Strategies for Microsphere-Mediated Cellular Delivery

By

Juanma Cardenas-Maestre

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SCHOOL OF CHEMISTRY

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Abstract for Doctor of Philosophy

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Amino-functionalised polystyrene microspheres are promising candidates as delivery systems due to their unique features, tunable surface functionalities, and controllable release of the cargo. Herein several strategies for the conjugation of biologically relevant cargoes to these microspheres and their biological evaluation are described. Firstly, dispersion and suspension polymerisation methods were applied for the synthesis of these devices. Subsequently, these polymeric particles were employed in multistep solid phase synthesis to conjugate a broad range of cargoes. The capability of the resulting constructs to cross the cell membrane and deliver the desired cargo was evaluated by flow cytometry and confocal microscopy. Additionally, the effect of these particles on cell viability was determined. Moreover a chemical strategy for dual functionalisation allowed the production of microspheres capable of carrying two cargos simultaneously (e.g. a biologically relevant cargo and a tracking fluorophore). Several strategies were used to transport biomolecules such as peptides and oligonucleotides inside cells. Cell-impermeable peptides with neuroprotective activity were conjugated to microspheres to facilitate their internalisation and they were efficiently delivered into neuroblastom cells (SH-SY5Y) without affecting their therapeutic activity. In addition, several microsphere-mediated oligonucleotide delivery strategies were investigated. As a first approach, siRNA was successfully attached to microspheres via thiol linkage or via electrostatic interaction (by formation of polycationated microspheres-siRNA microplexes). Using both strategies EGFP expression was efficiently down-regulated in cervical cancer cells permanently expressing EGFP (HeLa-EGFP) following beadfection. Additionally embryonic stem (ES) cells were beadfected with siRNA linked to microspheres by amide formation and essential transcription factors implicated in cell renewal and differentiation were successfully silenced, exceeding the silencing capabilities of commercially available lipofection products. Furthermore, a novel approach for the intracellular delivery of plasmid DNA was designed. Following an easy protocol for the linearisation and functionalisation of the plasmid DNA, this was covalently coupled to beads and cells were homogeneously ‘beadfected’. Finally, the coupling of fluorogenic substrates for caspase-3 to microspheres allowed the in situ monitoring and quantification of apoptotic process within cells. In conclusion, these small particles are excellent devices for the efficient intracellular delivery of a broad range of cargoes.
No matter how high is the hill, there is always a footpath towards its top. So, when you believe that life is about to finish because overwhelmed you feel already, fight even more for what you want to reach.

Juanma C-M.
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Declaration of Authorship

I, Juanma Cardenas-Maestre, declare that the thesis entitled “Strategies for Microsphere-Mediated Cellular Delivery” and the work presented in it are my own work.

I confirm that:

- The research described in this thesis was carried out under the supervision of Dr. Rosario Sanchez-Martin at the University of Edinburgh;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- Parts of the work presented herein have been published or are in the process of being published as:

Book Chapter:

Articles:


Signed:………………………….

Date:…………………………
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ABBREVIATIONS

µg= micrograms
µL= microliter
µM= micromolar
aa-dUTP= 5-(3-aminoallyl)-dUTP
AGO2= Argonaute 2
Ahx= Aminohexanoic
AMD= Age-related macular degeneration
Boc= tert-Butyloxy carbonyl
br= Broadened (NMR assignment)
BSA= Bovine Serum Albumine
Buffer B= Blue buffer
CMV.GFP= Cytomegalovirus-Green Fluorescein Protein
CoCl2= Cobalt(II) chloride
CSK= C terminal Src Kinase
Cy5= Cyanine 5
d= Doublet (NMR assignment)
DC= Dabsyl Chloride
DCU= Dicyclohexylurea
dd= Double of doublets (NMR assignment)
Dde= 4,4-dimethyl-2,6-dioxacyclohexylidene
DIC= N,N'-Diisopropylcarbodiimide
DIPEA= N, N-Diisopropylethylamine
DLS= Dynamic Light Scattering
DMA= Dimethylacetamide
DMEM= Dulbecco's Modified Eagle Medium
DMF= Dimethylformamide
DMSO= Dimethylsulfoxide
DNA= Deoxyribonucleic acid
DSC= N,N'-Disuccinimidyl carbonate
dsRNA= Double strand Ribonucleic acid
DTT= dithiothreitol
DVB= Divinylbenzene
EDC= 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA= Ethylenediaminetetraacetic acid
eGFP= enhanced green fluorescent protein
eNOS= Endothelial Nitric Oxide synthase
ES= Embryonic Stem
ES-E14TG2a= mouse pluripotent embryonic stem cell
FACS= Fluorescence Activated Cell Sorting
FAM= 5(6)-Carboxyfluorescein
FBS = Foetal Bovine Serum
FCS = Foetal Calf Serum
Fmoc = 9-Fluorenlymethyloxycarbonyl
g = gram
GMEM = Glasgow's Modified Eagle Medium
HATU = 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate Methanaminium
HBS = Hank's Buffered Salt Solution
HOAt = 1-Hydroxy-7-azabenzotriazole
Hz = Hertz (NMR assignment)
IC50 = half maximum inhibitory concentration
ICM = Inner Cell Mass
iNOS = Inducible Nitric Oxide synthase
IR = Infrared
Kb = Kilobase
Lck.YFP = Lymphocyte-Specific protein tyrosine Kinase-Yellow Fluorescein Protein
IDNA = linear Deoxyribonucleic acid
LIF = Leukaemia Inhibitory Factor
LNCaP = human prostate adenocarcinoma cells
M = Molar
mESC = mouse embryonic stem cells
mg = miligram
MIF = Mean fluorescence Intensity
miRNA = MicroRNA
mL = mililiter
mM = milimolar
Mp = Melting point
MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEAA = Non-essential aminoacids
NIBS = Non-Invasive Back-Scatter
NMDA = N-Methyl-D-Aspartate
NMDARs = N-Methyl-D-Aspartate receptors
NMP = N-Methyl-2-Pyrrolidone
NMR = Nuclear Magnetic Resonance
nNOS = Neuronal Nitric Oxide synthase
NO = Nitric Oxide
NOS = Nitric Oxide synthase
NSCs = Neural Stem Cells
o/n = Overnight
ODNs = Antisense Oligonucleotides
Oxyma = Ethyl 2-cyano-2-(hydroxyimino)acetate
PALS = Phase Analysis Light Scattering
PAPDK= Protein activator of protein kinase  
PBS= Phosphate buffered saline  
PCD= Programmed Cell Death  
Pci I= Planococcus citreus SE-F45 Type I  
PCS= Photon Correlation Spectroscopy  
pDNA= plasmid Deoxyribonucleic acid  
PEG= Polyethylenglycol  
PEP= PEST-domain-enriched tyrosine phosphatase  
pmol= picomol  
PSD-95= Postsynaptic density protein  
PSMA= Prostate-specific membrane antigen  
q= Quintuplet (NMR assignment)  
QELS= Quasi Elastic Light Scattering  
RHO= 5(6)-Carboxytetraethylrhodamine  
RISC= RNA-Induced silencing complex  
RNA= Ribonucleic acid  
RNAi= Ribonucleic acid interference  
RPMI= Roswell Park's Memorial Institute medium  
RSV= Respiratory syncytial virus  
rt= room temperature  
RT-PCR= Real time-Polymerase Chain Reaction  
s= Singlet (NMR assignment)  
SD= Standard deviation  
shRNA= Short hairpin Ribonucleic acid  
siRNA= Small interfering Ribonucleic acid  
SNLP= Stable nucleic-lipid particle  
SphI= Streptomyces phaerochromogenes Type I  
SPPS= Solid Phase Peptide Synthesis  
ssRNA= Single strand Ribonucleic acid  
t= Triplet (NMR assignment)  
TAE= Tris-Acetate-EDTA buffer  
TAMRA= Tetramethyl-6-Carboxyrhodamine  
TBE= Tris/Borate/EDTA  
TBP= TATA binding protein  
TdT= Terminal deoxynucleotidyl transferase  
TEA= Triethylamine  
TES= Triethylsilane  
TFA= Trifluoroacetic acid  
TLC= Thin layer chromatography  
TP3= TO-PRO-3 Iodide  
TR= Texas Red or Sulforhodamine 101  
TRBP= TAR RNA-binding protein
U= Unit
v/v= volume/volume
w/w= weight/weight
YFP= Yellow Fluorescein Protein
Chapter 1: Introduction

Biological membranes are natural barriers that build cells’ outer boundaries (plasma membranes) and their inner compartments (organelles). Being selectively permeable, membranes control the movement of substances in and out of the cell, regulating the composition of the fluid within individual cells.

1.1-The cellular membrane

As said above, biological membranes isolate the content of cells from the extracellular medium and are also responsible for compartmentalisation within cells. Various proteins are distributed throughout cell membranes, which are responsible for the controlled efflux/influx of solutes from cells and cellular organelles, regulating intracellular concentrations of ions and small molecules according to the needs of cells as well as being otherwise impermeable to most molecules.

**Figure 1.1:** Structure of a cellular membrane and its components.

In recent years a large number of potentially active molecules for therapy and diagnosis have been developed, but they have failed due to their lack of bioavailability inside the cell. It is essential for a drug to enter the cell to be able to exert its effect; a drug or molecule could be very active *ex-vivo* but if it is not able to go inside the cell it is completely useless. The cellular membrane is the main obstacle for entry of therapeutic molecules into the cell. Therefore, it is of fundamental
importance that drugs are able to go through the cell membrane in order to perform their therapeutic action; sometimes they are not able go through this biological membrane and they need to be helped by carrier systems.

1.1.1 The lipid bilayer

The lipid components of cell membranes are amphiphatic (or amphiphilic). The vast majority of membrane lipids are phospholipids. Their arrangement in cell membranes has been deduced by X-Ray diffraction data.¹ These phospholipids have a hydrophilic ("water-loving") polar head group and two hydrophobic ("water-fearing") hydrocarbon tails or non polar end (Figure 1.1). These tails are generally fatty acids, and they can differ in length. Tails can have one or more cis-double bonds (unsaturated tail), whereas other tails may be completely saturated. Differences in the length and saturation of the fatty acid tails are crucial because they influence the ability of phospholipid molecules to pack against one another, thereby affecting membrane fluidity.

1.1.1.1 The lipid bilayer as bidimensional fluid

A molecular understanding of the interaction of biomolecules with lipid bilayers requires a detailed knowledge of the constitution of the membrane bilayer, the transbilayer location of bound biomolecules, the structures that biomolecules adopt and the changes that happen in the bilayer structure as a result of partitioning. Because cellular membranes must be in a fluid state for regular cell function, it is the structure of fluid (Lα-phase) bilayers that is relevant to understanding the interactions between molecules and cell membranes in detail. The 'structure' of a fluid bilayer is subsequently defined operationally as the time-averaged spatial distributions of the principal structural (quasi-molecular) groups of the lipid (carbonyls, phosphates, etc.) projected onto an axis normal to the bilayer plane,² as shown in Figure 1.2 for the phosphate group of a phosphatidylcholine molecule.
1.1.1.2 Relationship between lipid bilayer and its components

The fluidity of cell membranes is precisely controlled by its composition. Some membrane transport processes and enzyme activities for example, fail when the bilayer viscosity is experimentally increased beyond a threshold level. The fluidity of a lipid bilayer depends on both its composition and its temperature, as it has been readily demonstrated in studies of synthetic bilayers. A synthetic bilayer made from a single variety of phospholipid changes from a liquid state to a two-dimensional rigid crystalline (or gel) state at a characteristic freezing point. This change of state is called a phase transition, and the temperature at which it happens is lower (the membrane becomes more difficult to freeze) if the hydrocarbon chains are short or have double bonds. A shorter chain length lessens the ability of the hydrocarbon tails to interact with one another and cis-double bonds produce twists in the hydrocarbon chains that make them more difficult to pack together, so that the membrane remains fluid at lower temperatures. However the lipid bilayer of many cell membranes is not composed uniquely of phospholipids, it often also contains cholesterol and glycolipids (see Figure 1.1). Cholesterol molecules enhance the permeability-barrier properties of the lipid bilayer. By decreasing the mobility of the
first few CH₂ groups of the hydrocarbon chains of phospholipid molecules, cholesterol makes the lipid bilayer less deformable in this area and thereby decreases the permeability of the bilayer to small water-soluble molecules. Although cholesterol tends to make lipid bilayers less fluid, at high concentrations it also prevents hydrocarbon chains from associating and crystallising. In this way, it inhibits possible phase transitions.

1.2-Principles of cellular membrane transport

Due to its hydrophobic interior, the lipid bilayer of cell membranes serves as a barrier to the transport of most polar molecules. This barrier capacity is of vital importance because it maintains solute concentration in the cytosol, which is different from that of the extracellular medium and in each of the intracellular membrane-enclosed compartments. The transport of inorganic ions and small water-soluble organic molecules across the lipid bilayer is achieved by specific membrane proteins, each of which is responsible for the transfer of a specific ion, molecule, or type of closely related ions or molecules. Cells can also transfer macromolecules and even large particles across their membranes. However, in most of these cases the mechanisms are different from those used for the transport of small molecules (Figure 1.3).

![Figure 1.3: Different types of transport across the cell membrane.](image-url)
1.2.1 Types of cellular membrane transport

The simplest route by which a molecule goes into the cell is by simple diffusion, with only a few molecules allowed to diffuse liberally through the lipid bilayer according to their concentration gradient. This is the case for small non-polar molecules (such as O₂, CO₂, N₂) and small uncharged polar molecules (such as urea, glycerol) which can diffuse slowly through the cell membrane. However, cell membranes also have to allow the crossing of a range of polar molecules, such as ions, sugars, amino acids, nucleotides, and many cell metabolites that cross synthetic lipid bilayers in only negligible amounts. Specialised membrane transport proteins are responsible for transferring such solutes across cell membranes. Different forms of these proteins are present in all types of biological membranes.

Channel proteins and carrier proteins are the two main classes of membrane transport proteins. Channel proteins interact with the solute to be transported, they form an aqueous pore that extend across the lipid bilayer; when these pores are open, they allow specific solutes (usually inorganic ions of appropriate size and charge) to pass through them and thereby cross the membrane (see Figure 1.3). Carrier proteins, in contrast, bind the specific solute to be transported by a relatively strong interaction and undergo a series of conformational changes to transport the bound solute across the membrane. Unsurprisingly, the rate of transport through channel proteins is higher than through carrier proteins.

1.2.1.1 Endocytosis

Mammalian cells take up extracellular material by a variety of different mechanisms that are collectively termed endocytosis (see Figure 1.4). Endocytic mechanisms serve many important cellular functions including the uptake of extracellular nutrients, regulation of cell-surface receptor expression, maintenance of cell polarity and antigen presentation.
Phagocytosis or “cellular eating” is the internalisation of large particles and microorganisms (typically >0.5 µm) into a cell. A particle that is endocytosed by this mechanism may be recognized directly by the receptors on the phagocyte surface, or it may be first “opsonised” by coating the particle with “opsonins.” This process is receptor- and actin-dependent and clathrin-independent and happens usually in phagocytes.

Macropinocytosis or “cellular drinking” is a form of endocytosis that causes cell surface ruffling. It is different in many ways from the more thoroughly characterised micropinocytosis, which includes clathrin-coated vesicle endocytosis and small uncoated vesicles. Because macropinosomes are relatively large, they offer an efficient route for non-selective endocytosis of solute macromolecules.

Clathrin-dependent endocytosis relies on the formation of clathrin-coated vesicles which occurs through the interactions of cytosolic proteins with components of the inner leaflet of the plasma membrane. The process normally occurs at specific sites, where a complex structure (called a coated pit) is assembled in order to concentrate surface proteins for internalisation.

Caveolin-dependent endocytosis: Caveolin is an integral caveolar protein that gives a striated aspect to caveolae and allows them to be distinguished from other smooth invaginations. Caveolar endocytosis is predominantly induced and can lead...
to the formation of ‘caveosomes’ from which endocytosed molecules can arrive at the endoplasmic reticulum and/or the nucleus.\textsuperscript{12}

\textit{Clathrin- and caveolin-independent pathways:} Although researchers around the world accepted that there are a multitude of entry mechanisms, it has taken a long time for this clathrin-independent mechanism to become recognized. In 2007 Mayor and Pagano\textsuperscript{8} subdivided the different mechanisms into four types: RhoA-regulated, cdc42-regulated, Arf6-dependent mechanisms and caveolar uptake. Of these, the RhoA-regulated mechanism and caveolar endocytosis also require the GTP-binding protein dynamin. However, there are already reports of mechanisms that may not necessarily fit into any of these four categories.\textsuperscript{13, 14} More work in this research area needs to be carry on, because one can still ask the question: How many endocytic mechanisms does a cell have, what are their functions, and how are they regulated.

1.3- Mediated Cellular Delivery

As stated previously cellular membrane poses a formidable barrier to the intracellular flux of drugs, nucleic acids, proteins, and other investigative constructs, which are unable to translocate the cellular membrane unaided and they often require the mediation of a delivery vehicle to be able to cross and carry out their intracellular function and/or activity.

1.3.1 Delivery devices

There are a multitude of delivery vehicles available, which allow the intracellular introduction of foreign material into cells. Each one of these devices has associated advantages and disadvantages. Some of them are briefly summarised here.

1.3.1.1 Liposomes

Liposomes are colloidal, vesicular structures (20 nm to 10 \( \mu \)m in diameter) based on phospholipid bilayers. These devices can vary from unilamellar (meaning only one bilayer surrounding an aqueous core) to multilamellar (several bilayers oriented concentrically around an aqueous core), with the structure depending on the processing protocol, concentrations, and choice of bilayer components.\textsuperscript{15} The idea of liposomes as drug-carriers was first proposed and demonstrated in the 1970’s\textsuperscript{16,17},
when it was shown that soluble molecules could be entrapped within the core of the liposomes which could be endocytosed. Their cellular uptake can be highly efficient (depending on the cell line). However, poor release of the material from the endosomal compartments (especially for high-molecular-weight species) can be a serious restriction. Endosomal escape can be enhanced by modification of the liposomes, by the use of pH-sensitive monomers (e.g., phosphatidylethanolamine), the addition of a lipid helper with “fusogenic” properties (e.g., DOPE•Q1), or complexation of the liposome with a membrane disruptive agent. Liposome-based delivery is an extensively applied method, which has been used to deliver many low-molecular-weight drugs, peptides, enzymes, and oligonucleotides.

1.3.1.2 Cell Penetrating Peptides

Cell-penetrating peptides (CPPs), of which HIV TAT sequence is possibly the most well-known example, have been used to deliver an assortment of cargos into cells. An essential characteristic of these systems, that allow them to cross cell membranes, is a high region of basic amino acids. As an example CPPs based on TAT sequence (GRKKRRQRRR) typically have a cationic cluster of arginine and lysine residues. Over the past decade several CPPs that enable the intracellular delivery of active compounds into the cells have been described, typically made up of 10–30 amino acids, but they all share some common characteristics, including hydrophilicity, helical moment and the capacity to interact with the cellular membrane. Furthermore, a range of peptidomimetics, structurally inspired by CPPs, have been successfully developed (such as polylysines, polyarginines and cationic peptoids), which potentially represent a highly efficient means of trafficking a variety of materials across the cell membrane, enhancing the pharmacokinetic properties of any drug, sensor, or protein.

1.3.1.3 Dendrimers

Dendrimers and hyper-branched polymers are a relatively new class of materials with unique molecular architectures and dimensions in comparison to traditional linear polymers. Highly branched functionalised dendrimers have the
potential to work as efficient drug-carrier systems\[^33\] in two different ways: (a) the encapsulation of soluble drugs within the dendritic core (which serves as a reservoir for drug molecules)\[^34\] and (b) the conjugation of drug molecules to surface functional groups.\[^35,36\] These functional groups can also be coupled to molecules that direct the polymer to a specific type of tissues or cells (targeted delivery) or enhance solubility. Up to date the research on dendrimers has been predominantly focussed on the delivery of “DNA drugs”, where its use has enhanced the transfection of DNA into the cellular nucleus,\[^37,38\] but they have also been used to deliver drugs.\[^39\]

1.3.1.4 Nanomaterials

Nanomaterials, such as carbon nanotubes, have been exploited as delivery systems.\[^40\] These devices were popularised by Kam et al\[^41\] whose publication spawned work focussed on their intracellular use, for example in the delivery of cell impermeable dyes and short interfering RNA (siRNA).\[^42-44\] They may be prepared by several methods, including chemical vapor deposition and laser ablation, and have additionally been manipulated to form magnetic nanotubes to reach site-specific targeting issues \textit{in vivo}.\[^45\] However, some controversy surrounds their intracellular uptake with both endocytic-based mechanisms\[^46\] and passive diffusion\[^47\] being considered along with additional concerns over their \textit{in vivo} toxicity. Therefore, the design of a novel delivery vehicle that overcomes the drawbacks of current available delivery methods could be very welcome by the broad scientific community involved in intracellular delivery. This system should be easy to prepare and functionalise, stable and non-toxic as well as being able to enter efficiently a range of cells.

1.4-Microspheres

The term microspheres (MS) typically refers to uniform spherical polymer particles (diameters ranging from nanometers to several microns) prepared from various materials such as polystyrene, silica and methacrylates.\[^48\] Microspheres were introduced in 1984 when Tseng \textit{et al} first reported the synthesis of highly uniform polymer particles.\[^49\] They suggested a mechanism known as dispersion polymerisation for particle formation and growth of uniform polystyrene particles in a 1-10 \(\mu\)m size range. Microspheres were prepared by polymerising styrene in ethyl
alcohol with azo-type initiators and polyvinylpirrolidone as a polymeric stabiliser. In addition, they successfully incorporated a variety of functional groups such as hydroxyl, carboxyl, amine, amide, silane, polydimethylsiloxane and silacrown to the particles by copolymerisation. It was not until 1994 when another synthetic approach was developed. Delair et al obtained 200-1000 nm amino-functionalised latex particles by batchwise emulsion copolymerisation of styrene and vinylbenzylamine hydrochloride (VBAH), which provides amino functionality in the presence of 2-2’azobis(amidinopropane). To enhance the size, monodispersity and spherical morphology a third monomer called divinylbenzene was added, which had the purpose of crosslink the growing polystyrene chains together to yield a final product that was robust and stable (Figure 1.5).

Due to the spherical nature of these latex products the term microspheres was agreed to denominate them and since then they have been routinely generated using a range of methods. The most spread and well-known methods are suspension, dispersion and emulsion polymerisation. Suspension polymerisation normally yields the largest microspheres particles, ranging from 50-500 µm in diameter. In this technique a monomer that is relatively insoluble in water, is dispersed as liquid droplets within a steric stabiliser along with vigorous stirring (which is maintained during polymerisation) to produce solid polymer particles dispersed within solution. The stirring speed dictates particle size. Dispersion polymerisation produces particles in the region of about 0.1-10 µm. In this technique the initiator and the monomer are both soluble in the polymerisation medium, but the medium is a poor solvent for the resulting polymer. Emulsion polymerisation yields particles in the range of 50-1000 µm.
nm, more typically 50-100 nm. The technique comprises water, an initiator (usually water soluble), a water-insoluble monomer and a colloidal stabiliser. Polymerisation occurs within the monomer-swollen latex particles. Microspheres have previously been used in a variety of applications including flow cytometry, markers of phagocytosis and DNA arrays. However, their use as delivery vehicles of therapeutic or diagnostic cargo in non-phagocytotic cells has not been widely studied.

1.5-Importance of the field

Cellular uptake of foreign materials can often be severely limited by their physical properties such as solubility, charge or size. This means that understanding processes governing cellular entry are important for the delivery of potential drugs, small molecule modulators and sensors. Delivery of biosensors into cells allows the study of a diverse range of intracellular events. The efficient introduction and delivery of membrane impermeable molecules into mammalian cells has been an invaluable tool to elicit, induce or control specific biological functions and for the study of intracellular processes. In addition such studies have helped hugely in gaining an understanding of molecular events at the cellular and sub-cellular level. A problem arises because most delivery devices studied to date such as nanotubes or cationic lipids rely on an energy-dependent endocytotic cellular uptake mechanism, resulting in the device itself along with any cargo being trapped within a vesicle. Consequently, endosomal disrupting agents have to be applied to facilitate release of the potential cargo from the endosome, which carries the risk of partial deactivation or degradation of cargo. There are presently no general methods for transportation of exogenously added molecules such as proteins, double-stranded DNA, peptides or siRNA into cells which deposit cargo directly into the cytoplasm without further treatment or showing no toxicity at all. One of the most promising cellular delivery devices are polymer particles, surprisingly their use as a novel carrier system has hardly been studied and its applicability as intracellular process sensors is an unexplored and promising area of research. Recently some research has been carried out in our research group to investigate the use of in house-made polystyrene microspheres as delivery systems. What is remarkable is that these beads are
rapidly taken up non-phagocytotically by cells and that the uptake of beads is in fact a very general and efficient process among all the cell lines studied so far.

