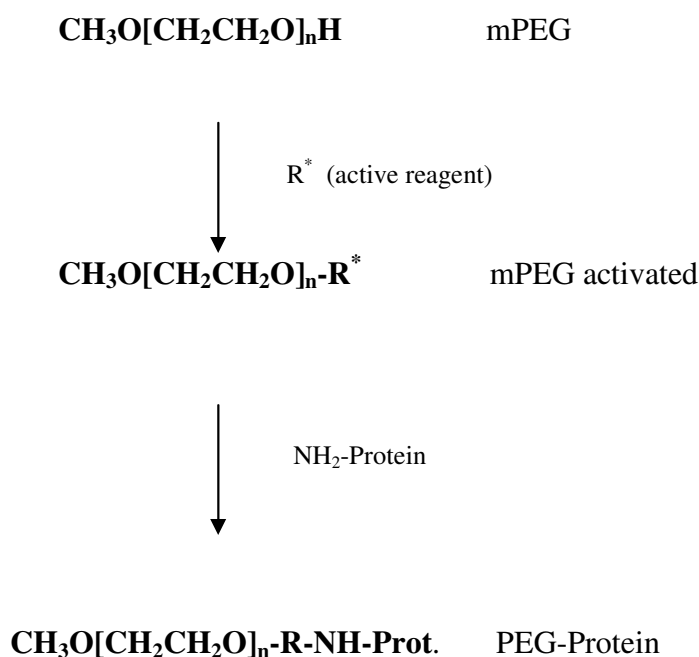


Fig. 7: Metodi di attivazione del PEG per la derivatizzazione proteica

3.2.3 Uses and applications

Polyethylene glycol is used in the pharmaceutical field to obtain bioconjugates as it modifies the chemical and physical characteristics, the pharmacokinetic and pharmacodynamic therapeutic agents without drastically altering the biological activity [54,55]. Generally, the following are observed in the pegylated active molecules:

- Increased plasma half life;
- Reduction of renal excretion and biodistribution for the increase of the molecular weight;
- Reduction of the hydrolytic and enzymatic degradation;
- Reduction of uptake into the reticulo-endothelial system [56]
- Increased solubility in water [57];
- Reduction of immunogenicity and antigenicity [31,58,59].

The pegylation was initially used to conjugate peptides and proteins, however there were some problems. Mild chemistry is needed so not to disable or denature the protein. The presence of many functional groups means that it is difficult to determine the exact position of

binding, then is necessary to use PEG mono-functional, and the active sites of the protein will be left intact. [16.34]. However there are several conjugates in both clinical trials and market which include:

- ADAGEN: conjugate between PEG and bovine adenosine deaminase [60] is marketed by Enzon, Inc., for the treatment of combined immunodeficiency syndrome;
- ONCOSPAR ®: conjugate between PEG and L-asparaginase [61], used in the treatment of cancer Acute lymphocytic leukemia;
- PEG-INTRON: conjugate between PEG 12000 Da and α interferon (α -IFN) to treat hepatitis C.

In this advanced stage of research there are PEG conjugated with insulin, superoxide dismutase, interleuchine- 2, hemoglobin and many others.

The pegylation technique also has been widely used in cancer therapy in order to direct the drug specifically within the tumor tissue, preventing the distribution to other areas to avoid side effects, and also to avoid circulation inactivation.

Examples of PEG conjugated with anticancer drugs include camptothecin, which is an antitumor alkaloid whose solubility is increased by binding to the polymer. There is also doxorubicin, which substantially reduced the cardiotoxic effects.

In addition, there are AraC, methotrexate and taxanes conjugates currently being researched aiming to target the antineoplastic agent in the tumor tissue, thus reducing systemic toxicity and improving the pharmacodynamic and pharmacokinetic profiles [60].

The pegylation technique is used with antiviral, antimalarial and anti-AIDS drugs to improve their pharmacological properties [60].

However, PEG has significant applications in other areas than in the pharmacological discipline [57].

PEG is very flexible and has a strong ability to coordinate water molecules [46] and a high hydrodynamic volume that is exploited in the "two phases partitioning" purification technique, which usually employs PEG and dextran.

In cyclic form it can form complexes with the transition metals; this characteristic is exploited to transfer the metals in the organic phase by allowing a new type of catalysis called phase-transfer catalysis.

The PEG is also used for the precipitation of proteins and nucleic acids, and for the synthesis of peptides and oligonucleotides in liquid and solid phase for the enzymatic catalysis in

organic solvents. It can induce cell fusion and can make the bound surface materials biocompatible thus reducing the thrombogenicity. It also used in cosmetic material production.

Lately it is also widely used in the pharmaceutical industry for the preparation of drug delivery systems such as liposomes, nanoparticles, nano-and microspheres, dendrimers and hydrogels [61] and for the preparation of prodrugs with the bioconjugation technique of peptides, proteins and low molecular weight drugs [64].

3.4 Folic Acid

3.4.1 General characteristic

The site of action of the cancer active targeting often consists of a cell membrane antigen expressed only in tumor cells. Research in this field has recently shown the receptor for vitamin B9 (also called folic acid (FR)) is a membrane protein with high folate affinity. FR is a glycoprotein with a molecular weight about 38 kDa, and in humans, there have been three isoforms indentifies; these are α , β and γ / γ' :

- FR- α and FR- β are membrane proteins anchored to glycosylphosphatidylinositol (GPI)
- -FR- γ and FR γ' are in soluble form, as they are devoid of GPI [65].
- FR- α is the isoform with a higher affinity for the folate and with a constant

dissociation Kd \sim 0.1 nM, whereas FR- β has a higher Kd, \sim 1 nM and FR- γ intermedia Kd \sim 0.4nM [65,66]. While high expression of FR has been regularly observed in a range of human cancers, the receptor is usually absent in most normal tissues with the exception of the choroid plexus, placenta and lower levels in lung, thyroid and kidney [66,67]. FR is frequently over-expressed in tumor cells in culture and in epithelial tumors, in particular, is over-expressed in ovarian cancer (90% of cases) making it a useful marker [69,69]. Other types of tumors that over-express this receptor include those found in the endometrium, brain, lung, breast and kidney [70]. The FR- α isoform is over-expressed in malignant epithelial tumors, while FR- β is over-expression in myeloid leukemias. This can allow a targeting effect for a broad range of malignancies [65]. It should be noted however that the receptor is present only in the apical membrane of epithelial cells which are inaccessible to the bloodstream.

Further the cell is protected from action of drugs coupled with folic acid. After the malignant transformation the cell polarity is lost and the receptor becomes accessible to the blood [65].

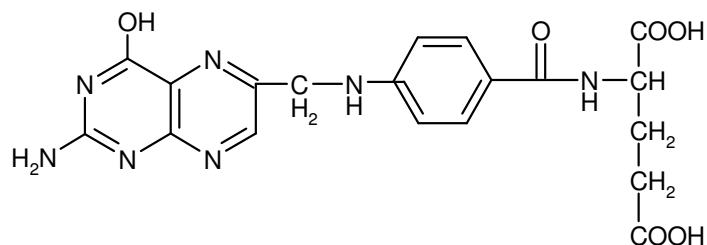


Fig 8: Structure of folic acid

The cause of this over-expression of FR in tumor tissues is not entirely clear. Folate is essential for the survival of the cell. Following activation to tetrahydrofolates, they are required for numerous biochemical processes including synthesis of DNA and RNA transmethylation, and so therefore are important for rapidly dividing cells. Recent studies have shown that high levels of FR not only induce cell proliferation which is mediated by the uptake of folate, but that they also generate regulatory signals. It is hypothesized that FR participates in a macromolecular complex that generates intracellular signals which are involved in modulating the cell survival and proliferation processes [71]. Through specific receptors, the cells are capable of the endocytosis of different types of macromolecules. There are two types of endocytosis using the clathrin-coated pits and uncoated pits called caveoli. FR internalizes folic acid using the last method called potocytosis. The receptors are grouped in the caveoli which are small uncoated invaginations of the membrane, and these form vesicles as a result of folic acid binding to its receptor i.e. creating a seal that remains bound to the plasma membrane. The content of the vesicle is rapidly acidified with the dissociation of folic acid to FR, allowing the vitamin to pass through the membrane to the cytoplasm by a protein carrier. Finally the caveolis are opened and displayed the FR again towards the extracellular space [72,73,74]. There have been two different strategies developed for targeting drugs to FR; by binding to the FR monoclonal antibodies or binding to folic acid. The first approach has two major drawbacks when compared to the second. The size of the antibody conferring a low capacity for diffusion through biological barriers and immunogenicity does not allow for another administration. Also folic acid is stable, inexpensive, has high affinity for the endocytic receptor and is very effective [75]. In fact,

when folate is covalently bound to a macromolecule carrier such as polyethylene glycol using the residual γ -carboxylic, its affinity for the FR remains unchanged. Therefore is better to use the endogenous ligand compared to the monoclonal antibody [76].

3.5 Epirubicin

3.5.1 General aspect

Epirubicin (4'-or epidoxorubicin) is an anthracycline antibiotic that has antineoplastic activity similar to its epimer. More importantly doxorubicin is now used alone and in combination with other cytotoxic agents in a variety of leukemias and solid tumors [77,78,79]. Anthracyclines are formed by a core planar anthraquinone (aglicole) that is linked to aminoglycosidic (daunosamine).

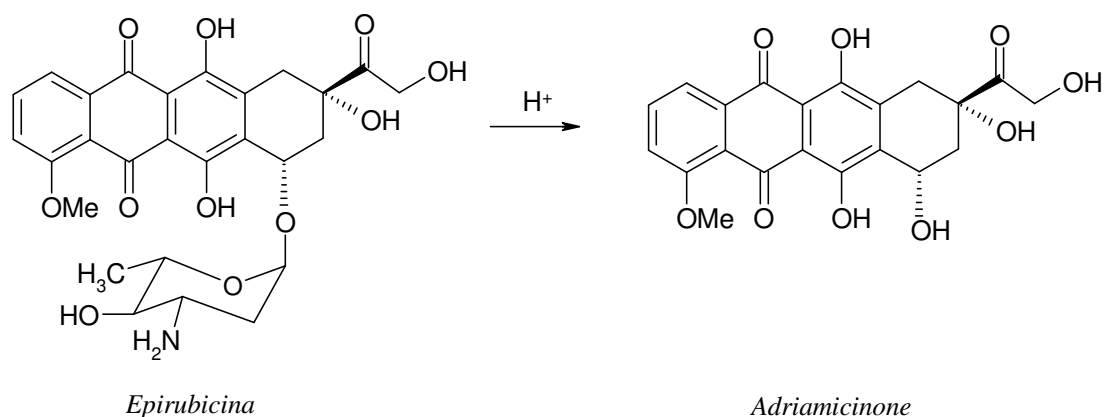


Fig. 9: Structure of epirubicin and adriamicinone.

Epirubicin is closely related to doxorubicin with regard to its chemistry, which differs only by the spatial orientation of the 4'-hydroxyl group. In the epirubicin this is equatorial [80]. Epirubicin was marketed in 1984 and approved by the FDA in 1999. In clinics it is most

commonly used in the form of hydrochloric acid, which is stable in both the solid state and in aqueous solution at a pH between 3 and 6.5. It decomposes at an increasing rate when the pH increases to a range of 6.5 to 12.

3.5.2 Mechanism of action

There are many biological actions of anthracycline antibiotics, all leading to a cytotoxic drug effect which is expressed predominantly at the level of tumor cells [77].

The mechanism of action of this drug is expressed at various levels:

- DNA intercalation with enzyme inhibition of DNA and RNA polymerases, topoisomerases, DNA helicases and DNA repair enzymes
- Intercalation in the cell and mitochondria membrane (vesicle formation, structural changes and interference with membrane activities)
- Production of free radicals

Intercalation between the two strands of DNA is possible due to the ability of the anthraquinon planar system to fit between the base pairs. When the drug intercalates, the aromatic system planar inserts perpendicular to the axis of the double helix and establishes interactions between the nucleotide bases and anthracycline rings B, C, D. The aminoglycosidic remains outside and adds stability to this complex by ionic interactions with the sugar and phosphate backbone of DNA. This binding induces stiffening, bending and elongation of the double helix, and forms the basis of the inhibition of nucleic acid synthesis in cancer cells, and induction of DNA fragmentation by inhibition of repair mechanisms.

The intercalation of the anthracycline antibiotics has been shown to interfere with the topoisomerase-DNA complex, with the formation of a ternary complex comprising of the drug leading to the inhibition of the topoisomerase catalytic activity. This is an intranuclear enzyme that transiently breaks one of the strands of DNA, rearrange and allowing relaxation of the double helix required for replication and transcription processes. The action of anthracyclines at this level stabilizes the cutting of DNA.

3.5.3 Pharmacokinetics, distribution, metabolism and elimination

Following intravenous administration, Epirubicin shows triphasic elimination in plasma. It has a rapid initial distribution phase (α) ($t_{1/2 \alpha}$ from 1.8 to 4.8 minutes), followed by an intermediate phase (β) ($t_{1/2 \beta}$ from 0.5 to 2.6 hours) and a slower terminal phase of elimination (γ) ($t_{1/2 \gamma}$ of 15 to 45 hours).

Epirubicin produces an intense tissue distribution and a high volume of distribution, ranging from 13 to 52 l / kg (1000l/m²). This is clearly demonstrated by the increased drug tissue concentration compared to its plasma concentration. This is evidence that tumor tissues accumulate larger amounts of epirubicin.

The AUC values were approximately 30-70% higher for doxorubicin compared with epirubicin after a single intravenous administration.

Epirubicin is abundantly and quickly transported to the liver, and is metabolized into two glucuronides (epirubicinolo-glucuronide and epirubicin-glucuronide) in addition to five other metabolites (aglycones, epirubicinolo and others) as illustrated in Figure 4.9.

The metabolic pathway converting epirubicin and epirubicinol to 4-O-glucuronidation does not occur in doxorubicin as it is only permitted by the position of the equatorial hydroxyl group in 4'.

Epirubicin is primarily eliminated by the hepatic system, whereas only 11-15% of a single dose is eliminated via the urine as the unchanged drug and metabolites.

3.5.4 Therapeutic uses

Epirubicin is usually administered by intravenous injection of various doses, either alone or in combination with other anticancer drugs [78,80].

The epirubicin alone is now used for the treatment of breast cancer; administered in the usual doses ranging from 75 to 90 mg/m² every three or four weeks. Doses over 180mg / m² are also used.

It is effective against many types of cancer including ovarian, stomach and lung cancer, non-Hodgkin's lymphoma and hepatocellular carcinoma.

3.5.5 Toxicity

The epirubicin most important dose-limiting toxicity was myelosuppression, manifested primarily as leukopenia, which is related to dose and is reversible, and less commonly as thrombocytopenia and anemia [78,80].

Comparative studies *in vitro* using the same doses of doxorubicin and epirubicin has shown that epirubicin is less myelotoxic than doxorubicin. The lower hematologic toxicity of epirubicin allows for an intensified therapeutic program, which is particularly important to define the dose-effect of anthracyclines.

The most important toxic chronic effect dose-limiting is cardiotoxicity, which manifests clinically as irreversible damage to the heart muscle, or as cardiomyopathy. Anthracycline cardiac damage can develop in several weeks after stopped of cancer therapy. The epirubicin has a lower cardiotoxic capacity when compared to doxorubicin, therefore its maximum recommended cumulative dose ($1000\text{mg}/\text{m}^2$) is almost twice that dxorubicina ($550\text{mg}/\text{m}^2$). This allows a greater number of cyclic treatments or an increased dose of the drug. The cardiac toxicity hypothesis could be explained by the transformation of anthracyclines in active radical species causing the formation of a superoxide which is highly toxic and harmful. It was observed that the presence of membrane P -450 reductase can promote this particular transformation in radical. Furthermore, the heart tissues lack the enzyme catalase, which converts hydrogen peroxide into water and oxygen; therefore there is no defence mechanism against the toxic metabolites of anthracyclines.

Other variable side effects that can occur following the administration of epirubicin include vomiting, nausea, alopecia, mucositis, stomatitis, diarrhoea, fever and hyperpigmentation.

3.6 Tri-dimensional cells culture system

These biodegradable systems have been introduced for the ability to stimulate the production of extracellular matrix and provide a valid structural support for cell growth. It usually made by natural source as collagen, agarose, alginate or synthetic materials, like PLLA or PLGA. [80]. Also in this research area, is having success a class of self assembling peptides, able to provide the optimal support characteristics for the three-dimensional culture.

Peptides self-assembling: This class of polymers is characterized by ability to organize independently, once placed in the middle of physiological or saline solution, in a tri-dimensional structure. Furthermore, for these features are classified as self-assembling peptides or sapeptide. From the perspective of molecular self-assembly is a spontaneous organization of molecules, under conditions of thermodynamic equilibrium, in structures well-defined and potentially stable, thanks to formation of a series of non-covalent interaction. [81] One of the first peptides to be used to this purpose was: EAK-16 II, (glutamic acid, alanine, lysine), it consists of 16 AA and was derived from a sequence repeatedly, found in zuotin a yeast protein. [82] Subsequent changes have led the synthesis of two derivatives: RADA 16-I and RADA 16 -II (arginine, alanine, acid aspartic) where residues of arginine and aspartic acid replace glutamic acid and lysine. [83] RAD sequence has been introduced to mimic the sequence RGD responsible of adhesion in many cell. (RAD) 16-I-ACN RADARADARADARADA-CNH₂ (Pure Matrix) (RAD) 16-II-ACN RARADADARARADADA-CNH₂ two end groups are protected because they do not join together the ends .

Self-assembly mechanism: The mechanism of self assembly has its explanations in the particular chemical structure of molecules. These peptides, highly soluble in water, forming stable structures in β sheets, characterized by two surfaces: a polar, formed by side chains ion, and a non-polar, formed by residues of alanine. Study of the self assembly mechanism has led to the identification of three stages of the process: the first, characterized by the formation of ionic links, hydrogen bonds and interaction between hydrophobic surfaces of different molecules peptide later, given the increase in molecules interaction, forming the real nano-fibers that are organized in a double layer propeller in the third wave, the fibers interact together to form a real gel. The diameter of the fibers appears to be around 10-20 nm, while the pores of the gel has a size about 250 nm. According to the saline solution used, are formed gels with different characteristics, followed studies on membranous structures stability prepared in different salt solutions (Li, Na, K, Cs) have shown that lithium salts provide best assembly of the gel, while cesium salts determine production of large precipitate.

3.7 Microsphere

3.7.1 Introduction and historical perspectives of drug delivery by PLGA devices

In order to avoid the inconvenient surgical insertion of large implants, injectable biodegradable and biocompatible polymeric particles (microspheres, microcapsules, nanocapsules, nanospheres) could be employed for controlled- release forms of dosage [84]. Microparticles sizes below 250nm are suitable for this purpose, but less than 125 nm is ideal [85]. Biodegradable polymers are natural or synthetic in origin and are degraded *in vivo*, either with or without enzymes, or by both methods to producing biocompatible and toxicologically safe by-products which are further eliminated by the normal metabolic pathways [86]. Drugs formulated in polymeric devices are released either by diffusion through the polymer barrier, by erosion of the polymer material or by a combination of both diffusion and erosion mechanisms [87]. The polymers selected for the parenteral administration must meet several requirements including biocompatibility, drug compatibility, suitable biodegradation kinetics and mechanical properties, and ease of processing [87,88]. A wide variety of natural and synthetic biodegradable polymers have been investigated for drug targeting or prolonged drug release. However, few are actually biocompatible. Natural biodegradable polymers like bovine serum albumin (BSA), human serum albumin (HSA), collagen, gelatin, and haemoglobin have been studied for drug delivery [84]. The use of these natural polymers is limited due to their higher costs and questionable purity [84]. Throughout the last two decades, synthetic biodegradable polymers have been increasingly used to deliver drugs, since they are free from most of the problems associated with the natural polymers [84,89]. Poly(amides), poly(amino acids), poly(alkyl-n-cyano acrylates), poly(esters), poly(orthoesters), poly(urethanes), and poly(acrylamides) have been used to prepare various drug loaded devices [84]. Amongst them, the thermoplastic aliphatic poly(esters) like PLA, PGA, and especially PLGA have generated tremendous interest due to their excellent biocompatibility and biodegradability [84,95]. The discovery, and the resulting synthetic work on the low molecular weight oligomeric forms of lactide and/or glycolide polymers was initially carried out several decades ago [86,88]. The methods to synthesize the high molecular weights of these polymers were first reported by Lowe [86]. During the late 1960s

and early 1970s a number of groups published pioneering work on the utility of these polymers to make sutures/fibers [85,86,88,92]. These fibers had several advantages such as good mechanical properties, low immunogenicity and toxicity, excellent biocompatibility and predictable biodegradation kinetics [85,86,88,92]. The wide acceptance of the lactide/glycolide polymers as suture materials made them an attractive candidate for biomedical applications, example include ligament reconstruction, tracheal replacement, ventral herniorrhaphy, surgical dressings, vascular grafts, nerve, dental, and fracture repairs [85,88,90]. Many researchers have investigated and documented the biodegradation, biocompatibility, and tissue reaction of PLA and PLGA [88,94]. The first work on parenteral controlled release of drugs using PLA was reported by Boswell, Yolles, Sinclair, Wise, and Beck [85,88]. Since then there has been a wealth of published literature regarding the use of PLA and especially PGLA for drug delivery. Various polymeric devices like microspheres, microcapsules, nanoparticles, pellets, implants and films have been fabricated using these polymers for the delivery of a variety of drug classes. In addition, they are easy to formulate into drug carrying devices for various applications, such as orthopaedic drug delivery and they have also been approved by the FDA for drug delivery use [84,95].