1.6-Aims of the project

The main goal of this PhD was to carry on the design of novel strategies for the conjugation of biologically relevant cargoes to polystyrene microspheres. It was expected that this approach would allow their use as an effective tool for the delivery of therapeutic molecules such as peptides with biological activity and oligonucleotides to perform gene silencing and gene expression. Additionally it was of interest to develop a novel approach for in situ monitoring of intracellular processes such as cellular apoptosis using these polymeric particles. All these strategies were developed with the main objective of giving solutions to specific biological problems. The effectiveness and efficiency of these microspheres to facilitate the delivery of a variety of cargos to some of the most problematic and difficult to translocate cell lines such as mouse Embryonic Stem Cells, human neuronal cells and T cells was carried out. For this purpose, in the first part of the project synthetic organic chemistry was applied, firstly in the synthesis of the microspheres and secondly in the coupling of spacers, linkers, fluorescent organic dyes and different cargos to the microspheres. The chemistry for each one of these applications was optimised. After synthesis and loading, several biological tests were carried out to confirm the efficiency of the developed strategy, such as cellular uptake in different cell lines and toxicity studies. The degree of cellular entry and evaluation of intracellular process was measured quantitatively and qualitatively by flow cytometry and microscopy.
Chapter 2: Microspheres

2.1-Introduction

Polymer beads or microspheres often have an apparent smooth exterior surface, under which a porous matrix lies capable of capturing and transporting molecules. Additionally their surface may be easily functionalised to allow the covalent linkage of compounds to the bead surface.

![Figure 2.1: SEM image of a in-house synthesised polystyrene microsphere. Scale bar is 0.5 µm.](image)

In recent years, functional polymer microspheres have been widely used in biomedical fields for bioseparation, enzyme immobilisation, and controlled release. Their widespread application is due to microspheres having a unique set of characteristics that include large specific surface area, stability and capability to undergo coupling with biological molecules. Among various types of microspheres, fluorescent microspheres have attracted great attention in various fields, such as labeling, immunoassay study, and a few cytometric analyses. Furthermore, their synthesis and functionalisation is cheap and they can be produced from a diverse range of materials and in a multitude of sizes. Once synthesised, they possess a long shelf-life. In terms of intracellular investigations, it is fair to point out that up to date, biodegradable microspheres have been studied in greater depth than their biostable counterparts, mainly due to the fact that therapeutic cargos can be released from a microsphere by leaching from the polymer or by degradation of the polymer matrix.
2.2-Biodegradable Microspheres

A key factor in the design of biodegradable microspheres as a delivery system is the choice of an appropriate biodegradable polymer. Biodegradable microspheres are made of synthetic or natural polymers which degrade in vivo, either enzymatically or non-enzymatically, to produce biocompatible or non-toxic byproducts along with the progressive release of dissolved drug. These can be further metabolised via normal physiological pathways.⁷⁰

2.2.1 Preparation

Biodegradable polymer microspheres can be prepared by a number of methods, each of them having its own advantages and disadvantages. The preparation method has significant influence on the properties of microspheres and therefore the desired properties should be kept in mind during the selection of a particular synthetic strategy. Some remarkable methods are phase separation-coacervation, double emulsion technique, spray drying, interfacial deposition, phase inversion microencapsulation, in situ polymerisation, chemical and thermal crosslinking. Three of the most widely used techniques for preparation of biodegradable microspheres are spray drying, double emulsion and phase separation-coacervation.

*Spray drying* is a well-known process, which yields dry powders, granules, or agglomerates⁷¹ from drug-excipient solutions and suspensions. In this technique the biodegradable polyester is dissolved in a volatile organic solvent (such as dichloromethane or acetone) and the drug is dispersed (in the form of solid) in the polymer solution by high speed homogenisation. This dispersion is then atomised in a stream of heated air. Solvent from the resulting droplets evaporates almost instantaneously, yielding microspheres in typical size ranges from 1 to 100 µm depending upon the atomising conditions. Residual solvents are removed from microspheres by vacuum drying after their subsequent collection from the airstream by a cyclone separation.⁷²
The double-emulsion solvent extraction/evaporation technique is one of the most popular methods for encapsulating hydrophilic drugs, in particular proteins and peptide drugs, into microspheres.\textsuperscript{73, 74} A drug in aqueous solvent is emulsified within a non-miscible organic solution of polymer to form a w/o emulsion. Dichloromethane is mainly used as the organic solvent and the homogenisation step is carried out using either high speed homogenisation or sonication. This primary emulsion is then rapidly transferred to an excess of an aqueous medium, containing a stabiliser (usually polyvinyl alcohol). Again either homogenisation or intensive stirring is necessary to initially form a double emulsion of w/o/w.\textsuperscript{75}

In phase separation-coacervation the drug is dispersed in solid form into a solution containing dichloromethane and polymer. Silicon oil is added to this dispersion at a defined rate, reducing the solubility of the polymer in its solvent. The polymer-rich liquid phase (coacervate) encapsulates the dispersed drug particles and “embryonic” microspheres are subjected to a hardening and washing step using heptane.\textsuperscript{76}

### 2.2.2 Applications

Over the last 25 years, the use of these polymers as vehicles has been exploited in research and commercial areas, the most notable example being for the treatment of prostate cancer, where a single once-a-month injection has replaced the tedious 30 daily injections that were previously administered. Biodegradable microspheres are being investigated for treatment of cancer, bacterial and viral infection, for birth control and AIDS.\textsuperscript{77-80} Delivery of genetically engineered products such as soluble recombinants HIV proteins,\textsuperscript{81} plasmid DNA\textsuperscript{82, 83} or antisense oligos and synthetic double-stranded DNA\textsuperscript{84, 85} has also been successfully improved thanks to these materials.

### 2.3-Bio-stable Microspheres

The fact that biodegradable polymer undergo a significant change in chemical structure under specific environmental conditions (such as chemical reactions involving organic solvents) make them very sensitive and limit the chemistry that
can be performed on them, moreover these changes result in a loss of physical and mechanical properties affecting massively their drug transport capacity. Because of this, research has been recently focused in the modification of traditional biodegradable polymers to make them more chemical-friendly. Thus, cross-linked microspheres prepared with stable monomers may be more desirable for some applications where multistep chemistry need to be carried out before cellular delivery of the cargo.

2.3.1 Preparation

There are several techniques that can be used for the preparation of bio-stable microspheres and these are: suspension, dispersion and emulsion polymerisation, all of them being part of a process called heterogeneous polymerisation whose mechanism is important to understand as it is involved in the generation of monodisperse particles.

2.3.1.1 Dispersion Polymerisation

Dispersion Polymerisation is an efficient polymerisation method that yields micron-sized monodisperse particles in a single batch process. In this method the reagents are mixed in an organic phase in which polymerisation of the monomer occurs in the presence of a suitable polymeric stabiliser that is soluble in the reaction medium. The solvent selected as the reaction medium must be a good solvent for both the monomer and the steric stabiliser polymers, but a non-solvent for the polymer being formed, which will precipitate from solution over a period of several hours. Under favourable circumstances, the polymerisation can yield, in a batch step, polymer particles of 0.1-10 \( \mu \)m in diameter, often with excellent monodispersity. Dispersion polymerisation therefore involves several steps (Figure 2.2). A homogeneous solution of monomer(s), initiator and stabiliser where polymerisation is induced by heating the solution which leads to initiator radicals being formed. These radicals react with the monomer(s) leading to formation of macroradicals or macromolecules which in turn leads to nucleation and further formation of primary particles. Thus, primary particles formed are swollen by the polymerisation medium and/or the monomer. At the early stage, there is a rapid nucleation phase where
mono-dispersed nuclei are swollen with monomer, short oligomers and initiator radicals. Therefore polymerisation takes place mainly within individual particles, leading to the formation of spherical particles.

![Diagram of particle nucleation and growth](image)

**Figure 2.2**: Schematic model showing particle nucleation and growth of sterically-stabilised particles in dispersion polymerisation.

**Particle stabilisation**

Stabiliser is essential, playing a crucial role in this process by adsorbing or becoming incorporated onto the surface (hairy layer) of the newly formed precipitated polymers (Figure 2.3). The stabiliser acts as a steric influence and facilitates precipitation of mono-dispersed primary particles, which continue their growth by swelling with more monomer or short oligomers to produce microspheres.
Particle size control

Particle size in dispersion polymerisation can be influenced to a greater or lesser extent by many factors including polymerisation temperature, monomer and initiator concentrations and the type and concentration of stabiliser. Additionally, the solvency of the polymerisation medium has a notable influence in the final size. 91

2.3.1.2 Emulsion-Polymerisation

The first successful theory to explain the mechanism of emulsion polymerisation was developed in the 1940s by Smith and Ewart, 92 and Harkins, 93 but recently because of the complex chemistry that occurs during an emulsion polymerisation process this mechanism has been reconsidered using computer simulations. 94 Emulsion polymerisation takes places over a number of steps and comprises monomer, water, surfactant and a water soluble initiator. The polymerisation is characterised by the insolubility of monomer in the polymerisation medium (generally water) which is dispersed by the aid of an emulsifier, forming 1-10 µm monomer droplets in water. Excess emulsifier will create micelles in water whereby small amounts of monomer can freely diffuse from the monomer droplet into the water (Figure 2.4). The water-soluble initiator is introduced into the water where upon heating it will form radicals which will react with monomer within micelles. The initiator reacts with the monomer in micelles in preference to reacting with the monomer droplets because the surface area of the micelles is much greater than the total surface area of the scarcer and larger monomer droplets. Monomer in

Figure 2.3: Sterically stabilised particles synthesised with the aid of a stabiliser. Size: 0.1-10 µm.
the micelle rapidly polymerises to form macroradicals, also called oligoradicals. It is at this point when the swollen micelle has turned into a polymer particle. These primary particles then either absorb more oligoradicals and monomer molecules from the medium or more monomer will diffuse from the droplets to the growing particle, where more initiator will eventually react. These polymer particles become the main loci of the polymerisation and the particles grow gradually until free monomer droplets disappear and the monomer is completely consumed.

![Diagram of emulsion polymerisation](image)

**Figure 2.4:** Schematic diagram for the emulsion-polymerisation.

### 2.3.1.3 Emulsifier-free emulsion polymerisation

Emulsifier-free emulsion polymerisation was first reported by Kotera et al,\(^5\),\(^6\) who demonstrated that emulsion polymerisation of styrene may be carried out without the addition of any stabiliser or surfactant in the polymerisation medium. The formation of the growing particles is explained by a homogeneous nucleation mechanism\(^7\),\(^8\) where the stabilisation of such particles comes from the orientation
of their own polymer chains, notably the chain ends originating from initiator molecules. The most studied emulsifier-free system is styrene/persulfate/water where it is believed that polymerisation initiated by a persulfate initiator starts in the aqueous phase and not in the emulsifier micelles (as what happens in emulsion-polymerisation) and causes the formation of radicals. As the particle grows, the surface charge (generated by the initiator end groups) increases until reaching a point where the particles are stabilised by their own electrostatic charge which also brings about static repulsions between particles, preventing flocculation.99

2.3.1.4 Emulsifier-free emulsion polymerisation and emulsion-polymerisation compared.

It has been demonstrated that monodisperse particles synthesised in the absence of emulsifier tend to have much cleaner surfaces.100 The reason is because during emulsion-polymerisation, stabilisers or surfactants tend to remain on the surfaces of polymer particles and their removal is quite tedious and can easily lead to flocculation. In fact, whether complete removal can be achieved is still a matter of debate by the scientific community. Moreover, the surfactant may affect the properties of the latex in suspension (size increase) and can also affect the magnitude of the surface charge. To overcome these potential problems, emulsifier-free emulsion-polymerisation is often preferred for the synthesis of monosized latex particles (in the range of nanometers) suitable as drug delivery agents (Figure 2.5).
2.3.2 Polystyrene microsphere applications

Polymer microspheres present a flexible platform for applications in diagnostics and bioseparations. They may be coated with recognition molecules, such as antibodies, antigens, peptides or nucleic acid probes, and can be loaded with hydrophobic dyes and other compounds. Unmodified polymer spheres also find extensive use as standards for instrument set-up and calibration. Polystyrene microspheres are ideal for protein adsorption, and have been utilised in a range of diagnostic tests and assays.¹⁰¹

2.3.2.1 Cellular uptake of polystyrene microspheres

Cellular uptake of polystyrene microspheres has been widely explored with notable results in different research groups. A remarkable example of work in this field has been carried out by Zauner et al.¹⁰² They characterised the ability of several cell lines to internalise labelled polystyrene microspheres of different sizes. In their investigation, all the cell lines tested ingested 20 nm microspheres avidly. With larger microspheres (93, 220, 560 and 1010 nm) cell type as well as growth associated differences were observed. Various cell lines (HUVEC, ECV 304 and HNX 14C) took up microspheres up to 1010 nm even when the cells were confluent.
As well as these cell lines, microspheres of defined sizes have been used previously to determine the pinocytic/phagocytic activity of certain cell types.\textsuperscript{56, 103-108}

2.3.2.2 \textit{Fluorescent polystyrene microspheres}

Significant attention has been paid to fluorescent probes for their extensive range of applications in biological research and clinical diagnosis.\textsuperscript{109, 110}

Traditionally, employment of fluorescent small molecules (organic dyes) has led to considerable limitations including photobleaching, low signal intensities and blinking characteristics.\textsuperscript{111, 112}

Composite polymer particles by the incorporation of organic dyes into polymer particles have effectively addressed these problems and have been used as fluorescent probes in various biomedical applications.\textsuperscript{113, 114}

2.3.2.3 \textit{Amino-functionalised polystyrene: Microspheres as carrier systems.}

The Bradley group is pioneer in the synthesis and application of multi-functionalised, cross-linked, polystyrene microspheres, which as mentioned previously, are taken up by the great majority of cell types studied so far.\textsuperscript{115, 116}

Importantly, the nature of these synthetic beads allows multistep solid-phase chemistry and the possibility to bind any molecule/sensor/nucleic acid to them. Populations of cells containing beads can be readily sorted from other cells for subsequent analysis with very high, but controllable, uptake rates that can be modulated through changing the bead size and incubation time.\textsuperscript{117}

To be able to follow the movement of microspheres inside cells and to study their cellular uptake, microspheres have been covalently labelled with several dyes (\textit{Figure 2.6}). The fact that the microspheres could be labelled with different fluorescent molecules was very important because it meant that standard organic and solid-phase protocols to microspheres coupling could be successfully applied. This opens a great number of possibilities for the application of these microspheres because it means that many different molecules could be bound to them.
Some of the applications that the Bradley group has been studying over the last years include:

- **Microspheres for Cellular Delivery:** By means of the cleavage of an ester bond between the molecule and the delivery system by an intracellular esterase. It was proven that microspheres could be used to deliver molecules into cells, but in addition that enzymatic reactions on microspheres could take place intracellularly. This feature of microspheres allows the use of microspheres as carrier systems for our enzyme substrates in order to study and monitor enzymatic activity.

- **Using Microspheres for Multiplexing Assays:** To assess the potential use of these microspheres as bio-markers, a time course study of cellular uptake followed by confocal microscopy was undertaken. 72 h after uptake all cell types tested were shown to have retained fluorescent microspheres, making these excellent candidates as cellular markers and opening up possibilities in the area of cellular multiplexing. These studies suggest that microspheres can be used as effective bio-markers for enzymatic activity *in situ* assays.
Analysis of Microsphere-Based Intracellular Calcium Sensing: By using amino functionalised polystyrene microspheres changes in the concentration of intracellular Ca\(^{2+}\) ions in single living cells in real time could be measured. The fact that microspheres are firmly retained for several days also allows changes in the concentration of intracellular Ca\(^{2+}\) ions to be followed in a single cell for much longer periods of time than when using established sensors. These results open the door to a range of possible applications, such as microspheres acting as carriers of sensors for enzymatic activity assays.\(^ {115}\)

pH Sensing in Living Cells Using Fluorescent Microspheres: The employment of fluorescein-loaded microspheres as intracellular pH sensors in living cells has been successfully proven by using a number of different techniques such as spectrofluorometry, fluorescence microscopy and flow cytometry. Covalent binding of fluorescein to microspheres significantly improves the stability of the indicator over time and eliminates escape. At the same time it retains the properties of fluorescein for pH sensing. These fluorescein-loaded microspheres provide a reliable way to complete long-term cell monitoring studies.\(^ {118}\)

Cellular uptake of Fluorescent Labelled Biotin-Streptavidin Microspheres: The use of streptavidin-loaded microspheres as a carrier system able to enter living cells was successfully demonstrated using a biotinylated fluorophore by fluorescence microscopy and flow cytometry. Moreover, the cellular uptake of a biotinylated oligonucleotide was successfully carried out. The fact that there are several biotinylation strategies to attach different molecules such as proteins, peptides, antibodies, nucleic acids, etc. to the streptavidin-loaded microspheres opens the door to a huge variety of applications, such as intracellular detection of a diverse number of targets.\(^ {119}\)

Microsphere Mediated Protein Delivery into Cells: A study was carried out to evaluate the ability of protein-loaded microspheres to go through cell membranes and to demonstrate that protein activity is maintained while attached to the beads “expressed” within the cellular environment.\(^ {120}\)

Knocking Anti-Sense into Cells: Green fluorescent protein (GFP) expressed in human ovarian cancer (HeLa) cells has been successfully silenced using amino
functionalised siRNA linked to 500 and 200 nm microspheres via cleavable and non-cleavable linkers.  

✓ **Microsphere-based tracking and molecular delivery in embryonic stem cells:** Amino functionalised polystyrene microspheres were employed for the coupling and efficient delivery of a variety of cargos into both undifferentiated and differentiated embryo derived stem cells. Cargos included proteins, siRNAs and fluorescent dyes. Thus, microspheres represent an effective means to introduce biological cargos into real time differentiating ES cell cultures, opening a new area of research in the transfection of Stem Cells.

✓ **Microspheres as a vehicle for biomolecule delivery to neural stem cells:** Mouse and human NSCs were beadfected with polystyrene-based microspheres without obvious toxic effects and without compromising the stem cell and differentiation property. Based on this research, microspheres may be considered as an excellent delivery vehicle for biomolecules and proteins in difficult to transfect cell lines such as neural stem cells.

In summary, polystyrene microspheres have a wide range of advantages over traditional drug delivery methods, including the following:

✓ High concentrations of the captured molecule on the bead, making analysis less complicated.

✓ Flow cytometry may be employed to sort cells containing beads from unloaded cells providing that a dye molecule has been linked to the microspheres.

✓ Specifically desired loadings may be achieved.

✓ A variety of cell lines have been successfully “beadfected”, including adherent, suspension and primary cells.

✓ An assortment of compounds may be linked to the microspheres for transportation, including proteins, dye molecules and sensors.

✓ These beads can be visualized by standard microscopy techniques such as confocal microscopy.
2.3.3 Synthesis of aminomethyl crosslinked polystyrene microspheres

Amino functionalised microspheres have been used for covalent couplings of a broad range of cargos such as with dyes, spacers or biomolecules. Crosslinked microspheres offer greater advantages over non-crosslinked microspheres because they exhibit much improved mechanical stability in various organic solvents, allowing a wide spectrum of possible reactions that can be carried out on them. Therefore, the free radical copolymerisation of amino-functionalised monomer vinylbenzylamine hydrochloride (VBAH) with styrene along with p-divinylbenzene (DVB) (acting as cross-linking agent) was used to generate robust particles for the production of aminomethyl crosslinked microspheres in this project. The process was carried out either by dispersion-polymerisation or emulsifier-free emulsion polymerisation depending on the desired microsphere size.

2.3.3.1 Synthesis of the amino functionalised monomer (VBAH)

The synthesis of functionalised monomer vinylbenzylamine hydrochloride (VBAH) (2.5) was performed as previously described\(^5\) where 4-vinylbenzyl phthalimide (2.3), resulting from the reaction of 3-vinylbenzyl chloride (2.1) with potassium phthalimide (2.2) in DMF, was heated in the presence of hydrazine to give the amino derivative (2.4), which was then converted to the salt VBAH (2.5) in an overall yield of 38% (Scheme 2.1).

\[
\begin{align*}
\text{(2.1)} + \text{(2.2)} &\xrightarrow{(i)} \text{(2.3)} + \text{(2.4)} \xrightarrow{(ii)} \text{(2.5)} \\
\text{(2.1)} &\text{Reagents and conditions. (i) DMF, 50°C, 17 h, 67%; (ii) N}_2\_\text{H}_4, \text{N}_2\text{ atmosphere, EtOH, 70°C, 3 h, 70%; (iii) HCl 6N, 0°C, 82%, 38% overall.}
\end{align*}
\]

\[\text{Scheme 2.1: Synthesis of vinylbenzylamine hydrochloride}\]
### 2.3.3.2 0.5 and 2 µm microsphere synthesis by dispersion-polymerisation

As said previously, dispersion-polymerisation is an attractive route for producing uniform particles with diameters ranging from 0.5-10 µm. It was decided for this project to synthesise monodisperse poly(styrene-co-divinylbenzene) microspheres. Microspheres were synthesised by dispersion polymerisation in pure ethanol (2.6) or ethanol/water (2.7) as dispersion medium with poly(N-vinyl pyrrolidone) (PVP) MW 29,000 as a stabiliser, VBAH as the amino functionalised monomer and 2,2′-azobisisobutyronitrile (AIBN) as a radical initiator in an inert atmosphere (nitrogen) (Scheme 2.2). When the reaction was completed, the microsphere suspension was cooled over 30 minutes and subsequently washed in water and methanol to collect the desired polymer particles. The method was performed on a Radley carousel where up to 12 glass reaction tubes (1 to 20ml) can be set-up simultaneously for polymerisation providing heating/cooling, stirring speed and refluxing with inert gas (nitrogen) control (Figure 2.7).

![Scheme 2.2: Aminojfunctionalised latex microspheres synthesis by dispersion-polymerisation.](image)

**Reagents and conditions.** (i) PVP, AIBN, ethanol/water, 65°C, N₂ atm., 16 h. (2.7);
(ii) PVP, AIBN, ethanol, 65°C, N₂ atm., 16 h. (2.6)

**Scheme 2.2:** Amino-functionalised latex microspheres synthesis by dispersion-polymerisation.

![Figure 2.7: Radley Carousel for microspheres preparation.](image)
2.3.3.3 0.2 µm microspheres synthesis by emulsifier-free emulsion-polymerisation

In this project, a facile emulsifier-free emulsion polymerisation approach was conducted in water by an adaptation of the protocol described by Delair et al., whereby VBAH was copolymerised with styrene in “boiled water purged with nitrogen” with magnesium sulphate as a form of stabiliser. After stirring at 350 rpm for 30 minutes at 80°C, the water-soluble initiator 2,2'-azobis(2-amidinopropane) dihydrochloride (V50) was added. The resulting emulsion was stirred for 15 hours while maintaining a constant stirring speed and temperature to produce the desired 0.2 µm crosslinked aminomethyl microspheres (2.8) in 28% yield (Scheme 2.3) following centrifugation and washing (methanol and water).

\[
\text{Reagents and conditions: (i) MgSO}_4, \text{H}_2\text{O}, \text{V}_{50}, 80^\circ\text{C}, 350 \text{ rpm, N}_2 \text{ atm., 15 h, 28%}. 
\]

Scheme 2.3: Synthesis of Amino functionalised latex microspheres by emulsifier-free emulsion-polymerisation.

2.3.4 Microsphere characterisation

Prior to characterisation, latex microspheres were washed by repeated centrifugations with methanol followed by water to eliminate any residual monomer and water-soluble polymers. The size and morphology of the beads were assessed by Dynamic Light Scattering and scanning electron microscopy (SEM). The colloidal stability and the loading of the obtained particles were also determined.

2.3.4.1 Particle size by Dynamic Light Scattering

Microsphere particle size was measured using a Zetasizer Nano ZS which allows particle characterisation by giving accurate, reliable and repeatable measurements of particle size and zeta potential (Figure 2.8). This system relies on a technique termed Dynamic Light Scattering (DLS), also known as Quasi Elastic...
Light Scattering (QELS) and Photon Correlation Spectroscopy (PCS). This technique has been used in polymer and colloidal science for about 30 years.\textsuperscript{124, 125} It is applicable to particles suspended in a liquid (such as microspheres), which are in a state of random movement due to Brownian motion.\textsuperscript{126} The instrumentation allows the rapid determination of particle size as well as providing information on relaxation time distributions for the macromolecular components of complex systems.

\textbf{Figure 2.8:} Zetasizer Nano ZS for particle characterisation.

Theory

In DLS a beam of light passes through a sample particle within a cell. The electric field of the particles or droplets scatter some of the light in all directions. Thus, the molecules provide a secondary source of light and subsequently scatter light (\textbf{Figure 2.9/B}). The frequency shifts, angular distribution, polarisation and the intensity of the scattered light are determined by the size, shape and molecular interactions in the scattering material. In practise, particles suspended in a liquid are never stationary, the particles are constantly moving due to Brownian motion. An important feature of Brownian motion regarding DLS is that small particles move quickly and large particles move more slowly. As the particles move around, the intensity appears to fluctuate and Zetasizer measures the fluctuation in scattering intensity and uses this to calculate the size of particles within the sample.
The intensity of the scattered light must be within a specific range for the detector to successfully measure it. If too much light is detected then the detector will become overloaded. To overcome this, an “attenuator” is used to reduce the intensity of the laser and hence reduce the intensity of the scattering. Subsequently, the scattering intensity signal for the detector is passed to a digital signal processing board called a correlator. The correlator compares the scattering intensity at successive time intervals to derive the rate at which the intensity is varying. This correlator information is then passed to a computer, where the Zetasizer software will analyse the data and will derive size information. Zetasizer Nano ZS measures the scattering information at 173°. This is known as backscatter detection, which relies on Non-Invasive Back Scatter (NIBS) technology (Figure 2.9/A). There are several advantages for doing this: i) As backscatter is being measured, the laser beam does not have to travel through the entire simple. ii) It reduces multiple scattering, where the scattered light from one particle is itself scattered by other particles. iii) Contaminants or dust particles, which are normally larger in size, mainly scatter in
the forward direction. Therefore, by measuring the backscatter, the effect of dust is highly reduced.