3.7.2 Physico-chemical and biological properties of PLGA

The understanding of the physical, chemical and biological properties of the polymer is essential before formulating a controlled drug delivery device. The various properties of the polymer and the encapsulated drug directly influence other factors like the selection of the microencapsulation process and the drug release from the polymer device etc. [1]. The polymer PLA can exist as an optically active stereoregular form (L PLA) and an optically inactive racemic form (D,L-PLA) [84,88,90]. L-PLA is found to be semicrystalline in nature due to high regularity of its polymer chain while D,L-PLA is an amorphous polymer because of irregularities in its polymer chain structure [86,90]. Hence the use of D,L-PLA is preferred over L-PLA as it enables more homogeneous dispersion of the drug in the polymer matrix [9,13]. PGA is highly crystalline because it lacks the methyl side groups of the PLA [86,90]. Lactic acid is more hydrophobic than glycolic acid and hence the lactide-rich PLGA copolymers are less hydrophilic, absorb less water and subsequently degrade more slowly [84,86,93]. The physical properties such as the molecular weight and the polydispersity index

affect the mechanical strength of the polymer and its ability to be formulated as a drug delivery device [86,88,92]. Also these properties may control the polymer biodegradation rate and hydrolysis [86,92]. The commercially available PLGA polymers are usually characterized in terms of intrinsic viscosity, which is directly related to their molecular weights [86]. The mechanical strength, swelling behaviour, capacity to undergo hydrolysis and subsequently the biodegradation rate are directly influenced by the crystallinity of the PLGA polymer [86]. The resultant crystallinity of the PLGA copolymer is dependent on the type and the molar ratio of the individual monomer components (lactide and glycolide) in the copolymer chain [84]. PLGA polymers containing 50:50 ratio of lactic and glycolic acids are hydrolyzed much faster than those containing higher proportion of either of the two monomers [88,92]. PLGAs prepared from L-PLA and PGA are crystalline copolymers while those from D,L-PLA and PGA are amorphous in nature [86,88]. Gilding and Reed pointed out that PLGAs containing less than 70% glycolide are amorphous in nature [96]. The degree of crystallinity and the melting point of the polymers are directly related to the molecular weight of the polymer [86,88]. The T_g (glass transition temperature) of the PLGA copolymers are above the physiological temperature of 37°C and hence they are glassy in nature [86,88]. Thus, they have a fairly rigid chain structure which gives them significant mechanical strength to be formulated as drug delivery devices [86,88]. Jamshidi *et al.* have reported that T_g of PLGAs decrease with decreasing lactide content in the copolymer composition and with a decrease in their molecular weight [97]. The PLGA polymers should have considerable mechanical strength since the drug delivery devices that are formulated using them are subjected to significant physical stress [86,88]. Different factors such as the molecular weight, copolymer composition (lactide/glycolide ratio), crystallinity, and geometric regularity of individual chains significantly affect the mechanical strength of the polymer [84,86,88]. Both *in vitro* and *in vivo*, the PLGA copolymer undergoes degradation in an aqueous environment (hydrolytic degradation or biodegradation) through cleavage of its backbone ester linkages [84,86,88,92,93]. The polymer chains undergo bulk degradation and this degradation occurs at uniform rate throughout the PLGA matrix [86,93]. Thies and Bissery reported that the PLGA biodegradation occurs through random hydrolytic chain scissions of the swollen polymer [98]. The carboxylic end groups present in the PLGA chains increase in number during the biodegradation process as the individual polymer chains are cleaved; these are known to catalyze the biodegradation process [86,88]. The biodegradation rate of the PLGA copolymers are dependent on the molar ratio of the lactic and glycolic acids in the polymer

chain, molecular weight of the polymer, the degree of crystallinity and the T_g of the polymer [86,88,93]. A three-phase mechanism for the PLGA biodegradation has been identified:

1. Random chain scission process. The molecular weight of the polymer decreases significantly but there is no appreciable weight loss and no formation of soluble monomer products.
2. During the middle phase there is a decrease in molecular weight accompanied by rapid loss of mass. Soluble oligomeric and monomer products are formed.
3. Soluble monomer products are formed from soluble oligomeric fragments. At this phase there is complete polymer solubilization.

The role of enzymes in any PLGA biodegradation is unclear [86,88]. Most of the literature indicates that the PLGA biodegradation does not involve any enzymatic activity and is purely through hydrolysis [86]. However, some investigators have suggested an enzymatic role in PLGA breakdown based upon the difference between the *in vitro* and *in vivo* degradation rates [88]. The PLGA polymer biodegrades into lactic and glycolic acids [84,86,88,92,93]. Lactic acid enters the tricarboxylic acid cycle and is metabolized and subsequently eliminated from the body as carbon dioxide and water [84,86,88,90]. In a study conducted using the C-labeled PLA implant, it was concluded that lactic acid is eliminated through respiration as carbon dioxide [99]. Glycolic acid is either excreted unchanged in the kidney or it enters the tricarboxylic acid cycle and eventually eliminated as carbon dioxide and water [86].

3.7.3 Microparticles

Although a number of microencapsulation techniques have been developed and reported to date, the choice of the technique depends on the nature of the polymer, the drug, the intended use and the duration of the therapy [84,85,87,88,91]. The microencapsulation method employed must include the following requirements [84,85,100]:

- (i) The stability and biological activity of the drug should not be adversely affected during the encapsulation process or in the final microsphere product.
- (ii) The yield of the microspheres having the required size range (up to 250 nm but ideally 125 nm) and the drug encapsulation efficiency should be high.
- (iii) The microsphere quality and the drug release profile should be reproducible within specified limits.

(iv) The microspheres should be produced as a free flowing powder and should not exhibit aggregation or adherence.

3.7.4 Spray drying

As discussed in the previous sections, injectable biodegradable PLA and PLGA microparticles have been successfully prepared by double-emulsion and phase separation methods. The coacervation method tends to produce particles which are agglomerated. There is difficulty in mass production as the method requires large quantities of organic solvent, and it is difficult to remove residual solvents from the final microsphere product [101]. The double-emulsion method requires many steps and a rigid control of the temperature and viscosity of the inner w/o emulsion. It is also difficult to encapsulate higher concentrations of hydrophilic drugs [84,101]. Contrary to these methods, the spray drying method is very rapid and convenient. It is easy to scale-up, involves mild conditions and is less dependent on the solubility parameter of the drug and the polymer [84,101,102]. A solution of PLGA, hexanuro-2-propanol, benzene and the drug is sprayed producing microcapsules of less than 125 nm [103]. Bodmeier and Chen prepared microspheres by spray drying where a water-soluble drug (theophylline) was suspended, or a water-insoluble drug (progesterone) was dissolved in a PLA/DCM solution and then spray dried to produce particles of less than 5 nm [104]. Due to incompatibility between the hydrophilic drug and PLA, needle-shaped crystals grew on the microsphere surface, whereas the progesterone}PLA solution gave smooth particles. The nature of the solvent used, temperature of the solvent evaporation and presence of PLA microspheres during the spray-drying process affected the polymorphic form of progesterone. A major problem encountered with this technique was the formation of fibres due to an insufficient force present to break up the polymer solution. Efficient dispersion of the filament into polymer droplets was dependent on the type of polymer and the viscosity of the spray solution. Other groups have also reported successful preparation of PLGA and PLA particles using the spray-drying technique [102,105,107]. A solution of the polymer, DCM and the drug piroxicam was spray-dried yielding microspheres that were hollow (no solid core) [102]. D,L-PLA microparticles were more spherical and smooth than those made from D,L-PLGA. The size range of the microspheres was 1-15 nm with a high drug encapsulation efficiency of 99.0% [102]. Men *et al.* have shown that PLGA microparticles prepared using spray-drying technique produced particles in the size range of 1 to 15 nm and drug loading of

4.4-6.6 ng/mg microspheres, while PLA microspheres prepared using coacervation technique yielded particles with diameters in the range of 20-90 nm with relatively low drug loading of 3.5 ng/mg microspheres [106]. In order to protect a hepatitis vaccine from the harmful effects of the solvent, a mixture of the antigen powder and an hydrophilic polymer Hydroxypropyl cellulose (HPC) was initially spray-dried to produce core microparticles [107]. These were then suspended in PLGA/ethyl acetate solution and spray dried to yield double-walled microparticles in the size range of 4-22 nm. The first coating layer of HPC protected the antigen from solvent during the second encapsulation process with PLGA [107]. There may have been a significant loss of the product during spray-drying due to adhesion of the microparticles to the inside wall of the spray-drier apparatus, which can also produce agglomeration of the microparticles [101]. To counteract these problems, a novel double-nozzle spray-drying technique was developed which involved use of mannitol as an anti-adherent [101]. A solution or dispersion (w/o emulsion) of the drug in PLGA solution was sprayed from one nozzle, while an aqueous mannitol solution was sprayed simultaneously from another nozzle. This process gave the final microspheres. The surfaces of the spray-dried microspheres were coated with mannitol and the extent of agglomeration was decreased [101]. This method also produced microspheres with a higher yield and encapsulation ratio when compared to those prepared from the double-emulsion method [101]. A novel low temperature spraying method for preparing PLA and PLGA microspheres has been reported by *Khan et al.* [108] and the research group at Alkermes Inc. (ProLease technology) [109,110]. The protein powder and optional excipients were initially suspended in the PLA/PLGA solution in acetone, ethyl acetate or DCM. This suspension was then sprayed into a vessel containing liquid nitrogen overlaying a frozen extraction solvent such as ethanol. The liquid nitrogen was subjected to evaporation causing the polymer solvent from the frozen droplets to be extracted by liquid ethanol. Microspheres were then filtered and the residual solvents evaporated by filtration. The microspheres were 50-60 nm in size with drug encapsulation efficiency more than 95% [108,110].

3.8 BMP: bone morphogenetic proteins

The BMP family are multifunctional factors that belong to TGF- β super-family [111]. Their existence and their functions were discovered in the 1960s and 1970s by an

orthopedic surgeon Marshall Urist. He studied the ability of decellularized bone tissue to stimulate the formation of new bone when implanted *in vivo*. Other studies have shown that this capacity was due to an organic component, specifically to a set of proteins that subsequently took the name of BMP [112]. Following from this, there has been a great interest in this family of proteins. Gradually the studies have shown that BMP plays an important role in embryonic development, and in adult organisms they are involved in the formation and maintenance of cardiac tissue, neuronal and muscle tissues [111]. Also the BMP stimulates migration, proliferation and differentiation of mesenchymal stem cells during embryogenesis, tissue repair and regeneration in adult organisms [113].

Until now, there have been 20 members of this family identified and characterized, the most extensively studied BMP are 2 to 8. All proteins show amino acid sequence homology with TGF- β in that the active form is represented by the dimer and have three disulfide bonds and a free cysteine. All members of this family are synthesized as long precursors, and following protease specific cutting the mature portion is released, resulting in the active protein [114].

3.8.1 BMP mechanism of action

The understanding of the BMP regulation and mechanism of action is the focus of much research, especially the receptors class associated with them. The protein activation involves the BMP binding to membrane receptor such as serine - threonine kinase which consists in two separate units (I and II). There have been 3 types of unit I identified (BMPR-IA, BMPR-IB, ACTR-IA) [115] and three types of unit II (BMPR-II, ACTR-II, ACTR-IIB) [116]. Receptors BMPR-IA, IB, II are specific for BMP while the receptors ACTR-II, IIB can act with other proteins. The binding of the ligand to the receptor allows the formation of an active tetrameric receptor complex consisting of two units I and two units II, [117]. An important role in this mechanism is performed by proteins SMAD 1, 5, 8 found on the unit I [118]. The binding of BMP to this receptor causes phosphorylation of SMAD protein unit followed by detachment from unit I and passage into the cytoplasm where it can bind with SMAD 4. The newly formed complex moves to the nucleus where it activates different transcription factors, depending on the type of cell.

Obviously this complicated signal transmission mechanism has different regulatory systems. The membrane has a BMP receptor antagonist called Noggin [119] in the cytoplasm SMAD 6 can bind to unit I and prevent the phosphorylation of SMAD 1, 5, 8 [120]. TOB is also always present in the cytoplasm and can interact with activated SMAD proteins and prevent their nucleus achievements [121], and

Smurf 1, a ubiquitin ligase E3 enzyme which promotes the protein degradation of SMAD 1 and 5, and finally osteogenic transcription factors and the type I units receptor [122] (Figure 10).

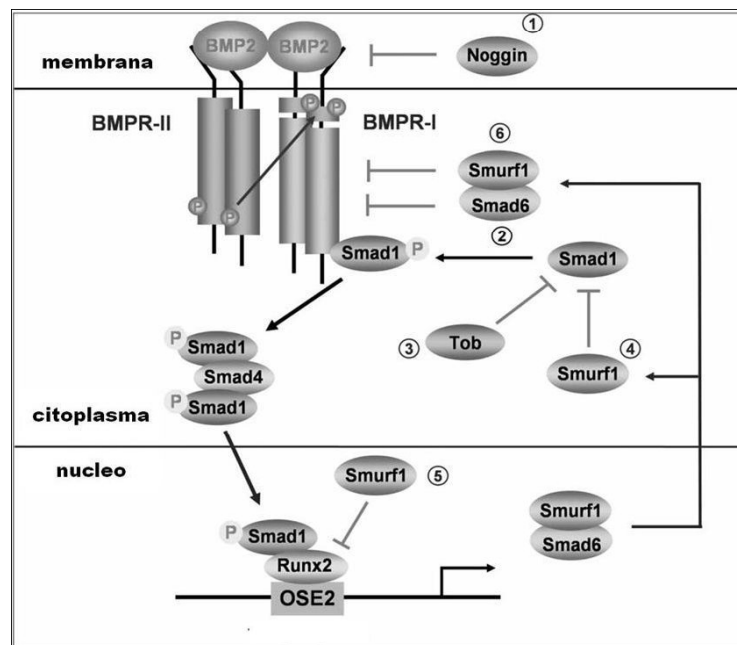


Fig. 10 Outline of the process of BMP dependent signal transmission. The inhibitors and their locations within the cell are illustrated [111].

3.8.2 Physiological role and pharmacology of BMPs

To understand the BMPs and the physiological role in the bone tissue formation have been used mature cut recombinant protein, or was induced an overexpression of receptor units or their antagonists. This led to an understanding that this class of proteins has an important role in the formation of bone tissue, before and after birth and for normal or post-trauma growth [123]. Furthermore, it is understood that these proteins are able to stimulate mesenchymal stem cells *in vivo* leading to osteogenic differentiation [124]. Through

the use of transgenic animals, it has been possible to understand the importance of BMP genes for healthy body development, especially the lack of BMP-2 and 4 genes which lead to spontaneous abortion for non-skeletal and placental appendages development [125- 126]. The absence of BMP-7 gene leads to premature death and serious skeletal, kidneys and eye defects [127]. The *in vivo* study on the effect of the proteins is complicated because the active form is a dimer, which can be made by two different BMP and, given the large number of BMP, there are many possible combinations. The heterodimers are difficult to distinguish because both the expression and the purification are much more difficult compared to individual proteins. However, it was possible to study some heterodimers and they have different functions than the respective homodimers [127]. There still remains a lot to understand on the action mechanism of these proteins and their interactions, but it has been noted that although the BMP contained in the matrix act synergistically *in vivo*, *in vitro* is sufficient by using a single protein to begin the osteoinductive process [128]. This has made the clinical use of BMP easier when it is required the new bone formation. There are already medicines based on BMP-2 and 7 and obtained by recombinant DNA technology [129] there is another product used for animal experiments based on BMP extracted from bovine bone matrix. These products may have different effects depending on the mode of use. Better results are obtained when the delivery systems of these molecules are based on collagen or biodegradable polymers in order to obtain more localized and less loss of protein in the bloodstream [123]. The studies performed have shown that, once inoculated, these proteins are able to stimulate the host mesenchymal stem cells, so it is not necessary to perform extraction and isolation of *ex vivo* cells from the patient and their subsequent replanting, unlike other growth factors. Indeed, the BMP are the only way to stimulate *in vivo* growth, cell differentiation and tissue remodelling by simply using the appropriate drug delivery systems. Before they are put to clinical use it is still necessary to know any side effects. In animals, the use of these proteins was generally found to be safe, with the exception in some cases where an onset of erythema and inflammation at the injection site was noted [130]. However there is a limit on BMPs clinical use including the high production costs of both proteins and systems transport [131]. In fact, today the BMP with better activities are produced in *E.coli* and then re-naturation *in vitro*. This technique has a low yield and high cost of implementation. For these reasons, the clinical use of these molecules still very limited [132,133].

3.9 Osteogenic protein 1: OP-1

The first human BMP cloned in 1990 was OP-1 (Osteogenic Protein 1) which is also called BMP-7 [134]. Later progress showed that recombinant OP-1 was able to induce the new bone formation through stimulation of the mesenchymal stem cells [128]. Studies of the gene sequences have shown that this protein has 7 cysteines in C-terminal domain characteristic on the TGF- β family members (C ... CC CXGXC cxcx). The correct configuration includes the formation of three disulfide bridges between cysteines in position 300-396, 359-428 and 363-430, with the cysteine in position 395 remaining free. Moreover, like all members of this family, OP-1 is secreted by 4 times longer precursor than mature - active form comprised from serine 300 to histidine 431. The active portion has a high sequence homology with the other protein in this family, but this is not valid for the N-terminal portion. Between the precursor and the 7 cysteines domain there is a small peptide of 6 amino acids (serine 300 - lysine 305) which is the cutting signal for trypsin-like protease [135] (Figure 11). The function is performed by C-terminal domain but the upstream portion is vital in allowing the proper configuration of the *in vivo* active part. Also, like all other BMP, OP-1 should become a dimer through the formation of a disulfide bridge between the cysteines in position 395 of the two different monomers. Many studies have been conducted to understand the OP-1 tri-dimensional structure; this is indispensable in understanding the action mechanism and the exact interaction with the receptors Figure 3 CDNA sequence and corresponding amino acid sequence of OP-1. The numbering of amino acids and nucleotides starts with the first ATG of the coding sequence. The colored portion of the sequence indicates the active portion of OP-1. The sequence is shown in green the cutting signal site and in blue the domain containing the 7 cysteine [134]. The correctly folded OP-1 mature form results in a greek key shape and size approximately 60A x 20A x 15A [136]. The characteristic of greek key shape is when the order of antiparallel β strands do not follow the order that they have in the peptide chain. Due to this, one of the loops of this shape is extended.

OP-1 is composed by 8 sheet filaments giving two distinct portions, finger 1 - finger 2, and a α -helix strand between the third and fifth cysteine residues, arranged spatially as shown in Figure 11.

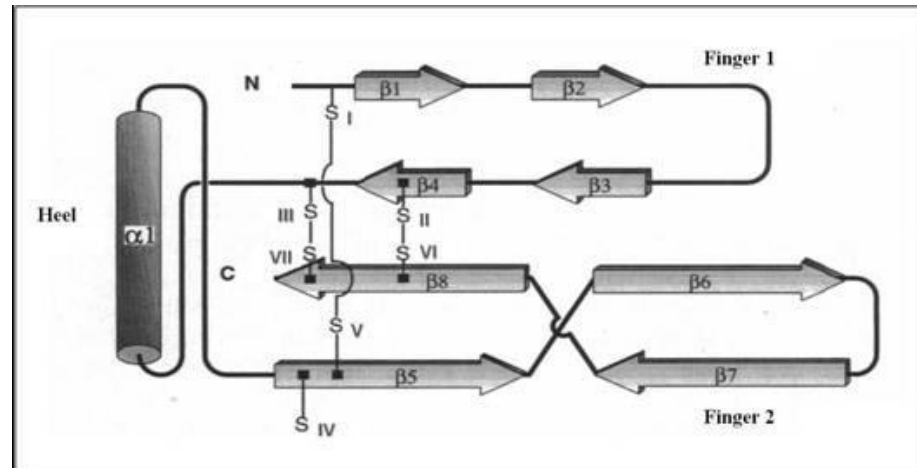


Fig 11: Graphic representation of mature OP-1 β package (residues 299-431). in the correct configuration: the 7 cysteines forming 3 disulfide bridges (II-VI, III-VII, IV) around 8 fragments These give rise to two protrusions (Finger 1 Finger and 2) that originate from the portion to α -helix (Heel) (Griffin *et al.*, 1996).

The β filaments are composed of: β 1, lysine 331 - histidine 333; β 2, tyrosine 336 - serine 3385, β 3, glutamic acid 352 - alanine 355; β 4, tyrosine 357 - glutamic acid 362; β 5 cysteine 395 - asparagine 402; β 6, isoleucine 404 - aspartic acid 410; β 7, asparagine 414 - tyrosine 420 and finally ; β 8, valine 424 - histidine 431. In particular, the filaments from β 1 to β 4 result in the portion finger 1, while β 5 to β 8 form finger 2. The α -helix portion extends from tyrosine 82 to isoleucine 94 and result in a long-helix with 3.5 turns and amphipathic characteristics, therefore containing both hydrophilic to hydrophobic amino acid residues. The helical structure also helps to stabilize the dimer formation [136]. Therefore the dimer is formed by the association of α -helix region and a monomer with the finger regions 1 and 2 of another monomer. This binding is stabilized by the formation of the disulfide bridge between the cysteines in position 395 of both monomers.

3.9.1 Protein transduction domains: TAT

The forging of new frontiers in medicine means it is necessary to use cell proteins or peptides for therapeutic action. The most widely used technique is the gene therapy. This term is referred to the genetic insertion of DNA material into cells to ensure that they can express the gene of interest. There are several ways to achieve this, including several viral transfection techniques involving the use of adenovirus [137], viruses adenoassociated

[137], lentivirus [138] and herpes virus [139]. All these systems have limitations for *in vivo* use because they can trigger an immediate or long-term immune response. In some cases they can cause neurotoxicity or impose a size limitation for the insertion of genetic material. Also, any possible long term effects for the patient are unknown [140].

Therefore other systems were used, including the recombinant proteins. These are produced, purified and renaturation *in vitro* and subsequently administered. This system is not very efficient because the recombinant proteins often do not possess the same activity of natural proteins nor produced directly by the cells. It is very difficult to get enough native protein by using *in vitro* techniques, as the process generally gives a low yield and it can also be very expensive. Moreover, the administration of proteins *in vivo* has problems associated with normal processes of transport and degradation inside the body [141]. However, with the discovery of peptides with the proteins transport function called protein transduction domains (PTD). The PTD have the ability to deliver drugs and proteins of sizes up to 110 kDa within different cell types [142]. The effectiveness of these domains was also demonstrated in the systemic transport of proteins *in vivo* [141].

The most commonly used is the domain belonging to the virus called HIV TAT. This domain was discovered and characterized in 1988 by two separate research teams: Green and Loewenstain and Frankel and Pabo [143,144]. In HIV, TAT is a protein of 86 amino acids and is responsible for the transport of viral proteins inside the cell. The active portion is involved in the protein transport and is composed of 11 amino acids, many of these are basic: YGRKKRRQRRR [145]. The presence of basic amino acids is a characteristic shared by all PTD that have been characterized. Subsequent studies have shown that this domain is capable of delivering proteins other viral. In particular, more efficient transport was obtained when the proteins are in denatured state. The cell provides the renaturation and subsequent activation of the protein once inside [144]. This system avoids all *in vitro* renaturation steps, but generally the yield is low. By using these carriers, the protein of interest can be inoculated directly to the desired location and importantly, the internalization process is much faster than with undelivered proteins. It is thus possible to avoid most of the processes of proteolytic degradation in the body. Despite numerous studies focusing on this, the PTD mechanism that allows the carrying of the cargo inside the cell is still unknown. It is understood that an important role is played by basic amino acids; they are involved in the hydrogen bonding between the hydrophobic phospholipid of the plasma membrane and the hydrophilic groups of

basic amino acids [146]. However this is not sufficient to explain the entire mechanism. In similarity to other PTD, TAT does not use the classic transport systems as there is no specific carrier or systems such as endosomes [147]. The high charge density possessed at physiological pH by these peptides exclude passive transport due to the difference in gradient concentration [148]. Experiments have shown that energy transport in the form of ATP is present in some cell types, while in other cells this phenomenon is completely absent [149].