Results

Size distributions of microspheres obtained from either emulsifier-free emulsion polymerisation (2.8) or dispersion polymerisation (2.6 and 2.7) were determined by Dynamic Light Scattering in Zetasizer Nano ZS (Figure 2.10). The obtained values of mean particle size, coefficient of variation and standard deviation are summarized in Table 2.1. These results indicate that the microspheres were highly monodispersed with a narrow particle-size distribution.

![Figure 2.10: Particle size distribution of synthesised microspheres.](image)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mean Diameter (nm)</th>
<th>Coefficient of variation (%)</th>
<th>Standard deviation (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8</td>
<td>190.1</td>
<td>19</td>
<td>0.035</td>
</tr>
<tr>
<td>2.6</td>
<td>458.7</td>
<td>14.4</td>
<td>0.069</td>
</tr>
<tr>
<td>2.7</td>
<td>1983.9</td>
<td>16.9</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 2.1: Values of Mean Diameter, Coefficient of Variation and Standard Deviation of the synthesised microspheres. Data generated from samples containing between 80,000-100,000 particles.
2.3.4.2 Colloidal stability by “Zeta potential measurements”

Zeta potential is a physical property which is exhibited by any particle that is in a suspension. It is well established that zeta potential is a very good indicator of the magnitude of the interaction between colloidal particles and measurements of zeta potential are regularly used to calculate the stability of colloidal systems. The zeta potential value of a sample is calculated by determining the Electrophoretic Mobility using a Zetasizer Nano. The Electrophoretic Mobility is obtained by performing an electrophoresis experiment on the sample which measures the velocity of the particles using Phase Analysis Light Scattering (PALS).

Theory

Colloidal particles dispersed in a solution are electrically charged due to their ionic characteristics and dipolar attributes. The development of a net charge at the particle surface influences in the distribution of ions in the liquid layer surrounding the particle. The liquid layer surrounding the particle exists as two parts, a fixed layer, full of oppositely charged ions, where the ions are strongly bound and an outer area, diffuse, forming a cloud-like area where the compositions of ions of opposite polarities are varying and ions are less firmly attached. The potential in the outer area decreases with the distance from the surface, until a point where it becomes zero. When a voltage is applied to the solution in which particles are dispersed, particles are attracted to the opposite polarity electrode, followed by the fixed layer and a part of the diffuse double layer (Figure 2.11/A). The potential at the boundary between this unit is known as the Zeta Potential. Then, Zeta potential is generally speaking a function of the surface charge of a particle, any adsorbed layer at the interface and the nature and composition of the surrounding medium in which the particle is suspended.
The measurement of a zeta potential in a Zetasizer Nano comprises six main components. As in DLS a laser is used to provide a light source which passes through the centre of the cell (folded capillary cell, equipped with electrodes on both sides). However the light source for zeta potential measurements is split to provide an incident and a reference beam. Scattering now is set up about 17° angle. When the electric field is applied to the cell, any particles moving through the measurement volume will cause the intensity of light detected to fluctuate with a frequency directly proportional to the particle speed. A detector sends this information to a digital signal processor, which will then pass this to a computer. Finally, the Zetasizer Nano software produces a frequency spectrum from which the electrophoretic mobility and hence the zeta potential information is calculated. (Figure 2.11/B). As in DLS, an attenuator is required to graduate the intensity of the laser.
In order to study the colloidal stability of the beads and carry out a novel procedure for reaction control performed on the microspheres, based in the zeta potential values, it is necessary to obtain a range of microspheres with different functionality in their surface. For this purpose, a series of couplings with Fmoc-type spacer, which was previously synthesised as detailed in Scheme 2.4/A, and further deprotections were carried out (as detailed in Scheme 2.4/B) applying a standard Fmoc solid phase protocol using 200 nm amino beads (2.8). Thus, colloidal stability of microspheres was measured by Phase Analysis Light Scattering in the Zetasizer Nano ZS with the following results (Figure 2.12). It can be observed that the values of zeta potential change significantly after each step. These results confirm that each step of a number of coupling steps to microspheres can be efficiently monitored using this technique.

Reagents and conditions: (i) acetonitrile, rt 3 h; (ii) FmocDOSu, acetonitrile, DIPEA, rt, 12 h, 65% overall.

Reagents and conditions: (i) acetonitrile, rt 3 h; (ii) Fmoc-OSu, acetonitrile, DIPEA, rt, 12 h, 65% overall.

Reagents and conditions: (i) Fmoc-PEG spacer (5eq), Oxyma (5eq), DIC (5eq), DMF, 2 h, 60°C. (ii) 20%piperidine, DMF, 3x20m. (iii) Adipic acid (5eq), DIC (5eq), DIPEA (0.1eq).

Scheme 2.4: A) Synthesis of PEG-type spacer; B) Preparation of 200 nm microspheres via PEG coupling (2.9), Fmoc deprotection (2.10) and Adipic acid coupling (2.11).
Beads stability

Figure 2.12: Colloidal stability and reaction control of 200 nm aminofunctionalised beads (2.8), Fmoc protected beads (2.9), Amino beads after Fmoc deprotection (2.10) and carboxyfunctionalised beads (2.11).

2.3.4.3 Amine group quantification

Several methods have been reported for determining the amino loading on the surface of microspheres including tritylation\textsuperscript{130} of the uncoupled amino groups with an active tritylating agent and spectrophotometric methods.\textsuperscript{131} However these methods have been proven to be unreliable when particles are in the nanometer size range.\textsuperscript{132} Classical methods typically used for the determination of free primary amino groups on aminomethylated polystyrene resin in solid phase synthesis, such as the Ninhydrin\textsuperscript{133} and the Fmoc\textsuperscript{134} tests, were considered.

Ninhydrin test

The Ninhydrin test, also known as the Kaiser test was first developed by Moore and Stein\textsuperscript{135} before being adapted by Kaiser\textsuperscript{136} for solid phase chemistry synthesis and finally optimised by Sarin,\textsuperscript{133} who designed conditions for a rapid and quantitative test for the determination of free primary amino groups on resin. The amount of free primary amine can be readily detected and quantified by reaction with ninhydrin. As shown in Scheme 2.5, ninhydrin reacts with a primary amine which eventually yields Ruhemann’s purple, which can be quantified spectrophotometrically by UV/Vis at 570 nm.
Ninhydrin test results

Quantitative ninhydrin tests were performed on the equivalent of 3 mg of dry microspheres. Once centrifuged, microspheres were washed with 60% ethanol aqueous solution and the absorption measured at 570 nm. The amount of primary amino groups on the microspheres calculated by this method range from 15 to 200 µmol/g depending on the amount of aminofunctionalised monomer used and the polymerisation technique applied. In particular the values obtained for samples 2.6, 2.7 and 2.8 were 17 µmol/g, 76 µmol/g and 26 µmol/g, respectively.

Fmoc test

The Fmoc test is a colorimetric test to quantify the loading of free primary amino groups on solid-phase. The test consists of coupling an Fmoc (9H-fluoren-9-yl-methoxycarbonyl)-protected molecule (generally amino acids) and measuring the amount of fulvene-piperidyl adduct by UV/Vis spectroscopy at 302 nm that is released following a deprotection step under basic conditions (Scheme 2.6).
Scheme 2.6: Fmoc deprotection by piperidine

**Fmoc test Results**

Fmoc tests on in-house synthesised microspheres were performed following an Fmoc-Aminohexanoic coupling and further Fmoc deprotection (Scheme 2.7) using a triple cycle of 20 minute treatments using piperidine. The supernatants were collected and combined and the amount of fulvene-piperidyl adduct measured at 302 nm giving loadings of 15 µmol/g for (2.6), 69 µmol/g for (2.7) and 21 µmol/g for (2.8).

![Scheme 2.7: Fmoc test performed on in-house synthesised beads via aminohexanoic spacer unit.](image)

**Reagents and conditions:** (i) Fmoc-Aminohexanoic (5eq), Oxyma (5eq), DIC (5eq), DMF. 2 h, 60°C; (ii) 20%piperidine, DMF. 3x20m.

2.3.4.4 **Particle appearance by Scanning Electron Microscopy**

Scanning electron microscopy (SEM) is a technique that relies on electrons rather than light to form an image and was developed in the early 1950’s. Since then it has helped to develop new areas of study for researchers mainly in the areas of medical and physical sciences. The signals that are obtained by SEM reveal
information about the sample including external morphology (texture), chemical composition and crystallinity.

The size and shape of the obtained polymeric microspheres were analysed by SEM using a Philips XL30CP microscope. For this purpose, before analysis, the samples were dried and coated with a nanofilm of gold. The micrographs obtained using this technique (Figure 2.13) show that these microspheres appeared as populations of monodisperse particles confirming the results obtained by Dynamic Light Scattering (section 2.3.4.1). Additionally, SEM gave crucial information about the particle shape, showing a spherical morphology with a smooth surface.

![Figure 2.13: Scanning electron microscopy. Micrographs of in-house synthesised microspheres. A) 0.5µm (2.6); B) 2 µm (2.7); C) 0.2 µm (2.8). Scale bar is as represented.](image)

2.3.5 Cellular uptake of polystyrene microspheres

2.3.5.1 Flow cytometry

Flow cytometry is a technique that simultaneously measures and analyses multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle’s relative size, relative granularity or internal complexity and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence.

A flow cytometer is made up of three main systems: fluidics, optics, and electronics.

- The fluidics system transports particles in a stream to the laser beam for interrogation.
- The optics system consists of lasers, to illuminate the particles in the sample stream, and optical filters to direct the resulting light signals to the appropriate detectors.
The electronics system converts the detected light signals into electronic signals that can be processed by the computer. For some instruments equipped with a sorting feature, the electronics system is also capable of initiating sorting decisions to charge and deflect particles.

In a flow cytometer, particles are carried to the laser intercept in a fluid stream. Any suspended particles or cells from 0.2–150 micrometers in size are suitable for analysis. Cells from solid tissue must be disaggregated before analysis. The portion of the fluid stream, where particles are located, is called the sample core. When particles pass through the laser intercept, they scatter laser light. Any fluorescent molecules present on the particle fluoresce. The scattered and fluorescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them. List mode data are collected on each particle or event. The characteristics or parameters of each event are based on its light scattering and fluorescent properties. The data is collected and stored on the computer. This data can be analysed to provide information about subpopulations within samples.  

Flow cytometry analysis

Fluorescence Activated Cell Sorting (FACS) is a method that can separate cells that are phenotypically different from each other. The process begins by adding the cells in a sample into a flask and forcing the cells to go into a tiny nozzle one at a time (Figure 2.15). The cells travel down the nozzle which is vibrated at an optimal frequency to produce drops at a fixed distance from the nozzle. As the cells flow down the stream of liquid, they are scanned by a laser (pink and turquoise lights in Figure 2.15) in all directions. Light that scatters axial to the laser beam is called forward scatter (FSC) and indicates relative differences in the size of the cells or particles and side scatter (SSC) indicates relative differences in the internal complexity or granularity of the cells or particles (Figure 2.14).
The FACS machine sorts single cells based on their fluorescent properties. Inside the machine, cells are suspended in a stream surrounded by sheath fluid, which races past a laser at a rate of 5,000 to 10,000 cells per second. The stream breaks into tens of thousands of drops, which are charged and then sorted. The laser detects fluorescence and translates its readouts to a computer, which charges the drops, after which charged plates shuttle cells into the appropriate bin.

**Figure 2.14:** Schematic representation of forward and side scatter generated by a cell.
Figure 2.15: Schematic representation of flow cytometry concept.

BD FACSARia Cell-Sorting System

The BD FACSARia™ cell sorter sets a model for high performance flow cytometry. This system delivers high-speed sorting and multicolour analysis. The BD FACSARia instrument incorporates a fixed-alignment cuvette flow cell. This flow cell provides superior fluorescence sensitivity. The fixed optical system offers freedom from instrument maintenance and alignment. ¹⁴⁰
Apart from mentioned above, some characteristics of FACS Aria Machine are:

- Accommodates up to four air-cooled lasers at 488-nm, 633-nm, 405-nm, and 375-nm wavelengths
- Digital acquisition rates of up to 70,000 events/second
- Multicolor analysis of up to 15 parameters
- Two- and four-way bulk sorting devices for a variety of tube sizes
- Optional Automated Cell Deposition Unit (ACDU) for sorting to BD™ multiwell plates or microscope slides

**Interpretation of results**

The data obtained using flow cytometry are generally displayed by 2 different forms of graphical output: dot plots and histograms. In dot plots each event is represented by a dot which is located along the x and y scales in accordance to the intensity detected for that event. Correlation data from any two parameters measured by the flow cytometer (the x and y axis representing different fluorescence intensities or the light scattering) **(Figure 2.17)** is plotted. One of the most used dot plot is FSC/SSC because, as previously mentioned, it detects any size distinction or internal complexity of cell population.
**Figure 2.17**: Dot plot from FACS analysis of HEK-293T cells. Each dot represents one event, where different colours represent different densities (red=higher density; blue=lower density). Sample size of 10,000 events.

In contrast, in a histogram populations are represented as peaks where the x axis represents the fluorescence intensity of events counted and the population heterogeneity is represented by the width of a peak: The wider the peak, the more heterogeneous the population (Figure 2.18). A multiple-peak histogram means that there are several populations of cells with different intensity at that particular wavelength. Although, there is more than one parameter that could be obtained from the histogram the fluorescence median is preferred because it is a robust estimate of intensity which characterises a population of cells.
Figure 2.18: Histogram from FACS analysis of HEK-293T cells. The curve represents the mean fluorescence of the full population. Sample size of 10,000 events.

2.3.5.2 Confocal microscopy

Confocal microscopy is a powerful technique in which high resolution images of real-life samples can be obtained in three dimensions rather than two dimensions (as happens in standard fluorescence microscopy). The main difference between confocal microscopy is that only light in-focus is detected, while most of the light from the specimen that is not from the microscope’s focal plane is excluded. The theory behind confocal microscopy is the idea of point-by-point illumination of the specimen and rejection of out-of-focus-light. This is achieved with the use of a pinhole. A pinhole is put at the image plane and an electronic light detector is placed behind the pinhole. Doing this allows only one point in the specimen to be focused upon at a time. The 3D image of the entire specimen is constructed by having a laser beam scan over the entire focal plane and can mechanically move the specimen to change the depth of the optical plane through the specimen. Thus, the combination of sharp optical sections obtained at different focus points will represent the final 3D image of the full specimen (Figure 2.19).
2.3.5.3 Cellular uptake results

In order to study the cellular uptake of the obtained polystyrene microspheres a fluorophore was coupled to the beads to allow their detection by microscopy or flow cytometry studies.

Preparation of Fluorescent Microspheres

Amino-functionalised microspheres of different sizes (0.5 µm, 2 µm and 0.2 µm, (2.6), (2.7) and (2.8) respectively) were fluorescently labelled with 5(6)-carboxyfluorescein via an aminohexanoic spacer unit as detailed in Scheme 2.8.
Reagents and conditions: (i) Fmoc-Aminohexanoic (5eq), Oxyma (5eq), DIC (5eq), DMF. 2 h, 60°C; (ii) 20%piperidine, DMF. 3x20m; (iii) 5(6)-carboxyfluorescein (5eq), Oxyma (5eq), DIC (5eq), DMF, 2h, 60°C.

Scheme 2.8: FAM-Microspheres. Preparation of 0.5 µm, 2 µm and 0.2 µm fluorescein microspheres (2.15), (2.16) and (2.17) via aminohexanoic microspheres (2.12), (2.13) and (2.14).

The reason behind the use of an aminohexanoic unit as a spacer between the microsphere and fluorophore, is that the incorporation of a spacer decreases steric repulsions between the bulky dye molecules and possible amino residues present within the polymeric matrix. Moreover, it has been previously reported that some spacer units increase the biocompatibility of foreign material entering into cells and can aid the interactions between microspheres and cell surfaces.116

Flow cytometry results

Thus, several cell lines such as mouse melanoma (B16F10) cells, human embryonic kidney (HEK293T) cells and human cervical cancer (HeLa) cells, which are known to be very keen to uptake foreign materials, were beadfected with 0.2 µm, 0.5 µm and 2 µm fluorescein labelled microspheres, (2.17), (2.15) and (2.16). The influence of incubation time and particle size on the cellular uptake of these polymeric particles was investigated. For this purpose, incubations were run over 3 – 24 hours with a range of size of microspheres at a concentration of 86 µg/mL (Graphic 2.1/A) in order to study cellular uptake rates and how microsphere diameter affects its internalisation. Unlabelled beads were used as negative control. Following incubation, the excess microspheres were removed, and analysis by flow cytometry showed that cellular uptake of the microspheres was effective in all cases (Graphic 2.1/A-B). All sizes of bead could be delivered into the cells, though with varying degrees of success depending on the cell type investigated. Cellular uptake was also found to be bead-size and time dependent.
Graphic 2.1: A) Uptake results at different incubation times of 0.2 (2.17), 0.5 (2.15) and 2 µm (2.16) fluorescein microspheres by B16F10 cells (1), HEK293T cells (2) and HeLa cells (3). % uptake was measured as the % of cell with a fluorescent emission exceeding untreated control cells, which were taken as 0% uptake. B) Histograms representing fluorescent signal by B16F10 cells, (1) beadfected with 2 µm unlabelled beads (2.7) and 2 µm fluorescein beads (2.16), HEK293T cells (2) beadfected with 0.2 µm unlabelled beads (2.8) and 0.2 µm fluorescein beads (2.17) and HeLa cells (3) beadfected with 0.5 µm unlabelled beads (2.6) and 0.5 µm fluorescein beads (2.15). Errors bar representing the standard deviation. n=6.

Confocal microscopy results

In addition, to confirm the result obtained by flow cytometry, the effective intracellular uptake of polystyrene beads into cells was assessed running a confocal microscopy study. As representative example, Figure 2.20 shows a confocal image of HeLa cells following incubation with 0.5 µm fluorescein beads (2.15) over a 12 hours time period at a microsphere concentration of 86 µg/mL. These results clearly show that the microspheres are inside the cells and consequently they confirm that these particles have successfully crossed the cell membrane.
2.3.6 Microspheres and Solid Phase Chemistry

Depending on the particular use of the microspheres, different chemical approaches have been applied to the coupling of cargos to the polymeric particle. In this section some solid phase protocols are described.

2.3.6.1 Fmoc Chemistry on Microspheres

These polymer particles could be readily employed in multistep solid-phase synthesis and the resulting constructs are rapidly taken up by cells and exploited for a variety of applications. From a synthetic chemistry point of view, the beads are comparable with the use of a protecting group while also acting as a mode for traversing the membrane of living cells and are able to deliver cargos ranging from sensors to proteins and siRNA. Our strategy consists of an Fmoc solid-phase synthesis protocol (Scheme 2.9), which is based on simple coupling and deprotection steps, where coupling is normally carried out in DMF (dimethylformamide) with standard coupling agents such as ethyl 2-cyano-2-(hydroxyimino) acetate (oxyma) and N,N′-diisopropylcarbodiimide (DIC). Fmoc chemistry allows spacers of a variety of different lengths and hydrophilicities, such as Fmoc-aminoheptanoic acid (Fmoc-Ahx-OH) or Fmoc-polyethylene glycolic acid (Fmoc-PEG-OH), to be
coupled to the microspheres in order to alter the physical properties of the microspheres (e.g., hydrophilicity), and to distance the cargo from the bead itself.\textsuperscript{141}

\subsection*{2.3.6.2 Dual Functionality on Microspheres}

\textit{Fmoc-Dde full orthogonality}

Orthogonality between protecting groups is essential for the design of a solid-phase strategy. For the controlled synthesis of our scaffold, both the \textit{N}-\textit{\alpha}-amino group and the side chain amino functionality of the lysine derivative should be blocked with orthogonal protecting groups. Where the classic strategy is based on a base-labile \textit{N}-Fmoc group for protection of the \textit{\alpha}-amino and an acid-labile side chain protecting group (e.g., Boc), our modified protocol is based on the use of a nucleophilically labile protecting group on the side chain (1-(4,4-dimethyl-2,6-dioxacyclohexylidene) ethyl, Dde).\textsuperscript{142} Dde has been used previously as a protecting group for primary amines in solid-phase chemistry, but its standard conditions for deprotection (3\% v/v hydrazine in DMF)\textsuperscript{143} also cleave Fmoc. However, recently complete orthogonality between Fmoc and Dde has been demonstrated using hydroxylamine hydrochloride and imidazole in NMP to afford the removal of Dde without deprotecting Fmoc.\textsuperscript{144} Applying this methodology yields a powerful approach to facilitate dual functionalisation of microspheres.
Multi-functionalisation strategy

Dual functionalised microspheres allow the production of microspheres capable of carrying two cargos simultaneously (e.g., a biologically relevant cargo and a tracking fluorophore) (Scheme 2.11 and Figure 2.21). Subsequently, selective cleavage of Fmoc or Dde permits the directed coupling of the cargo so that its position can be known, and facilitates the introduction of a scaffold that could be easily attached to the microspheres and elongated using Fmoc protocols in a directed manner.
Reagents and conditions: (i) DCC, DMAP, CH$_3$COH, 36 h, 81.37%; (ii) Fmoc-Lys-OH, TFA (0.1%), EtOH reflux, 60 h, 54.4%; 45.1%

Scheme 2.10: Synthesis of Fmoc-Lys(Dde)-OH.

Figure 2.21: Dual functionality. Structure of Fmoc-Lysine(Dde)-OH (2.20).
Scheme 2.11: Multifunctionalisation. General solid-phase synthesis strategy applied to microspheres.

2.4-Conclusions

In this chapter, a brief description of the different methods for the preparation and characterisation of microspheres has been reported. Additionally, developments in microsphere chemistry have been described in order to broaden the application of microspheres as carrier systems. The synthesis and characterisation of mono-dispersed populations of robust cross-linked microspheres of defined sizes (from 200 nm to 2 µm) have been successfully carried out. Also, the evaluation of cellular
uptake performed on different cell lines for different incubation times using flow
cytometry and confocal microscopy analysis have been presented. Cellular uptake of
these polysterene microspheres has been found to be concentration, time and size-
dependent. Additionally, thanks to their stability in organic solvents provided by the
cross-linking structure; numerous chemical reactions have been carried out easily on
these materials and the general standard solid-phase multistep protocols which are
applied to microspheres have been briefly introduced.
It has been demonstrated that microspheres provide a reliable way to perform cellular
delivery and long-term cell monitoring and encourage the possibility of the use of
this approach for alternative applications, such as monitoring of enzymatic activity or
for the delivery of therapeutic macromolecules (pDNA, siRNA, etc.) and small
molecules such as drugs.
CHAPTER 3: *Inhibition of neurodegeneration mediated by microspheres*

**3.1-Introduction**

Nitric oxide (NO) plays a key role in nervous, immune and cardiovascular function. The neurotransmitter function of NO is dependent on dynamic regulation of its biosynthetic enzyme, nitric oxide synthase (NOS). There are three types of NOS: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). NO biosynthesis in the brain is preferentially activated by calcium influx through N-Methyl-D-aspartate (NMDA)-type glutamate receptors and regulated by the nNOS, suggesting that there is a specific link between these receptors and nNOS. It has been reported that Postsynaptic Density Protein (PSD-95) assembles a postsynaptic protein complex containing nNOS and NMDA receptors. The activation of NMDA receptor allows the entrance of calcium and activates the nNOS by calcium/calmodulin interaction. Consequently, the levels of NO is regulated for the formation of the ternary complex between the NMDA receptor, nNOS and PSD-95 protein (Figure 3.1). The importance of this, from a physiopathological point of view, is that NO biosynthesis activation is mostly regulated by NMDA receptor activation.

*Figure 3.1:* Ternary complex formation for the biosynthesis of nitric oxide.
Over-excitation of these receptors is involved in a number of human diseases such as stroke, Alzheimer, Huntington’s disease and amyotrophic lateral sclerosis. Additionally, nNOS inhibitors selectively protect from post-ischemic brain injury. Therefore, alteration of the nNOS-NMDA receptor interaction has been established as a therapeutic target because over-excitation of NMDA receptors, such as during a brain injury ischemic process, yields neurotoxic NO levels. This might be achieved by the intracellular introduction of peptides that bind to either the PDZ domain of nNOS which mediate nNOS-PSD-95 protein binding or the PDZ domain from PSD-95 protein which binds to NMDAR. Targeting PSD-95 protein represents a therapeutic approach for diseases that involve excitotoxicity and ischemic brain damage. However recently research has been focussed in the interaction of the PDZ domain of nNOS with PSD-95 because it has been predicted that the inhibition of this interaction will be less detrimental to other cell functions regulated by protein-protein interaction to PSD-95 and the probability of causing side effects will be reduced.

3.2-Neuropeptides design

Cell-permeable small organic modulators of protein-protein interactions are highly desirable tools both for the study of physiological cellular processes and for the treatment of numerous diseased states. Developing small molecules which modulate protein-protein interactions is difficult, where one of the biggest, if not the major issue, is lack of well-defined binding pockets. Historically, large peptides and natural products have been considered the main source of compounds capable of modulating protein-protein interactions.

3.2.1 Design background

It has been established that inhibitors of protein-protein interactions mediated by the nNOS PDZ domain could behave as neuroprotective drugs. An X-Ray crystallography study of nNOS has established that specific interaction of a peptide with the PDZ domain is largely determined by the final four residues of the peptide. Previous studies in the area have confirmed the selectivity of the PDZ domain from nNOS to different peptides. In this sense, a research study involving
thirteen billion peptides modified at the C-termini deduced the specific common sequence that binds to the PDZ domain from nNOS. Binding peptides all contain a common terminating sequence at the C-terminus: D-X-V-COOH. Also, it has been established that PDZ domain functionality requires additional amino acids apart from those previously mentioned, suggesting that peptides-PDZ or PDZ-PDZ interactions arise from a similar three-dimensional structure. Consequently the peptide VSPDFGDAV has been described as a potent ligand of the nNOS PDZ domain.

3.2.2 General synthesis

The synthesis and characterisation of the neuropeptides, both nona- and hexapeptides, was performed by Dr. Perez-Lopez from Gomez-Vidal’s research group at the Organic and Medicinal Chemistry department of the University of Granada in Spain.

A convergent synthetic route based on Fmoc Solid Phase Peptide Synthesis (SPPS) was carried out to obtain the nonapeptide VSPDFGDAV. Standard coupling conditions and reagents were used (HATU, HOAt and DIPEA in NMP at room temperature for 2 hours with 2-chlorotrityl chloride resin as solid support. An improved synthetic strategy was developed whereby the hexapeptide sequence DFGDAV was synthesised as before, and coupled to the tripeptide (VSP), providing the desired nonapeptide. This strategy was a clear improvement in synthetic efficiency, avoiding the incorporation of a second Hmb protecting group and resulted in improved nonapeptide purity.

3.2.2.1 Synthesis of hexapeptide segment: DFGDAV

The methodology was optimised for the synthesis of this segment, incorporating a Hmb protecting group in the Ala residue using HATU/HOAt as peptide coupling reagents (Scheme 3.1).
Reagents and conditions: (i) Fmoc-Val-OH, DIPEA, DCM; (ii) 20% Piperidine/DMF; (iii) Fmoc-Ala-OH x 2, HATU, HOAt, DIPEA, NMP; (iv) 20% Piperidine/DMF; (v) AcOH (0.2%), anh DMF; (vi) NaBH₄ (0.05M), anh MeOH/anh DMF; (vii) DIC, HOAt; (viii) Fmoc-Asp(OtBu)-OH, DCM/DMA 1:1; (ix) 20% Piperidine/DMF; (x) Fmoc-Gly-OH x 2, HATU, HOAt, DIPEA, NMP; (xi) 20% Piperidine/DMF; (xii) Fmoc-Phe-OH, HATU, DIPEA, NMP; (xiii) 20% Piperidine/DMF; (xiv) Fmoc-Asp(OtBu)-OH, HATU, DIPEA, NMP; (xv) 20% Piperidine/DMF; (xvi) TFA, TES, DCM.

**Scheme 3.1:** Solid phase synthesis of hexapeptide fragment.

**3.2.2.2 Synthesis of hexapeptide derivatives**

Research carried out by Cull et al found that the common peptide binding motif, D-D-X-V-COOH, was required for binding to nNOS PDZ domains. Based on this fact, several hexapeptide derivatives were synthesised where the amino acid Phe was replaced by different Phe derivatives due to Phe being the only Aminoacid on the sequence with aromatic properties, aromaticity is important in the peptide sequence due to their interactions with the lipophilic part of proteins. Also, aromatic
molecules typically display enhanced chemical stability, compared to similar non-aromatic molecules (Table 3.1).

<table>
<thead>
<tr>
<th>R</th>
<th>APL-HP 1</th>
<th>APL-HP 5</th>
</tr>
</thead>
<tbody>
<tr>
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<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
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<td>APL-HP 6</td>
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<tr>
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<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
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<td>APL-HP 8</td>
</tr>
<tr>
<td>NH</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>F</td>
<td>APL-HP 4</td>
<td>APL-HP 9</td>
</tr>
<tr>
<td>NH</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Table 3.1:** Serie of hexapeptide derivatives obtained by modification of phenylalanine residue on the hexapeptide DFGDAV.

3.2.2.3 *Synthesis of the tripeptide segment: VSP*

The synthesis of the tripeptide segment was achieved by using the previous coupling conditions (HATU/HOAt) (Scheme 3.2).
**Scheme 3.2:** Solid phase synthesis of tripeptide segment

3.2.2.4 *Convergent synthesis between tripeptide and hexapeptide*

Coupling of tripeptide segment (3.3) to hexapeptide-containing resin (3.1) was carried out using HATU/DIPEA in NMP (Scheme 3.3).

Reagents and conditions: i) Fmoc-Pro-OH, DIPEA, dry DCM; ii) 20% Piperidine/DMF; iii) Fmoc-Ser-OH, HATU, HOAt, DIPEA, NMP; iv) 20% Piperidine/DMF; v) Fmoc-Val-OH, HATU, HOAt, DIPEA, NMP; vi) 0.5% TFA, TES, DCM.
Scheme 3.3: Convergent synthesis between hexapeptide fragment (3.1) and tripeptide segment (3.3).

3.3-Microspheres cellular uptake studies on neuroblastoma cells

The physicochemical features of peptides make them poor drugs due to compromised bioavailability and pharmacokinetics. The hydrophilic nature of peptides impedes their passage across the blood brain barrier, which makes any action over pharmacological targets very difficult. Also, the character of these peptides makes them unlikely to cross the cell membrane. A strategy based on conjugating these potential drugs into carrier systems might be an answer to overcoming these problems. To assess the biological activity of these peptides, in vitro studies were carried out using neuroblastoma cells (SH-SY5Y) as a model. The intracellular activity of these non-permeable peptides was tested thanks to the use of in-house synthesised polystyrene microspheres as delivery vehicles.
The first step in this project was to determine whether in-house synthesised microspheres could be taken up by neuroblastoma cells. Consequently, primary studies were focused on evaluate if fluorescein labeled microspheres of different sizes could be internalised by SH-SY5Y cells. Cellular uptake of 2.15-2.17 (see Section 2.3.5.3) was analysed by flow cytometry and represented in terms of fluorescence intensity (Figure 3.2).

![Figure 3.2: Flow cytometry: A) Mean fluorescence intensity (MFI) of SH-SY5Y cells beadfected with 200 nm (2.17), 500nm (2.15) and 2µm (2.16) fluorescein labelled beads at two different concentrations. B) Histogram representation of Untreated cells (black line), cells treated with 500nm beads (2.15) at Concentration 1= 43 µg/ml (light green line) and at Concentration 2 = 86 µg/ml (dark green line). The incubation time was 12 hours.]

These results showed that cellular uptake of the microspheres was effective in all cases. It was found that cellular uptake was concentration and size-dependent. Concentration was found to have a positive effect on cellular uptake, but the most important influence observed was particle size: better uptake was demonstrated with smaller particles. These results match previous results obtained using other cell lines (see section 2.3.5.3). However, the fluorescence intensity obtained from 200 nm microspheres (2.17) labelled cells was extremely high and it need to be considered that this level of uptake could be causing toxicity due to cell overloading.

### 3.3.1 Toxicity studies

Cell viability studies were carried out to evaluate the effect of cellular uptake of different sizes of microspheres in neuroblastoma cells. The cytotoxicity of 200 nm, 500 nm and 2 µm polystyrene microspheres was tested by MTT ((3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) assay which is a quantitative colorimetric method that determines cell viability.\(^{155}\) It is based on the metabolism of
the “succinate-tetrazolium reductase” system. The latter belongs to the respiratory chain of the mitochondria and is only active in metabolically intact cells, which cleave the yellow MTT to its corresponding formazan (Scheme 3.4). Formazan absorbance can be measured spectrophotometrically at 570 nm directly from 96-well plates where its amount is proportional to the number of viable cells.

Scheme 3.4: Reduction of the tetrazolium salt in living cells.

**Figure 3.3** shows the result for SH-SY5Y cells treated with several sizes of unlabelled microspheres (2.6-2.8) and fluorescein-loaded microspheres (2.15-2.17) at two different concentrations. After 24 hours, microspheres were found to be non-toxic at the concentrations tested (compared to untreated cells, taken to be 100% viable), reinforcing the use of these polymeric particles as delivery agents. Entry of microspheres into neuroblastoma cells did not appear to reduce cell
viability.

**Figure 3.3: Cell viability study:** Cytotoxicity of microspheres in SH-SY5Y cells measured by MTT assay. Viability was expressed respect to the percentage of untreated cells (100 %). Each bar represents the mean ± SD). C1= 43 µg/ml and C2 = 86 µg/ml. Unlabelled microspheres (2.6, 2.7 and 2.8) and fluorescein-loaded microspheres (2.15, 2.16 and 2.17) were tested. The errors bar represents the standard error of the average. n=6. Statistical studies performed by Dunnett's test (α=0.05) showed that 2.15 C1, 2.15 C2 and 2.7 C2 were statistically significantly different to Untreated.

### 3.4-Optimisation of neurodegeneration protocol: Looking for neurotoxicity on neuroblastoma cells.

The next step in this project was to optimise the protocol to induce neurotoxicity on neuroblastoma cells and, consequently, to cause neurodegeneration in order to test the efficiency of these peptides as potential neuroprotective agents in neuronal cells. Following a previously reported protocol, a number of MTT assays were carried out in SH-SY5Y cells, to determine the optimal concentration of NMDA and glycine needed to induce neurotoxicity. As can be seen in **Figure 3.4** better results were generally obtained when low concentrations of NMDA were used. However, it can be observed that the use of low concentration of NMDA require the use of higher amount of glycine. This can be explained by the fact that glycine binds to NMDA receptor in a different place than NMDA ligand, and therefore, its role is to boost the neurotoxic effect. The best results were obtained with cells which had been treated with 0.1 µM NMDA and 1mM glycine for 3 hours.
Knowing the optimal cytotoxic cocktail and incubation time, different neuropeptide-loaded microspheres were prepared in order to test their effect as potential neuroprotective agents on neuroblastoma cells.

**3.5-Microspheres preparation**

As previously stated in chapter 2, microspheres are bio-compatible polymeric particles of defined sizes that can be employed in solid phase multi-step synthesis and taken into different cells rapidly. In order to evaluate the influence of the nature of the spacer and type of linker, neuropeptides (hexa and nonapeptide) containing either aminohexanoic or PEG units as spacers were conjugated to 500 nm polystyrene beads, previously modified with either cleavable or non-cleavable linkers.
3.5.1 Neuropeptides chemical development

Neuropeptides, while still on solid support, were modified firstly with spacer units such as aminohexanoic acid and PEG and then with Fmoc-Lys(Dde)-OH which allowed the labelling of neuropeptides with 5(6)-carboxyfluorescein through the amino side chain, while still keeping the α-amino group for further coupling to microspheres (Scheme 3.5).

Scheme 3.5: Spacer coupling and labelling of neuropeptides

3.5.2 Spacers and linkers coupling into microspheres

Regarding to microspheres, 4 different samples starting from 500 nm polystyrene microspheres (2.6) were prepared where either aminohexanoic (Ahx) or polyethyleneglycol (PEG) spacers were coupled to study the influence of the type of
spacer used on accessibility and interaction of the neuropeptides with the PDZ domain and, consequently, on the neuroprotective activity. Also, two different types of linkers were used (cleavable and non-cleavable) in order to investigate whether hydrolysis of a disulfide bond and subsequent release of neuropeptide into the cytoplasm had any effect on neuropeptides activity (Scheme 3.6).

![Scheme 3.6](image)

**Scheme 3.6:** Spacer and linker couplings into 500 nm polystyrene microspheres following by microsphere-neuropeptide conjugation.

3.5.3 Neuropeptide-microsphere conjugation

All conjugations between neuropeptides and microspheres were based on simple amide bond formation (an example of the general coupling is showed in Scheme 3.6). Following this strategy, fourteen neuropeptides were obtained, of which eight were derived from the natural hexapeptide. These fourteen peptides were coupled onto four different microsphere samples to finally obtain a 56-membered library of peptide-microsphere conjugates (Table 3.2), which were then delivered into neuroblastoma cells in order to test their activity as potential neuroprotectors.
3.6-Study of biological activity of the library of peptides-microsphere conjugates

Once the efficiency of microspheres as a carrier system for neuroblastoma cells was proven (see section 3.3), the next step was to assess the biological activity, by evaluation of the neuroprotective and cytotoxic activities, of the above synthesised library of peptide-microspheres conjugates (Table 3.2).

### 3.6.1 Neuroprotective studies

SH-SY5Y cells were grown 24 hours before addition of the library of peptide-microsphere conjugates at two different concentrations. After 24 hours of beadfection to allow a uniform and efficient cellular uptake of the microspheres, the neurotoxic cocktail was added to all samples for a period of 3 hours. After removal of the cocktail, cells were allowed to recover from the shocked environment. Their

![Table 3.2: Library of peptide-microsphere conjugates](image)
neuroprotective capacity was evaluated following recovery time (24 hours) by MTT assay (Figure 3.5).

![Graph](image)

**Figure 3.5: MTT assay**: Selected samples from the 56 samples library of microsphere-neuropeptide conjugates. MU= Unlabelled microspheres; MF= Fluorescent microspheres; N= Naked Nonapeptide; H= Naked Hexapeptide; DMSO= DMSO solution. The error bars represent the standard error of the average. n=6. Statistical studies performed by Dunnett’s test (α=0.05) showed that all the samples were statistically significantly different to Untreated.

As can be seen in **Figure 3.5**, some neuroprotection (30%) was achieved using a concentration of 1.24 µM hexapeptide with a non-cleavable linker (ANHP). However, at the same concentration the nonapeptide only showed activity when conjugated to microspheres via a cleavable linker (ACNA) that will allow the release of the nonapeptide in the cytoplasm. The absence of affinity data to the PDZ domain from nNOS for these peptides-microspheres conjugates makes quite difficult to explain the fact that hexapeptide and nonapeptide have different requirements to exhibit neuroprotective activity. It can be hypothesised that when a non-cleavable linker is present in the conjugate, the nonapetide stay attached to the microsphere and, consequently, it is highly localised and this will result in a high concentration in the cytoplasm of this nonapeptide and this concentration can be excessive for neuroprotection and become cytotoxic. In other hand, the optimal concentration of hexapeptide to display neuroprotective effect is higher than for the nonapeptide and
when a cleavable linker is used for conjugation, the hexapeptide get diluted in the cytoplasm and the resulting concentration is to low to display any significant neuroprotective effect.

Whilst moderately successful, it was thought that these experimental results could be noticeable improved if a higher ratio of cell death could be achieved using NMDA/Glycine cocktail. In order to optimise the neurotoxic cocktail SH-SY5Y cells were treated with NMDA/Glycine concentrations for longer periods of time (3-5 hours), also these neurotoxic cocktails were applied to cells where cell culture medium had not been removed in order to test the effect on cell viability (Figure 3.6).

![Figure 3.6](image.png)

**Figure 3.6: MTT assay:** Optimisation of treatment with NMDA/Glycine to induce neurotoxicity in neuroblastoma SH-SY5Y cells. Errors bar representing the standard deviation. n=9.

*Figure 3.6* shows that a treatment with 0.1 µM NMDA and 1 mM Glycine for a longer period of time (5 hours) effectively decreased cell viability. It can be observed that the addition of this cocktail in the presence of medium affect detrimentally its neurotoxic effect. These results show that an increase of the incubation time and removal of medium before addition of the cocktail can improve its efficiency.

Based on previous results we focused on the most successful samples so far (ANHP and ACNA) and their efficiency as neuroprotective agents was evaluated under these
optimised stressed cell-environment conditions at different concentrations (Figure 3.7).

**Figure 3.7: MTT assay:** Evaluation of neuroprotective activity of peptide-microsphere conjugates in neuroblastoma cells. The error bars represent the standard error of the average. n=6. Statistical studies performed by Dunnett's test (α=0.05) showed that samples ANHP C1, ANHP C2 and ACNA C1 were statistically significantly different to ACNA C2.

It was observed that the hexapeptide-microsphere conjugate (ANHP) at a concentration of 1.27 µM generates neuroprotection (44%). However, it seems that nonapeptide-microsphere conjugate (ACNA) at the same concentration shows a slightly lower activity (34%). As previously suggested, a significant influence of the linker choice can be observed. Future work could be focussed in the optimisation of the amount of nonapeptide to be coupled to the microspheres. It can be expected that lower loading of nonapeptide to the microsphere via a non-cleavable linker (ANNA) will allow tuning the optimal concentration to display neuroprotective effect.

### 3.6.2 Cytotoxicity studies

As seen in Figures 3.5 and 3.7 it seemed that rather than having a positive effect in terms of neuroprotection, increasing neuropeptide concentration actually had a detrimental effect on their neuroprotective activity. On the other hand, it has been proved that nNOS can be implicated in the regulation of genes related to cell
grow and differentiation. In order to study the cytotoxic effect of these conjugates, a further series of experiments were carried out using concentrations ranging from 2.8-13 µM to calculate the half maximal inhibitory concentration (IC$_{50}$) of these conjugates on neuroblastoma cells (SH-SY5Y) (see Figure 3.8/A). Additionally, in order to evaluate the relationship between the cytotoxic effect and the interaction with PDZ domain from nNOs, the IC$_{50}$ was calculated in a cell line that do not produce nNOS such as melanoma cells (ARN8) (see Figure 3.8/B). These results show that the IC$_{50}$ value in SH-SY5Y is lower than in ARN8 (Figure 3.8/C). These results suggest that there is a specific interaction of these conjugates with nNOS and this interaction is responsible for the cytotoxic effect of these conjugates at high concentration. This effect has been previously reported for the interaction of nNOS to CtBP.

![Figure 3.8](image.png)

Figure 3.8: IC$_{50}$ for ANHP and ACNA conjugates on SH-SY5Y cells (A) and ARN8 cells (B). (C) Values table for the IC$_{50}$.

### 3.6.3 Microscopy studies

The last step in this project was a microscopy study to explore whether polystyrene beads containing fluorescent neuropeptides could be efficiently taken up by neuroblastoma cells. SH-SY5Y cells were beadfected with ACNA and ANHP conjugates. As a representative example, Figure 3.9 shows a confocal image of SH-SY5Y cells after incubation with ANHP.
Figure 3.9: Confocal microscopy analysis of SH-SY5Y cells after 24 h incubation with fluorescein-labelled neuropeptide-microsphere conjugate ANHP. Concentration= 1.24 µM. Cytoplasm and nucleus were labelled with Cell Tracker Red (Invitrogen) and Hoestch 33342 (Sigma-aldrich) respectively.

3.7-Conclusions

To conclude can be said that to date, no work had been previously reported where these specific peptide ligands of the nNOS PDZ domain (nonapeptide and hexapeptide) had been chemically synthesised and evaluated as neuroprotective drugs inside neuronal cells. The impact of this research to the scientific community is twofold, firstly a general convergent strategy for the synthesis of a family of ligands of the nNOS PDZ domain using an efficient solid phase approach has been described and secondly microspheres have been chosen as delivery system as they are biocompatible polymeric particles that were taken by different neuronal cells rapidly and they have been successfully used to deliver these non-permeable peptides into neuronal cells. The result showed that hexapeptide and nonapeptide conjugated to microspheres displayed neuroprotective effects at low concentrations and cytotoxic effects at high concentration in neuroblastoma cells. It has been determined that the nonapeptide is bound tighter than the hexapeptide to the PDZ domain. These results offer a huge range of potential application of this methodology for the preparation of libraries of analogues of these peptides to be evaluated in vitro and in vivo as novel ligands of PDZ domain and consequently open a door to find new therapies to combat severe neurodegenerative diseases such as Parkinson and Alzheimer. Additionally, cell viability results confirmed that the microspheres themselves did
not significantly affect cell viability at the concentrations analysed demonstrating that such micro-beads are efficient biocompatible cellular delivery devices in neurons.
CHAPTER 4: Microsphere-mediated siRNA delivery strategies for gene silencing

4.1-Introduction

Since Fire and Mello discovered the ability of double-stranded oligonucleotides to silence gene expression in 1998\textsuperscript{160} gene delivery has become a research area of huge interest due to its possible clinical applications to treat or to prevent many diseases related to gene expression.\textsuperscript{161} Since then billions of dollars have been invested in the therapeutic application of gene silencing in humans, where the first clinical applications of RNAi have been directed at the treatment of wet, age-related macular degeneration (AMD)\textsuperscript{162, 163} and respiratory syncytial virus (RSV) infection.\textsuperscript{164} However, the widespread use of RNAi therapeutics for disease prevention and treatment requires the development of clinically suitable, safe and effective drug delivery vehicles.

4.1.1 Comparison of gene silencing approaches

A number of different types of devices that act by silencing gene expression by sequence-specific targeting of mRNAs have been developed in the hope to creating therapeutic agents.\textsuperscript{165} Some of these approaches are:

\textit{Antisense Oligonucleotides (ODNs):} A short sequence of DNA (~20 nucleotides in length) that act by hybridising to pre-mRNA and mRNA to produce a substrate for ribonuclease H (RNase H), which specifically degrades the RNA strand of RNA-DNA duplexes.\textsuperscript{166}

\textit{Ribozymes:} RNA molecules that act as enzymes that bind to RNA through Watson-Crick base pairing which hydrolyse the phosphodiester backbone and subsequently degradation of the target RNA.\textsuperscript{167}

\textit{DNAzymes:} Small DNAs capable of site specifically cleaving RNA targets via Watson and Crick base pairing binding, resulting in 2’,3’-cyclic phosphate and 5’-OH termini.\textsuperscript{168}

\textit{miRNA:} Small, non-coding RNAs that regulate gene expression in a sequence-specific manner. Once inside a cell, single-stranded miRNA binds through partial
sequence homology to the 3′ untranslated region (3′ UTR) of target mRNAs, and causes either block of translation or, less frequently, mRNA degradation.\textsuperscript{169}

\textit{siRNA}: Derived from the cytoplasmic processing of long dsRNA by the RNase-III type enzyme termed Dicer.

### 4.1.2 The mechanism of RNA interference

RNAi pathway is a fundamental process in eukaryotic cells where sequence-complementary siRNA is able to recognise and cleave a target mRNA.\textsuperscript{170} RNAi pathway is triggered by the presence of long fragments of double-stranded RNA (dsRNA), which are cleaved into the fragments known as siRNA (21-23 nucleotides long) by a RNAase enzyme known as Dicer.\textsuperscript{171} The process starts with the introduction of dsRNA into the cytoplasm by a physical or chemical mechanism. This dsRNA will be processed by a complex consisting of Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT) into small interfering RNAs (siRNAs), which are loaded into RNA-Induced silencing complex (RISC). Argonaute 2 (AGO2), a multifunctional protein contained within RISC unwinds the siRNA, after which the sense strand (or passenger strand) of the siRNA is cleaved.\textsuperscript{172} The activated RISC, which contains the antisense strand (or guide strand) of the siRNA, localises and recognises target sites to direct mRNA cleavage complementary to the guide strand, which is carried out by the catalytic domain of AGO2 (Figure 4.1). The cleavage of the mRNA happens in a position between nucleotides 10 and 11 on the complementary antisense strand, relative to the 5′-end.\textsuperscript{173}
Figure 4.1: RNAi pathway. Dicer cleaves double stranded RNA to form small interfering RNA or microRNA. These processed RNAs are incorporated into the RNA-induced silencing complex (RISC), which targets messenger RNA to prevent translation.

4.1.3 Synthetic materials for siRNA delivery

One of the main issues for oligonucleotides-based therapeutics involves effective intracellular delivery of the active molecules so that oligonucleotides do not permeate intact cell membranes to any significant degree via simple diffusion, primarily because of the hydrophobic nature of the membrane lipid bilayer. To overcome this barrier, synthetic materials have demonstrated potential as effective non-viral siRNA delivery carriers.\textsuperscript{174}
4.1.3.1 Liposomes and lipid-like materials

Liposomes have been used for the delivery of nucleic acids for over 20 years. In a recent study for example, a stable nucleic acid-lipid particle (SNALP) was used to formulate SNALP containing a siRNA which target the polymerase gene of the Ebola virus.\textsuperscript{175}

4.1.3.2 CPPs and CTLs

Cell penetrating peptides (CPPs) and Cell targeting ligands (CTLs) have been used to attain cell-type selective targeting or to enhance the uptake of oligonucleotides. In 2004, Muratovska \textit{et al} formulated a disulphide-linked CPP-siRNA which was complementary to a region for the firefly luciferase or GFP transgene, suppressing their expression, the coupling to the CPP increased the cellular uptake of siRNA dramatically.\textsuperscript{176} On the other hand, an aptamer-siRNA chimera targeting prostate-specific membrane antigen (PSMA) was able to effectively deliver the associated siRNA to LNCaP prostate cancer cells, resulting in cell death.\textsuperscript{177}

4.1.3.3 Polymeric nanocarriers

Nanotechnology, in the form of efficient delivery vehicles, plays a key role in the area of gene trasfection. Cationic polymeric particles including PEGylated polycations,\textsuperscript{178} polyethyleneamine (PEI) complexes,\textsuperscript{179} cationic block co-polymers\textsuperscript{180} and dendrimers\textsuperscript{181} have been used over the last few years to deliver antisense and siRNA oligonucleotides. Another widespread approach for oligonucleotide delivery is the use of polymeric nanoparticles,\textsuperscript{182} polymer micelles,\textsuperscript{183} quantum dots\textsuperscript{184} and lipoplexes.\textsuperscript{185} It is important to recall that carrier systems themselves can have significant effects on gene expression, and may cause toxicity. Choosing an appropriate delivery vehicle is therefore a key factor in gene silencing assays and will likely depend on the therapeutic context.

4.2-Polystyrene Microspheres as siRNA delivery vehicles

As reported in \textit{chapter 2} polymeric amino functionalised microspheres have shown high uptake efficiency into a variety of cell lines including adherent,
suspension and primary cells. Additionally, these nanoparticles have been successfully used for the delivery of a broad range of biological cargoes such as sensors and proteins. These devices have been found to be non-cytotoxic and easily functionalised. While the mechanism of uptake is now completely known, it has been suggested that microspheres go into cells following an endocytosis-independent uptake mechanism that results in the cytoplasmic localisation of microspheres. In addition, these particles are released in the cytoplasm without using an endosome-disrupting agent. All these properties make microspheres a promising candidate for siRNA delivery which requires a cytoplasmic location to be efficient. A chemical strategy has been recently developed in our group for the conjugation of these microspheres to amino functionalised siRNA targeted against EGFP expressed in human cervical cancer (HeLa) cells. These conjugates have shown to efficiently silence protein expression over 72 hours, without detrimental cytotoxicity.