Different behaviours were observed by changing the characteristics and size of the transported protein. All these aspects limit the use of these systems *in vivo* because each PTD fusion product requires an appropriate characterization both in the transport or activity efficiency once inside the cell.

Also the toxicity aspects are varied between cases. Thus a great limit in the use of PTD is the amount of research and development required for each product. This leads to extended periods of time needed for the experimentation and validation of the constructs [150].

3.9.2 The recombinant protein TAT-OP1

The recombinant protein used in this thesis consists of 162 amino acids and can be divided into two portions.

The N-terminus is made by 30 amino acids and is comprised of the 11 TAT amino acids and six histidines necessary for the protein purification process.

The C-terminal consists of 132 amino acids corresponding to the residues 300 to 431 of the native protein and this constitutes the active portion of OP-1. This portion thus includes both the 6-amino acid peptide that acts as a cutting signal for enzyme trypsin-like and the domain with seven cysteines (Fig. 12).

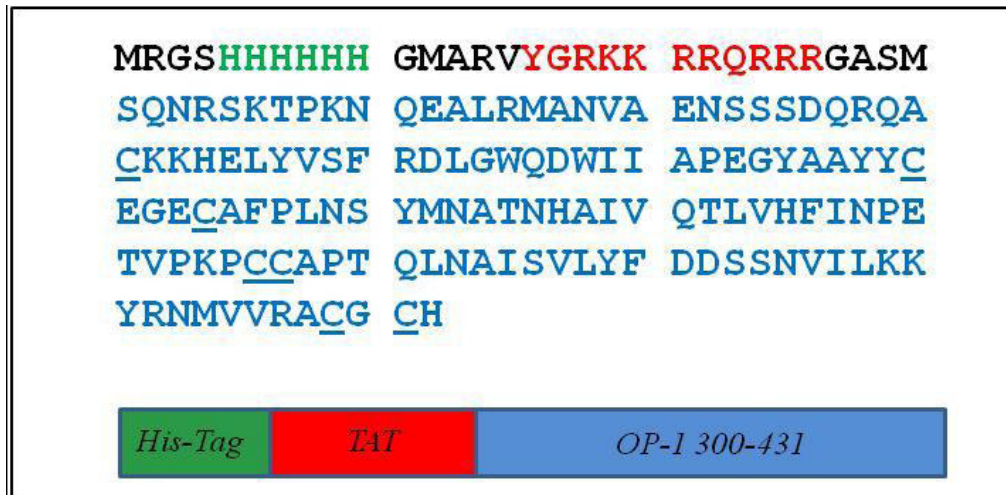


Fig12 Sequence and schematic representation of TAT-OP1.

The TAT-OP1 designed is presented as an ideal construct for tissue engineering applications in the regeneration of bone tissue.

There is the active portion of the OP-1 protein which is a powerful factor in the stimulation of osteogenic differentiation.

Moreover, it has the TAT sequence making it possible for the intracellular translocation of the protein.

The presence of the TAT peptide is a major advantage for the *in vitro* construct studies and especially for the *in vivo* application. The recombinant protein OP-1 can be used without need of *in vitro* renaturation processes, which are very expensive and have very low yields. The domain TAT will also make the administration of the protein possible, thus avoiding the many degradation processes by enzymes normally present in the body.

4. Materials and Methods

Materials:

Chemical compounds used in this thesis, like sodium chloride, sodium phosphate monobasic and dibasic, potassium chloride, potassium phosphate mono phosphate, EDTA, DTT, hydrochloric acid, acetonitrile, TFA, NHS, DCC, etc. were purchased from Fluka (Basel, Switzerland), from Sigma - Aldrich (Saint Louis, Missouri) or from Merck (Darmstadt, Germany) and had an analytical grade. The plastic material for cell biology was provided by BD Falcon (San Jose, California), while medium, FBS serum and antibiotic from Sigma-Aldrich. Puramatrix HydrogelTM was purchased from 3DM inc. Other materials are specified with a commercial source in the text.

4.1 Synthesis and purification of FOL-PEG-EPI

4.1.1 Preparation of the FOL-PEG-COOH conjugate.

The carboxylate group of folic acid was activated by *N*-Hydroxysuccinimide (NHS) and *N,N'*-Dicyclohexylcarbodiimide (DCC) as described earlier [Schiavon et al, 2008]. Briefly, 500 mg (1.13×10^{-3} mol/L) of folic acid were dissolved, via stirring, in 10 mL of anhydrous dimethylsulfoxide (DMSO) and 0.24 mL (1.7×10^{-3} mol/l) of triethylamine (Et₃N); this was reacted with 260.79 mg of NHS (2.2×10^{-3} mol/L) and 467.54 mg of DCC (2.2×10^{-3} mol/L) at room temperature overnight in dark conditions.

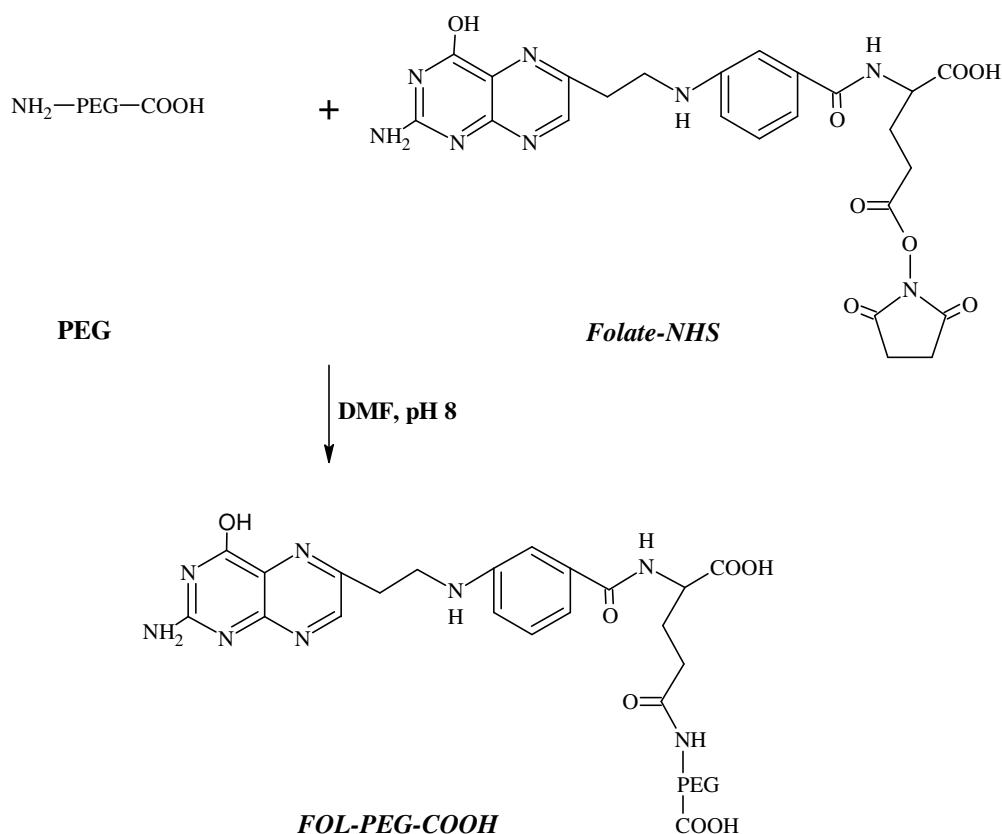


Fig. 13 FOL-PEG-COOH synthesis

The insoluble dicyclohexylurea was filtered and the solution was added drop by drop into 200mL of diethyl ether. After 4 hours at 4°C the precipitate, formed by folate-NHS, was filtered and vacuum dried.

The activated folic acid (172.31mg, 3.2×10^{-4} mol/L), dissolved in 6ml of anhydrous DMSO, reacted with 400mg (8×10^{-2} mol/L) of $\text{H}_2\text{N-PEG}_{5000}\text{-COOH}$ (5 kDa MW), previously dissolved in 2 mL of anhydrous DMSO. The pH was brought up to pH 8 using 40 μl (2.8×10^{-4} mol/L) Et_3N (triethylamine). The reaction was performed at room temperature via stirring in dark conditions for 4 h. The compound FOL-PEG-COOH was extracted by adding 5mL of water to the solution, by adjusting the pH to pH 7.5 and by adding 5mL of dimethylformamide (DMF) and chloroform (CHCl_3) (8X80mL non-solvent for folic acid). The organic phase collected was dried over Na_2SO_4 , reduced to a small volume and added to 200mL of diethyl ether. The intermediate was recovered by filtration and vacuum dried.

The percentage of bound folic acid was calculated via the determination of uncoupled PEG amino groups, using the Snyder-Sobocinski colorimetric assay [T.G.Park et al, 2005]. The

reaction of folic acid and H₂N-PEG₅₀₀₀-COOH was followed by RP-HPLC chromatography using a Phenomenex C18 column (250 x 4,6 mm, 5µm, 300 Å). Chromatography was performed with the following parameters: 1mL/min flow, 420nm UV.VIS. detector and the solvents used were (A) H₂O + 0.05% trifluoroaceticacid, (B) acetonitrile (ACN) 0.05% trifluoroaceticacid. The gradient settled is shown in table 1.

Run Time (minutes)	% Eluent B
0	5
30	60
35	90
40	5

Table 1 RP-HPLC elution gradient used to detect FOL-PEG-COOH

4.1.2 Determination of uncoupled amino group percentage in FOL-PEG-COOH conjugate.

The percentage of the uncoupled amino group was determined indirectly by using the Snyder-Sobocinski (TNBS) assay.

The reaction between 2,4,6- trinitrobenzene sulfonic acid and the primary ammine group of H₂N-PEG-COOH, in an alkaline solution, formed trinitrophenil derivates that absorbe at 420 and 340nm.

Briefly, two equimolar solutions (2.157×10^{-3} mmol/mL) of FOL-PEG-COOH(sample) and H₂N-PEG-COOH (control) were made.

25µL of the sample solution were added to 950µL of borate buffer pH 9.3 as well as 19µL of TNBS solution. The same procedure was used to make the blank solution but the amount of phosphate buffer to start the reaction was 975µL.

The reaction was kept at room temperature for 30 minutes and its absorbance at 420nm was determined.

To obtain the percentage of the free ammine group, the sample absorbance, multiplied by a factor of 100, was divided by the absorbance of the control solution. The result, as shown below, was the free ammine group.

Sample O.D._{420nm} : Control O.D._{420nm} = x : 100

Sample O.D._{420nm} : medium absorbance of FOL-PEG-COOH

Control O.D._{420nm} : medium absorbance of H₂N-PEG-COOH

4.1.3 Epirubicin Conjugation to FOL-PEG-COOH

The carboxylic group of FOL-PEG₅₀₀₀-COOH was activated, so it could react with the glycosidic amine group of epirubicin. Briefly, 300 mg (6×10^{-5} mol/l) of FOL-PEG₅₀₀₀-COOH were dissolved in dichloromethane (CH₂Cl₂). To this solution 10.43 mg (9.06×10^{-5} mol/L) of NHS and 37.13 mg (1.8×10^{-4} mol/L) of DCC were added. Under stirring 53 mg (9.06×10^{-5} mol/L) of epirubicin were added and the pH was brought to pH 8 with 25 μ L of Et₃N.

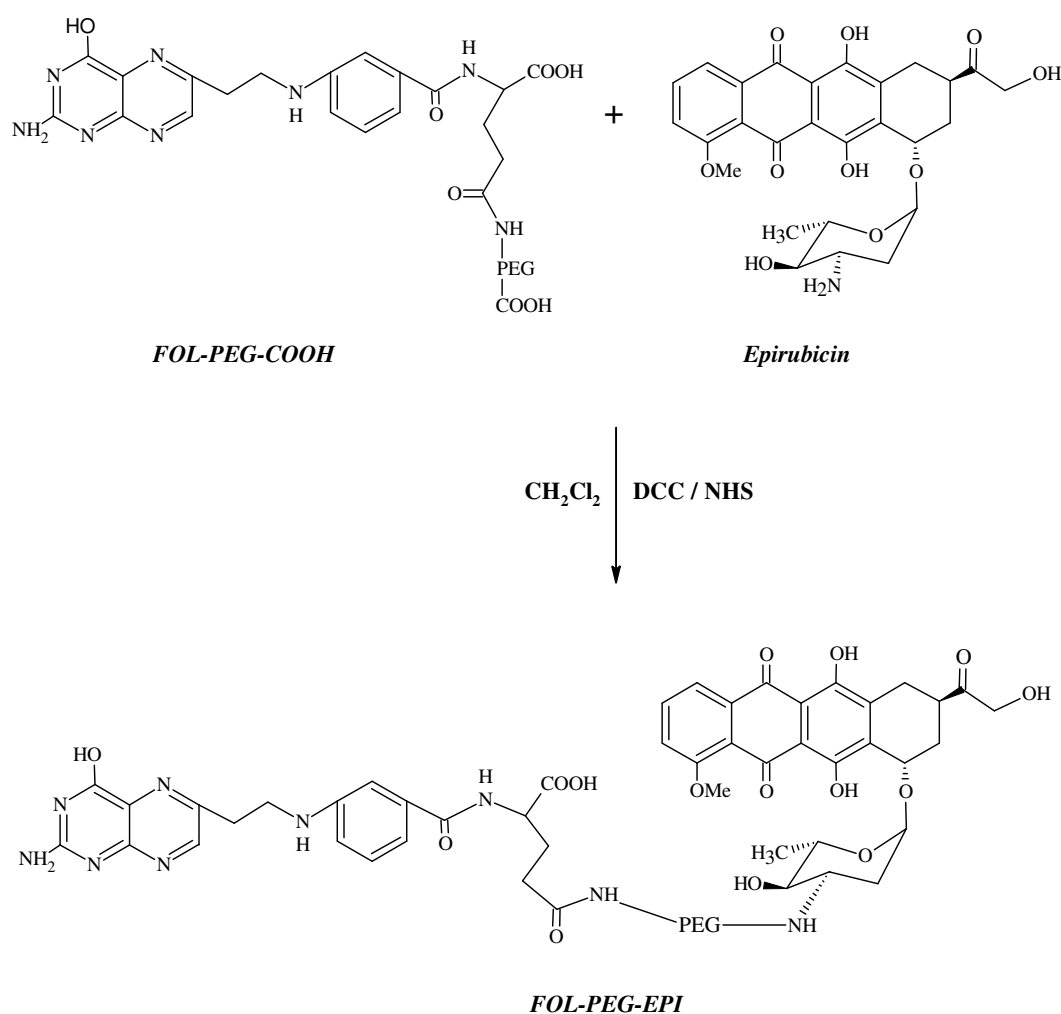


Fig. 14 Synthesis of FOL-PEG-EPI

The reaction, obtained after 4h under stirring in dark conditions, was purified with 5-6mL of CH₂Cl₂ (extraction solvent), and HCl 0.1N (4 x 60mL). The recovered organic fraction was dried with Na₂SO₄, reduced to a small volume and precipitated in 200mL pre-cooled diethyl ether, in the manner previously described. The conjugation yield of epirubicin and the free drug were calculated as reported in a previous study for doxorubicin [Pasut et al, 2008] and is reported in chapter 1.5, 1.6. The epirubicin conjugation to FOL-PEG₅₀₀₀-COOH, was monitored by RP-HPLC chromatography using a Phenomenex C18 column (250 x 4,6 mm, 5µm, 300 A). Chromatography was performed with the following parameters: 1mL/min flow, 480nm (for epirubicin conjugation) UV.VIS. detector and (A) H₂O + 0.05% trifluoacetic acid (TFA), (B) acetonitrile (ACN) + 0.05% TFA as solvents. The gradient settled is show in table 2.

4.1.4 Epirubicin determination

The quantification of free and conjugate epirubicin was performed using a calibration curve obtained at different epirubicin concentration as show below:

- solution 1: 0.001 mg/mL
- solution 2: 0.004 mg/mL
- solution 3: 0.008 mg/mL
- solution 4: 0.02 mg/mL
- solution 5: 0.05 mg/mL
- solution 6: 0.1 mg/mL
- solution 7: 0.5 mg/mL

The solutions were analysed by RP-HPLC C18 chromatography using a Phenomenex C18 column (250 x 4,6 mm, 5µm, 300 A). Chromatography was performed with the following parameters: 1mL/min flow, 480nm UV.VIS. detector and (A) H₂O + 0.05% tryfluoroacetic acid (TFA) (B) acetonitrile (ACN) + 0.05% TFA as solvents. The gradient settled is show in table 2.

Run time (minutes)	% Eluent B
0	25
10	30
15	80
20	25

Tab 2 RP-HPLC C18 elution gradient

After that, the peak area at retention time (R_T) of 10.9 minutes due to free epirubicin, was quantified by using the standard curve.

4.1.5 Conjugated epirubicin determination

To quantify the conjugated drug, a standard curve was obtained at different epirubicin concentration as reported below:

- solution 1: 0.001 mg/mL
- solution 2: 0.004 mg/mL
- solution 3: 0.008 mg/mL
- solution 4: 0.02 mg/mL
- solution 5: 0.05 mg/mL
- solution 6: 0.1 mg/mL
- solution 7: 0.5 mg/mL

200 μ l of these solutions were hydrolyzed by adding 200 μ l of HCl 2N and placed at 50°C in a thermostatic bath for 2 hours. The hydrolysis removes the glucosidic part of epirubicin giving adriamicinon, which has a different retention time of the free drug and is also insoluble in a water solution.

After 2 hours, to achieve the adriamicinon solubilisation, 1200 μ L of DMSO (dimethylsulfoxyde) were added to each one of these solutions, which were then analyzed via RP-HPLC with the same conditions as described in the previous paragraph (1.4).

The same procedure was used to make 1mg /mL FOL-PEG-EPI hydrolysis. The solutions were analysed by RP-HPLC C18 chromatography using a Phenomenex C18.

By using the peaks area of the adriamicinon solutions the standard curve was made, and the conjugated epirubicin was calculated.

4.2 Biological activity

4.2.1 Cell line

In this study three different cell lines were used, KB-31, MCF-7 and HT-29, distinguished by the well known different surface expression of folic acid receptor FR α (Sovinco F et al, 2005).

4.2.1.1 KB-31

KB-31 cell line was obtained from human oropharyngeal carcinoma. These cells have the typical morphology of epithelial cells and present a multilayer growth. They replicate within 24 to 36 hours and it has been reported that they posses a high level of FR α expression (FR++). (Sovinco F et al, 2005)

4.2.1.2 MCF-7

MCF-7 is a breast cancer cell line The characterised by an epithelial morphology. The replication time is between 24 and 36 hours and the FR α expression is slightly higher than that of normal human cell (FR+).(Sovinco F et al, 2005)

4.2.1.3 HT-29

The human colon adenocarcinoma cell line HT29 displays an epithelial morphology and Folic Acid Receptor normal expression.

The cells were seeded, with a density of 7.5×10^{-3} cells/cm², in Petri dishes with Dulbecco's modified medium (DMEM-LG), fetal bovine serum (FBS) 10% and an antimicrobic antimicotic solution (10000 U/mL penicillin, 10000 μ g/mL streptomycin sulphate, 25 μ g/mL anphotericin B) 1% [[media 1](#)].

The cells were incubated at 37 °C in an atmosphere of 95% air-5%CO₂ and examined under an optical microscope. When the cells reached 80% confluency they were detached using a PBS solution of trypsin (0.25%) and EDTA (0.02%). After centrifugation at 1200rpm for 5minutes the cells were re-suspended in the culture medium and seeded in Petri dishes at optimal density.

4.2.2 Sample preparation

The cytotoxicity localization and up-take of FOL-PEG-EPI were investigated in two different culture systems, bi-dimensional (2D) and tri-dimensional (3D).

-Bi-dimensional culture system (2D): the cells, KB-31, MCF-7 and HT-29, were detached by using enzymatic treatment (EDTA 0.02%-trypsin 0.25%) and seeded (1×10^4 cells/cm²) in monolayer on a 96 well plate (Falcon). After 24 hours from cells seeding, the conjugate FOL-PEG-EPI and the negative (samples without treatment) and positive (samples treated with epirubicin solution) controls were added. Three experiments were conducted for each condition.

-Three-dimensional culture system (3D): the self assembling peptide RADA-16 called puramatrix hydrogel was used to grow the cells line in 3D. The peptide matrix with a concentration of 1% was purchased from 3DM Inc. This solution had a high intrinsic density, so it had to be sonicated for 30 minutes in a ultrasound bath before use and subsequently diluted to 0.5% with 20% of sucrose water solution. The cells were trypsinized, spun down at 1200rpm for 5 minutes and rinsed three times with a 10% sucrose water solution to eliminate all the salt residuals present in the media. The cells (2×10^5), were collected in 100µl of 10% sucrose solution, diluted in 100µl of 0.5% (0.5mg/ml, 6.43×10^{-5} mol/l) puramatrix hydrogel and gently mixed. The puramatrix cell mixture was pipetted into 24 well polytetraphenylethylene membrane inserts with a porosity of 0.4µm. The inserts were placed in a 24 well plate and the membrane was previously immersed in medium before being put inside the puramatrix mixture.

The self assembling of the scaffold was achieved by adding for each well 300µl of culture medium and for each insert 20µl as well. After incubation at room temperature for 10 minutes, the medium in the well on top of the insert was removed and another 300µl of media was pipetted on the insert. The complete assembling was obtained by incubation at 37°C for

30 minutes. After ten minutes of incubation the medium was changed and removed from the well. The sample was observed with LEICA DMR optical microscope. The cells were grown by adding 400µl and 300µl respectively in the well and inserts. After 24 hours of incubation at 37°C the treatments with epirubicin (positive control), with medium alone (negative control) and with FOL-PEG-EPI were performed in triplicate.

4.2.3 Cell treatment

4.2.3.1 FOL-PEG-EPI and Epirubicin solutions

1mg/ml of FOL-PEG-EPI and epirubicin solutions were made, and the effective concentration was measured via spectrophotometer analysis (Beckman) in correspondence of the maximum absorbance of epirubicin at 488nm. After that, different concentration solutions for each cell line were made by the dilution in medium of the stock solution. In tab 3. the concentrations used for the cells treatment are shown.

<i>KB-31</i>	<i>Concentration (µM)</i>		
	1	2	3
Epirubicin	0,017	0,021	0,04
FOL-PEG-EPI	0,46	0,58	0,8

<i>MCF-7</i>	<i>Concentration (µM)</i>		
	1	2	3
Epirubicin	0,065	0,09	0,13
FOL-PEG-EPI	1,32	2,65	2,81

<i>HT-29</i>	<i>Concentration (µM)</i>		
	1	2	3
Epirubicin	0,12	0,16	0,32
FOL-PEG-EPI	1,95	2,65	15,8

Tab. 3 Concentration used of FOL-PEG-EPI and Epirubicin for the cytotoxicity assay.