4.2.1 Knocking down on stem cells

Gene silencing on embryonic stem (ES) cells is a powerful method to study mammalian genetics; including clarifying the role of key factors involved in stem cell maintenance and fate decisions such as Oct-3/4, a master regulator of self-renewal and pluripotency. The major obstacle for effective gene silencing in general is the poor transfection efficiency of interference RNA. This problem become more problematic in embryonic stem cells where the conventional cellular delivery strategies for nucleic acids, such as electroporation and microinjection, retroviral transduction and cationic liposomes are often characterised by high cell toxicity and low efficiency. Reports of stem cell transfection have ranged from less than 1-20% of cells, which may not allow for efficient knockdown of the gene of interest. Previous work shows that in-house synthesised polystyrene beads can be effectively taken up by mESC and human and mouse NSCs without affecting ES pluripotency. Knocking down of EGFP in ES cell line that expresses a membrane-specific farnesylated version of EGFP (aEGFP) mediated by microspheres has been recently achieved in our group. These results show that microspheres are a promising candidate for silence significant proteins related to self-renewal and pluripotency in stem cells.
4.2.1.1 Embryonic Stem cells

ES cells are cell lines derived from the Inner Cell Mass (ICM) of the mammalian blastocyst. They are capable of dividing indefinitely and able to differentiate into all the lineages of the fetus and adult.191-193

![Diagram of the embryonic stem cell development process](image)

**Figure 4.2: ES cells.** Undifferentiated cells from the embryo that have the potential to become a variety of specialised cell types.

4.2.1.2 Beads preparation

In order to prepare the beads for ES cell beadfection and further gene silencing assay an Fmoc solid phase protocol as described in chapter 2 (Scheme 2.11) was followed. Following the protocol, 500 nm polystyrene beads (2.6) were
pegylated using a PEG spacer, bifunctionalised by Fmoc-Lys(Dde)-OH and pegylated further using PEG to yield the “main bead construct” (4.2.1.1). Beads were then labelled with different dyes such as 5(6)-carboxyfluorescein (FAM), 5(6)-carboxytetraethylrhodamine (RHO), Dabsyl chloride (DC), Texas Red (TR) or Cyanine 5 (Cy5), this labelling allowed us to track and localise the beads once inside cells. The last step was the carboxyfunctionalisation of the beads by adipic acid in order to have an end carboxylic group which will form a covalent bond with the amino-siRNA (Scheme 4.1).

![Scheme 4.1: Preparation of Dual-Labeled 500 nm Dye-co-siRNA.](image)

**Frozen beads**

While coupling siRNA into beads is an easy process, we thought that would be very desirable to be able to freeze these devices following siRNA coupling onto the beads, in order to increase their shelf-life for subsequent experiments. After being defrosted they were added to cells and the transportation ability of the polystyrene
beads tested, looking at the ability of siRNA to silence a target gene. It is important to note that the freezing process cannot be carried out in pure water as this water would result in the formation of ice crystals which would then form hydrogen bonds inside the polystyrene structure of our beads damaging their network and affecting their ability to translocate the cellular membrane (see Figure 4.3). Polystyrene beads were thus frozen using different dilutions of dimethylsulfoxide (DMSO) in water and their capability to deliver siRNA inside cells and consequently silence their target gene was tested. The reason behind the use of a DMSO solution in water is that DMSO avoids the form of large ice crystals, maintaining the volume and protecting the polystyrene network.

![Figure 4.3: Scanning Electron Microscopy. Beads after being frozen in -80°C freezer for at least 48 hours in pure water where aggregation has occurred resulting in bead clusters (left) and 10% DMSO in water where beads remain spherical and ordered (right).](image)

SEM images (Figure 4.3) proved that a 10% DMSO solution in water is capable to freeze microspheres without affecting their shape.

4.2.1.3 Cellular uptake studies

This work was carried out in collaboration with Dr. Brickman’s group at the Institute of Stem Cell Research (ISCR) and MRC Centre for Regenerative Medicine of the University of Edinburgh.

The first step was to test the ability of our microspheres to be taken up by ES cells. For this purpose, mouse pluripotent embryonic stem cells (ES-E14tg2a) were beadfedected with fluorescent-labelled beads (4.2.1.2-5) for 48 hours and their cellular uptake capability was analysed by flow cytometry. Additionally, the same experiment was carried out on HeLa cells in order to compare the cellular uptake ratios between ES and adherent cells.
As can be seen in Figure 4.4 the ES cellular uptake is not as high as cellular uptake in HeLa cells. Despite ES cellular uptake being slightly inconsistent, it can be clearly seen how generally the ES cellular uptake is improved following freezing, increasing uptake by about 20% for beads frozen in 5% DMSO/H$_2$O solution and around 25% for beads frozen in 10% DMSO/H$_2$O solution in comparison with untreated beads. An explanation for this could be that after being frozen, beads surface is more regular and smooth, facilitating translocation across the cellular membrane.

### 4.2.1.4 Gene silencing assay

Following the successful beadfection into ES cells using beads that had been previously frozen and defrosted, the next step was the beadfection of an ES cell line which continuously expressed GFP under the control of mouse Oct4 gene regulatory elements (Oct4-DGip). GFP expression in this cell line correlates with the ES cell identity; consequently, the self-renewal and differentiation status of the cells can be monitored. Oct4-DGip is a mouse ES cell line that contains a GFP transgene driven by Pou5f1 (Oct3/4) promoter. In self-renewing ES cells Oct3/4 and Sox2 transcription factors bind Pou5f1 promoter and activate the transcription of Oct3/4 and of the GFP reporter. Upon Oct3/4 silencing the transcription of the factor itself and its reporter are reduced and, as a result, differentiation of the Oct4-DGip cells is induced. Therefore we use the loss of GFP fluorescence as a measure of silencing efficiency. With that in mind, Oct4-DGip cells were incubated with microspheres coupled to siRNA against Oct3/4 or Sox2 (Scheme 4.1/A) and analysed for GFP expression by flow cytometry and confocal microscopy.
**Flow cytometry studies**

Thus, Oct4-GiP cells were beadfected with a range of siRNA-microsphere conjugates. Incubations were run over 24 – 72 hours with a microsphere concentration of 86 µg/mL. The silencing efficiency of these constructs were analysed by flow cytometry (Figure 4.5).

![Flow cytometry results](image)

**Figure 4.5: Silencing efficiency results obtained by Flow cytometry:** % of cell population showing a GFP+ activity, expressing the GFP (dark colored columns) or a GFP- activity, complete knocking down of the GFP (light colored columns) incubated for 24 (A), 48 (B) or 72 hours (C). Untreated Oct4-GiP cells and Lipofectamine-siRNAs were used as a positive control while untreated E14tg2a cells, naked Lipofectamine, naked Oct3/4 siRNA, naked Sox-2 siRNA, naked Scramble siRNA, Lipofectamine-scramble siRNA, naked beads (2.6), beads without siRNA (4.2.1.2 and 4.2.1.6) and beads with scramble siRNA (4.2.1.9 and 4.2.1.10) were used as negative controls. The errors bar representing the standard error of the average. n=6. Statistical studies performed by Dunnett’s test (α=0.05) showed that all the samples in 24 h and 48 h studies were statistically significantly different to LipSox2.

Silencing of GFP in Oct4-GiP cells was achieved after 24 hours using Cy5-labelled beads loaded with Oct3/4 siRNA (4.2.1.7) with a silencing efficiency of 14%. This silencing efficiency seems to improve up till 44% after 72 hours of beads co-
cultivation with cells. However, an opposite tendency was detected when beadfection was carried out with Sox2 loaded microspheres (4.2.1.8) which silence 38% of cells after 24 hours, this silencing efficiency appearing to decrease till 25% after 72 hours co-cultivation. These values look quite promising when compared with a commercial transfection agent as Lipofectamine 2000 which silencing efficiency is about 30% over all time courses studied. Interestingly, when microspheres loaded with Sox2 (4.2.1.8) and Oct3/4 (4.2.1.7) were co-cultivated with cells simultaneously the silencing efficiency seems to be stable around 30%. The same experiment was repeated with beads which had been frozen at -80°C in a 10% DMSO/H2O solution. The obtained results by flow cytometry are represented in Figure 4.6. It can be observed that the silencing efficiency is not significantly affected by the frozen process. These results are pretty similar to the ones obtained with unfrozen siRNA-microsphere conjugates (represented in Figure 4.5). All these results were repeated by Dr Livigni who obtained similar results.
Figure 4.6: Silencing efficiency results obtained by Flow cytometry. Samples are the same as figure 4.5 but all the microsphere samples were frozen in 10% DMSO/H$_2$O solution. Error bar representing the standard deviation. n=6. Statistical studies performed by Dunnett’s test ($\alpha=0.05$) showed that all the samples in 24 h and 48 h studies were statistically significantly different to LipSox2.

As said previously, along with other properties ES cells can choose to become any type of cell present in the body by adopting different morphologies according to the cell type being adopted. As detailed in chapter 2 cells which are phenotypically different from each other can be detected and sorted or separated if required by FACS. Based on this, cells which had been beadfected and the expression of GFP suppressed, start a differentiation process which can be easily detected by flow cytometry. In Figure 4.7 a migration from population 1 (intact or untreated cells; in black line) to population 2 (cells which GFP has been knocked down and consequently adopting different morphology; shown in turquoise) was observed. If we analyse in detail rows one (4.7/A) and two (4.7/B) it can be observed that there is no population 2 when cell has not been beadfected and therefore no GFP silencing.
was produced and neither PE nor AP signals from population 2 were detected (4.7/B). Nevertheless when cells were beadfected with Cy5-labeled Oct3/4 siRNA microspheres (4.7/C), with RHO-colabeled Sox-2 siRNA microspheres (4.7/D) or both samples at the same time (4.7/E) different signals from population 2 (turquoise line) in AP filter, PE filter and in both filters respectively can be detected. Moreover, in the three beadfected samples (4.7/C, D and E) a significant reduction in the GFP intensity from population 2 can be observed demonstrating that gene silencing was successful only in cells that had been taken up beads and had been consequently transfected with siRNA.

**Figure 4.7: Flow cytometry. A) Untreated E14tg2a cells; B) Untreated Oct4-G iP cells; C) Oct4-G iP cells incubated with 4.2.1.7; D) Oct4-G iP cells incubated with 4.2.1.8; E) Oct4-G iP cells incubated with 4.2.1.7 and 4.2.1.8. Microsphere concentration of 86 µg/mL co-cultivated for 72 hours. The second, third and fourth columns represent the filters for FITC (for GFP silencing detection), PE (for RHO-Sox2 samples (4.2.1.8) detection) and AP (for Cy5-Oct3/4 sample (4.2.1.7) detection) respectively.**
To reinforce this theory, samples 4.7/C and 4.7/D were analysed separately with respect to cell population using Flowjo® software and either AP or PE intensity represented against FITC in order to co-localise GFP silencing and beads uptake. It can be observed that differentiated cells are simultaneously fluorescently labelled (Figure 4.8).

**Figure 4.8: Flow cytometry.** A) Oct4-GiP cells incubated with Cy5 labelled microspheres loaded with Oct3/4 siRNA (4.2.1.7); B) Oct4-GiP cells incubated with Rho labelled microspheres loaded with Sox2 siRNA (4.2.1.8); C) Dot plot representing the two populations, turquoise for population 2 (GFP- and Cy5+); D) Dot plot representing the two populations, pink for population 2 (GFP- and RHO+) and black for population 1 (GFP+ and RHO-).

**Microscopy and real-time polymerase chain reaction (RT-PCR) studies**

These studies were carried out by Dr. Alessandra Livigni from the Institute of Stem Cell Research (ISCR) at the University of Edinburgh to confirm the results obtained by flow cytometry that suggest that polystyrene beads effectively transfected siRNA in mES cells.

For this purpose a microscopy study was carried out where cells which had been incubated with Cy5 labelled microspheres loaded with Oct3/4 siRNA (4.2.1.7) and rhodamine labelled microspheres loaded with Sox2 siRNA (4.2.1.8) were observed (Figure 4.9).
Figure 4.9: Pseudoconfocal microscopy. A) Untreated Oct4-GiP cells; B) Oct4-Gip cells treated with microspheres coupled to a scramble siRNA (4.2.1.9); C) Oct4-Gip cells treated with Cy5 labelled microspheres coupled to Oct3/4 siRNA (4.2.1.7); D) Oct4-Gip cells treated with Rhodamine labelled microspheres coupled to Sox2 siRNA (4.2.1.8); E) Oct4-Gip cells treated with 4.2.1.7-8. Not frozen samples. Photographed 48 h after incubation and 48 h before sorting. Scale bar is 100 µm. Data courtesy of Dr. Alessandra Livigni from the Institute of Stem Cell Research (ISCR) at the University of Edinburgh.

It can be observed that cells incubated for 48 hours with Oct3/4 or/and Sox2 siRNA-coupled microspheres (4.2.1.7-4.2.1.8) show large areas of differentiating cells (red outline) characterised by a distinct flattened morphology (Figure 4.9/C-E), such population of cells are genetically modified to express a marker or antibody when becoming liver or lung cells. ES cells incubated with microsphere coupled to a negative control scramble siRNA (4.2.1.9) show no sign of differentiation (Figure 4.9/B) and look indistinguishable from normal ES cell cultured in self-renewing conditions (Figure 4.9/A). Based on these results, FACS sorting was performed on cultures beadfected with siRNA coupled microspheres for 48 hours. Cy5 positive and
Cy5 negative fractions were recovered for culture 4.9/C. In sample 4.9/E cells were sorted in either Cy5 or RHO positive and Cy5 or RHO negative fractions. After being sorted, the two fractions were replated and grown for a period of 48 hours (Figure 4.10).
Figure 4.10: Pseudoconfocal microscopy. Replating of sorted cells photographed 2 days after sorting. Bright field in the left, pseudocolored images in the right. In green, Oct3/4 reporter and in red Sox-2 immunostaining. A) Untreated Oct4-GiP cells; B) Cy5+ fraction
from 4.9/C; C) Cy5- fraction from 4.9/C; D) RHO+ fraction from 4.9/D; E) RHO- fraction from 4.9/D; F) Cy5+ or RHO+ fraction from 4.9/E; G) Cy5- or RHO- fraction from 4.9/E. Scale bar is 100 µm. Data courtesy of Dr. Alessandra Livigni from the Institute of Stem Cell Research (ISCR) at the University of Edinburgh.

It can be appreciated in Figure 4.10 that cultures replated from a Cy5 or RHO positive fractions (4.10/B, D and F) show reduced GFP fluorescence in comparison to the Cy5 or RHO negative fractions (4.10/C, E and G). Moreover, Sox2 immunostaining shows reduction in the number of Sox2 positive cells when both siRNA (Oct3/4 and Sox2) are delivered by beadfection (4.10/F, G).

In the next experiment in order to quantify the effect of knock-down on both the target gene and its partner (Oct3/4 and Sox-2 are known to regulate each other) cultures which were globally Cy5 positive (4.10/B) or RHO positive (4.10/D) were analysed by RT-PCR (Figure 4.11). This technique allows the amplification and simultaneous quantification of a targeted DNA after reverse transcription of the transcriptome.

![Figure 4.11: RT-PCR analysis. A) Oct3/4 expression. Cells treated with Cy5 coupled beads for 48 hours. Beads were linked to either Oct3/4 (4.2.1.7), Sox-2 (4.2.1.11) or scramble Control (4.2.1.9) siRNA; B) Sox2 expression. Cells exposed to Cy5 coupled beads for 48 hours. Beads were linked to either Oct3/4 (4.2.1.7), Sox-2 (4.2.1.11) or scramble Control (4.2.1.9). RNA was prepared from sorted fractions that did uptake labelled beads, transcript numbers were normalized to TAT binding protein (TBP). Relative expression to the control treated ES cells. Data courtesy of Dr. Alessandra Livigni from the Institute of Stem Cell Research (ISCR) at the University of Edinburgh.](image_url)

Oct3/4 transcripts were reduced by 70% with Oct3/4 siRNA beadfection and by 50% with Sox2 siRNA when compared to transcript number in cells treated with scramble control siRNA (4.11/A and B, second column). Sox 2 transcripts knock-down was of 40% and 60% with Oct3/4 and Sox2 siRNA respectively (4.11/A and B, third column).
4.2.1.5 Toxicity studies

As said in chapter 3 MTT assay is a simple and reliable method to evaluate cell viability. It is of vital importance for a transfection method do not exhibit cytotoxicity in cells. To confirm that microsphere uptake did not affect viability of ES cells a MTT assay were performed to estimate the percentage of viable cells in these cultures following treatment with a range of microspheres. Figure 4.12 shows that E14tg2a cell contained around 60% viable cells following microsphere treatment, indicating that microspheres exhibits much lower toxicity that a well-known tranfection reagent as Lipofectamine 2000 which contained around 20% viable cells.

![MTT Toxicity assay](image)

Figure 4.12: MTT assay graphic showing cell viability (%). E14tg2a cells incubated with different samples for an incubation time of 72 hours. The error bars representing the standard error of the average. n=6. Statistical studies performed by Dunnett’s test (α=0.05) showed that all the samples were statistically significantly different to Lip.

4.2.2 Thiol-mediated siRNA delivery

In the last decade, several chemical strategies have been developed for the intracellular delivery of siRNA. A relatively unexplored strategy for the delivery of siRNA from the nanocarrier is the incorporation of cleavable bonds into the functionalisation to enable controlled molecular releasing from the particle surface, thus creating “smart” delivery vehicles with useful functionality in chemical and biological settings. The efficiency of this strategy is that the delivery process is
significantly facilitated because release of biological cargos from the nanoparticle surface is achieved by triggered bond cleavage of nanoparticles complexes. Naturally occurring enzymes or cellular factors could be harnessed to cleave a chemical bond, e.g. a disulphide bond. This could be a more attractive avenue as it rarely results in undesired cytotoxicity. Glutathione is a tripeptide that is naturally present in cells as a protective agent it reduces disulphide bonds by acting as an electron donor and is itself oxidised to glutathione disulphide. Dai et al have described recently a strategy for the functionalisation of carbon nanotubes via cleavable disulfide bond for the conjugation of siRNA. However, the use of these nanotubes as delivery system require the use of a membrane disrupting agent such as chloroquine to release the siRNA from endosomes and be able to deliver it efficiently. Based on this strategy the delivery of thiol-functionalised siRNA from the microspheres was evaluated.

4.2.2.1 Synthesis of 5(6)-(N-thioethyl)tetramethylrhodaminamide

To demonstrate that a disulphide bridge could be used to facilitate the intracellular cleavage of a cargo from microspheres, the first step in this project was the synthesis of a thiol-functionalised dye which could be coupled further to our thiol-functionalised microspheres through a disulfide bond in order to obtain a fluorescent construct to be deliver into cells. Based on this system, the cellular uptake could be studied by flow cytometry, and also the fluorescence of the beads allowed us to track and study the dye release process depending on cleavable or non-cleavable linkers attached into microspheres.

With that in mind, an efficient solid phase strategy for the functionalisation of 5(6)-carboxytetramethylrhodamine was designed where in two steps the dye 5(6)-(N-thioethyl)tetramethylrhodaminamide was obtained without requiring further purification (Scheme 4.2). The first step was the reaction of cysteamine with the 2-chlorotrityl polystyrene resin (step i) via a nucleophilic substitution (S_N1) mechanism under basic conditions using a polar aprotic and resin-friendly solvent as DMF. The commercial available dye 5(6)-carboxytetramethylrhodamine was then functionalised. Following preactivation using standard solid phase coupling reagents (oxyma/DIC), the dye was reacted with the functionalized resin (step ii). After cleavage from the resin using acidic conditions and precipitation using cold diethyl
ether the desired compound $5(6)-(N\text{-thioethyl})$tetramethylrhodaminamide (4.2.2.1) was obtained in good yield (81%) and high purity (97%) without the need of further purification. The crude compound was fully characterised through $^1$H-NMR, $^{13}$C-NMR, high-resolution mass spectrometry (HRMS) and UV-visible spectroscopy (see Chapter 7. Experimental part for details).

Scheme 4.2: Route for the synthesis of $5(6)-(N\text{-thioethyl})$tetramethylrhodaminamide (4.2.2.1).

The optical properties of this rhodamine derivative 4.2.2.1 were then determined using UV-visible spectroscopy and spectrofluorimetric analysis. The obtained data of maximum absorbance for this compound are summarised in Fig. 4.13. These results confirmed that this derivative behaved in similar manner than carboxytetramethylrodhamine (TAMRA). Only a small difference of 4 nm in the emission maxima can be observed but retain its fluorescence profile.

Figure 4.13: Spectral properties of thiol funcionalized derivative 4.2.2.1 compared to carboxytetramethylrodhamine (CBR). The excitation spectra are represented in the right side.
(λ<sub>em</sub> = 572 nm) and the emission spectra in the left side (λ<sub>exc</sub> = 543 nm) of the diagram. Measurements performed in PBS (pH = 7.4).

4.2.2.2 Microspheres preparation

To be able to evaluate the efficiency of delivery of any thiol modified biomolecule to microspheres it was necessary to modified them chemically to be reactive with thiol group in the biomolecule and allow an efficient conjugation. For this purpose, a glutathione-reducible disulfide linker was introduced on microspheres by using a very well-known thiolation reagent called N-succinimidyl 3(2-pyridyldithio)propionic acid (SPDP) (4.2.2.2). Additionally, a non-cleavable maleimide linker that is non-cleavable by glutathione was added by using N-succinimidyl 3-(maleimido)propionic acid (4.2.2.3) (Figure 4.14).

![Cleavable (4.2.2.2) and non cleavable (4.2.2.3) crosslinkers.]

As said in chapters 2 and 3 our strategy consists of an Fmoc solid-phase synthesis protocol. Using this strategy, firstly spacers of a variety of different lengths and hydrophilicities, such as aminohexanoic (Ahx) or polyethylenglycol (PEG) were coupled to the microspheres. The use of some spacer units can result in enhanced biocompatibility and can aid the interaction between labelled beads and cell surfaces.116 For instance, the incorporation of PEG at the nanoparticle surface can shield the net charges of the nanoparticles, minimise non-specific cellular interaction with the nanoparticles and result in better protection of siRNA molecules.197-199

Then, the disulfide and maleimide moieties were introduced into microspheres by reacting a cleavable (4.2.2.2) and non-cleavable (4.2.2.3) crosslinkers with 500 nm microspheres (4.2.2.4 and 4.2.2.5). Preactivation wasn’t required because these linkers were already activated as N-hydroxsuccinimide esters. The resulting pyridylidthio and maleimido derivatives (4.2.2.6-4.2.2.9) were then coupled to the...
fluorescent thiolated rhodamine derivative 4.2.2.1 using standard solid phase reagents (oxyma/DIC) (Scheme 4.3) to give a range of fluorescent constructs (4.2.2.10-4.2.2.13) that will allow the evaluation of this strategy by fluorescence techniques. As a negative control, amino functionalised microspheres (4.2.2.4.-4.2.2.5.) were coupled to carboxytetramethylrhodamine.

Reagents and conditions: (i) Fmoc-Ahx spacer (5eq), Oxyma (5eq), DIC (5eq), DMF, 2 h, 60°C; (ii) Fmoc-PEG spacer (5eq), Oxyma (5eq), DIC (5eq), DMF, 2 h, 60°C; (iii) 20%piperidine, DMF, 20 min x 3 times; (iv) 4.2.2.2 (10 eq), DIPEA (15eq), DMF, o/n rt; (v) 4.2.2.3 (10 eq), DIPEA (15eq), DMF, o/n rt; (vi) 4.2.2.1, PBS buffer at pH 6.9, o/n rt; vii) 5(6)D carboxytetramethylrhodamine (1.2eq), Oxyma (1.2eq), DIC (1.2eq), DMF, o/n, rt.

Scheme 4.3: Preparation of Rhodamine labelled 500 nm microspheres with cleavable (4.2.2.10 and 4.2.2.11), non-cleavable linker (4.2.2.12 and 4.2.2.13) and without linker (4.2.2.14 and 4.2.2.15).

4.2.2.3 Proof of principle

The next stage of this project was the evaluation of the cellular uptake of cleavable and non-cleavable linkers loaded microspheres. A study was conducted using HeLa cells. These cells were treated in duplicate with microspheres at a concentration of 43 µg/ml per well. Following incubation excess microspheres were
removed and analysis by flow cytometry (Figure 4.15) showed that cellular uptake of microspheres was effective.

![Hela fluorescence signal](image)

**Figure 4.15**: Flow cytometry results of Hela cellular uptake with different microspheres at different incubation times.

These cellular uptake studies were based on flow cytometry. As can be seen in Figure 4.15 a decrease in the fluorescence signal from cells incubated with glutathione-reducible microspheres (4.2.2.10) was detected after 24 hours. This result suggested that after 12 hours incubation the cells began expelling part of the fluorescent dye outside the cell. However, the mean fluorescence from cells incubated with the control microspheres (4.2.2.14) as much as the non-cleavable maleimide ones (4.2.2.12) was higher after 24 hours incubation than after 12 hours. These results demonstrate that the dye was effectively released from the microspheres by a reduction of the disulfide linker.