4.2.3.2. 2D Culture System

After 24 hours of incubation at 37°C of the cells lines, KB-31, MCF-7, HT-29, the media was removed and replaced with 100µl of epirubicin in medium 1 or with 100µl of FOL-PEG-EPI solubilised in medium without folic acid FDMEM(gibco), fetal bovine serum FBS (10%), AF (10000 U/ml penicillin, 10000µg/ml streptomycin sulphate, 25µg/ml anphotericin B) (1%) [medium 2].

Cytotoxiciy was assayed at 24, 72 hours and 7 days of incubation at 37°C and 5% CO₂.

4.2.3.3. 3D culture system

After 24 hours at 37°C, the cells encapsulated in the puramatrix hydrogel were treated by replacing the culture medium with 400µl in the well and 300µl inside the insert with epirubicin solubilised in media 1 or with conjugate solubilised in media 2.

After 24, 72 hours and 7 days the cytotoxicity was assayed.

4.2.4 Cytotoxicity assay

The colorimetric assay MTT (3-[4,5-dimethylthiazole-2-il]-2,5-diphenyltetrazolebromure) was used to determinate cell viability. The reduction of this substrate, due to succinate dehydrogenase on the mitochondria membrane, forms formazane crystals which have an intense blue colour. The crystal extraction was performed by using isopropanol acid and the extraction solution was analysed with a spectrophotometer at 570nm. The data was compared to the negative control (cell without treatment) to achieve the IC₅₀ for each cell lines.

4.2.4.1 2D Culture system

After 24, 72 hours and 7 days of treatment the medium was removed and replaced with 100ul of MTT solution made at 10% in DMEM-LG without serum or antibiotics. The cells were incubated at 37°C for 2.5 hours and subsequently 100ul of isopropanol acid extraction solution were used. The plate was kept at room temperature on a stirring plate for 15 minutes. The absorbance of the formazane solutions, of the negative control and of all the treated samples was analysed by spectrophotometer at 570nm (ELISA Elx 508).

4.2.4.2 3D culture system

The 3D puramatrix cell mixture was taken off the inserts, placed in 1.5ml eppendorfs and re-suspended in 1 ml of media with 10% MTT without serum or antibiotic. The eppendorfs were vortexed and incubated at 37°C for 2.5 hours. After that the sample was centrifuged at 7000rpm for 5 minutes, the media were removed and the pellet was resuspended in 100ul of isopropanol acid extraction solution. This solution was incubated in shaker plate for 10 minutes at room temperature. After centrifugation in the same conditions reported before, the supernatant formazane solutions were pipetted in a 96 well plate for spectrophotometer analysis at 570nm.

4.3 Localization and Up-take Study

The localization and up-take studies were carried out in all cell lines, KB31, MCF7, HT29, by using confocal laser scanning microscopy (CLSM) and flow cytometry. The first technique was used to localize the fluorescence signal of epirubicin in the sample after laser (Argon) excitation at 488nm. The quantitative measurement of free drug and conjugate uptake inside the cell populations was achieved using the second technique by using a Moflo high speed cell sorter laser 488nm. The data was analysed using summit 4.0 Dako software.

4.3.1 Confocal laser scanning microscopy

Epirubicin has an auto fluorescence due to the aglyconic part of the molecule with an emission at 570nm and an excitation at 488nm. The confocal laser scanning Leica SP5 was used to localize the fluorescence signal obtained by laser argon excitation at 488nm.

4.3.1.1 2D Culture system

Five hundred μL of KB-31, MC-F7, HT-29, were seeded at 5×10^4 cell/cm² in 0.69 cm² chambers slides (Falcon). After 24 hours the cells were treated with 20 μM free drug and FOL-PEG-EPI for 10, 30, 60, 90 minutes and 24 hours. At the end of each time point the

media were removed and the wells were rinsed with 0.1M PBS. Cultures were fixed in HEPES 10mM / p-formaldehyde 4% / 7% sucrose pH 7.2 and kept at 4°C for 20 minutes. The cover slips were placed on the top of the slides with a 50 : 50 solution of PBS and glycerol. Samples were kept in the fridge.

4.3.1.2 3D culture system

2×10^5 cells for each kind of cells line were included in puramatrix hydrogel as previously described, and after treatment for 10, 30, 60, 90 minutes and 24 hours with 20 μ M conjugates and epirubicin the cultures were rinsed in PBS and fixed in HEPES 10mM / p-formaldehyde 4% / 7% sucrose pH 7.2 for 24 hours at 4°C. Afterwards part of puramatrix cell mixture was placed on a glass slide. The cover slips and solution 50 : 50 PBS/glycerol were used as early reported. Samples were kept at 4°C.

4.3.2 Cytofluorimetry

4.3.2.1 Uptake of bioconjugates

1.5×10^6 cells from KB-31, MCF-7, HT-29 cultures were prepared as modisperse cellular suspension using 5% BSA in PBS and submitted to the treatment with 20 μ M FOL-PEG-EPI for 30 minutes at 37°C. In parallel, samples were prepared also using 20 μ M mPEG-EPI, or 20 μ M epirubicin. Untreated cells were used as negative control. After a centrifugation at 1200rpm for 5 minutes, the supernatants were discarded and pellets were resuspended in 250 μ l 5% BSA solution. The samples were kept at room temperature on ice in dark conditions. In parallel, the same treatment was performed using cultures prepared in bidimensional in vitro system. The uptake study was done using Moflo High Speed Cytometer (Dako-Beckman Coulter) and the acquisition (400 events/second) was performed by Summit 4.0 software.

4.3.2.2 Trafficking study by confocal microscopy

KB-31, MCF-7, HT-29 were seeded at 1×10^4 cell/cm² on glass rounded cover slips kept in tissue culture 24-well plates and encapsulated in puramatrix hydrogel (2×10^5 cells/ml). After 24 hours 20 μ M bioconjugates and free drug were added to the culture medium and the treatment

was performed for 30 minutes and 72 hour. The cells were fixed using 10mM Hepes / 4%p-paraphomaldehyde/7% sucrose pH=7.2 for 20minutes at 4°C. The samples were mounted using 50% glycerol (Sigma) solution in PBS and kept at 4°C till the analysis on Leica SP5 Confocal Microscope (Leica-Mycrosystems).

4.4 Preparation and characterization of TAT-OP1 microspheres

The osteoinduction was achieved by using poly(lactic-*co*-glycolic acid) PLGA loaded with the recombinant human TAT-OP1. The protein was purified and characterized by Prof. Grandi and Prof. Negro from the Università degli Studi di Padova.

4.4.1 Preparation of microspheres

Three hundred mg (1.2×10^{-5} mol/l) of PLGA 50:50 polymer RG503H (Boehringer Ingelheim) in 10 ml of dichloromethane (DCM) were emulsified in an aqueous solution of 0.2% w/w polyvinylalcohol (PVA Sigma) and with 1% w/w of TAT-OP1. The water/oil emulsion was spray dried by Mini Spray Dryer B-290 (Buchi) using a nozzle tip aperture of 0.7mm.

The tested process parameters were: inlet temperature 50° C, aspirator 100%, compressed air flow 600 NI/h, feed rate 20.

4.4.2 Release study

The *in vitro* protein release was carried out at 37°C in a phosphate buffer solution (0,1M PBS, pH 7.4). The microspheres (15 mg) suspended in 500 µl of buffer solution and 10 µL of 20mM dithiothreitol (DTT) were incubated in a shaking water bath. At different time points half the solution was removed and replaced with 240 µL of fresh PBS and 10 µL of DTT. The TAT-OP1 content was evaluated by RP-HPLC. Before the injection on RP-HPLC, the disulfide bonds in the protein were reduced with 8 M guanidium at pH 8.3 and 20 mM DTT in nitrogen atmosphere for 20 minutes at 37°C. After that the sample was acidified with TFA / H₂O 50% / 50%, centrifuged for 3 minutes and injected in RP-HPLC (Vydac C4 column) with an inner diameter of 4 mm and length of 15 cm. The eluants used were (A) H₂O + 0.05% v/v trifluoroacetic acid (TFA), (B) acetonitril (ACN) + 10% H₂O + 0.05% v/v TFA.

The vyd_op1 gradient was used as reported in tab 4.

Time (min)	Flow (ml/min)	B %	Detector on (nm)
0	1.00	1,5	220
20	1.00	80	220
22	1.00	100	220
24	1.00	100	220
25	1.00	1,5	220
30	1.00	1,5	220

Tab 4 vyd4_op1 gradient used with Vydac-C4 column in RP-HPLC chromatography.

Both solvents were sonicated for 20 minutes before use. The UV.VIS detector was settled at 220nm.

4.4.3 Encapsulation efficiency

Ten mg of the microspheres were dissolved in 200 μ L of dichloromethane (DCM) and 500 μ L of 8 M GuHCl / 20 mM DTT and shaken for 15 min. The aqueous phase containing the extracted protein was clarified via centrifugation at 4000 rpm for 5 min. The protein content of each sample was determined using RP-HPLC (Vydac C4) at room temperature as reported before (paragraph 1.2).

4.4.4 Scanning electron microscope

The shape, morphology and diameter of the PLGA empty and loaded with rhTAT-OP1 microspheres were determined by scanning electron microscopy (SEM). The samples were sprinkled onto carbon tape attached to aluminium mounts followed by silver coating obtained using a Technics Hummer II sputter coater. Images were obtained using a JEOL JSM-6300 Scanning Electron Microscope.

4.5 Cell culture

For our study we used the MC3T3-E1 cell line (murine pre-osteoblasts) cultured with α -MEM medium (Sigma) supplemented with 10 % FBS (Fetal Bovine Serum, Sigma), 2 mM glutamine (Sigma), 100 μ g / mL penicillin and 100 μ g / mL of streptomycin (both Sigma). During proliferation cells were seeded at 7000 cell / cm² in 100mm Petri dish and cultured at 37°C with 5 % CO₂. For the experiments the cells were washed with PBS (Phosphate Buffer Sample: 137 mM NaCl, 2,7 mM KCl, 8 mM NaH₂PO₄, 1,5 mM KH₂PO₄, pH 7,2), detached from the Petri dish with a solution 0,25% trypsin - EDTA 0,02% (Gibco) and seeded in 96 or 24 well plates (0.63 or 1.76 cm² respectively, Falcon) with the same seeding density. For immunocytochemistry, the cells were seeded on glass cover slips, sterilized with UV overnight.

The cell treatment in which the culture media and TAT-OP1 microspheres were removed and replaced with fresh media or media plus microspheres, was repeated every three days.

The cytotoxicity assay was performed at 24 hours and 12 days. Furthermore, the osteogenic differentiation induction was done at 7, 14, 21 days. The cells grown in culture media were used as control.

The UCBMSC cells were obtained from Interdepartmental Centre of Research and Service for Biology and Regenerative Medicine S. Luca Hospital, Trecenta (RO).

4.5.1 Cytotoxic assay MTT

The cytotoxicity assay was done by using 10 % CellTiter 96[®] Cell Proliferation Assay (MTT) (Promega) in α -MEM medium (Sigma). The tetrazolium component was converted by mitochondrial dehydrogenase of living cells in the coloured product, formazan salt. The salt was solubilised with an isopropanol acid solution and the solution's optical density (O.D.) was measured at 570 nm with an ELISA plate reader.

The assay was performed on MC3T3-E1 cells treated with culture media as a control, with empty microspheres and microspheres loaded with TAT-OP1. The cells were seeded in 96 wells plate in culture media. After 24 hours the culture media were removed and replaced with media containing 200nM TAT-OP1 microspheres (the higher microspheres concentration) or microspheres alone at the same concentration (w/w). The treatment was

performed in triplicate. The MTT assay was performed after 24 and 12 days of treatment. At the end of the treatment the media were removed and 200 μL / well of media with 10 % of MTT were added. After 2 hours of incubation at 37°C the media were removed and 100 μL of 0.2% v/v isopropanol extraction solution were added. After 15 minutes 100 μL of formazane solution from each sample was placed in a new 96 well plate and the optical density was measured with an ELISA Ultra Microplate Reader (Bio-Tek Instruments) at 490 nm. The results were expressed as the percentage of cells viability compared to the control. The data analysis was carried out using Student *t-test*.

4.5.2. Osteogenic differentiation induction by TAT-OP1 microspheres.

The biological activity of TAT-OP1 loaded in the microsphere drug delivery system was tested on MC3T3-E1 and UCBMSC cells with different culture systems.

In the first case with MC3T3-E1 the TAT-OP1 microspheres were added inside the insert well culture system (Millipore). The microspheres were re-suspended in culture media with 50mM L-Arginine (Sigma) as a suspension stabilizer and then pipetted inside the insert well (culture system 1).

The second culture system used for UCBMSC was made by using Puramatrix peptide hydrogels (3DM) where the cells and microspheres were included in the 3D scaffold (culture system 2).

4.5.3 ALP BCA analysis

The bone alkaline phosphatase (ALP) enzyme is a bone mineralization marker and it is expressed on the mature osteoblast membrane.

To measure quantitatively the amount of this enzyme, the alkaline phosphatase yellow kit substrate (Sigma) was used (ALP kit). Owing to the enzyme activity, the substrate becomes yellow after incubation at 37°C and the optical density of each sample can be read using spectrophotometer analysis at 405 nm.

The bicinconinic acid assay (BCA, Pearce) was done to obtain the total amount of protein for each well. BCA uses the oxidation of copper from (Cu^+) to (Cu^{++}) by the primary ammine group of the protein. As a result of this the bicinconinic acid forms a blue coloured complex with bivalent cations, such as Cu^{++} .

The MC3T3-E1 cells were seeded in 24 wells plate in culture media and after 24 hours the culture media were removed and replaced with culture system 1 containing inside the insert 300 μ L culture media, empty microspheres as a control, or 200 nM, 27 nM of free protein and loaded TAT-OP1 microspheres, while 400 μ L of culture media were put in the well. The treatments were performed in triplicate by using the treatment previously described. The ALP assay was performed after 7 and 14 days of treatment. At these time points the media were removed and 300 μ L / well of ALP kit solution were added. After 30minutes of incubation at 37°C, 100 μ L of substrate for each well were pipetted in a 96 well plate and the optical density was measured with an ELISA Ultra Microplate Reader (Bio-Tek Instruments) at 405 nm. Subsequently the wells were rinsed with 0.1M PBS and 0.2% sodium dodecyl sulphate (SDS) solution was added.

After 15 minutes in a shaking plate the cells were detached and pipetted 10 times. Ten μ L of this protein solution were placed in a 96 well plate. This was repeated for each well.

At this point 200 μ L of the BCA reagent kit were added to each well and the plate was incubated at 37°C for 30 minutes. A BCA standard curve made by using known concentrations of albumin was also obtained.

The data was analysed with Student t test and expressed as alkaline phosphates per μ g of total protein per each well.

4.5.4 Mineralization analysis

This assay was performed with Alizarin Red S (Sigma) to quantify the calcium presence in the extracellular matrix. Alizarin Red S is an anthrachinonic derivate that can complex the calcium ions giving a red coloured compound soluble in cetilpiridinium clorure (CPC) (Sigma).

The MC3T3-E1 cells were seeded in 24 well plate in culture media. After 24 hours the culture media were removed, replaced with culture system 1 containing inside the insert 300 μ L of 200 nM, 27 nM of native protein, with 200 nM, 27 nM of TAT-OP1 loaded into microspheres and medium alone or empty microspheres both as controls, while 400 μ L of culture medium was put on well. Every treatment was performed in triplicate.

The assay was performed after 7, 14, and 21 days of treatment and incubation at 37°C.

At the end point the medium was removed and the cells were washed with 0.1 M PBS and then fixed in ethanol 70 % (v/v) for 1 hours at -20°C. After that the cells were rehydrated

with deionised water for 2 times and subsequently the 40 mM Alizarin Red S pH 4,2 in deionised water was added for 10 minutes at room temperature. The dye was removed, the cells were washed 5 times with deionised water and kept for 15 minutes in 0.1 M PBS under slow agitation. To solubilise the alizarine red S a 10 % (p/v) of CPC solution in phosphate buffer 10 mM pH 7.0 was made. This solution was placed on the cells for 15 minutes. One hundred μ L extraction solution for each sample were pipetted into a 96 well plate and read by an ELISA Ultra Microplate Reader (Bio-Tek Instruments). The optical density was measured at 570 nm.

The data was analysed with Student t test and expressed as optical density at 570 nm.

4.5.5 Immuno fluorescence analysis

The osteogenic markers osteocalcine, osteopontine and Cbfa1/Runx2 were qualitatively identified via immunofluorescence staining. The MC3T3-E1 cells were seeded in culture media on cover slips placed in a 24 well plate and left to grow for 24 hours. The cells were then treated as shown before (paragraph 2.1.5).

The assay was performed after 24, 48 hours and 12 days of treatment and incubation at 37°C. At these time points the cells were fixed with cooled methanol for 10 minutes at -20°C. To identify the citoplasmatic or nuclar marker the cellular membrane was treated with 1 % Triton X100 (Sigma) for 6 minutes at room temperature. The wells were washed with 0.1 M PBS and incubated with 10 % horse serum (HS) in PBS at 4°C for 1 hour and 15 minutes at room temperature to block the aspecific sites. After that the primary antibody diluted 1:100 in PBS – 10 % HS was added and incubated at room temperature for 60 minutes. The primary antibodies used were: osteocalcin, osteopontin and Cbfa1/Runx2 (Santa Cruz Biotechnology, Inc).

The primary antibody was removed and the plate was washed three times for 15 minutes with PBS – 10 % HS. The diluted secondary biotinylated pan-specific (Vector)antibody was added to PBS – 1.5 % HS (followed the maker manual) for 30 minutes at room temperature. The cells were then washed in PBS and treated in dark conditions for 10 minutes with Fluorescein Avidin DCS from Fluorescent Avidin Kit (Vector) diluted 1:500 in 10 mM HEPES solution, 0.15 M NaCl.

The samples were mounted with or without DAPI (4-6 diamidino-2-fenilindolo) (Vector).

As a negative control we used cells that were not incubated with the primary antibody.

Images of the samples were obtained via a fluorescence microscope Leica DMR and captured with a Leica DC500 camera.

4.5.6 UCBMSC 3D cell culture

It is well known from literature that 3D culture systems are capable of creating an appropriate microenvironment in which the complex cell-cell and cell-matrix interactions, that are really important for metabolic cell function, can occur (Hwang *et al.*, 2006).

For this reason this kind of system was very useful to study the biological activity of bioactive proteins such as growth factors and differentiative factors.

For this work we used the tridimensional matrix Puramatrix Hydrogel TM (Zhang *et al.*, 1993) obtained from a self assembling peptide sequence widely used for *in vitro* cellular differentiative studies (Semino, 2003).

The samples were made with 3×10^5 cells in 300 μ L of puramatrix peptide hydrogel as described in paragraph 2.2. The TAT-OP1 microspheres were included in the tridimensional scaffold at the concentration of 200 nM. We also performed the treatment with 200nM free protein and differentiative medium. As control we used cells in culture media.

4.5.6.1 TEM analysis

The osteoinductive activity of TAT-OP1 on the UCBMSC cells in 3D culture system was evaluated after 27 days of treatment with 200nM TAT-OP1 microspheres and 200nM of free protein.

At this time point the samples were fixed at 4°C with 2.5 % of glutaraldehyde in 0.08 M phosphate buffer pH 7.3, washed in 0.08 M phosphate buffer and post-fixed in 1 % tetra osmium oxide in 0.1 M phosphate buffer. The cells were also included in epossidic resin Epon 812 (Società Italiana Chimici).

Sixty nm sections were obtained using a ultramicrotome SuperNova LKB (Richert, Austria) and they were coloured with uranyl nitrate and lead acetate using the Sato procedure (Sato, 1968). They were also observed by transmission electron microscope (TEM) Hitachi H-300 (Hitachi, Tokyo, Japan).

5.Results

5.1 Synthesis and purification of FOL-PEG-EPI

The FOL-PEG-EPI synthesis was done by reaction of the folic acid carboxylic group activated to ester with *N*-Hydroxysuccinimide (NHS) and the amino group on H₂N-PEG-COOH. This was followed by epirubicin coupling with NHS activated carboxylic group on the other side of bi-functional PEG.

5.1.1 Synthesis and characterization of FOL-PEG-COOH

Activated folic acid was coupled to H₂N-PEG-COOH. The end point reaction was monitored by RP-HPLC C18 with the parameters described before (paragraph 4.1.1 materials and methods).

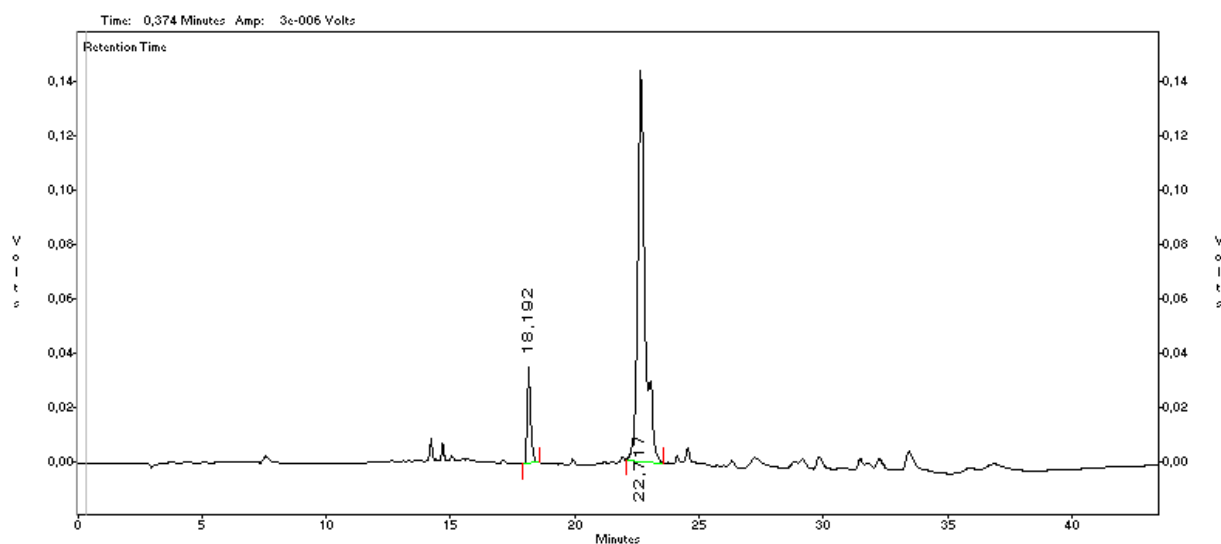


Fig. 1 Chromatogram obtained by RP-HPLC of 1mg /ml FOL-PEG-COOH solution. The peak at R_T 22.7 minutes was relative to the conjugate.

Fig. 1 shows the chromatogram of FOL-PEG-COOH in which it is easy to recognise the peak the conjugate, R_T 22.7 minutes, and the peak of free folic acid, R_T 18 minutes. The coupling reaction conversion was 97%, whereas the recovery yield was 75 %.

5.1.2 Epirubicin Conjugation to FOL-PEG-COOH and characterization

The carboxylic group on FOL-PEG-COOH was reacted with epirubicine, (recovery yield was 56 %).

The amounts of free and bound epirubicine in the purified conjugate was evaluated by RP-HPLC chromatography, (see table 1)

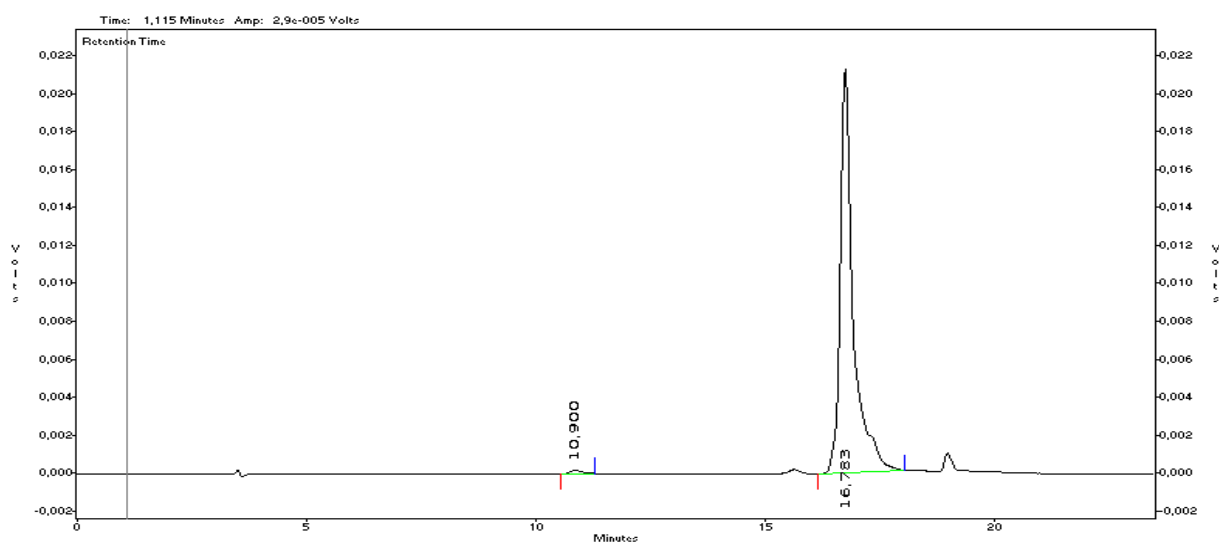


Fig. 2 Chromatogram obtained by RP-HPLC of 1mg /ml FOL-PEG-EPI solution. The peak at R_T 16.9 minutes was relative to the conjugate.

Fig. 2 shows the FOL-PEG-EPI elution profile in RP-HPLC. The conjugate retention time (16.9 minutes) was completely different from that of free epirubicin (10.9 minutes). The efficacy of the purification process is demonstrated by the low free drug amount 0.8 % (w/w). The total drug amount (free and bond)was determinate after acid hydrolysis of a solution of conjugate at the same concentration.

In Tab. 4 we have the final data regarding epirubicin.

% Free Epirubicin (w/w)	Total Epirubicina for mg of conjugate (μg)	Coupling yield %
0,8	57,44	63,02

5.2 Studies on FOL-PEG-EPI conjugate

5.2.1 Citotoxicity

The cytotoxicity of epirubicin and the conjugate was studied in different human tumoral cell lines (HT-29, MCF-7 and KB-3-1) differing for the level of folic acid receptor (FR) expression. FR expression was low expressed in HT-29 (FR), medium expressed in MCF-7 (FR+) and over-expressed in KB-3-1 (FR++) cells. Every cell line was cultured through two different systems: the normal 2D and the 3D system obtained by using the puramatrix peptide hydrogel.

The evaluation of conjugate biological activity on human tumoral cell lines in 2D system is well known (Pasut et al., 2005). Clearly this system does not resemble the structure of an *in vivo* tumor, where the cells are organized in a 3D fashion. In the latter case the cells in the inner of the mass are less exposed to the drug, this leading to a great difference in terms of IC₅₀. For this reason it is really interesting to study the cellular response in a 3D system. Moreover, I investigated the folate targeted conjugate FOL-PEG-EPI, to evaluate the role of specific targeting in 3D in vitro culture system.

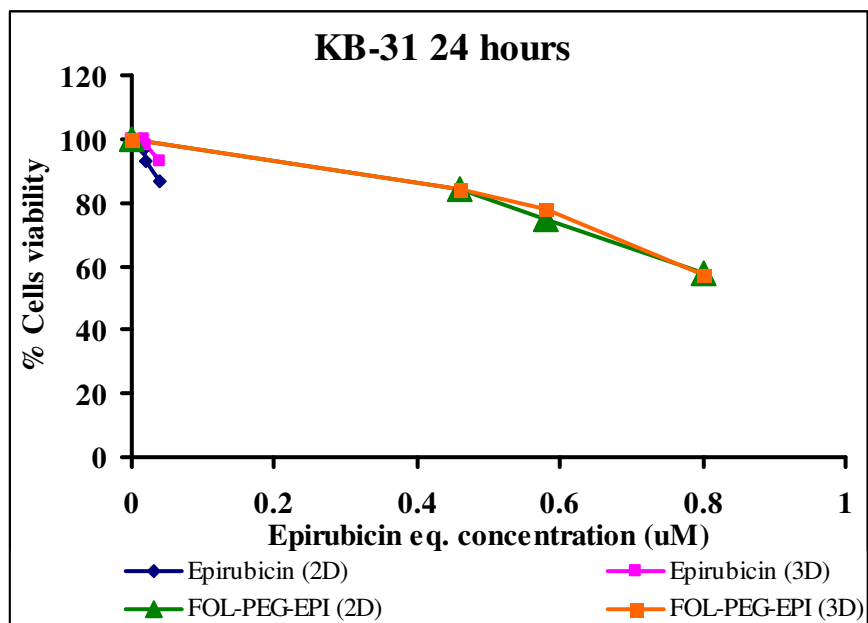
The Puramatrix hydrogel (3D) scaffold was obtained by the self assembling of the charged aminoacid monomer RAD-16. It is made up of 99% water and 1% of peptide. The peptide sequence is a repetition of arginin positively charged, aspartic acid negatively charged and in between the neutrally charged adenine (Zhang *et al.*, 1993).

The cells were encapsulated in 0.25% of Puramatrix, then treated with epirubicin and FOL-PEG-EPI solubilised in culture media. The IC₅₀ values of each cell line for the two culture systems are following reported.

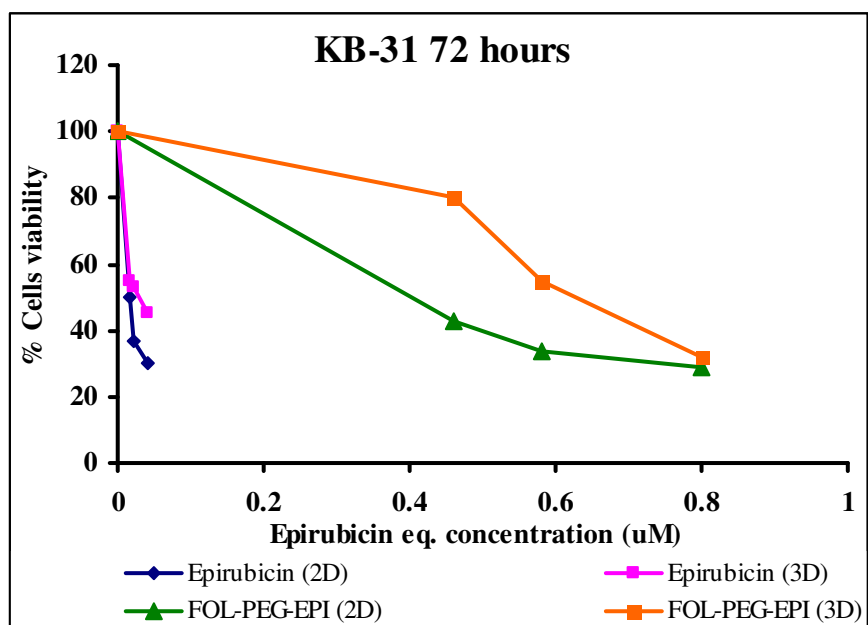
5.2.1.1 KB-31

Fig. 3 shows the cytotoxicity effect on KB-31 cells treated with epirubicin and FOL-PEG-EPI at 24, 72 hours and 7 days.

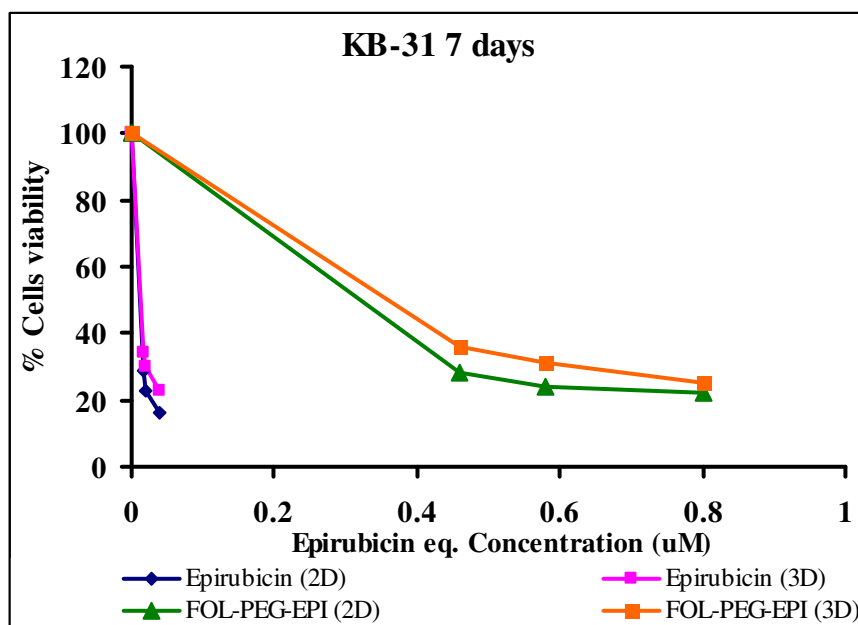
The conjugate had a lower cytotoxicity than free drug in both culture systems. The treatment at 72 hours and 7 days clearly displayed a 20 fold lower IC₅₀ for FOL-PEG-EPI compared to the free epirubicin tab 2 (p<0.05).



A



B



C

Fig. 3 Cytotoxicity curve of epirubicin and FOL-PEG-EPI after treatment for 24 hours (A), 72 hours (B), 7 days (C) both in 2D and 3D systems.

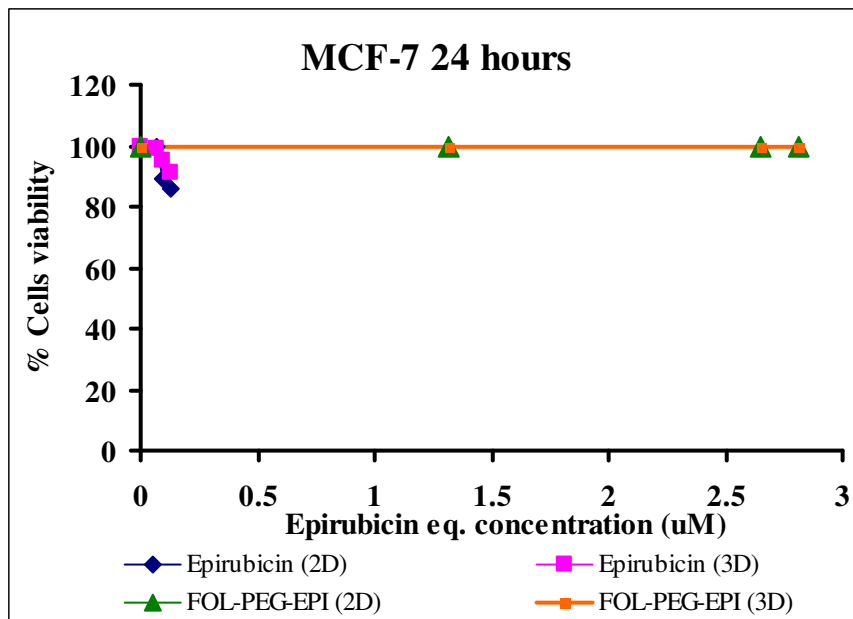
Compound	<i>IC₅₀ for KB-31 (μM)</i>			
	<i>2D culture system</i>		<i>3D culture system</i>	
	72 hours	7 days	72 hours	7 days
Epirubicin	0,021± 0,003	0,016 ± 0,002	0,031± 0,004	0,018 ± 0,005
FOL-PEG-EPI	0,405 ± 0,006	0,335 ± 0,003	0,635 ± 0,002	0,357± 0,003

Tab. 2 IC_{50} value of epirubicin and FOL-PEG-EPI at 72 hours and 7 days, both in 2D and 3D systems.

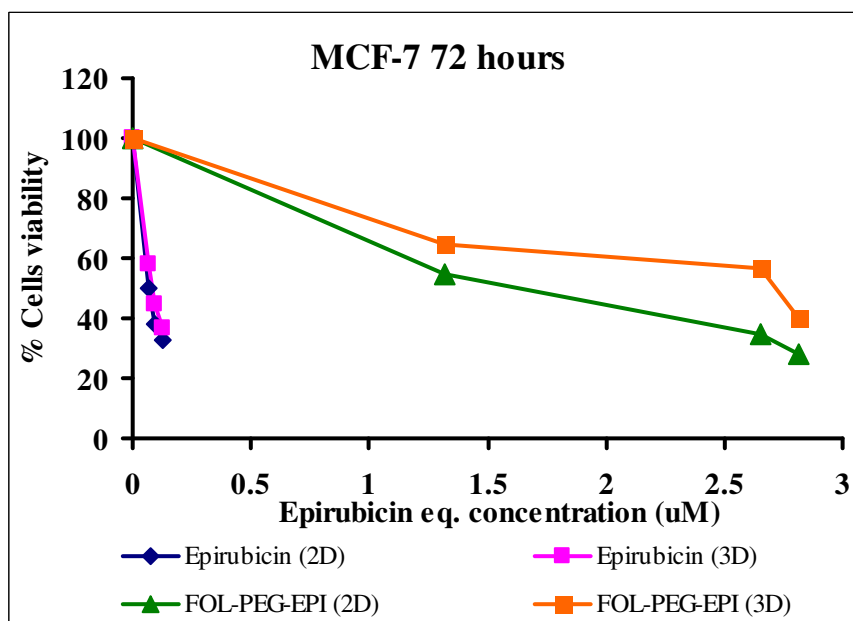
5.2.1.2 MCF-7

Fig. 4 shows the cytotoxic curve relative to MCF-7 cells treated with FOL-PEG-EPI and epirubicin.

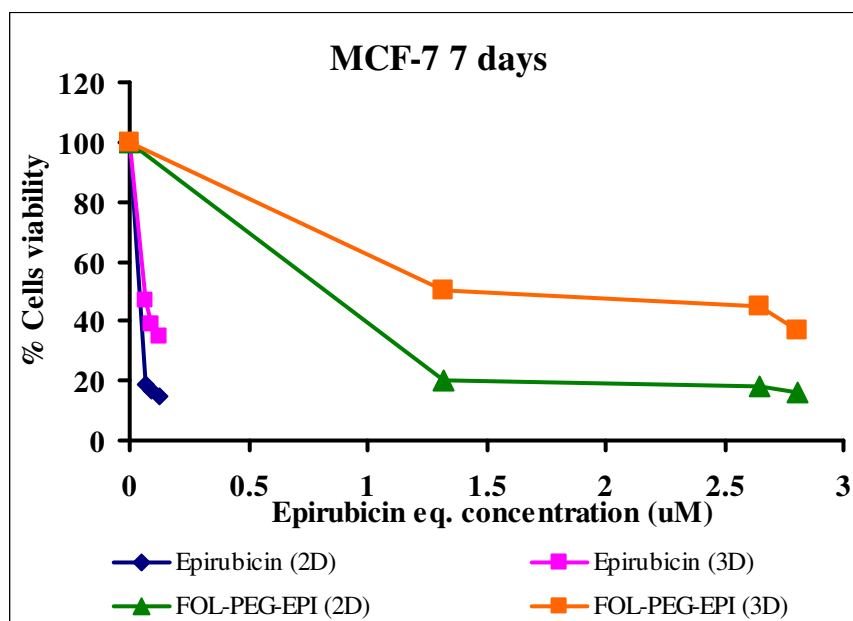
The free drug showed at 72 hours and at 7 days an higher cytotoxic effect than the conjugate in both culture systems. Furthermore, at 72 hours and at 7 days of treatment, the conjugate IC_{50} in 2D was found to be 26 and 19 fold lower than epirubicin (Tab 3), while in 3D the IC_{50} ratio was 35 and 22 fold lower ($p < 0.05$) (Tab. 3).



A



B



C

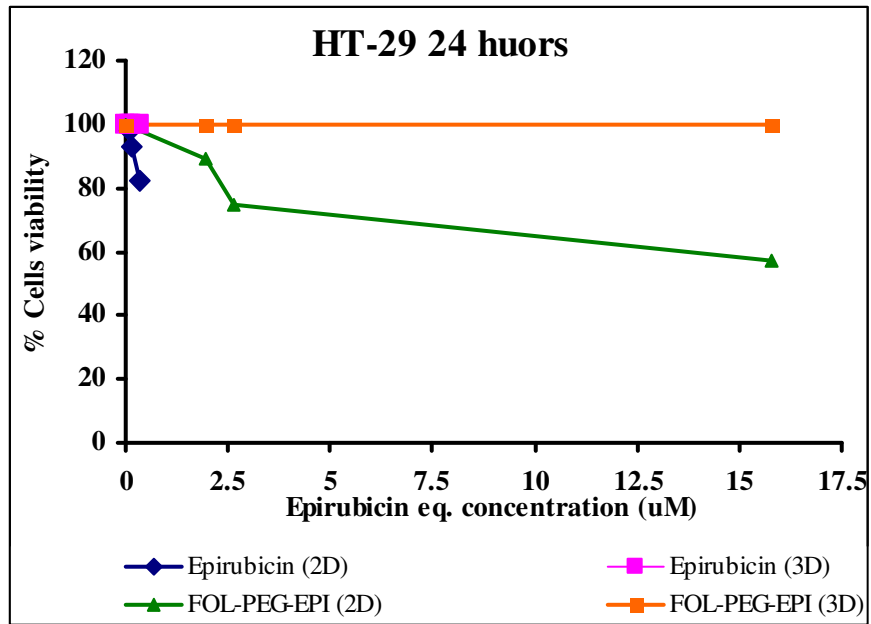
Fig. 4 Cytotoxicity chart of epirubicin and FOL-PEG-EPI after treatment for 24 hours (A), 72 hours (B), 7 days (C) both in 2D and 3D systems.

Compound	<i>IC₅₀ for MCF-7 (μM)</i>			
	<i>2D culture system</i>		<i>3D culture system</i>	
	72 hours	7 days	72 hours	7 days
Epirubicin	0,062 ± 0,003	0,041 ± 0,005	0,08 ± 0,007	0,061 ± 0,001
FOL-PEG-EPI	1,59 ± 0,001	0,81 ± 0,006	2,78 ± 0,004	1,31 ± 0,005

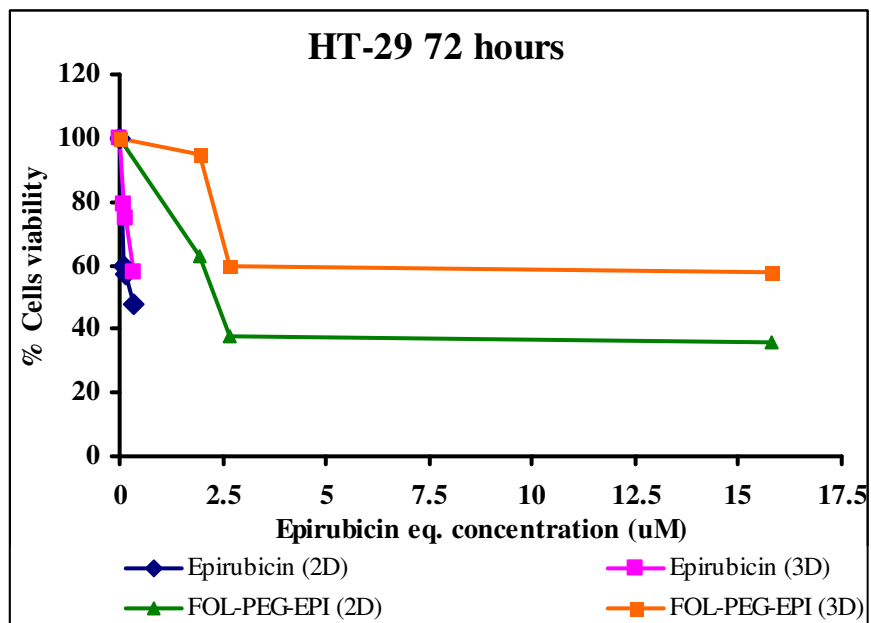
Tab. 3 IC_{50} value of epirubicin and FOL-PEG-EPI at 72 hours and 7 days, both in 2D and 3D systems.