### 4.2.2.4 Deprotection of disulfide-modified siRNA and conjugation to microspheres

Thiol-modified oligonucleotide was supplied in the protected form with the disulfide linkage intact to minimise the potential for oxidation, which results in oligo dimmer formation. To be able to use the free thiol (-SH) in our application the disulfide linkage had to be reduced with a reducing agent. In our case, dithiothreitol (DTT) was used (Scheme 4.4).
Free thiol derivative (4.2.2.16) was desalted on a desalting column prior to conjugation to microspheres in order to remove salts which could otherwise interfere with bioconjugation or even worse, adversely affect cellular beadfection and lead to poorer gene silencing. After being desalted, the free thiol siRNA was conjugated to pyridylthio and maleimido functionalised microspheres (4.2.2.6-9) in very mild conditions using slightly acid PBS (pH 6.9) (Scheme 4.5).

Scheme 4.5: Bioconjugation of free thiol derivative into microspheres.

4.2.2.5 GFP silencing assay

Following successful HeLa beadfection with rhodamine labelled beads and once proved that thiolated cargos could be delivered and released cytoplastically...
by polystyrene microspheres, several studies using a thiolated siRNA were carried on using HeLa-GFP cells to study the ability of this system as an effective suppressor of gene expression. The detection of GFP was based on fluorescent techniques (fluorescent microscopy and flow cytometry).

Flow cytometry studies

Hela-GFP cells were co-cultivated with different samples at different concentrations. Incubations were run over 48-72 hours where scrambled siRNA was used as negative control and a well-established transfection reagent, Lipofectamine 2000, was employed as a positive control (Figure 4.16).

Knocking down of GFP expression in HeLa-GFP cells was achieved after 72 hours with a silencing efficiency of up to 80%. It is important to recall that the well-known
transfection reagent, Lipofectamine 2000 showed a silencing efficiency of 60%, which is already a good result. Surprisingly, it was found that reduction of siRNA had a detrimental effect in terms of silencing efficiency. It was noticed that free thiol siRNA (Lip+HS-siRNA) demonstrated a poorer silencing when compared with the protected form of siRNA (Lip-siRNA) suggesting a loss of functionality during the reduction procedure. It was found that best result in terms of silencing efficiency was produced by 4.2.2.19 which contained a PEG spacer and a non-cleavable thioester crosslink in its structure. These results suggest that the introduction of a PEG spacer increases the biocompatibility with cell membrane for a successful membrane translocation. In the other hand, the coupling of the siRNA to the microspheres via a non-cleavable linker has given to the construct the ability to deposit siRNA cytoplasmatically without being dispersed (as would happen with cleavable linkers) and this fact has improved dramatically the efficiency of the silencing.

Microscopy studies

To confirm that silencing of GFP was really due to the activity of siRNA delivered by microspheres inside the cells, a fluorescently labelled siRNA was used to be able to track it inside the cells. Tetramethyl-6-Carboxyrhodamine (TAMRA) labelled thiol siRNA was conjugated to sample 4.2.2.9 (which showed the best silencing efficiency) following the approach described above (see Scheme 4.5) and subsequently co-cultivated with HeLa-GFP cells and analyse by fluorescence microscopy. As a representative example Figure 4.17 shows a composed image of Hela-GFP cells following beadfection with TAMRA-labelled siRNA loaded microspheres after 24 hours of incubation. It can be observed that TAMRA-labelled siRNA loaded microspheres were inside some cells and already started the process of silencing of GFP expression.
Figure 4.17: Fluorescence microscopy: HeLa-GFP cell incubated with TAMRA-labelled siRNA loaded microspheres at a concentration of 1.4 µM. Nucleus was labelled with Hoechst 22242.

4.2.2.6 Toxicity studies

As can be seen in Figure 4.18 thiol-mediated siRNA delivered through polystyrene microspheres into HeLa-GFP cells did not show any toxicity when compared with untreated cells. Additionally cell viability was not detrimentally affected by microsphere concentration.
4.2.3 Microplexes-based gene silencing using spermine functionalised microspheres

The introduction of oligoamines as a terminal group for the polymers is a promising strategy for the construction of transfection reagents for the delivery of oligonucleotides. Previous work has shown that although the introduction of oligoamines of different nature such as spermidine or tetramine gives very stable complexes, only the spermine derivatives have been found to be highly efficient transfection reagents.

4.2.3.1 Spermine functionalised microspheres preparation

A novel transfection reagent was built by incorporation of spermine residues into amino functionalised microspheres (Scheme 4.6). These polycationic microspheres should be able to interact with the anionic phosphate backbone of siRNA to complex it by electrostatic interaction. Additionally, to investigate the influence of spacer length between the microsphere core and the spermine’s lateral chains either on the ability of the conjugate to cross the cell membrane or to complex the siRNA, several derivatives were synthesised with different numbers of PEG as spacer. PEG was chosen as spacer due to its cell friendly nature and its hydrophilic properties that improve the interaction with the aqueous environment. Short hydrophobic moieties were introduced into the spermine-functionalised microspheres in order to increase the hydrophobic interaction between cationic copolymer and oligonucleotides and to increase endosomal release. For this purpose, amino functionalised microspheres were firstly modified with different units of PEG spacer and subsequently functionalised with either 100% spermine moieties (4.2.3.1, 3, 5) or 50-50% ratio of spermine:ethanolamine moieties (4.2.3.2, 4, 6).
Reagents and conditions: 1) PEG spacer (5eq), Oxyma (5eq), DIC (5eq), DMF, 3h 60 °C; 2) 20% piperidine, DMF, 3x20 min; 3i) DSC (4eq), Dry DMF, DIPEA, 3h 40 °C; 3ii) Spermine (10eq), Dry DMF, DIPEA, o/n rt; 4i) DSC (4eq), Dry DMF, DIPEA, 3h 40 °C; 4ii) Spermine (5eq), Ethanolamine (5eq), Dry DMF, DIPEA, o/n rt.

Scheme 4.6: Chemical structure of all the spermine functionalised microspheres.

Guanidination of spermine functionalised microspheres

The introduction of a guanidinium group into the structure of polycations has been reported to moderately increase moderately transfection efficiency and reduce the toxicity. It is predicted then that the addition of a guanidinium moiety in the spermine functionalised microspheres (4.2.3.1-6) can improve the formation of the complex. The idea was that the more positively terminal charged groups present on the surface of the delivery agent, the more successful should be the ionic interaction with the anionic siRNA. Spermine functionalised microspheres were converted into the polyguanidine derivatives as shown in Scheme 4.7 by a straightforwardly coupling of samples 4.2.3.1-6 with pyrazole-1-carboxamide which supplies the leaving group (imidazole) required for the nucleophilic aromatic substitution.
Reagents and conditions: 1) Sodium carbonate (10eq), Pyrazole-1-carboxamide (10eq), H_2O, o/n 50°C.

**Scheme 4.7:** Chemical structure of all the guanidilated functionalised microspheres.

*Spermine-functionalised microspheres-siRNA complexes formation.*

The formulation of these microplexes was achieved very easily by simple mixing of spermine-functionalised microspheres samples (**4.2.3.1-6**) and the siRNA in OPTI MEM medium (**Scheme 4.8**).
Scheme 4.8: A) Complex formulation by simple electrostatic interaction; B) siRNA anti-GFP full sequence; C) RNA base pairing with polar, hydrophilic phosphate groups which facilitates complex formation by electrostatic interactions on the surface.

4.2.3.2 siRNA complexation studies

Gel retardation assay

The ability of spermine-functionalised microspheres to complex siRNA was investigated by using a gel retardation assay. In this case gel electrophoresis was used to check the capability of siRNA to migrate through the gel. If the siRNA is free, it will migrate to the top of the gel due to its negative charged structure, but if the microplex is stable, the siRNA should lose its migrating ability due to its retention in the well by the non-moving microsphere. As already mentioned, the complex was formed in OPTI MEM medium, by adding a fixed amount of siRNA to different amounts of microspheres to obtain different polycation:siRNA ratios (w:w) ranging from 0.5:1 to 50:1. Complexation was shown to be effective from a 5:1 ratio; even if the 5:1 ratio starts to allow the complexation of the siRNA and the microspheres, the 10:1 ratio appeared to be more efficient (Figure 4.19). After the 10:1 ratio, increasing the ratio did not seem to significantly increase the ability of microspheres to bind the siRNA. In addition, it can be observed that a small quantity of siRNA always seems to be released and is able to migrate on the gel and be localised at the top of the gel even when the ratio is strongly increased (50:1). This effect can be also observed when a commercial transfection reagent (Lipofectamine 2000) is used as a positive control.

![Figure 4.19: Electrophoresis gel with different ratios of microspheres:siRNA complex (w:w). LIP: Lipofectamine 2000-siRNA complex (positive control); microspheres:siRNA ratios 1:5, 1:1, 5:1, 10:1, 20:1, 50:1 and free siRNA (negative control).](image)

Biophysical properties

Biophysical properties of spermine-functionalised microsphere:siRNA complexes such as mean diameter and zeta potential were determined via DLS and
zeta potential measurements. For this purpose the complex was formed as previously described. Based on the gel retardation results, ratios ranging from 5:1 to 50:1 (w:w) were analysed. As expected, the value for microspheres without siRNA was positive. Additionally, zeta potential values were decreasing when increasing the ratio (Figure 4.20/A). Size of microspheres without siRNA was found to be 492 nm. From ratio 5:1, microspheres start to bind siRNA and the size of the microplex starts to increase (Figure 4.20/B).

**Figure 4.20:** A) Zeta potential curve for microspheres and different microspheres:siRNA complex ratios 5:1, 10:1, 20:1, 50:1. B) Size curve for microspheres and different microspheres:siRNA complex ratios 5:1, 10:1, 20:1, 50:1.

### 4.2.3.3 Gene silencing assay

To investigate the ability of this system to be used as a transfection reagent several studies were carried out using human cervical cancer cells (HeLa), where enhanced green fluorescent protein (eGFP) has been stably expressed (HeLa GFP cells). The detection of GFP was based on fluorescent techniques (fluorescent microscopy and flow cytometry).

**Influence of the quantity of siRNA**

To optimise the amount of siRNA needed to induce an efficient knocking down of eGFP expression, the best microsphere:siRNA ratio (w:w) suggested from electrophoresis gel, zeta potential and DLS results was used (10:1). Different amounts of siRNA (µg) were used (1, 2 and 4 µg of siRNA/well) using a 48-well
plate format. These results were analysed by fluorescence microscopy (Figure 4.21/A) and flow cytometry (Figure 4.21/B).

**Figure 4.21: A) Fluorescence microscopy.** Images of HeLa GFP cells co-cultivated for 72 hours with sample 4.2.3.9 with a 10:1 ratio of microsphere:siRNA using different concentrations of siRNA. Inset is brightfield. Scale bar is 350 µm. Inset are phase contrast images, showing approximately 70 – 80% cell confluency. **B) Flow cytometry.** % of cell population showing a GFP+ activity, expressing the GFP (dark green) or a GFP- activity, complete knocking down of the GFP (light green) of eGFP fluorescence expression in the HeLa GFP cells using only microspheres (4.2.3.1-6) or microspheres:siRNA (4.2.3.7-12), ratio 10:1 and 4 µg of siRNA.
The 2 µg samples show low or no silencing, suggesting that this concentration was not optimum. However it was observed that the silencing efficiency was clearly higher for the samples with 4 µg or siRNA. It is important to recall that the amount of siRNA used was not increased above 4 µg/well because the results were satisfying but also because an efficient delivery system should be able to induce silencing without consuming too much of an important, costly and difficult to produce on large scale quantity of siRNA. The amount required to induce an efficient GFP knocking down was then shown to be 4 µg of siRNA/well and the following experiments are carried out using this quantity.

**Influence of the ratio**

Knowing the optimal amount of siRNA needed to observe some silencing, ratios of 5:1, 10:1 and 20:1 were tested. The ability of the microplex to induce silencing could be either as efficient with a smaller amount of microspheres or more efficient when considering a stronger microsphere:siRNA complex (Figure 4.22).

![Figure 4.22: Flow cytometry results of eGFP expression in HeLa GFP cells incubated with 4.2.3.11 with 4 µg in different ratios. A) Mean relative fluorescence of the GFP; B) % of cell population showing a GFP+ activity, expressing the GFP (dark colored columns) or a GFP- activity, complete knocking down of the GFP (light colored columns); C) Histogram showing eGFP intensity in untreated cells (black) and in cells incubated with 4.2.3.11 at ratio 5:1 (dark red) and at ratio 10:1 (light red).](image)

As can be seen in Figure 4.23, the 20:1 ratio did not show any improvement compared to the 10:1 ratio. In fact, microscopy pictures show that the knocking down seems to be less efficient with the ratio 20:1. The explanation for this phenomenon could be that if the stability of the complex is very strong, then the siRNA can not be released in the cytoplasm to induce silencing.
Figure 4.23: Fluorescence microscopy. Images of HeLa GFP cells co-cultivated for 72 hours with a quantity of siRNA of 4 µg and various ratios of microspheres:siRNA. Scale bar is 350 µm. Inset are phase contrast images, showing approximately 70 – 80% cell confluency.

Influence of microsphere functionalisation

Now that the amount of siRNA and the ratio needed to be efficient was known the experiment was repeated using the optimal conditions. All of the samples were tested with a ratio microspheres:siRNA of 10:1 and 4 µg of siRNA.
Figure 4.24: Fluorescence microscopy. Microscopy images of HeLa GFP cells with only microspheres or using microspheres:siRNA, ratio 10:1 and 4 µg of siRNA; A) Sample 4.2.3.5; B) Sample 4.2.3.11; C) Sample 4.2.3.6; D) Sample 4.2.3.12; E) Sample 4.2.3.3; F) Sample 4.2.3.9; G) Sample 4.2.3.4; H) Sample 4.2.3.10; I) Sample 4.2.3.1; J) Sample 4.2.3.7; K) Sample 4.2.3.2; L) Sample 4.2.3.8. Scale bar is 350 µm. Inset are phase contrast images, showing approximately 70 – 80% cell confluency.

The microscopy images revealed a good knocking down efficiency (see Figure 4.24). Flow cytometry analysis showed that samples 4.2.3.11 and 4.2.3.12 (with two PEGs) worked really well showing a GFP silencing efficiency of 44 and 54% as well as the samples 4.2.3.9 and 4.2.3.10 with one PEG showing a GFP silencing efficiency of 53 and 54%. The samples 4.2.3.7 and 4.2.3.8 with no PEG showed a poorer efficiency with 34 and 0% of GFP knocking down respectively (see Figure 4.25/B-C). The samples with two PEGs seemed to be efficient, as well as the sample with one PEG (see Fig. 4.25/A). The samples with no PEG, 4.2.3.7 and 4.2.3.8 were as efficient as the others according to microscopy (see Figure 4.24) but are showing a poor capacity of GFP silencing compare to the other samples by flow cytometry analysis (see Figure 4.25/A-B). Even worse, these samples were less reliable and that is a real issue for a biological delivery system. It is one of the main requirements for any system with a therapeutic goal to be stable and reliable. This could be explained by a sterical effect. When the siRNA is closer to the microsphere (due to the absence of PEG spacer) complex stability could be weaker and be broken more easily before penetration into cells.
**Figure 4.25: Flow cytometry results of eGFP fluorescence expression.** A) Histogram representation showing eGFP intensity in untreated cells (black line), cells incubated with only microspheres (light colored lines) and cells incubated different samples of microspheres:siRNA at a 10:1 ratio for 4 µg for 72 hours (dark colored lines); B) % of cell population showing a GFP+ activity, expressing the GFP (dark green columns) or a GFP-activity, complete knocking down of the GFP (light green columns); C) Mean relative fluorescen of the eGFP.

The next step was a silencing assay with guanidine-functionalised samples. Based on microscopy results (**Figure 4.26**), the guanidine-functionalised microspheres seemed to be as efficient as the spermine functionalised one.

**Figure 4.26: Fluorescence microscopy.** Hela GFP cells co-cultivated with 4.2.3.9 (A) or with 4.2.3.9G (B) at a microspheres:siRNA ratio of 10:1 with 4 µg of siRNA. Scale bar is 350 µm. Inset are phase contrast images, showing approximately 70 – 80% cell confluency.

However, flow cytometry analysis was then performed and it was revealed that in fact none of the samples had worked, with no silencing at all for five out of six samples and a really poor efficiency (8%) for the sample 4.2.3.12G when compared with non guanidilated samples (**Figure 4.27**). Despite the disappointment of this failure, it proves that not only the charge is critical for system efficiency. Additionally, the nature of the group that carries this charge and supposedly binds the siRNA is crucial for activity.
Figure 4.27: Flow cytometry. Cells co-cultivated with different samples for an incubation time of 72 hours with a microspheres:siRNA ratio of 10:1 with 4 µg of siRNA; A) Mean relative fluorescence of the eGFP; B) % of cell population showing a GFP+ activity, expressing the GFP (dark green columns) or a GFP- activity, complete knocking down of the GFP (light green columns).

4.2.3.4 Controls

To prove that the knocking down of the GFP was really due to the entry of the microspheres:siRNA complex in the cellular cytoplasm and to the action of this siRNA, several control assays were carried out. HeLa GFP cells were incubated with naked microspheres (microspheres with no siRNA on them), free siRNA without a delivery system, microplexes of scrambled (or not sequence-complementary) siRNA (Figure 4.28/B-C). None of these samples worked demonstrating that GFP silencing is effective only when cells are incubated with sequence-complementary siRNA transported by spermine-functionalised microspheres.
Figure 4.28: Flow cytometry results of eGFP fluorescence expression. A) Histogram representation showing eGFP intensity in untreated cells (black line), cells incubated with only microspheres (light colored lines) and cells incubated with different samples of microspheres:siRNA (scrambled) at a 10:1 ratio for 4 µg for 72 hour (dark colored lines); B) % of cell population showing a GFP+ activity, expressing the GFP (dark green columns) or a GFP- activity, complete knocking down of the GFP (light green columns); C) Mean relative fluorescence of the eGFP.

Previous results suggest that microspheres are penetrating into the cells by formation of microscopic holes in the cell membrane that are efficiently repair by cells after a while. So, even though delivery of siRNA by naked microspheres has been unsuccessful reinforcing the idea that spermine moiety is a requirement for complexation, one may think that siRNA is able to translocate the cell membrane thanks to these microsphere-created holes. To check this, HeLa cells were incubated with fully naked microspheres (without PEG on them), which after a while was incubated equally with naked siRNA (Figures 4.28/B-C and 4.29). This experiment showed that the siRNA can penetrate into the cell’s cytoplasm only if it is bound to a delivery system and not only by using the microspheres-created holes when crossing the cellular membrane. The experiment was also carried out with carboxylic acid functionalised microspheres (2.11) to prove that these microspheres could not bind siRNA due to electrostatic repulsion between the negative charges of the carboxylic group on the microsphere and the phosphate backbone of the siRNA. In fact these negatively charged microspheres were unable to penetrate the cell’s membrane because of electrostatic repulsion with the negatively charged cell membrane (Figure 4.29).

Figure 4.29: Flow cytometry result represented by histograms showing eGFP intensity in untreated cells (black) and in cells incubated with several samples of microspheres:siRNA, ratio 10:1 with 4µg of siRNA (dark grey lines) and scrambled siRNA (light grey lines); A) Control with siRNA positive or negative without a delivery system; B) Amino-functionalised microspheres; C) Carboxy-functionalised microspheres.
The fact that amino functionalised microspheres as well as carboxylic acid functionalised microspheres were unable to silence GFP expression is strong evidence that spermine coupling was successful and also that the presence of the spermine terminal group is the key to build a successful delivery tool.

4.2.3.5 Cellular uptake studies

TAMRA-labeled siRNA

To check the entry of the complex in the cell, a Tetramethyl-6-Carboxyrhodamine (TAMRA) labelled siRNA was used. Microscopy results showed that the TAMRA-labelled siRNA could be localised in the cytoplasm of the cell (see Figure 4.30). It also allowed the co-localisation of siRNA and GFP knocking down. In the cytoplasm of the cell which showed the presence of siRNA, there is also some knocking down, while in the cell with no or less siRNA we could still see the eGFP expression.
Figure 4.30: Fluorescence microscopy. Microscopy image of HeLa GFP cells after 24 hours incubation with microspheres: TAMRA–labelled siRNA, ratio 10:1 with 4 µg of siRNA; Scale bar is 350 µm. Inset are phase contrast images, showing approximately 70 – 80% cell confluency. A) Magnification x10; B) Magnification x20; C) Detail of the image.

4.2.3.6 Cytotoxicity assay

Lack of toxicity is a very important property of a transfection reagent. Viability of the cells was tested in vitro by MTT assay. This allowed assessment of the viability of cells by cell counting directly in the culture well-plate and to determine cytotoxicity of different materials introduced into the cell. MTT analysis was carried out after 72 hours incubation of the polyplex at a 10:1 microspheres:siRNA ratio in HeLa-GFP cells. Toxicity data are shown in the MTT graph (Figure 4.31). It can be seen that all polyplexes resulted in low cell toxicity.
Figure 4.31: MTT assay graphic showing cell viability (%). Cell viability was tested for each sample of microspheres with siRNA, ratio 10:1 and without siRNA. Error bars representing the standard deviation. n=4. Statistical studies performed by Dunnett’s test (α=0.05) showed that samples 4.2.3.4, 4.2.3.1 and 4.2.3.2 were statistically significantly different to Hyp.

4.2.4 Conclusions

In this chapter, different chemistry strategies have been applied for microspheres-based gene silencing by the delivery of siRNA on either ES or HeLa-GFP cells. Firstly, Knock down of relevant self renewal genes (such as Oct4 and Sox2) have been efficiently achieved using microspheres as transfection system. Green Fluorescein Protein stably expressed in mES under the control of mouse Oct4 gene regulatory elements (Oct4-GiP) cells was successfully silenced (by approximately 40%) using siRNA linked to 500 nm microspheres, exceeding the silencing capabilities of commercially available lipofection products. In addition, a system employing dual-functionalised (Dye-co-siRNA) microspheres allowed independent evaluation of only those cells which had been beadfected with siRNA, yielding gene silencing data based only on those culture fractions that had received the delivery vehicle, generating a more accurate analysis of the gene silencing capabilities of microspheres. Also microspheres showed much lower toxicity that commercially available lipofection product as Lipofectamine 2000. These results confirm that microspheres are a great candidate for the knock down of genes implicated in self renewal and provide a safe tool to investigate the influence of silence these genes in differentiation and pluripotency of stem cell.
Secondly, a novel approach based on disulfide exchange chemistry was developed in order to design an efficient strategy for the delivery of thiolated siRNA. Green Fluorescein Protein (GFP) stably expressed in human ovarian cancer (HeLa) cells was successfully silenced using this strategy. Different spacer units were tested resulting in significant differences in terms of cellular uptake. In addition, cleavable and non-cleavable linkers were attached to microspheres in order to make a comparison between the effect of cargo released into the cytoplasm and cargo remaining attached on the delivery system. It was found that among the tested samples the one which comprised a PEG spacer and a non-cleavable linker achieved a silencing capability of approximately 80%, improving the silencing efficiency of commercially available lipofection products. Finally, the expression of GFP in HeLa cells has been proven to be successfully silenced using complex of spermine functionalised microspheres binding siRNA by electrostatic interaction between the positive charge of the delivery system and the phosphate backbone of the cargo. This microplex was able to deliver siRNA into the cell cytoplasm allowing GFP knock down of more than 50%. The formulation of the microplex is really easy to synthesise being fast and without requiring any chemical reaction due to its electrostatic action. In conclusion I would say that while the three chemical strategies has been proved being efficient when applied to microspheres, however the system based on polycationic chain has been found quite novel and without previous publications found on the scientific community opening in this sense a broad range of possibilities in the field of cell delivery reagents.
CHAPTER 5: Aminomodified Nucleobases: Novel Strategy for microsphere-mediated DNA transfection

5.1-Introduction

Efficient carrier systems for the delivery of plasmid DNA into cells is a key technology that allows research progression in biological sciences. In the last few years, different technologies and methodologies have been developed in order to efficiently deliver nucleotides at the cellular level. Examples include cell penetrating peptides, peptidomimetics as cationic liposomes, nanodevices such as nanoparticles and quantum dots or invasive techniques such as particle bombardement. As mentioned in previous chapters, polymer mediated delivery offers many advantages over more traditional methods. For instance, oligonucleotides can be encapsulated within polymer microparticles, which can then release their cargo intracellularly in a tunable manner. Such systems include cationic polymers, poly(amidoamine)s and copolymers. Microspheres have been shown to be efficient delivery agents which can be taken up by a wide range of cell lines. These devices have been used for delivery of therapeutic peptides on neuroblastoma cells (chapter 3), delivery of siRNA on adherent and embryonic stem cells (chapter 4) and delivery of proteins among others. The fact that these latex beads are easy to functionalise with high controllability over cargo loading and they are taken up by cells with any undesired cytotoxic effects observed, make them enormously attractive as carrier/delivery systems in molecular biology. The opportunity to sort cells individually based on the number of beads per cell, allowing defined cellular doses, is very important for the application of microspheres in the field of molecular biology. Therefore, microspheres are shown as a potential tool for the manipulation and long-term labelling of relatively hard to transfect cells as T cells.

5.1.1 Gene expression

Gene expression is the transformation of DNA information into functional molecules which comprises several steps. In the first step, termed transcription, RNA polymerase produces RNA copies of the DNA by adding one RNA nucleotide at a
time to a growing RNA strand. This RNA strands possess the same sequence as the gene being transcript. The resulting primary transcript of RNA is altered before being translated by removing RNA segments which are not found in the mature RNA (Introns) in a process called splicing. The second step involves protein synthesis, also known as translation. At this stage, the sequence in DNA (and RNA) is processed to amino acids (three nucleotides are required to specify one amino acid). The final chain of amino acid must pack it in to generate the final tertiary structure of the protein (See Figure 5.1).

**Gene expression**

![Gene expression diagram](image)

**Figure 5.1:** Simplified overview of gene structure and expression.

**5.2-F5.BW hybridoma cell line**

A hybridoma is a hybrid cell created by fusing two cells together, F5.BW cell line comes from a secreting cell from the immune system following activation for a specific antigen and a long-lived cancerous immune cell, within a single membrane. These individual hybridoma cells are subsequently cloned, producing many identical offspring. Their identical daughter clones will secrete, over a long period of time, a single specific antibody. In particular, F5.BW hybridoma cells were created by fusing a CD8 T cell expressing a F5 T-cell receptor (F5 TCR), which has a high
affinity for the peptide derived from the influenza virus nucleoprotein (NP68). Thus, it is an immortal CD8+ T cell that can be activated with a specific antigen.

5.3-Cellular uptake optimisation

All experiments using the F5.BW hybridoma cell line were performed by Dr. Jessica Borger from Prof. Zamoyska’s group at the Institute of Immunology and Infection Research, School of Biological Sciences in Edinburgh University.

In the first stage of this project, the uptake efficiency of microspheres into F5.BW hybridoma cells was assessed. For this purpose, this cell line was beadfected with previously prepared fluorescein-labelled polystyrene microspheres of different sizes (200 nm and 500 nm) (2.15 and 2.17).

5.3.1 Influence of size in cellular uptake

The initial optimisation experiments performed with the F5.BW hybridoma cell line were carried out to evaluate cellular uptake based on particle size after 24 hours of incubation.
Figure 5.2: A) Histograms representing F5.BW hybridoma cells which have been beadfected with different concentrations of 200 nm (left) and 500 nm (right) fluorescein labelled beads. B) Graph showing the percentage of cellular uptake of microspheres and evaluation of cell viability (by staining with Topro3). Purple bars are FITC negative and the lilac bars are FITC positive. Data courtesy of Dr. Jessica Borger.

The main finding was that bead uptake by the hybridoma cell line was bead-size dependent. Figure 5.2/A demonstrates that the smaller the beads, the increased MFI/fold-increase in FITC signal, thus more beads were taken up when cells were incubated with smaller beads (200 nm) and with increased concentrations of beads in the media. Cell viability was evaluated using TOPRO-3. An important note was that there was no increase in cell death when cells were beadfected with microspheres. Although the percentage of FITC positive cells do increase with increasing concentration of beads (Figure 5.2/B), the overall total of cell death does not change (Topro3+) when compare to untreated cells (number of beads = 10⁶), suggesting that cell death is not attributable to the internalisation of beads. These results suggest that 200 nm is the optimal size to achieve cellular uptake by F5.BW hybridoma cells. It was of considerable importance that the 200 nm microsphere size was selected in
view of the fact that the average size of this cell line ranges from 6-10 µm, significantly smaller than cell lines beadfected thus far.

5.3.2 Influence of time in cellular uptake

The second optimisation experiment concentrated on uptake over time using the 200 nm fluorescein beads (2.17), which demonstrated the best cellular uptake ratios from previous experiments in F5.BW cells.

![Figure 5.3](image1)

**Figure 5.3:** A) Histograms representing F5.BW hybridoma cells which have been beadfected with different concentrations of 200 nm over different incubation times. B) Confocal microscopy of F5.BW hybridoma cell beadfected with 2.17 for 8 hours. Top images show brightfield and bottom images were taken with DAPI channel. Inset represents images of a single cell. *Data courtesy of Dr. Jessica Borger.*

As seen in **Figure 5.3/A** there was a shift in MFI/fold-increase in FITC signal at 4 hours which was slightly increased at 8 hours, with only a small reduction seen after 24 hours. This slight reduction could be due to cell proliferation leading to dilution of the beads in the cell population. These results suggest that the time of incubation can be tune depending on the number of beads. It is important to take into account that
the ratio of bead(s) per cell in every experiment should be calculated with respect to the size of cells being used, where an excess on the number of beads could lead to cytotoxicity. Confocal microscopy was used to confirm these flow cytometry results. Figure 5.3/B shows a representative example where F5.BW hybridoma cells were beadfected with 200 nm fluorescein-labelled microspheres (2.17) after 8 hours of incubation.

**5.4-Plasmid DNA linearisation**

As stated in section 5.1, pDNA is not easy to transfect into target cells. In order to facilitate its further transfection into cells, a simple protocol was followed to linearise pDNA. Different pDNAs where chosen to be expressed on F5.BW hybridoma cells: Lymphocyte-Specific protein tyrosine Kinase (Lck), C terminal Src Kinase (Csk) and PEST-domain-enriched tyrosine phosphatase (PEP). These proteins are biologically relevant in T-cell activation and differentiation. These proteins were transfected as fusion proteins with Yellow Fluorescent Protein (YFP): Lck-YFP, Csk-YFP and PEP-YFP. This strategy allows to confirm the transcription of the desired protein by observation of expression of the fluorescent protein (YFP) that it is very easy to analyse using flow cytometry and/or fluorescence microscopy. Additionally Green Fluorescent Protein (GFP) was chosen to be expressed as model to prove this concept. These target pDNAs were cut in a specific region by *Streptomyces phaeochromogenes* Type I (SphI), in the case of GFP, or *Planococcus citreus* SE-F45 Type I (Pcil) restriction enzymes, in the case of Lck-YFP, Csk-YFP and PEP-YFP. In order to test if the restriction had been performed successfully, agarose gel electrophoresis was carried out (Figure 5.4).
As can be seen in Figure 5.4 digestion was successful for all samples as indicated by linear DNA (lDNA) and plasmidic DNA (pDNA) running differently through the gel. It is important to recall that even though the molecular weight of pDNA and lDNA is the same, they run differently because plasmid or circular DNA migrates faster through the gel than linear DNA.²¹⁶

### 5.5-Strategies for incorporation of DNA constructs onto microspheres

For the incorporation of previously linearised DNA into microspheres a strategy was designed where an amino functionalisation was carried out by incorporation of a modified nucleotide 5′-(3-aminoallyl)-2″-deoxyuridine 5″-triphosphate (dUTP).

#### 5.5.1 Microspheres preparation

200 nm polystyrene beads (2.8), were functionalised as described in Scheme 5.1. Beads were double pegylated with Fmoc-PEG-OH spacer before being carboxyfunctionalised with a cleavable linker. They are two reasons behind beads being double pegylated. Firstly, the addition of these units aids transport across the cell membrane by increasing beads biocompatibility and secondly, the distance between the IDNA and cellular vehicle is increased, which could otherwise lead to undesirable interactions.
Reagents and conditions: (i) Fmoc-PEG-OH spacer (5 eq), Oxyma (5 eq), DIC (5 eq), DMF, 2 h, 60 °C; (ii) 20% piperidine, DMF, 3 x 20 min; (iii) Fmoc-PEG-OH spacer (5 eq), Oxyma (5 eq), DIC (5 eq), DMF, 2 h, 60 °C; (iv) 20% piperidine, DMF, 3 x 20 min; (v) 3,3′-Dithiodipropionic acid (10 eq), DIC (5 eq), DIPEA (0.1 eq), DMF, 2 h, 60 °C.

Scheme 5.1: Microsphere preparation for conjugation to linearised DNA.

5.5.2 DNA-microspheres conjugation

The next step was the conjugation of linearised DNA with carboxyfunctionalised 200 nm polystyrene microspheres (5.1). As said previously, the conjugation involved a modified nucleotide 5-(3-aminoallyl)-dUTP (5.2), which when incorporated into DNA provided a reactive amino group, which subsequently reacted with the carboxylic group present on microspheres, to form an amide bond. The modified dUTP was readily incorporated by Terminal deoxynucleotidyl transferase (TdT). TdT is a specialised DNA polymerase which catalyses the addition of nucleotides to the 3’ terminus of a DNA molecule. The aminoallyl modification enables downstream reaction with amine-reactive compounds such as activated esters; thus aminoallyl-modified DNA could be conjugated into carboxyfunctionalised microspheres in two easy steps as detailed in Scheme 5.2.
5.5.3 Internalising lDNA-microsphere complex into hybridoma cells.

Once conjugation to give lDNA-microspheres had been performed the next step was to test if the linkage had been successful as well as to identify if such a lDNA-microspheres ‘complex’ could be intracellularly detected. For this purpose, a well known double stranded DNA intercalator, TO-PRO-3 Iodide\textsuperscript{217-219} was added to DNA-microsphere conjugates (5.5 to stain the DNA and consequently allow monitoring the uptake of these conjugates by fluorescence techniques). After 8 hours of incubation, beadfected cells were analysed by flow cytometry (Figure 5.5).

**Scheme 5.2:** Strategy for conjugation of DNA constructs to microspheres.

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**Reagents and conditions:** i) TdT (20 units), 10x TdT buffer, CoCl\textsubscript{2}, 37\degree C, 20 minutes; ii) PBS pH 7.4, Thermostat 700 rpm, o/n rt.
It can be observed that the fluorescence intensity increases an order of magnitude when DNA is conjugated to the microspheres compared to naked microspheres (see Figure 5.5). These results demonstrate that amino functionalised lDNA was effectively conjugated with polystyrene microspheres and this system was efficiently delivered into cells.

5.5.4 Comparison between pDNA and lDNA gene expression

At this stage it was a key point in the study to test the capacity of this lDNA to express the target protein. As such, the capacity of both DNA (linear and plasmid) to express GFP on two cell lines (human embryonic kidney (HEK293T) and human cervical cancer (HeLa) cells) was tested. Transfection with a well known transfection reagent, Lipofectamine 2000, was used to deliver the linear and plasmid DNA on both cell lines. The transfection efficiency can be assessed by quantification of GFP expression using flow cytometry analysis.

![Transfection efficiency](image)

Figure 5.6: Transfection efficiency by flow cytometry analysis. MFI of cells transfected with pGFP and lGFP using Lipofectamine 2000 after 24 hours incubation time. Errors bar representing the standard deviation. n=4.

The obtained results are summarised in Figure 5.6. It can be observed how both DNAs (plasmid and linearised) effectively increase the MFI in both cell line tested. However, plasmid DNA (pGFP) afforded higher MFI than its linearised counterpart in about 2.5 fold. It was also found that the fluorescence intensity on HEK 293T cells was significantly higher overall than in HeLa cells. This result was expected as this cell line is known to transfect very readily.
**5.6-GFP expression on HEK-293T cells using DNA-microsphere conjugates**

Following the successful linkage of DNA construct to 200 nm microspheres and assessment of the ability of the linearised DNA to express the target protein, HEK-293T cells were incubated with lDNA-microsphere conjugates (5.3) and analysed for GFP expression by flow cytometry (Figure 5.7). Naked microspheres without DNA attached (5.1) and unconjugated GFP-lDNA were used as negative controls.

![Figure 5.7: GFP expression analysed by flow cytometry.](image-url)

**Figure 5.7: GFP expression analysed by flow cytometry.** A) MFI on HEK-293T cells after incubation for 48 hours. Transfection using Lipofectamine was used as positive control and unconjugated GFP lDNA and naked microspheres (5.1) as negative controls. B) Histogram of Untreated cells (black line) and cells beadfected with 5.3 (turquoise line). The error bars represent the standard error of the average. n=4. Statistical studies performed by Dunnett’s test (α=0.05) showed that 5.3 was statistically significance different to Lipofectamine.

Expression of GFP on HEK 293T cells was achieved after 48 hours incubation using 5.3 where GFP intensity had increased 10 fold compared to untreated cells, exceeding a widely used commercial transfection reagent, Lipofectamine, which yielded 9 fold increased intensity.

**5.7-CSK-YFP and PEP-YFP expression on F5.BW hybridoma cell line**

Once the effects of bead size and incubation time on cellular uptake by F5.BW hybridoma cells had been optimised and encouraged by successful GFP expression on HEK 293T cells after pGFP transfection (which had been linearised by the protocol previously described), the next step was to transfect F5.BW hybridoma cells with lDNA encoding genes for Csk-YFP and PEP-YFP fusion proteins conjugated with 200 nm polystyrene microspheres (5.5 and 5.6). Gene expression
achieved by these IDNA sequences coupled to microspheres was then compared with electroporation using Amaza Nucleofactor System, which is a popular transfection system, particularly used in difficult to transfect cell such as ES$^{188}$ and T cells.$^{220}$

As demonstrated in Figure 5.9, beadfection effectively led to expression of the tagged protein YFP. When compared with electroporation, it can be seen that beadfection shifted the entire population, indicating that all cells were taking up a bead and expressing the protein, however electroporation shifted only a proportion of the population. In conclusion the results suggest that even though electroporation afforded higher values of MFI, beadfection would have more biological relevance as
the afforded MFI values came from the entire population and not only a proportional part of the population as what seemed to happen in electroporation. To evaluate the amount of protein expression per cell, a confocal microscopy study was performed looking at YFP expression after hybridoma cells were either beadfected or electroporated with PEP-YFP or Csk-YFP (Figure 5.10).

![Beadfection (24 hours)](image1)

![Electroporation (8 hours)](image2)

**Figure 5.10: Confocal microscopy:** Images of a single F5.BW hybridoma cell transfected by beadfection (Top images) or electroporation (Bottom images). Scale bar is 15 µm. Data courtesy of Dr. Jessica Borger.

As shown in Figure 5.10, when beadfection was used as the transfection method, protein expression per cell was lower than in cells transfected by electroporation. It is fair to say that electroporation pictures are not representative of the whole population and only the “best-cases” are showed. Surprisingly, it seems that when cells are beadfected, YFP expression is not uniformly dispersed into the cytoplasm. This can be due to lower expression of the protein.

**5.8-Conclusions**

In conclusion, a new approach for microsphere-mediated delivery of oligonucleotides, pDNA in this case, has been described. Following a simple
protocol for the linearisation and functionalisation of the plasmid DNA, this amino-modified IDNA was covalently coupled to 200 nm polystyrene beads and subsequently different cell types were “beadfected” where GFP (in HEK-293T cells) and biologically relevant proteins fused to YFP (in F5.BW hybridoma cells) have been successfully expressed. In addition, these microspheres showed no toxicity in F5.BW hybridoma cells, a sensitive and difficult to transfect cell line.
CHAPTER 6: Microsphere-based intracellular sensing of enzymatic activity

6.1-Introduction

Monitoring enzymatic activity in biological systems, such as in cells, is important in many fields of medicinal chemistry and chemical biology. For this purpose enzyme-activity assays are often used, for example screening enzyme inhibitors and activators to discover novel drug candidates. So far, the majority of enzymatic activity studies have been performed using cell lysate. Therefore, there is a lack of knowledge about enzymatic activity assays in situ. The capability to analyse the activity and behaviour of a particular enzyme inside the cell will provide valuable information and also display a more realistic picture of the genuine behaviour of the enzyme. There are different fluorescence-based techniques to detect enzymatic activity, such as the use of fluorogenic or FRET substrates. However, most of these substrates are not able to cross cell membranes. To solve this problem a range of carrier systems have been developed, each of them with different properties and features. Previously, in house-made microspheres have been reported as a carrier system, which has been successfully synthesised and their cellular uptake demonstrated in various cell lines (HeLa, HEK-293T, SH-SY5Y and Stem cells). Since then they have been used as delivery systems for different types of molecules, such as proteins, sensors, neuropeptides (chapter 3) and oligonucleotides (chapters 4 and 5). However, the design of one of the most novel carrier systems, microspheres, has hardly been studied and its applicability as an enzymatic sensor is an unexplored and promising area of research. Therefore, it was proposed that microspheres could be used as a highly competitive cellular delivery technique, due to their lack of toxicity, the ability to control uptake and the opportunity to sort at a single-cell level, thus rivalling current nanoparticle techniques in the area of enzymatic activity assays.
6.1.1 Apoptosis

Programmed cell death (PCD) or apoptosis is an evolutionarily conserved and meticulously coordinated type of cell death. Its function is the deletion of disused, infected, damaged or deformed cells during the normal life duration in various biological systems. Also it is a vital course of action in maintaining homeostasis in multicellular organism. It is normally implicated in natural processes such as embryogenesis, metamorphosis, immune system and normal adult tissue remodelling; moreover it has also been associated in various pathological disorders such as cancer, autoimmunity and degenerative diseases. Apoptosis may involve single cells or small clusters of cells.

6.1.1.1 Morphology of apoptosis

Light and electron microscopy have elucidated the various morphological changes that take place during apoptosis. In the early process of apoptosis, cell shrinkage and chromatin condensation occurs, the cell compacts in size associating in a dense cytoplasm where the organelles are more tightly packed. Next, membrane blebbing commences and subsequently nuclear collapse while membrane continued blebbing followed by formation of membrane bound bodies (apoptotic bodies). Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained and all of this is enclosed within an intact plasma membrane. These bodies are then phagocytosed by macrophages or lysated (Figure 6.1).
6.1.2 Role of Caspase-3 in apoptosis

Caspases are fundamental mediators of apoptosis. One set of mediators involved in apoptosis belong to the aspartate-specific cysteinyl proteases or caspases. So far, 13 caspases have been identified in mammals, which can be divided into 2 groups, initiator and effector caspases, based upon the lengths of their N-terminal prodomains. Ordinarily, caspases are present as zymogens in cells until pro-apoptotic signal activate initiator caspases. Once activated, the initiator caspases cleave downstream effector caspases, resulting in a proteolytic cascade that leads to cell death. All caspases cleave the peptide bond C-terminal to aspartic acid residues. Initiator caspases recognise the WEHD/(L/V)EXD sequences, whereas effector caspases (Caspases 3, 6 and 7) recognise the DEXD motif. Caspase-3 has been identified to be a key member of the caspase family and is characterised as an effector caspase which interacts with Caspases 8 and 9 as can be seen in Figure 6.2. Caspase-3 is of particular interest in cancer research, as it is down-regulated in different types of tumors, and its decreased activity is a prognostic indicator of chemosensitivity in breast and ovarian tumors.
Fluorometric methods based on the peptide substrate labelled with cleavable fluorophores have been extensively used for assaying various proteases, including caspases. As previously noted, the activation of caspase-3 plays an important role in the apoptotic process. To prove that polystyrene microspheres are a useful tool for in situ detection and monitoring of enzymatic activity, a well-known peptide substrate of caspase-3 (DEVD) coupled to a fluorophore (7-amino-4-trifluoromethylcoumarin, AFC) was chosen. This fluorogenic substrate, Ac-DEVD-AFC was coupled to 500 nm amino functionalised microspheres.

6.2.1 Fluorescent microspheres preparation

For the preparation of fluorescent polystyrene microspheres the general solid-phase synthesis protocol previously mentioned in chapter 2 (Scheme 2.11) was followed. Such strategy allowed us to bifunctionalise our 500 nm polystyrene beads, allowing fluorescent labelling of the side chain and subsequent coupling of the fluorogenic substrate on the N-α chain (Scheme 6.1).
**Scheme 6.1:** Preparation of fluorescent microspheres for fluorogenic-based studies

6.2.2 Coupling of fluorogenic substrate into polystyrene microspheres

Once the fluorescent labelled microspheres were prepared the final step for the preparation of the fluorogenic probe was the conjugation of the fluorogenic substrate (Ac-DEVD-AFC) onto microspheres. It is important to recall that the conjugation will take place in the γ-carboxylic group of glutamic acid because of its higher reactivity during carbodiimide-mediated coupling to primary amino groups as compared to that of the α-carboxyl group of aspartic acid.  

**Scheme 6.2:** Conjugation of microspheres with fluorogenic substrate.
This enzymatic substrate-microsphere nanoprobe could allow detection and monitoring of enzymatic activity of caspase, because its fluorogenic properties after degradation of DEVD sequence by caspase-3 and, additionally, this detection could be done in situ due to the facilitated internalisation of the substrate by microspheres.

6.3-Biological assays

The biological assays were based on detection of cleavage of substrate DEVD-AFC. The amide form of AFC when conjugated to the peptide sequence is not fluorescence; upon cleavage of the substrate by caspase-3, free AFC emits a yellow-green fluorescence ($\lambda_{\text{max}} = 505$ nm), which could be quantified by flow cytometry and fluorescence microscopy. Comparison of the fluorescence of AFC from an induced apoptotic cell sample with an uninduced control allowed the determination of the fold increase in caspase-3 activity.

6.3.1 Apoptosis induction

In order to induce cell apoptosis, a well known protein kinase inhibitor such as staurosporine was used. Staurosporine can rapidly trigger both the morphological changes and intranucleosomal DNA fragmentation typical of apoptosis.\textsuperscript{236} As the apoptosis-regulating caspases are activated during the apoptotic process the caspase-hydrolizable substrate (Ac-DEVD-AFC) is cleaved between an aspartic acid and coumarin by caspase-3, thus releasing the fluorescent dye (Scheme 6.3).
6.3.2 Flow cytometry results

To test the efficiency of polystyrene microspheres as potential devices for the study and monitoring of enzymatic activity a pilot experiment in HeLa cells was performed. In such study, enzymatic activity was based through evaluation of an enzyme involved in apoptosis as caspase-3 following ‘beadfection’ with different samples and subsequently apoptosis-induced by staurosporine. HeLa cells were co-cultivated with several samples of microspheres at a single concentration (43 µg/mL) for 24 hours before apoptosis was induced. After 3 hours of staurosporine exposure, cells were washed and a flow cytometry study was carried out.
Figure 6.3: Flow cytometry. A) MFI for AFC fluorescence in HeLa cells treated with different samples. The errors bar represent the standard error of the average. n=4. Statistical studies performed by Dunnett's test (α=0.05) showed that sample 6.7+ST was statistically significance (6.7 and 6.4+ST as reference). B), C) and D) Histograms of HeLa cells beadfected with different samples. (Unt cells= Untreated cells, ST= Staurosporine, 6.4= unlabelled beads without caspase substrate, 6.7= unlabelled beads loaded with caspase substrate, DEVD= unconjugated substrate free in solution.)

As illustrated in Figure 6.3 it seems that, as expected, for cells which were beadfected with microspheres conjugated to the apoptotic-sensing agent (DEVD-AFC) and subsequently treated with staurosporine (6.7 + ST), AFC was released affording an MFI increase of about 7-fold to untreated cells (Figure 6.3/A-C), however when such microspheres were not induced to apoptosis by staurosporine and consequently caspase-3 was not over expressed the peptide substrate could not be cleaved, there was no AFC release and therefore no MFI increase was detected (6.7). Likewise, when cells were beadfected with microspheres which were not coupled to the fluorogenic substrate (6.4) or cells incubated with unconjugated substrate in solution (DEVD) fluorescence intensity seems not to increase significantly after staurosporine treatment (Figure 6.3/A,B and D).
Based of these results DEVD-AFC loaded-microspheres function as an apoptosis-sensing agent was clear. However, it was considered that a more accurate assessment of this probe would be obtained by evaluating fluorescently labelled microspheres. For this purpose, previously prepared samples labelled with Cy5 (6.6) were tested for their ability to quantify staurosporine-induced apoptosis in HeLa cells. Equally, beads which were not coupled to the fluorogenic substrate DEVD-AFC (6.3) were used as negative control. As the previous experiment, HeLa cells were either apoptosis-induced with staurosporine or not to evaluate the effect of such treatment in terms of caspase-3 activity.

**Figure 6.4: Flow cytometry.** A) MFI for AFC fluorescence in HeLa cells treated with different samples. Errors bar representing the standard deviation. n=4. Statistical studies performed by Dunnett's test (α=0.05) showed that sample 6.7+ST and 6.6+ST were statistically significance (6.7, 6.4+ST, 6.3+ST and 6.6 as reference). B), C) and D) Histograms of HeLa cells beadfected with different samples. (Unt cells= Untreated cells, ST= Staurosporine, 6.3= Cy5-labelled beads without caspase substrate, 6.6= cy5-labelled beads loaded with caspase substrate, DEVD= unconjugated substrate free in solution).

As shown in Figure 6.4, HeLa cells which were treated with microspheres conjugated to the apoptotic fluorogenic probe and induced to apoptosis (6.6+ST) showed an increase in fluorescence intensity of 6-fold as compared with samples.
which were not apoptosis-induced (6.6) and the same result was obtained when cells were treated with staurosporine following beadfection with microspheres without the caspase-3 substrate (6.3) or with unconjugated substrate (DEVD).

This system employing dual-functionalisation (Cy5-co-Caspase-3 Substrate) allows co-localising fluorescent labelled beads (6.6) in cells which showing caspase activity. With that in mind, obtained results from flow cytometry experiment were analysed by Flowjo® and represented in Figure 6.5 as dot plots.

**Figure 6.5: Flow cytometry.** Dot plots representation of HeLa cells treated with staurosporine following beadfection. A) Untreated cells; B) Cy5-labelled beads without caspase substrate treated with staurosporine (6.3+ST); C) Unlabelled beads loaded with caspase substrate (6.7+ST); D) Cy5-labelled beads loaded with caspase substrate (6.6+ST) and; E) Combination of A, B, C and D dot plots.