5.2.1.3 HT-29

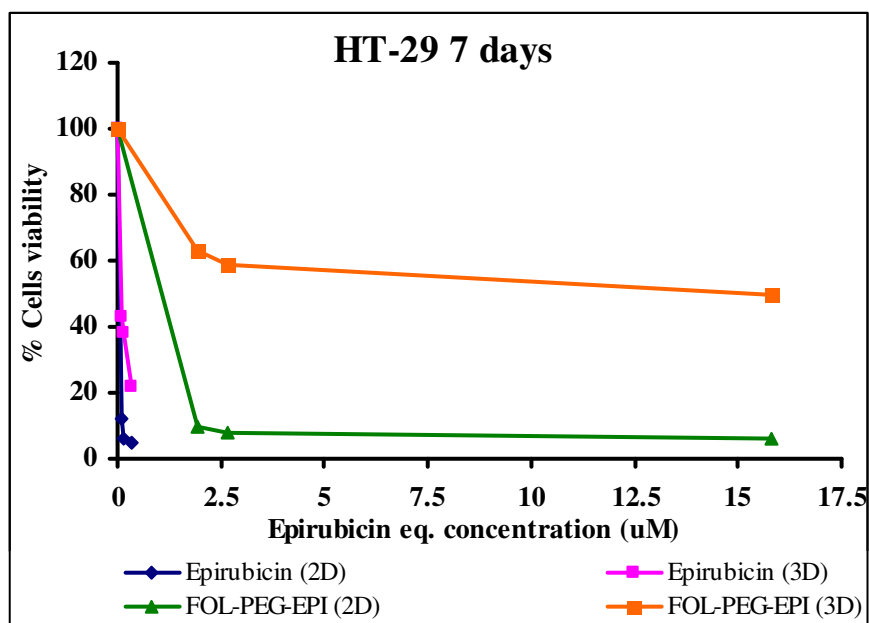
As reported before [paragraph 4.2.1.1, 4.2.1.2], free epirubicin had much higher cytotoxic effect compared to FOL-PEG-EPI (fig. 5) in both culture systems. It was also clearly found by analysing the data that significant IC_{50} differences existed between FOL-PEG-EPI to epirubicin in 2D and 3D culture systems as displayed in tab 4.; at 72 hours and at 7 days of treatment the difference was 11, 17 fold high for 2D system and 48, 108 fold high for 3D system ($p < 0.05$) respectively.



A



B



C

Fig. 5 Cytotoxicity chart of epirubicin and FOL-PEG-EPI after treatment for 24 hours (A), 72 hours (B), 7 days (C) both in 2D and 3D systems.

Compound	<i>IC₅₀ for MCF-7 (μM)</i>			
	<i>2D culture system</i>		<i>3D culture system</i>	
	72 hours	7 days	72 hours	7 days
Epirubicin	0,2 ± 0,01	0,061 ± 0,02	0,33 ± 0,03	0,1 ± 0,05
FOL-PEG-EPI	2,29 ± 0,01	1,06 ± 0,03	15,8 ± 0,06	10,85 ± 0,08

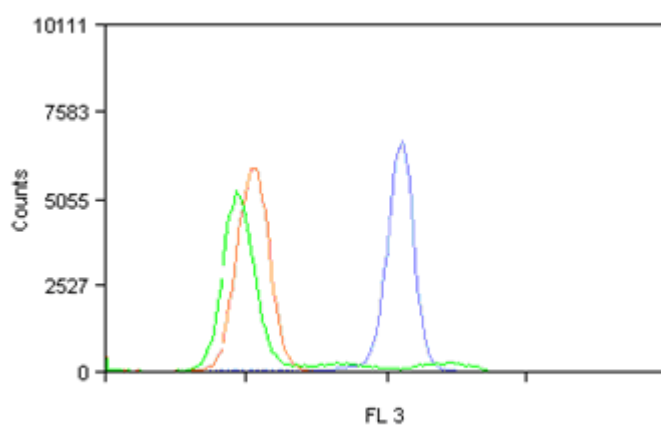
Tab. 4 IC_{50} value of epirubicin and FOL-PEG-EPI at 72 hours and 7 days, both in 2D and 3D systems.

The comparison of the IC_{50} data for each cell lines in the 3D cultures, have showed clearly that at concentration lower than $0.5\mu\text{M}$, the conjugate was 4 to 30 fold more active on KB-31 cells, in both treatments at respectively 72h and 7 days, compared to the MCF7 and HT29. In the 2D culture systems, the conjugate had a bigger cytotoxic effect in all cell lines but at a lower concentration ($0.4\mu\text{M}$ - $1\mu\text{M}$). Moreover, to achieve the same cytotoxic effect in 2D and 3D on the HT-29 cell line with the targeted conjugate, the concentration of FOL-PEG-EPI was shown to have to be 7 - 10 times higher in 3D culture system than in 2D system (tab 4). Therefore if the cytotoxic activity increased going from the HT-29 FR cells to the MCF-7 FR+ cells and then to the KB-3-1 FR++ cells, this must mean that the targeting

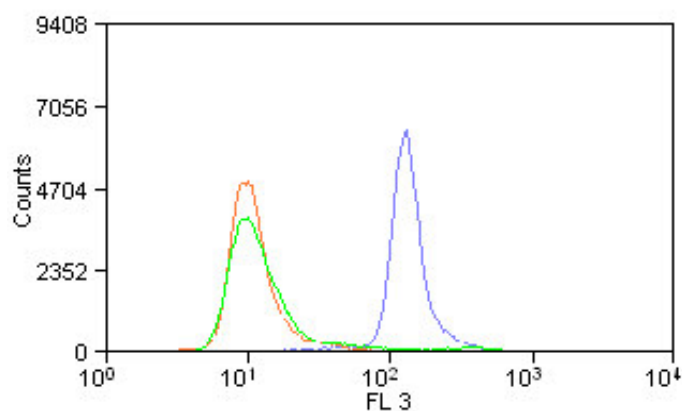
residue has an clear effect, this being more evident in the 3D culture system.

5.2.2 Up-take Study of FOL-PEG-EPI

The FOL-PEG-EPI and epirubicin up-take was analysed by cytometry using Moflo High speed instrument equipped with a laser Argon 488nm. The fluorescence mean value emitted by KB-31, MCF-7 and HT-29 cells after treatment with the conjugate (20 μ M drug equivalent) or the free drug (20 μ M drug equivalent) allowed to evaluate the intracellular up-take quantity. The fluorescence data for each cell line are reported in the following histograms, where the fluorescence signal is correlated to the number of events (cells) analysed (count).



A



B

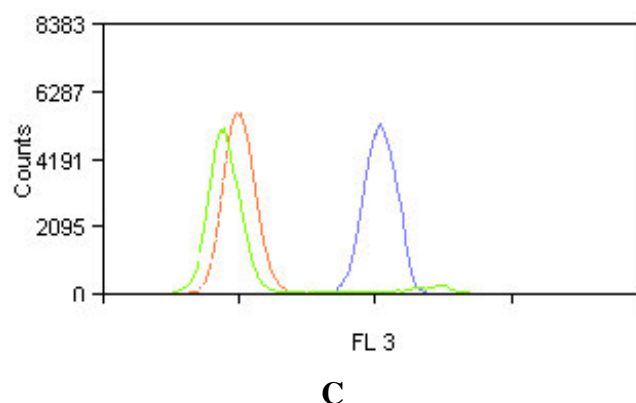


Fig. 6 Cytofluorimetry histograms obtained from treatment of KB-31 (A), MCF-7 (B), HT-29 (C), cells for 30 minutes with 20 μ M epirubicin (green), 20 μ M FOL-PEG-EPI (blue) and EPI-PEG (red).

Fig 6 shows the up-take of conjugate and free drug after 30 minutes by cells treated at monodisperse phase. The presence of FOL-PEG-EPI conjugate was detected inside the cells, while the samples treated with EPI-PEG conjugate showed no fluorescence signal. These data suggested the internalization of FOL-PEG-EPI by a receptor-dependent process. Furthermore, all three tumoral cell lines showed about totally (97%) to uptake fastly the bioconjugate. Only 8-14% cellular populations treated with Epirubicin showed the fluorescence signal after treatment: as Epirubicin is known to enter the cells by 5-10 minutes by diffusion, the low fluorescence detected in this work is probably due to a fluorescence quencing occuring.

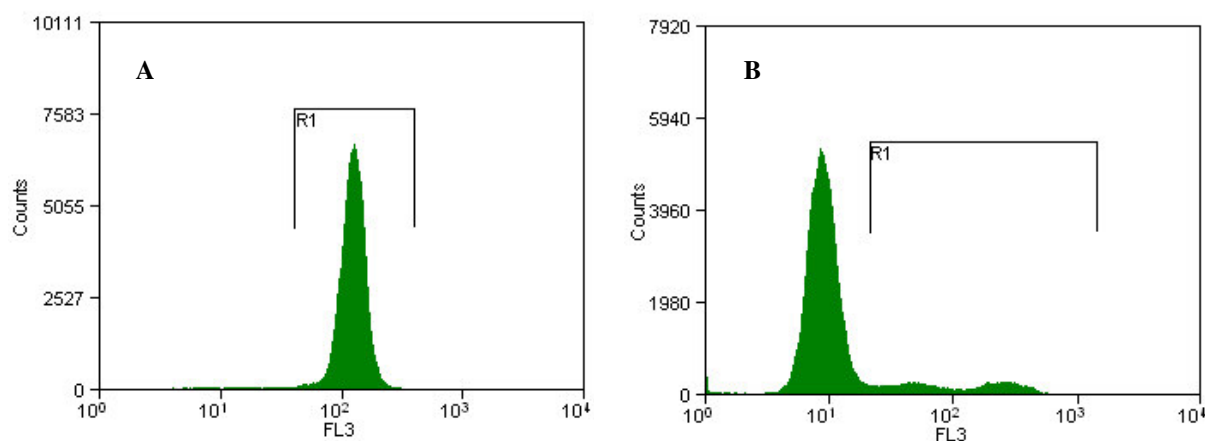
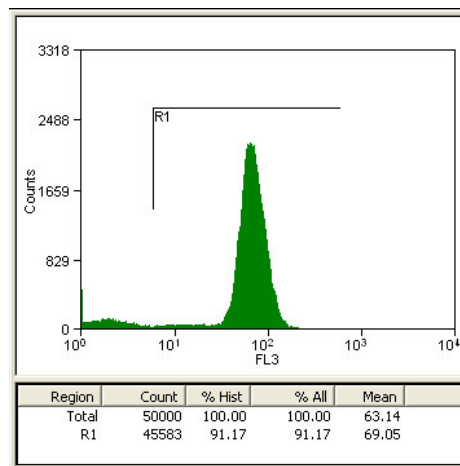
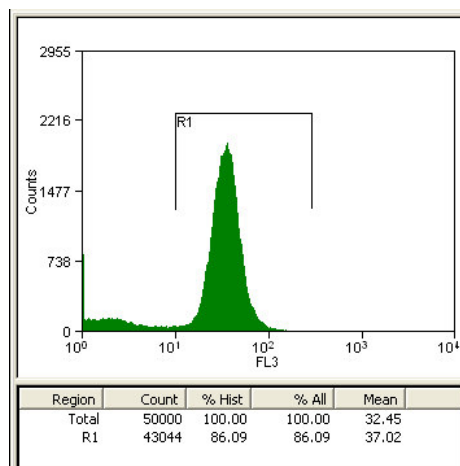
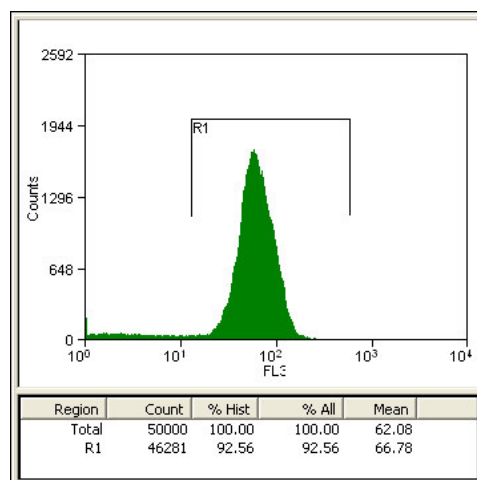


Fig. 7 Cytofluorimetry histogram performed on KB-31, the cells were treated in suspension for 30 minutes with 20 μ M FOL-PEG-EPI (A) and EPI-PEG (B).

The same treatment was carried out on cells cultured in 2D in vitro system. A lower mean fluorescence was detected in all cell lines after the treatment with conjugate: this result could be interpreted as a consequence of a minor conjugate quantity entering the cells because of a different exposition of the folate receptor in cells cultured in bidimensional system (fig 8).

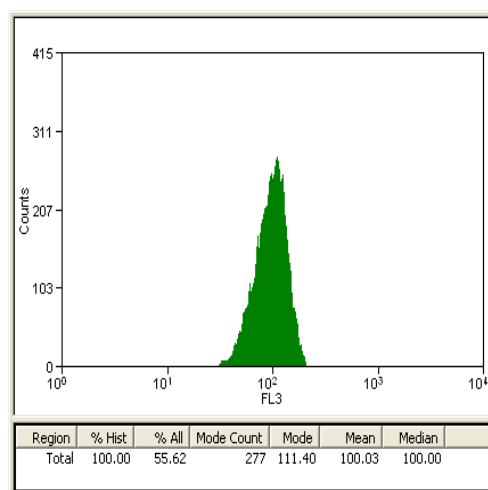
**A****B**



C

Fig. 8 Cytofluorimetry histogram performed on KB-31 (A), MCF-7 (B), HT-29 (C), the cells were seeded in 2D culture system and treated for 30 minutes with 20 μ M FOL-PEG-EPI.

After 72 hours of conjugate and epirubicin treatments in 2D, underwent further decrease of the mean fluorescence for both tumoral cells line. This was due to cell death and fluorescence quenching caused by the accumulation of the FOL-PEG-EPI inside the cell cytoplasm.



A

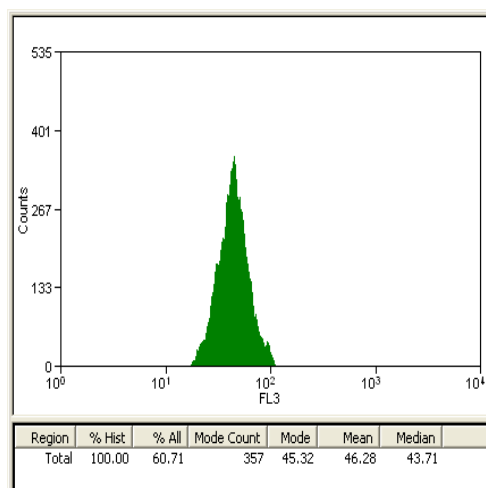
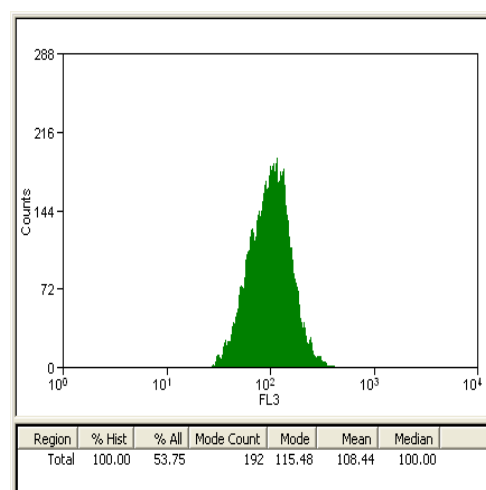
**B****C**

Fig. 9 Cytofluorimetry histogram performed on KB-31 (A), MCF-7 (B), HT-29 (C), the cells were seeded in 2D culture system and treated for 72 hours with 20 μ M FOL-PEG-EPI.

5.2.3 Localization study by confocal laser microscopy

Fig. 10 shows the emission spectra of epirubicin in solution at different wavelengths (476nm, 488nm, 514nm). The spectra were obtained with a “ λ Scan”. This is a specific function in the LAS AF software that is usually used to define the maximum emission of new fluorochrome by analysing the spectra. The “ λ Scan” captured few images in the wavelength interval where we wanted to work and it then extrapolated the emission spectra with maximum emission λ as well.

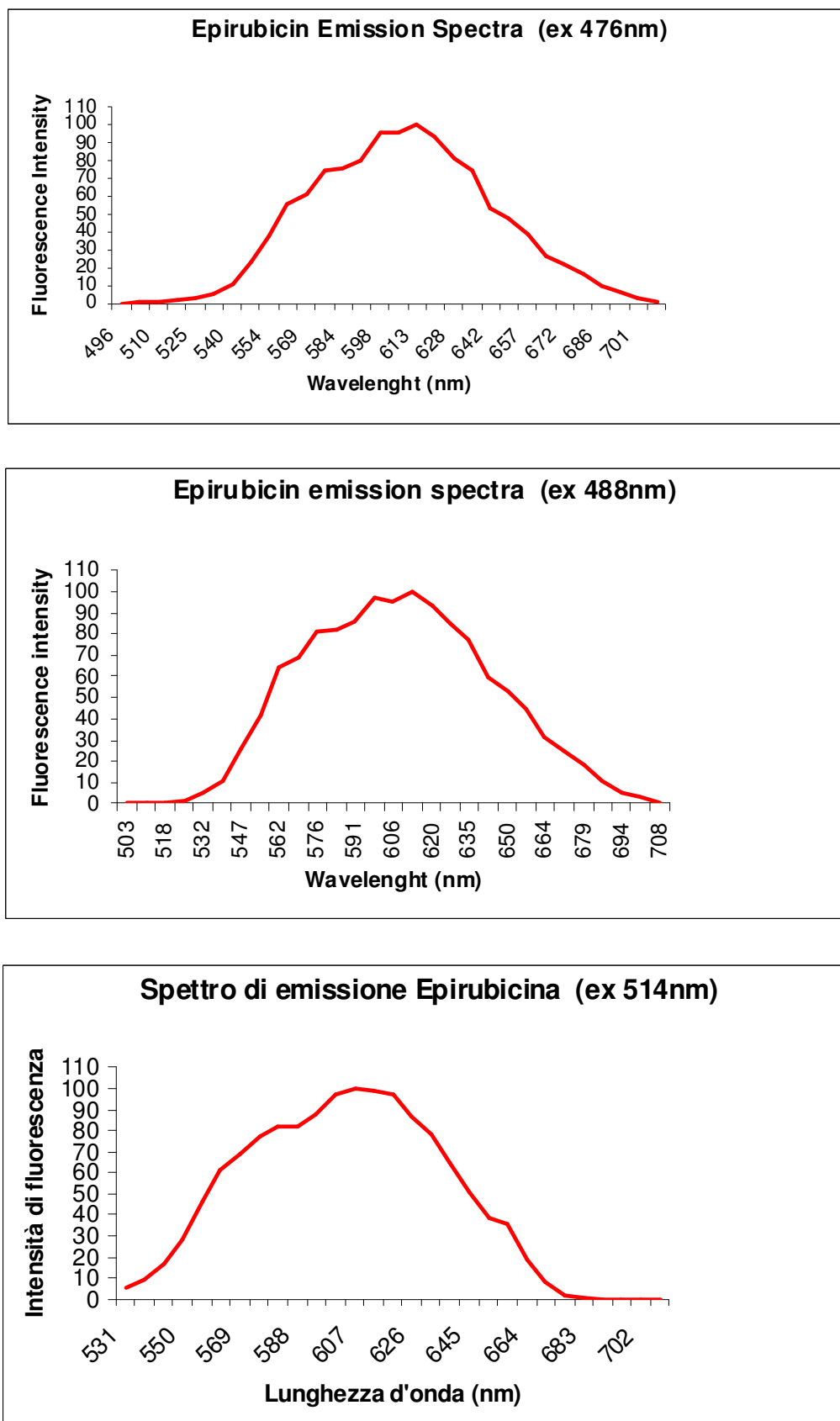


Fig. 10 λ emission spectra of Epirubicin after excitation with laser argon 488nm.

The auto fluorescence of the epirubicin anthrachinonic group (20 μ M), analysed by confocal microscope, displayed the diffusion to the nuclear compartment just after 10 min of treatment in all the cell lines tested (fig. 11). The image below shows that in the 2D (fig. 11: 2D) and 3D (fig. 11: 3D) culture systems the fluorescence signal was very bright and localized in the nuclear compartment, as expected. Some samples exhibited the fluorescent signal in the cytoplasmic membrane as well.

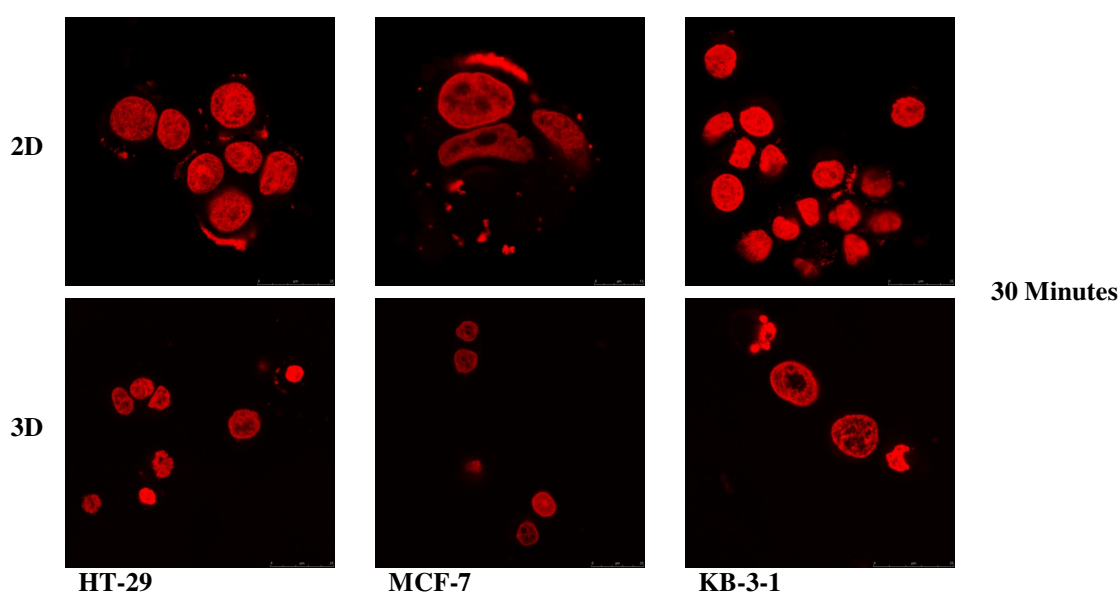


Fig. 11 Confocal laser scan microscopy performed on KB-31, MCF-7, HT-29 cells. They were seeded in 2D and 3D culture systems and treated for 30 minutes with 20 μ M Epirubicin.

After 30 minutes and 72 hours of treatment with FOL-PEG-EPI (20 μ M) in 2D culture system, the fluorescence presented a dot-like shape in the precise area near the nuclei. This was due to the formation of a fluorescent conjugate vesicle. If there had been free drug then all the cytoplasm would have been fluorescent, and not only the area around the nuclei.

Although this analysis was qualitative, the fluorescence intensity seemed to be bigger in KB-31, medium in MCF-7 and lower in HT-29 cells (fig. 12). Indeed, the image was achieved with laser argon 488, with the same power.

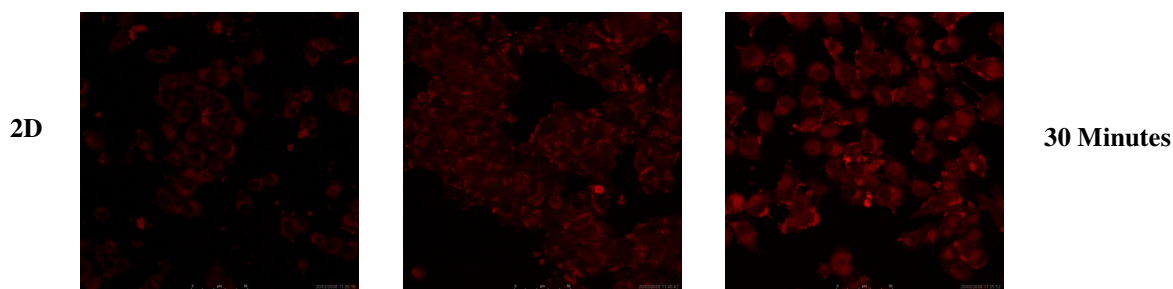


Fig. 12 Confocal laser scan microscopy performed on KB-31, MCF-7, HT-29 cells. They were seeded in 2D culture system and treated for 30 minutes with 20 μ M FOL-PEG-EPI.

In the 3D culture system a low intensity signal was obtained in all tumoral cell lines after 30 minutes of conjugate treatment (20 μ M). The fluorescence was localized near the nuclei (fig 13) as described before.

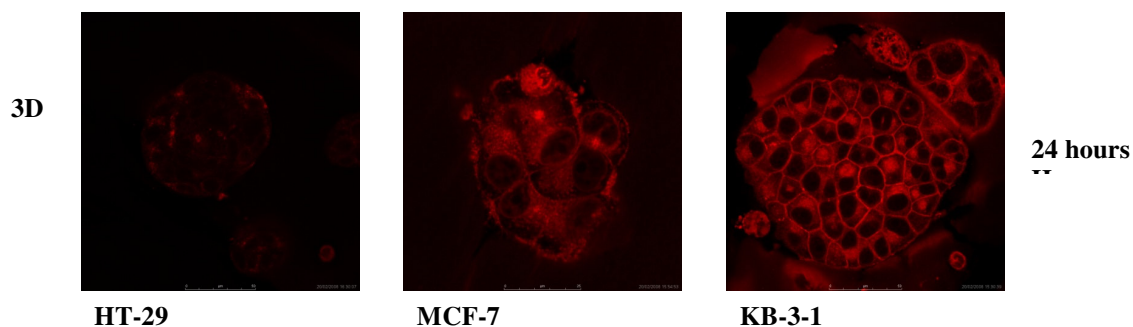


Fig. 13 Confocal laser scan microscopy performed on KB-31, MCF-7, HT-29 cells. They were included in 3D culture system and treated for 24 hours with 20 μ M FOL-PEG-EPI.

5.3 Microspheres production and characterization

5.3.1 Release study

The *in vitro* protein release was carried out at 37°C in a phosphate buffer solution (0,1M PBS, pH 7.4). The microspheres suspended in buffer solution were incubated by shaking in a water bath. At different time points half the solution was removed and replaced with fresh PBS. The TAT-OP1 content was evaluated by making a standard curve in RP-HPLC (Vydac C4) with different concentration of native protein Fig 14.

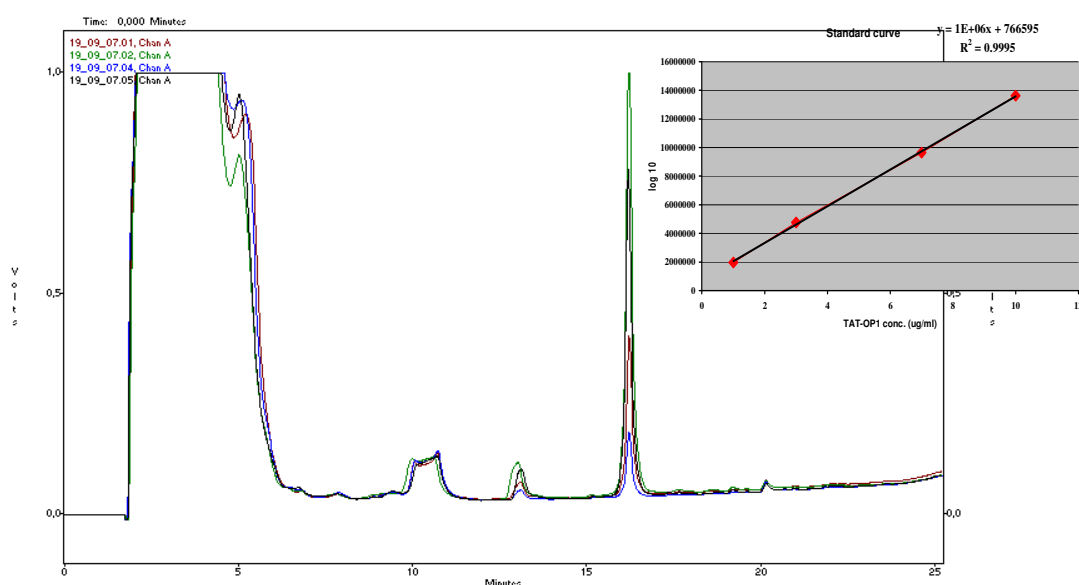


Fig. 14. TAT-OP1 standard curve by RP-HPLC: the protein have been eluted with gradient OP1(as reported in chapter1.2 materials and methods) by using Vydac-C4 column. The eluents used were: H₂O + MeCN both additionated with 0.05 % v/v of TFA. The operative flow was 1.00 ml/min. at room temperature.

The TAT-OP1 release profile from PLGA 50:50 microspheres is reported in Figure 15. It is characterized by a fast initial burst with about 64% release within the first 6 hours and a further slow release within the following 7 days.

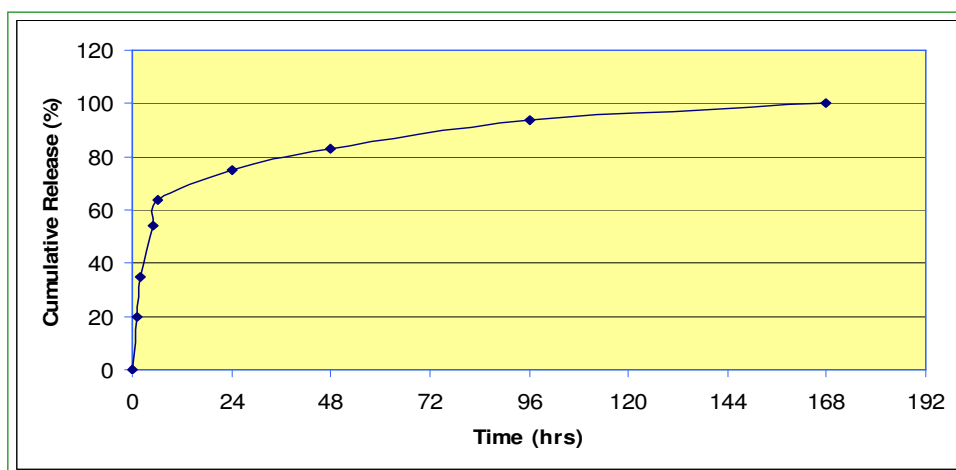


Fig. 15 TAT-OP1 microspheres cumulative release by RP-HPLC: the proteins have been eluted with gradient OP1 (as reported in chapter 1.2 materials and methods) by using Vydac-C4 column. The eluents used were: H₂O + MeCN both additionated with 0.05 % v/v of TFA. The operative flow was 1.00 ml/min. at room temperature.

5.3.2 Encapsulation efficiency

The formulation displayed protein loading efficiency of about 35%, expressed as the percentage of protein inside the microspheres obtained compared to the initial amount of TAT-OP1.

5.3.3 Scanning electron microscope characterization

The scanning electron microscopy (SEM) analysis was based on electron excitation of metallised surfaces and detection by a detector of the electron stream. This allowed us to investigate the microsphere surface and shape. Furthermore the diameter of the particles was determined by using the SEM size measurement software.

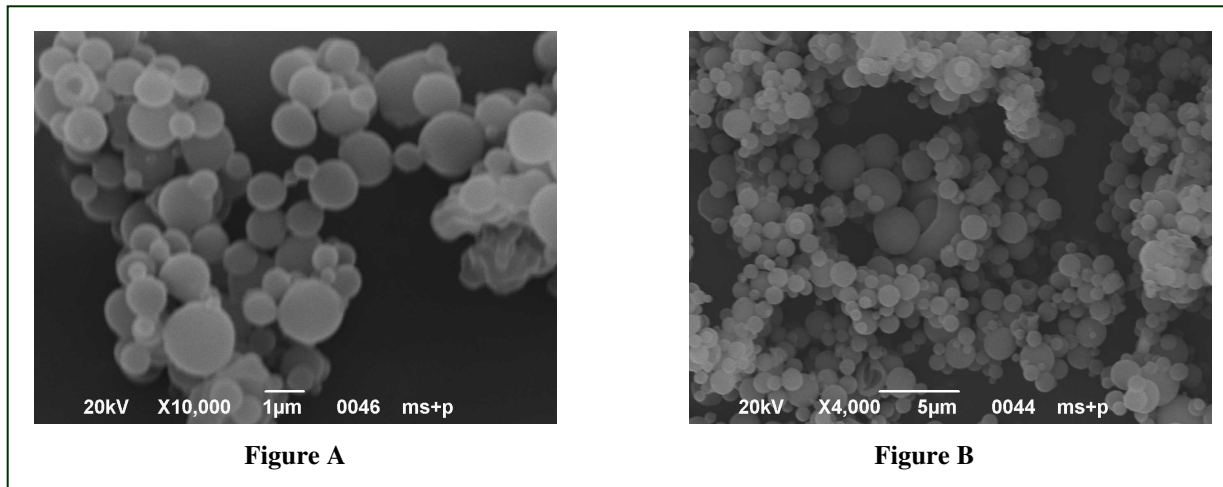


Fig. 16 TAT-OP1 microspheres SEM analysis: the microspheres TAT-OP1 (A, B) have been metalized with silver paste(as reported in chapter 1.4 materials and methods) and then covered by vacuum sublimation.

The microsphere diameters ranged from $\sim 0,2$ to $2 \mu\text{m}$ (Figure 16 A, B). They possessed a spherical shape and there weren't any highly aggregated particles. Their surfaces were smooth and there was no evidence of a porous surface.

5.4 Biological activity study of TAT-OP1 microspheres on MC3T3-E1

From our previous work (Boran et al) we found that the TAT-OP1 microspheres were non toxic at the concentration of 200nM or less, the protein can penetrate inside the cells and stay there for 7 days without any evidence of change in structural conformation and the protein is stable inside the cells. This condition is indispensable if the protein is to start the differentiation process.

The first cells used were the murine pre osteoblasts MC3T3-E1. To study the differentiation potential of TAT-OP1 loaded into microspheres quantitative and qualitative assay were done on it.

The qualitative assay performed was imunofluorescence for the typical antigen of osteogenic differentiation. The quantitative tests were done by using alkaline phosphatase kit and alizarin red assays.

5.4.1 Cytotoxicity study of TAT-OP1 microspheres on MC3T3-E1 cells

Cationic agents have been reported to cause serious cellular toxicity due to the electrostatic interaction with the negatively charged cellular membrane (Fischer et al., 2003). Also the organic solvent from the production method and the product obtained by polymer hydrolysis could decrease the cells viability.

For this assay the MC3T3E1 cells were used and treated for 24h and 12 days with TAT-OP1 PLGA 50:50 microparticles, and microparticles alone. The study was conducted at a higher microspheres concentration than that used for our tests. The control was done with cells alone treated just with culture media. Fig. 17 shows the results of the MTT assay.

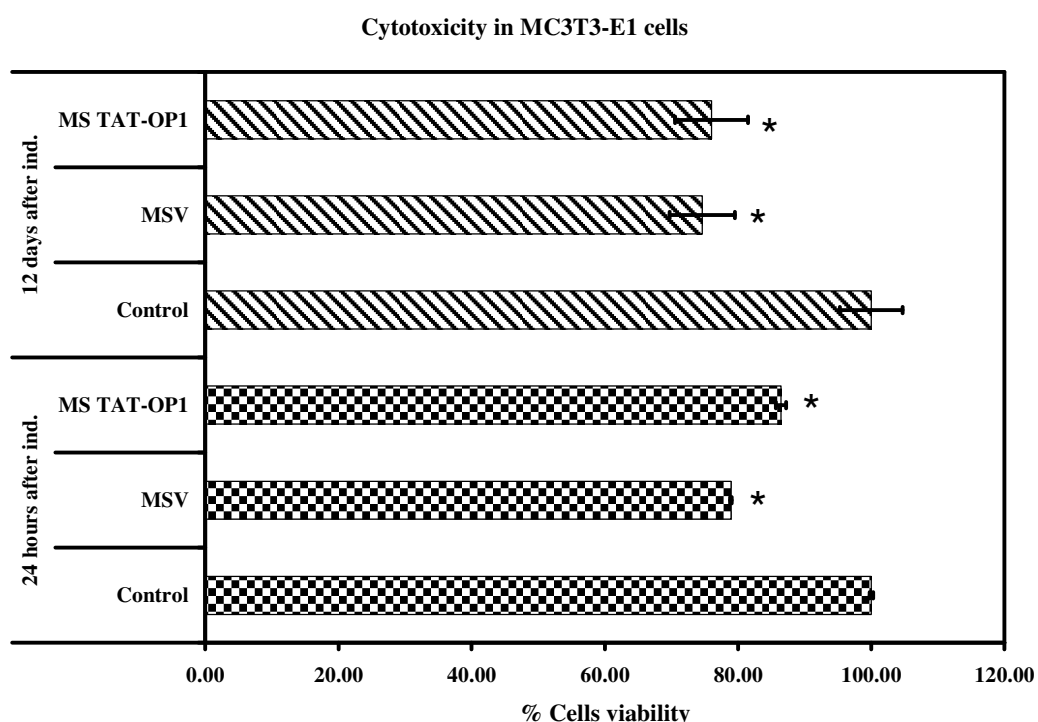


Fig. 17 TAT-OP1 microspheres cytotoxicity assay: the MC3T3E1 cells were seeded at 7000 cells /cm². after 24hrs the 200nM TAT-OP1 microspheres and empty PLGA RG503H microspheres were added at the same quantity. The MTT assay was carry out after 24hrs and 12 days. The data were expressed in % of cells vitality. Error bar: \pm SD * $P \leq 0,05$ respect to the controll (*t test*).

The microparticle formulations were not highly toxic at the concentrations studied as showed in fig17. Average cells viabilities were between 75 and 80% of that of the control at the concentration studied.

5.4.2 Immunofluorescence assay on MC3T3-E1 cells treated with TAT-OP1 microspheres.

The immunofluorescence staining, was used to characterize specific osteogenic markers like osteopontine (OPN), osteocalcine (OC) and Cbfa1/Runx2.

OPN is a phosphoglycoprotein of bone matrix, expressed in various kinds of cells that , when bounded to biologically active molecules, can activate adhesion, chemotaxis and signals transductional in osteoblasts cells.

OPN is produced also during bone metabolism and it is deposited into bone matrix by osteoblasts and then used by osteoclasts in the reabsorbing process.

OC is the most important not collagen-like protein in the bone matrix. It is produced and released by mature osteoblasts and has an important role during bone mineralization and re-modelling processes.

Cbfa1/Runx2, is a nuclear trascriptional factor that regulates the gene expression involved in the osteogenic differentiation.

For this experiment MC3T3-E1 cells was treated with TAT-OP1 loaded microspheres (200nM in TAT-OP1 equivalent). The differentiative induction stimuli, performed with the TAT-OP1 microspheres was carried out for 24, 48 hours and 12 days.

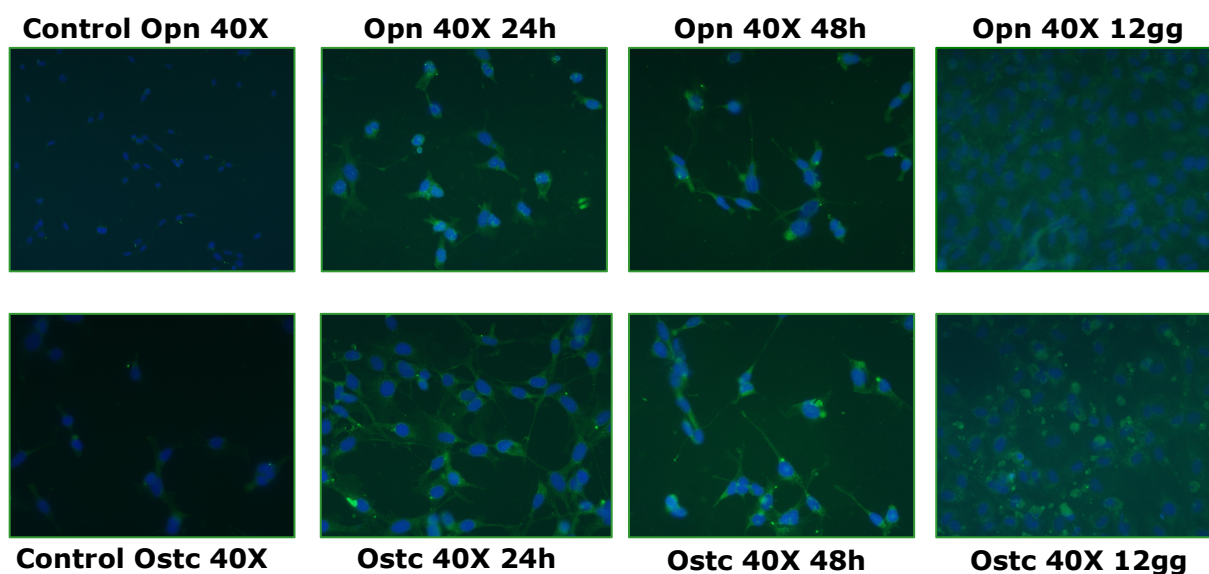


Fig. 18 Fluorescence microscopy of MC3T3-E1 cells performed by using anti-osteopontine (A-B-C-D) and anti-osteocalcin (E-F-G-H): A-E control cells treated with basic medium; B-C-D Cells treated with TAT-OP1 microspheres 200nM; F-G-H cells treated with TAT-OP1 microspheres 200nM. Enlarged to x200.

As showed in fig 18 the MC3T3-E1 cells treated for 7 days with TAT- OP1 microspheres had higher osteopontine and osteocalcin expressions compared to the untreated control cells. In all the samples the transcription factor Cbfa1 / Runx 2 was not expressed (data not shown). After 12 days of treatment, there was a significant signal increase in all TAT-OP1 samples. All the data showed that the protein could induce the expression of the osteogenic markers.

5.4.3 Mineralization quantification on MC3T3-E1 treated with TAT-OP1 microspheres.

The quantification of the bone matrix mineralization was performed using the *Alizarin Red S* assay. This kind of dye is able to complex the calcium in the osteoblast membrane and is extracted by using hexacetylpyridinium. It is then possible to quantify via spectrophotometric analysis the calcium concentration as well as the mineralization level of differentiated cells.

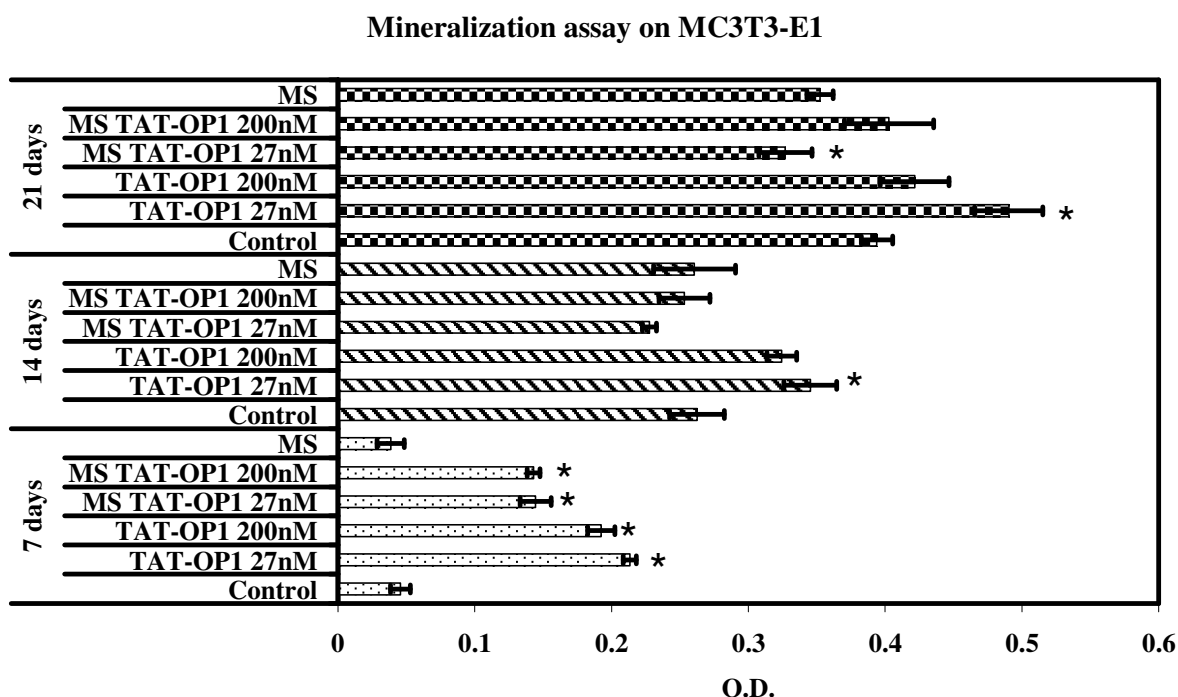


Fig. 19. Alizarin Red S assay to quantified the level of bone matrix mineralization. Absorbance value after 7, 14 and 21 days after treatment with 200nm TAT-OP1(MS 200), 27nm TAT-OP1(MS 27), TAT-OP1 (200nM, 27 nM) microspheres (MS) alone compared to the untreated cells as control (Control). Error bar: \pm SD. * $P \leq 0,05$ respect to the control (*t test*).

Fig. 19, shows the amount of Alizarin Red S at different time points (7, 14, 21 days). The formation of calcium deposit in the samples treated with TAT-OP1 microspheres and TAT-OP1 alone (both at the same concentration), compared to the controls, displayed clearly that TAT-OP1 microspheres give a good stimulus after 7 days but after 14 and 21 days the mineralization was lower. Differently, the native protein alone gave a progressive increase of the mineralization. Mineralization increased in the control cells without any treatment. This is due to the differentiation of MC3T3E1 into osteoblast when confluency is achieved. Therefore, the contribution of TAT-OP1 stimulation should be better evaluated at early time points where osteoblast formation due to confluency is not relevant. At 14 and 21 days, 200nM TAT-OP1 loaded microspheres induce higher mineralization effects compared to 27nM. Therefore, this last result showed that a controlled release system could optimise the biological activity of TAT-OP1 allowing a cellular appropriate response.

5.4.4 Quantification of Alkaline Phosphatase (ALP) on MC3T3E1.

The bone alkaline phosphatase, a.k.a. bALP, is usually present in the external membrane of osteoblast cells and in larger quantities in the bone matrix vesicles where hydroxyapatite crystal formation starts. For this reason it is considered a good maker for the bone mineralization and new bone formation.

The alkaline phosphatase yellow kit substrate (Sigma, ALP kit) was used to obtain the formation of bALP. This process is based on the ester bond hydrolysis of the organic phosphate substrate, which becomes yellow by the action of alkaline phosphatases.

The assay was performed on MC3T3E1 cells after 7, 14 days of treatment with native TAT-OP1, TAT-OP1 microspheres and by using as control the cells alone or the empty microspheres.

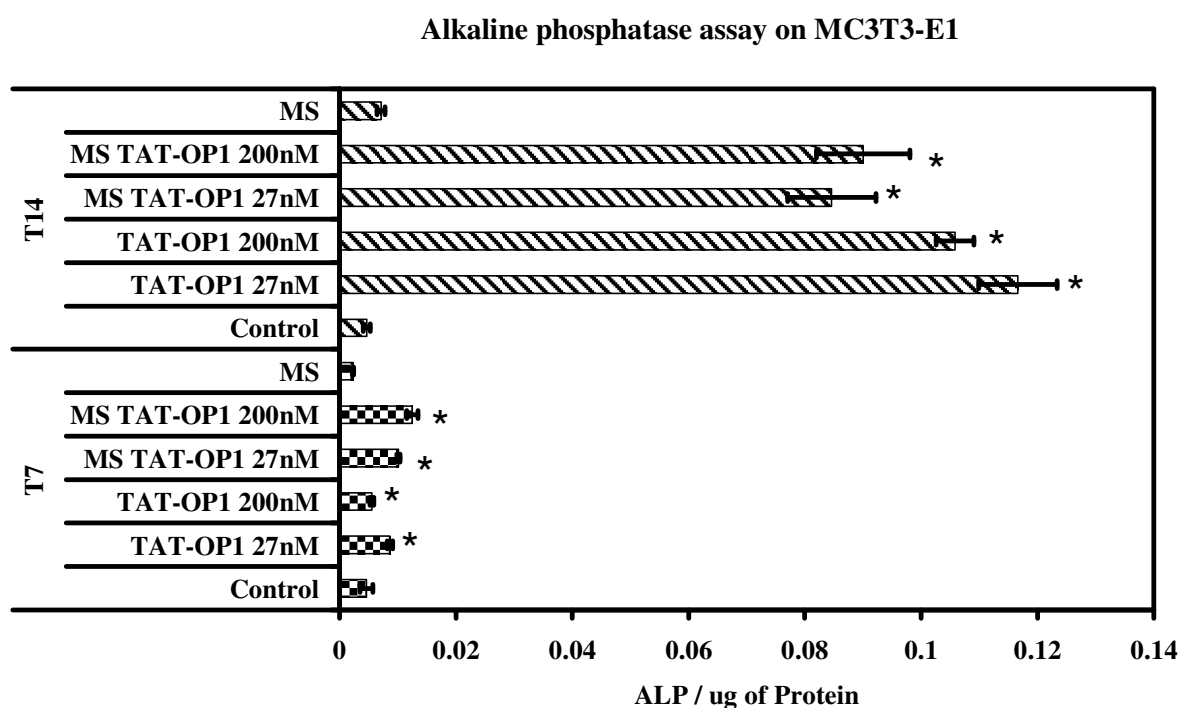


Fig. 20 Bone Alkaline Phosphatase assay to quantified the level of bone mineralization. Absorbance value after 7 and 14 days after treatment with TAT-OP1 alone and TAT-OP1 microspheres both at 200nm, 27nM; compared to the untreated cells and microspheres alone as controls. Error bar: \pm SD. * $P \leq 0,05$ respect to the control (*t test*).

The results displayed in the fig. 20 show that the alkaline phosphatase amounts, are greater in both treatments when compared to the controls. This means that the microspheres stimulate the osteogenic differentiation just as much as the TAT-OP1 alone. The result of the comparison between the native protein and the encapsulated protein shows that after 7 days the controlled release system had a better osteogenic stimulation than native protein. This situation changed after 14 days during which the native protein showed a higher stimulation compared to the encapsulated protein. This is probably due to the larger amounts of protein available to be used directly rather to the controlled release system where we only achieved the 75% of protein release in the culture media after 24 hours.

5.5 Biological activity study of TAT-OP1 microspheres on UCBMSC

Having obtain such positive results from this part of our study we began using the TAT-OP1 microspheres on mesenchimal stem cells from umbelical cord blood (UCBMSC) obtained from donors in San Luca hospital Trecenta (RO).

Literature data shows that bone morphogenetic proteins (BMSs) are able to induce the osteogenic differentiation in these kinds of cells (Reddi, 2001). Other studies have shown that rhOP-1 induces osteogenic differentiation (Sampath *et al.*, 1992).

For this reason we thought it would be interesting to investigate the osteogenic differentiation in stem cells by using TAT-OP1 loaded in PLGA microspheres.

To performe this assay we used a 3D culture system to simulate the *in vivo* enviroirment of the cells.

5.5.1 3D culture system in Puramatrix Hydrogel™ of UCBMSC

The evaluation of TAT-OP1 biological activity on UCBMSC in the 2D culture system is well know (Sampath *et al.*, 1992). For this reason it was really interesting to study the cellular response in a 3D culture system in which we have also used PLGA microspheres loaded with TAT-OP1 200nM. This drug delivery system releases the protein into the matrix to achieve an optimal stimuli condition.

The cells were encapsulated in 0.25% of puramatrix and treated with 200nM TAT-OP1 in culture media or loaded into the microsphere controlled release system. Cells alone were treated with differentiative culture media as control.

The transmission electron microscopy analysis of the culture after 27 days of treatment with 200nM TAT-OP1 native showed the micro fibrillar structure formation and the partial aggregation in collagen fibres (Fig 21 A). This was also present in the control differentiative media (Figure 21 B)

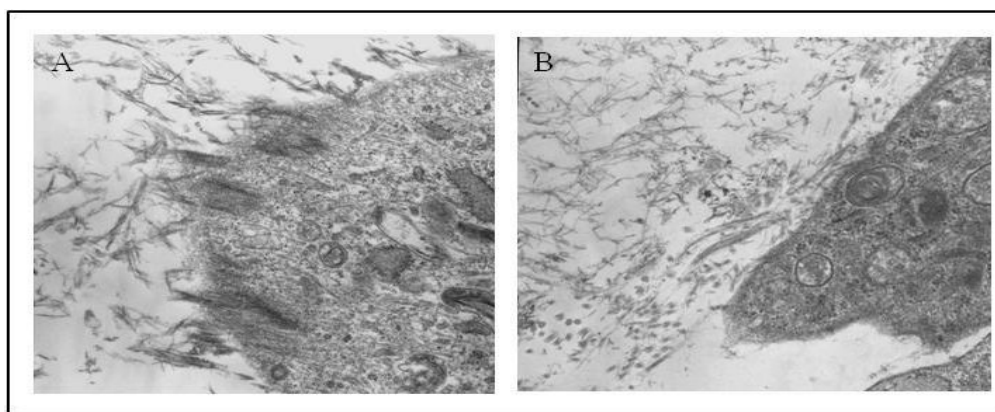


Fig. 21 Transmission electron microscopy image on cells UCBMSC encapsulated in Puramatrix Hydrogel™. **A:** Cell culture after 27 days of treatment with TAT-OP1 200 nM. **B:** Cell culture after 27 days of treatment with normal osteogenic medium. Enlarged x30.000.

Moreover after treatment with the 200nM TAT-OP-1 microspheres there were not only micro fibril structures but also extra cellular matrix vesicles with little hydroxyl apatite crystals (fig. 22)

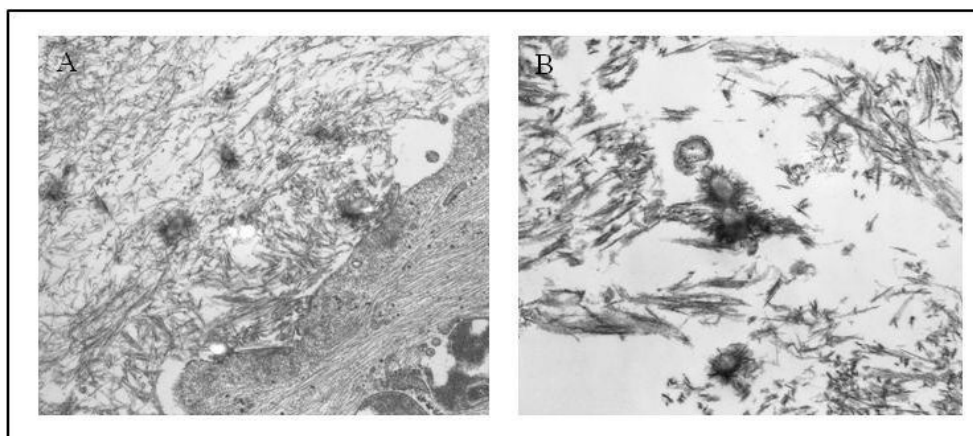


Fig. 22 Transmission electron microscopy image on cells UCBMSC encapsulated in Puramatrix Hydrogel™, and treated with TAT-OP1 PLGA microspheres: **A** Cell culture after 27 days of treatment with 200 nM TAT-OP1 microspheres. **B:** Cell culture after 27 days of treatment with normal osteogenic medium. Enlarged x30.000 (A), x50000 (B).

The results confirmed the TAT-OP1 stimulate UCBMSC cells to produce bone-like extracellular matrix inside the 3D microenvironment which can resemble the *in vivo* situation. We must also observe that the protein controlled release from the microspheres has optimized the stimulation of the cells due to its constant concentration in the 3D system.

6.Discussion

Drug delivery system (DDS) technology is particularly promising to improve the *in vivo* efficiency of active molecules.

During my PhD course I have used two different drug delivery systems focusing on site specific targeting, and growth factor controlled release.

PEG conjugate

The site specific targeting of drugs is a well know problem that needed to be resolved if we wanted to achieve selective delivery. For example in cancer therapy anti-tumoral drugs have various problems due to fast inactivation, short plasma life and non specific targeting that involves all cells and can cause many side effects. The consequence is a frequent administration schedule of the drug at high doses with consequently toxic effects for all cells. Drug conjugation with high molecular weight polymers is considered an important approach to overcome this problem and achieve a new chemical entity capable of selecting cells by active targeting. At the same time these conjugate can passively accumulate into tumors by enhanced permeability and the retention effect (EPR).

To investigate one system that combines both active and passive targeting we used folic acid as a targeting moiety and polyethylenglicol as high molecular weight carrier. Folic acid was selected because its receptor, folate receptor (FR), is overexpressed in several tumour cells. Epirubicin an anticancer drug, was used because is a good candidate due to its high cytotoxicity and fluorescence that allows to study the cell up-take and localization. We synthesized a conjugate based on heterobifunctional polyethylenglicol. Folic acid was conjugated at one side of the polymer, and epirubicin at the other one. The synthesis was made by bonding the activated ester of the folic acid to the ammine group of PEG and by conjugating the amino group on the glycoside ring of epirubicin to the activated carboxylic group of PEG. This kind of synthesis was well know in our laboratories because every single passage of reaction could be controlled by RP-HPLC and in our case the product obtained in the end of each reaction were clearly without impurity . This is important because the impurity presence like free folic acid and epirubicin give the conjugate not usable for *in vitro* tests. RP-HPLC analysis of the conjugate allowed us to calculate the amounts of free drug

(0.8 %) and after acid hydrolysis, the amount of conjugated drug. The cytotoxic effect of epirubicin was evaluated in three different cell lines, KB-31 that over express the folate receptor (FR++), MCF-7 that have slightly more folate receptor expression (FR+) than normal, and HT-29 cells that have normal expression of FR. The puramatrix hydrogel system was used as a 3D culture system to evaluate the biological activity of our conjugate in similar to *in vivo* conditions. The results obtained from these three cell lines cultured in 3D were compared to that obtained from the same cells cultured in normal 2D system. The IC₅₀ values for both epirubicin and the conjugate were higher in the 3D culture than the 2D culture. These data were expected because the cells seeded in a monolayer on a 2D system are over exposed to the free active molecule. In the 3D model the drug and the conjugate undergo a dilution effect due to the high diffusion area in the gel and to the low cytotoxic effect on the cells present inside the colonies. Moreover, the cytotoxicity results showed that the conjugate activity in both systems was about 20-26 times lower than that of the free drug in KB-31 and MCF-7. The FOL-PEG-EPI selective cytotoxic action was clearly shown in the 3D culture system, while in HT-29 cells the conjugate cytotoxicity was evident only at higher concentration. Another important consideration is that in 3D was showed that the cells cytotoxicity increase with the increasing of the FR expression, than the treatment on HT-29 have lower cytotoxic effect than MCF-7 and the high cytotoxic effect was observed in KB-31. The up-take and localization studies with the conjugate and epirubicin have shown that the higher cytotoxicity of epirubicin was probably due to the different mechanism of action rather than the internalization rate. The results obtained by cytometry analysis showed that all cell lines internalize FOL-PEG-EPI or epirubicine after 30 minutes of incubation. The cells treated in suspension with the conjugate were observed to have a mean fluorescence of 124.28 (KB-31), 141.46 (MCF-7), 113.34 (HT-29), about 11-14 times higher than the negative control (not treated) in 97% of the cells from each cell line. The lower mean fluorescence observed in the sample treated in the 2D culture system was probably due to the lower amount of receptors that could interact with the conjugate. The same study performed with EPI-PEG, verified the impossibility of the KB-31, MCF-7 and HT-29 to up-take epirubicin bonded to PEG by diffusion or by a non-specific receptor mechanism. The up-take study in cells seeded in the 2D system with epirubicin alone showed internalization just after 30 minutes but in a smaller number of cells (10-14 %) in which we observed a mean fluorescence of 151.56.

The results showed an accumulation of the conjugate inside the cells and this is cause of the cytotoxic effect probably due to the free radical production and lipoperoxidation of the mitochondrial membrane by the conjugated epirubicin as reported in literature. The localization study by confocal laser scanning microscopy showed that epirubicin could pass across the cell membrane in just 30 minutes and reach the nuclei which were red stained. This was obtained with all the cell lines either in 2D or in 3D culture systems. When the conjugate was analysed a completely different situation was observed, indeed the conjugate was internalized by receptor mediated (FR) endocytosis and it was localized in nano-vesicles near the nuclei in both culture systems, 2D and 3D.

Microspheres

The second part of this PhD thesis was focused to regenerative medicine and the possibility to control the delivery of bioactive molecules. Indeed, in tissue engineering the regeneration of new tissue on scaffolds required the action of proper growth factors. It has been highlighted that the simple administration of native growth factor does not adequately stimulate the formation of the new tissue. The controlled release of growth factor at the site of action over an extended time period is find aim on many studies dedicated to overcome the not adequate stimulation. Furthermore, the possibility to control the release offer the opportunity to tune the stimulation achieving a better response. In our case the protein studied was the recombinant TAT-OP1 which has the viral TAT portion and the BMP-7 active sequence bound together by a recombinant technique. The BMP-7 belongs to the BMP's family which is well known for its capacity to stimulate stem cells or pre-osteoblast cells for osteogenic differentiation. The protein was obtained from Prof. Grandi, Prof. Negro laboratories, University of Padova.

The main issue with this kind of construct is the plasma concentration when used *in vivo*. To allow an optimal stimulation time so that osteogenic differentiation *in vivo* can start it was essential to develop an appropriate controlled release system.

One well known system studied in the last few years is based on microspheres. These are a good system because it is possible to obtain the wanted release profile simply by changing some parameters such as the type of polymer, the concentration of the bioactive molecule / polymer, the polymer molecular weight or the emulsifying agents. In our case the microsphere release system loaded with TAT-OP1 was obtained with the spray drying technique. The

encapsulation method by spray-drying was simple and fast since spray-drying combines the drying of the feed solution and the embedding of the drug into the polymeric network process into a one-step operation.

In our case we know from previous studies that the protein internalization occurs after just 30 minutes. Therefore, we used a polymer with a fast release rate such as PLGA poly(lactide-co-glycolid) 50 : 50 RG503H which has a low inherent viscosity and a low molecular weight.

Release of TAT-OP1 encapsulated into microspheres is governed primarily by the molecular weight of the PLGA used. These polymers degrade by bulk hydrolysis forming progressively smaller polymer fragments which eventually are solubilised. Lower molecular weight polymers, being more hydrophilic, degrade faster releasing the encapsulated molecule. In vitro protein release, was 64 % in 6 hours due to the great hydrophilic nature coupled to the high surface area as a result of small size. Due to their physicochemical properties and release behaviour, the formulations prepared were selected for further studies. The formulation's protein loading efficiency displayed was about 35%, expressed as the percentage of protein inside the microspheres obtained with respect to the initial amount of TAT-OP1. In general, the amount of TAT-OP1 loading was better at high polymer (3 %) and protein (1 %) concentrations in the feeding solution of the spray drier. Also particles prepared with low molecular weight PLGA displayed higher loading efficiency compared to those prepared with higher molecular weight PLGA. By using scanning electron microscopy we also analysed the morphology of our microspheres. The mean particle size was found to depend on polymer molecular weight and other spray drying parameters, like injection temperature, aspiration amount and feed quantity. Microspheres prepared with lower molecular weight PLGA, like our polymer RG503H, had slightly lower mean sizes than particles prepared using higher molecular weight polymers. Increase in polymer molecular weight causes an increase in viscosity of the organic solvent phase which results in formation of larger particles. Similar results have been reported by other investigators on the influence of polymer molecular weight on particle size. Higher injection temperature resulted in particles with a higher mean diameter, irrespective of the polymer type or molecular weight. Higher injection temperature leads to formation of bigger particles due to the fast solidification of the polymer. For this reason we achieved lower mean diameter by using a low injection temperature (50°C).

SEM images revealed that all microparticles had a good morphology with a spherical structure. Moreover, the mean diameter observed ranged from 0.2 to 2 µm. This was due to the optimization of the formulation protocol.

To understand the possibility of achieving an osteogenic differentiation stimuli, studies have been conducted on the biological activity of TAT-OP1 microspheres. The first important thing to be seen was the cytotoxicity of our system on cells.

Cationic agents have been reported to cause serious cellular toxicity due to electrostatic interaction with negatively charged cellular membranes. For the TAT-OP1 to be kept stable it was also necessary to have an acid pH and acetonitrile. Both conditions can be toxic for cell cultures than *in vitro* toxicity was evaluated and our formulations were found to be non-toxic at the concentrations studied. There was a slight reduction in cell viability of about 20 to 25%. The average cell viability was between 75% and 80% compared to the control, confirming the results obtained in another study (Basakar et al.). *In vitro* investigation showed an increase of osteogenic markers, osteopontin (OPN) and osteocalcin (OSTC) after 24-48 hours compared to the control. This signal increased after 12 days in the sample treated with TAT-OP1 microspheres, which means that the cells are exposed to a prolonged differentiative process stimuli. To understand if cells differentiate into mature osteoblasts we investigated the matrix mineralization and alkaline phosphatase enzyme. The data obtained during the mineralization study showed a calcium production after 7 days in the sample treated with TAT-OP1 and TAT-OP1 loaded in microspheres. The same data, but after 14 and 21 days, showed a completely different result where just the free protein gave a larger calcium production stimulation. Whilst the microspheres loaded with protein and the control had more or less the same production. This was due to the differentiative process started when the cells reached confluency. From this we can say that the data was not influenced by cells confluence but just by the TAT-OP1 loaded or not was the one obtained after 7 days.

When osteoblasts reach maturity another important quantitative osteogenic marker is the alkaline phosphatase (ALP) enzyme. Data obtained shows that after 7 days and 14 days there was an increase in ALP expression on cells treated with TAT-OP1 microspheres and with free protein alone but not in the control.

All this data confirmed that the microsphere delivery system could give the appropriate stimulus for osteogenic differentiation. Furthermore after just 24 hours of treatment with TAT-OP1 loaded microspheres the markers expression began and this was followed after 7 days by the production of the matrix mineralization and the alkaline phosphatase enzyme. Another interesting analysis was the cells response to the loaded microspheres and to the free protein both at 200nM and at 27nM. In both the mineralization and alkaline phosphatase tests it was clear that the protein loaded inside the microspheres was an optimal way to stimulate the

cells since the cellular response at high concentration was higher than the one displayed at lower concentrations. This behaviour was not found for the cells treated with free TAT-OP1, where at low concentrations there was a higher response. This is probably due to the non controlled release profile and the cells being stressed at higher concentrations of TAT-OP1. This stress can decrease the osteogenic differentiation.

The positive results obtained led us to evaluate the possible osteogenic activities on stem cells, like UCBMSC. These cells were therefore used with TAT-OP1 loaded in PLGA microspheres in a Puramatrix hydrogel 3D culture system that simulates the cells *in vivo* environment. The transmission electron microscope observations demonstrated that TAT-OP1 alone and the differentiative medium begin the fibrillar structure formation whilst the cells treated with TAT-OP1 loaded inside the microspheres showed not only the microfibrillar structure but also the hydroxyapatite vesicle formation. The presence of hydroxyapatite crystals, even if only after a long treatment time, could be interpreted by knowing that in 3D culture systems cell response is delayed compared to traditional 2D culture systems. In the 2D culture system the cells were organized into a monolayer and therefore were more reactive to the extracellular stimuli because the protein expression was reorganized on the top surface which was not involved in adhesion to the culture plate.

7. Conclusions

PEG conjugates

In this work has been possible to prepare a PEG based conjugate, targeted with folic acid, loaded with epirubicin and study its activity on 3D culture system compared with 2D. Using a polymeric carrier have been possible prepare a conjugate with potentials targeting to tumor tissue due to both mechanisms passive EPR through high molecular weight and active targeting, through the use of a targeting molecules as folic acid. Moreover, the result show that FOL-PEG-EPI have a selective cytotoxicity, and this was better displayed thanks to the 3D system.

Furthermore, by confocal microscopy studies and flow cytometry measures has been possible understand the intracellular trafficking. However, remain to investigate the selective effect of this targeting carrier in vivo where the cells behaviour and blood stream, could influence the conjugate distribution in the tumour tissue.

Microspheres

The results obtained show clearly that the microsphere loaded with recombinant protein TAT-OP1 stimulate in vitro differentiation both osteogenic precursor line (MC3T3-E1) and stem cells (UCBMSC). This construct shows therefore a good candidate for application in tissue engineering with the potential use in both the preparation of bone substitutes and in vitro osteogenic differentiation induction. Indeed, the use of controlled-release PLGA microspheres ensure a continuous stimulation, with defined release kinetics as well as by using of a minimum effective dose. To confirm the biological activity of TAT-OP1 microsphere, will be in the future, conduct in vivo studies on animal models of bone tissue damage.

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