**Figure 6.5** shows the cell population distribution when Cy5 fluorescence intensity (using a 633 nm laser for excitation and a 660/20 nm band pass emission filter) is represented versus AFC fluorescence intensity (using a 405 nm laser for excitation
and a 510/50 nm band pass emission filter). As such, in Figure 6.5/A untreated cells are gated as Cy5 and AFC negative (Q4 = neither Cy5 nor AFC signal). However when cells were beadfected with 6.3, the cell population is Cy5 positive and AFC negative (Q1 in Figure 6.5/B), suggesting successful cell beadfection but without AFC release. This was expected as 6.3 was a sample which was not conjugated to DEVD-AFC. Then, when cells were beadfected with 6.7 (unlabelled beads with DEVD-AFC) cell population resulted Cy5 negative and AFC positive (Q3 in Figure 6.5/C), expected result showing AFC signal because of the substrate cleavage. Next, when cells were beadfected with 6.6 (Cy5 labelled beads conjugated to DEVD-AFC) population migrated till Cy5 positive and AFC positive area (Q2 in Figure 6.5/D), suggesting efficient cell beadfection and substrate cleavage releasing AFC. Thus, can be suggested that microspheres loaded with caspase-3 specific substrate can be used to monitor apoptosis in situ. This strategy allows the intracellular delivery of the fluorogenic substrate DEVD-AFC to study enzymatic activity of an enzyme involved in apoptosis as caspase-3. At the same time fluorescent labelling allow to track the location of the conjugate inside the cell.

6.3.3 Fluorescent microscopy results

To support our findings based in flow cytometry analysis, HeLa cells which were treated with different microspheres samples were analysed by fluorescence microscopy (Figure 6.6).
As it can be seen in Figure 6.6/1 when cells were treated with fluorescein-labelled microspheres delivering the apoptotic sensing probe (6.5) but which were not induced to apoptosis the DEVD-AFC could not be cleaved and consequently no AFC was detected in DAPI filter, however using this FITC filter the fluorescein-labelled microspheres could be seen. In Figure 6.6/2 which were treated with unlabelled beads (6.7) loaded with DEVD-AFC and apoptosis was induced, the morphology of...
the cells was affected and can be seen that the AFC was released, therefore can be suggested that the apoptosis-sensing probe was cleavage by caspase-3 when apoptosis was induced.

6.4-Conclusions

In summary, microspheres loaded with a fluorogenic substrate specific for caspase-3 enzyme have been successfully employed to monitor in situ intracellular enzymatic activity. The real-time monitoring of caspase activity was enabled because the fluorescence of microspheres-DEVD-AFC conjugates was activated by caspase-3 only in cells which were induced to apoptosis by staurosporine. No fluorescence was observed in treated cells with the fluorogenic substrate but in which apoptosis was not induced or in cells induced to apoptosis but in which the substrate was not attached to the microsphere (naked), confirming cleavage specificity. Following this can be clearly seen how cells could be co-localise depending if they became apoptotic or not, additionally cells which were beadfected with either Cy5-labelled or unlabelled beads could be sorted. As said previously, so far the majority of enzymatic activity studies had been performed using cell lysate, being a lack of knowledge about enzymatic activity assays in situ. In this project, a new competitive assay for measuring enzymatic activity and behaviour of a particular enzyme inside the cell, apoptotic cells in our experiments, has been developed providing in this sense valuable information and also displaying a more realistic picture of the genuine behaviour of the enzyme. To conclude can be confirmed that a proof of concept for the monitoring of enzymatic activity has been successfully developed opening a wide range of possibilities for microspheres-based enzymatic activity studies intracellularly.
CHAPTER 7: Experimental section

7.1 General information

7.1.1 Chemistry equipment

^H and ^13C-NMR spectra were measured on a Bruker DMX 500 spectrometer in the solvents indicated at 298 K. Chemical shifts are reported as δ in units of parts per million (ppm) relative to the indicated solvent d4 (δ 3.30, septet in ^1H and 49.15, septet in ^13C-NMR). Multiplicities in ^1H-NMR are reported as follows: s (singlet), d (doublet), t (triplet), q (quintuplet), dd (doublet of doublets), m (multiplet), br (broadened). Coupling constants are reported as a J value in Hertz (Hz). The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values.

Analytical HPLC was conducted on an Agilent 1100 series HPLC system coupled to a Polymer Lab PL-ELS 1000 Evaporative Light Scattering (ELS) detector with UV detection at 220, 254, 260, 282 and 495 nm, Supelco’s Discovery® C 18 (50 mm x 2.1 mm x 5 µm) was used, method S50D. Elution was performed with Solvent A (0.1% formic acid in HPLC-grade deionised water) and Solvent B (0.1% formic acid in HPLC-grade methanol) at 1 mL x min with a gradient of 5 to 95% B over 3 min, followed by 1 min isocratic at 95% B and ending with a gradient of 95 to 5% B over 1 min, then 1 min isocratic at 95% A.

Low resolution mass spectra were obtained using a Hewlett Packard LCMS 100 Chemstation with G1946B mass detector.

High resolution mass spectra were recorded by the MS Department of the University of Edinburgh on a Thermo MAT 900 XLP high resolution, double focussing mass spectrometer.

Thin layer Chromatography (TLC) was carried out using Merck silica gel 60 F_{254} (0.25 mm) foil-backed plates with visualisation by ultraviolet light. Flash
chromatography was carried out using Silica 60 Å, particle size 35-70 micron under positive pressure.

**Melting points** were determined using a Gallenkamp melting point apparatus and are uncorrected.

**Infrared (IR)** spectra were recorded neat using a Bruker Tensor 27 FT-IR spectrometer.

**Ultraviolet UV/Visible Spectrophotometry** was recorded on an Agilent 8453 spectrophotometer using 1 cm pathlength fused silica cuvettes using a 60% EtOH blank and recording absorbance at 570 nm for quantitative ninhydrin tests and a 20% piperidine /DMF blank measuring absorbance at 302 nm for Fmoc tests.

Scanning Electron Microscopy (SEM) analysis was performed on a Philips XL30CP with PGT Spirit X-ray analysis and HKL Channel5 Electron Backscatter Diffraction (EBSD) systems.

**Dynamic Light Scattering (DLS) and Zeta potential** were measured on a Zetasizer Nano ZS ZEN 3500 in molecular biology grade water in a disposable sizing cuvette for size measurements or clear disposable zeta cuvette for zeta potential measurements.

7.1.2 Biology equipment

**Cell cultures** were performed in a HERAcell 150 incubator from Heraeus.

**Cell experiments** were carried out in a HERAsafe KS 18 class II negative-flow cabinet from Heraeus.

**Flow cytometry** was performed on a BD Biosciences FACS Aria® system using the Flowjo® 7.5 software for analysis.
Flow cytometric sorting was done using a MoFlo MLS high-speed sorting flow cytometer (Beckman Coulter).

Cell viability was assessed using a Bio-Rad microplate reader (Version 1.15) measuring absorbance at 570 nm.

Microscopy and Confocal Microscopy were performed on a Zeiss Axiovert 200M pseudo confocal microscope with a 100 W Hg lamp or a Leica Inverted Confocal Microscope with a DM IRE2 microscope stand and analysed using Improvision Volocity acquisition software.

Carboxylated Cy5 fluorophore was from Bradley group.
7.2 General protocols

7.2.1 Washing of monomers

Prior to use, monomers divinylbenzene (DVB) and styrene were washed to clear any presence of 4-tert-butylcatechol inhibitor from them. Styrene (50 mL) and DVB (50 mL) were washed with 25% aqueous NaOH (2 x 100 mL, then 2 x 50 mL) and deionised water (2 x 100 mL, then 2 x 50 mL). The monomers were then dried over MgSO₄, filtered off and stored at 4°C.

7.2.2 Washing of the latex microspheres

For microspheres preparation, see the experimental to Chapter 2.

For 0.2 µm where polymerisation was performed on polymerisation vessel (70 mL of polymerisation mixture), microspheres were isolated by centrifugation in 1 mL aliquots for 1 hour at 17500 rpm and supernatant discarded, after centrifugation microspheres were washed sequentially with methanol (2 x 50 mL) and deionised water (2 x 50 mL). For 0.5 and 2 µm where polymerisation was performed on a Radley carousel (5 mL of polymerisation mixture), microspheres were isolated by centrifugation at 8000 rpm for 5 minutes in 15 mL capacity centrifuge tubes and supernatant discarded. Then, microspheres were washed sequentially with methanol (2 x 5 mL) and deionised water (2 x 5 mL). After washing, microspheres were stored at 4°C in deionised water. Prior to on-bead reactions, the microspheres were isolated by centrifugation and conditioned in the solvent of the reaction (3 x 1 mL).

7.2.3 Characterisation of the latex microspheres

7.2.3.1 Solid content of the emulsion

A known mass of a suspension of polystyrene latex microspheres (1-2 g, suspended in water) was placed in a Petri dish, covered with aluminium foil, dried at 80°C for 15 hours and reweighed to give the mass of microspheres. The solid content was then calculated according to the following equation:

\[
\%sc = \left( \frac{m}{V_s} \right) \times 100
\]

Equation 7.1: Analysis of microsphere solid content of suspension.
Where:

- \( sc \) = Weight % solid,
- \( V_s \) = Volume of suspension (mL),
- \( m \) = Mass of microspheres (g),

(e.g. 7% \( sc \) = 0.35 g microspheres for 5 mL suspension).

### 7.2.3.2 Preparation of polystyrene latex microspheres for scanning electron microscopy

Microspheres as a suspension in water (100 µL) were spread on a carbon disk (11 mm), mounted on SEM pins and dried at 50°C in the vacuum oven overnight. The samples were gold sputter coated prior to analysis (between 20-25 nm layer).

### 7.2.3.3 Measurement of particle size distribution

A sample of polystyrene microspheres in water (5 µL) was suspended in 995 µL of deionised water and placed in a disposable sizing cuvette.

### 7.2.3.4 Measurement of Zeta Potential

A sample of polystyrene microspheres in water (1 µL) was suspended in 999 µL of 10% PBS pH 7.4 solution in double processed tissue cultured water (Sigma-Aldrich) and place in a clear disposable zeta cell.

### 7.2.3.5 Calculation of coefficient of variation

The coefficient of variation was calculated according to the results obtained from the measurement of the particle size distribution using the following equation:

\[
CV = \frac{SD}{d}
\]

**Equation 7.2:** Determination of Coefficient of variation of microspheres suspension.

Where:

- \( CV \) = Coefficient of variation (size distribution of the microspheres population),
- \( SD \) = Standard deviation (µm)
- \( d \) = Mean diameter (µm)
7.2.3.6 **Calculation of Number of particles per gram**

\[
N = \frac{6 \times 10^{12}}{(\pi \times \rho_s \times d^3)}
\]

**Equation 7.3:** Calculation on the number of particles.

Where:

- \(N\) = Number of particles/g for dry powder,
- \(\rho_s\) = Density of solid spheres (g/cm\(^3\)), which is 1 g/cm\(^3\) for polystyrene,
- \(d\) = Mean diameter (µm),
  
  (e.g. \(N = 3.7 \times 10^{15}\) for 0.5 µm polystyrene beads).

7.2.3.7 **Qualitative ninhydrin test**

Small samples of microspheres in MeOH (12 µL, 4% sc) in a 0.5 mL capacity eppendorf were washed with methanol and centrifuged after which 6 µL of reagent A and 2 µL of reagent B were added. The mixture was heated at 100°C for 3 minutes and a resulting blue or yellow color indicated, respectively, a positive or negative test.

![Figure 7.1: Ninhydrin test results](image)

7.2.3.8 **Quantitative ninhydrin test**

Small samples of microspheres suspension (12 µL) were washed with methanol, centrifuged and the solvent discarded. Ninhydrin reagents were added to the beads (6 µL of reagent A and 2 µL of reagent B) and heated at 100°C for 3 minutes which was followed by the addition of a 60% aqueous ethanol solution (0.4 mL) and centrifugation. The microspheres were washed three times with the above ethanol solution, the supernatants were combined and the amount of primary amine on the resin was then calculated using the following equation:
Loading (mmol/g) = \( \frac{(A_{570} \times V)}{(\varepsilon_{570} \times W)} \times 1000 \)

Equation 7.4: Analysis of amine loading by Ninhydrin test

Where:
- \( A_{570} \) = Absorbance measured at 570 nm,
- \( \varepsilon_{570} \) = Molar extinction coefficient (15000 M\(^{-1}\)cm\(^{-1}\)),
- \( V \) = Volume of combined supernatants (mL),
- \( W \) = Mass of resin (mg).

7.2.3.9 Fmoc test

After washing, Fmoc-(aa)\(_n\)-microspheres were resuspended in 20% piperidine in DMF (3 x 20 minutes) after which the beads were washed by centrifugation three times, the supernatants combined and the loading was calculated according to the following equation:

Loading (mmol/g) = \( \frac{(A_{302} \times V)}{(\varepsilon_{302} \times W)} \times 1000 \)

Equation 7.5: Analysis of amine loading by Fmoc test

7.2.3.10 Ninhydrin Solutions

**Reagent A**

Solution 1: Reagent grade phenol (40 g) was dissolved in absolute ethanol (10 mL) with warming and then stirred over Amberlite mixed-bed resin MB-3 (4 g) for 45 minutes. The mixture was then filtered.

Solution 2: Potassium cyanide (65 mg) was dissolved in water (100 mL). A 2 mL aliquot of this solution was diluted with pyridine (freshly distilled from ninhydrin) and stirred over Amberlite mixed-bed resin MB-3 (4 g). The solution was filtered and mixed with solution 1 to form reagent A.

**Reagent B**

Ninhydrin (2.5 g) was dissolved in absolute ethanol (50 mL).
7.2.4 Amide couplings into microspheres using activate esters

Unless otherwise stated amino functionalised microspheres (1 mL; 1 equiv) were washed in DMF (3 x 1 mL) and suspended in DMF (1 mL). Separately, the carboxylic acid nature compound (5 eq) was dissolved in DMF (1 mL), then oxyma (5 eq; Apollo Scientific) was added and the solution mixture mixed for 4 minutes at rt before the addition of DIC (5 eq; Sigma-Aldrich) and mixed for 8-10 minutes at rt. The solution mixture was then added to amino microspheres and suspension mixed on the Thermomixer at 1400 rpm for 2 hours at 60°C.

7.2.5 DIC couplings into microspheres using acid anhydrides

Unless otherwise stated amino functionalised microspheres (1 mL; 1eq) were washed in DMF (3 x 1 mL) and suspended in DMF (1 mL). Separately, the carboxylic acid nature compound (Adipic acid or 3,3’-Dithiodipropionic acid; 10 eq) was dissolved in DMF (1 mL), then DIC (5 eq; Sigma-Aldrich) was added and the solution mixture mixed for 8-10 minutes at rt. The solution mixture was then added to amino microspheres and DIPEA (0.1 eq) was added. The suspension was mixed on the Thermomixer at 1400 rpm for 2 hours at 60°C.

7.2.6 Preactivation of Carboxyfunctionalised beads

Unless otherwise stated carboxy functionalised microspheres (1 mL; 1eq) were washed in DMF (3 x 1 mL) and suspended in DMF (1 mL). Next, DIC (5 eq) was added and the suspension mixed for at least 2 hours at rt. After this time beads were collected by centrifugation and a previously prepared solution of amino nature compound in DMF (1 mL) was added, subsequently DIPEA (0.1 eq) was also added. The suspension was mixed on the Thermomixer at 1400 rpm for 2 hours at 60°C.

7.2.7 Fmoc deprotection on microspheres

Unless otherwise stated Fmoc deprotection was achieved by treating microspheres with 20% piperidine/DMF (1 mL; 3 x 20 minutes). Microspheres were obtained by centrifugation and subsequently washed with DMF (3 x 1 mL), MeOH (3 x 1 mL), deionised water (3 x 1 mL) and finally DMF (3 x 1 ml). Next, ninhydrin
test as described in section 7.2.3.7 was performed to check the presence of primary amine or not.

7.2.8 Dde derotation on microspheres in presence of Fmoc

7.2.8.1 Deprotection mixture preparation

1.25 g (1.80 mmol) of NH2OH.HCl and 0.918 g (1.35 mmol) of imidazole were suspended in 5 mL of NMP, and the mixture was sonicated until complete dissolution. Just before reaction, 5 volumes of this solution were diluted with 1 volume of DMF.

7.2.8.2 Dde deprotection

Dde deprotection was facilitated by treating microspheres with the solution mixture prepared in section 7.2.8.1 (1 mL) for 1 hour at rt on a rotary wheel, then microspheres were washed with DMF (1 mL) and the entire process repeated under the same conditions. Microspheres were obtained by centrifugation and subsequently washed with DMF (3 x 1 mL), methanol (3 x 1 mL), deionised water (3 x 1 mL) and finally DMF (3 x 1 mL). Next, ninhydrin test as described in section 7.2.3.7 was performed to check the presence of primary amine or not.

7.2.9 Biolab protocols

7.2.9.1 Cell culture

B16F10 murine melanoma cells and HEK 293 T (human embryonic kidney) were grown in Dulbecco’s Modified Eagles Medium (DMEM, Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS, Biosera), 4 mM L-glutamine and 100 units/mL penicillin/streptomycin (complete Dulbecco’s medium) in T25 cell culture flask until 80% confluency. HeLa (human cervical carcinoma) cells were grown similarly in Roswell Park’s Memorial Institute (RPMI-1640, Sigma-Aldrich) medium supplemented with 10% foetal bovine serum, 4 mM L-glutamine and 100 units/mL penicillin/streptavidin (complete RPMI medium) in T25 cell culture flask until 80% confluency. E14tg2a and Oct-4Gip mouse embryonic stem cells were grown in Glasgow’s Modified Eagle Medium (GMEM, Sigma-Aldrich) supplemented with 10% Foetal Calf Serum (FCS), 0.1 mM non-essential aminoacids
(NEAA, Gibco), 2 mM L-Glutamine, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol and 1000 units/mL Leukaemia Inhibitory Factor (LIF, Gibco) (GMEM complete medium) in T-25 flasks coated with 0.1% gelatine/PBS until 70-80% confluency. SH-SY5Y neuroblastoma cells were grown in (DMEM)/F-12 (Ham) 1x supplemented with 75 mL FBS, 5 mL of penicillin/Streptomycin/L-Glutamine and 5 mL of non-essential amino acids (Complete DMEM/F12 (1:1) medium) in T25 cell culture flask until 70% confluency. At this stage, the old growth medias was removed and the cells were washed with PBS (5 mL) and harvested via trypsination (1% trypsin/EDTA, Gibco; 0.25% for SH-SY5Y cells) (1 mL) at 37° C for 3-4 minutes. The detached cells were collected in fresh growth media (4 mL) and diluted to the appropriate cell density for experiments in fresh growth media. An aliquot (about 1-1.5 mL) was re-seeded to a T-25 flask for re-growth.

7.2.9.2 Cell counting

Cell densities were determined by haemocytometry. 10 µL of cell suspension detached from a T-25 flask were resuspended in 40 µL 0.2% trypan blue and pipetted into a Bright Line™ haemocytometer (an etched glass chamber that holds a quartz coverslip in a total surface area of 9 mm²). Cell concentrations and densities required for experiments were determined by Equation 7.6 and 7.7.

\[
\text{Concentration (cell/mL)} = (N/Q) \times 5 \times 10^6
\]

\textbf{Equation 7.6: Concentration of cell/mL by haemocytometry}

Where:
- \(N\) = Total number of cells counted,
- \(Q\) = Number of quadrants counted,

\[
V_{\text{exp}} (\text{mL}) = (V_{\text{Tot}} \times C_{\text{Well}}) \times (1000/V_{\text{Well}})/C_{\text{Tot}}
\]

\textbf{Equation 7.7: Volume of cells detached from T-25 flask}

Where:
- \(V_{\text{exp}}\) = Volumen required in an experiment,
- \(V_{\text{Tot}}\) = Total medium volume,
- \(C_{\text{Well}}\) = Concentration per well,
V_{Well} = Volume required per well,
C_{Tot} = Concentration of cells/mL as calculated in Equation 7.6

7.2.9.3 Beadfection of adherent cells

Cells were suspended to the appropriate cell density in fresh growth media before seeding onto polystyrene well-plates (Nunc). Cells were incubated (37°C/5% CO\(_2\)) for 24 hours to allow adhesion. Subsequently, microspheres were dispersed in fresh growth media to a stated concentration. The old media was removed from cells and substituted with fresh media containing microspheres. Cells were incubated in the presence of microspheres (37°C/5% CO\(_2\)) for incubation time stated prior to analysis.

7.2.9.4 Analysis of cellular uptake/fluorescence intensity by flow cytometry

In order to prepare samples for flow cytometry analysis, the old media was removed from cell cultures and the cells were washed with PBS and trypsinated at 37°C for 3-4 minutes. Once detached, cells were collected in growth media and the cell pellet collected by centrifugation (1200 rpm, 4 min). Then, cells were re-suspended in 300 µL of 2% FBS/PBS or 300 µL of 0.2% trypan blue/HBSS (where extracellular quenching of fluorescein was desired). Samples were analysed by flow cytometry according to the experiment under investigation. Results were analysed by Flowjo® software.

7.2.9.5 Analysis of cellular uptake/fluorescence intensity by microscopy

Cells were cultured and beadfected as described in sections 7.2.9.1 and 7.2.9.3. In preparation for microscopy, cells were washed with PBS and, if no staining was required (see below), microscopy was directly performed in 2% FBS/PBS or 0.2% Trypan blue/HBSS.

Membrane staining with DilC\(_{18}\)

Following washing with PBS 75 µL of PBS containing DilC\(_{18}\)(5)-DS (Molecular Probes) at a final concentration of 2 µg/ml 5 minutes at 37°C and then for an additional 5 minutes at 4°C. Next, the solution is replaced by PBS.
Nuclei Staining with Hoechst 33342

Cell culture medium was removed and cell washed with PBS, then cells were treated with Hoechst 33342 (1 µ/mL, Sigma-Aldrich) for 10 minutes at 25°C. Cells were washed three times with PBS. Microscopy was performed in 2%FBS/PBS

Cellular Fixation

Cells which were washed with PBS were fixed with 4% para-formaldehyde (Sigma-Aldrich) solution (20 minutes, 25°C) and washed subsequently at least 5 times with PBS. Microscopy was performed in PBS.

7.2.9.6 Cell viability

Cells were seeded as described in section 7.2.9.1 and seeded onto a 96-well plates at a density of 1 x 10^4 cells/well (volume per well:100 µL). The last row of the well-plate was used as a blank (no cells seeded). Cells were incubated (37°C/5% CO₂) for 24 hours prior to beadfection (Section 7.2.9.3) at concentration of 75 and 150 µg/mL. After stated time, the old media was removed and replaced with fresh phenol red-free culture media (supplemented as described in section 7.2.9.1) (90 µL) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) (10 µL). Cells were incubated for at least 4 hours at 37°C/5% CO₂. After this time, MTT solubilisation solution (10% Triton-X, 0.1 N HCl in isopropanol) (100 µL) was added and the 96-well plates was gently shaken for at least 1 hour. Absorbance was measured at 570 nm. Untreated cells were considered to be 100% viable and cell viability was calculated from Equation 7.8.

\[
\% \text{Viable cells} = \frac{A_{570 \text{ sample}}}{A_{570 \text{ reference}}}
\]

Equation 7.8: Viability of cells by MTT assay.

Where:

\[A_{570 \text{ sample}}\] = Absorbance at 570 nm of the sample (treated cells)

\[A_{570 \text{ reference}}\] = Absorbance a 570 nm of the reference sample (untreated cells)
7.3 Experimental for Chapter 2

7.3.1 Synthesis of 4-vinylbenzylphthalimide (2.3)

\[
\text{(2.3)}
\]

\[N-p\text{-vinylbenzylchloride (15.3 g, 100 mmol, 1eq) and potassium phthalimide (18.5 g, 100 mmol, 1 eq) were dissolved in DMF (50 mL) resulting in an orange solution/suspension. Magnetic stirring for about 17 hours and a consequent colour change to pale yellows was noted. Upon completion, the reaction mixture was poured into a 1.7 N sodium hydroxide (1.000 mL) which facilitated precipitation of the crude product. The white powdery precipitated was isolated by vacuum filtration before re-solubilisation in ethyl acetate (300 mL) forming an opaque off-white solution. The solvent was removed and subsequently recristallised in MeOH (200 mL). The recristalisation process was repeated twice giving crystals after filtration. (17.49 g, 66.23 mmol, 39.2%).}

\[\text{Mp: 94 ºC (Lit.}^{237} \text{ 107 – 108ºC)}\]

\[\text{Rf 0.45 (3:1, hexane/ EtOAc)}\]

\[\text{HPLC (S50D, } \lambda=220 \text{ nm): purity 97.6%, retention time 4.07 minutes}\]

\[\text{\^H NMR (250 MHz, CDCl}_3\text{) } \delta \text{ (ppm): 4.80 (s, 2H), 5.23 (d, } J=11.00 \text{ Hz, 1H), 5.71 (d, } J=17.60 \text{ Hz, 1H), 6.68 (dd, } J=11.00 \text{ Hz, 17.60 Hz, 1H), 7.36 (d, } J=8.00 \text{ Hz, 2H), 7.40 (d, } J=8.00 \text{ Hz, 2H), 7.71 (dd, } J=3.00, 6.00 \text{ Hz, 2H), 7.83 (dd, } J=3.00 \text{ Hz, 6.00 Hz, 2H).}^{13}\text{C NMR (63 MHz, CDCl}_3\text{) } \delta \text{ (ppm): 41.4, 114.3, 123.5, 126.6, 129, 132.2, 134.1, 135.9, 136.4, 137.3, 168.1}}\]
7.3.2 Synthesis of 4-vinylbenzylamine (2.4)²³⁷

\[
\begin{align*}
\text{N-p-vinylbenzylphtalimide (8 g, 30 mmol, 1 eq), was suspended in nitrogen-purged} \\
\text{ethanol (50 mL) and heated to reflux under nitrogen until dissolution. After stirring} \\
\text{for 30 minutes, hydrazine hydrate (3 mL, 60 mmol, 2 eq) was added via a self-} \\
\text{equilibrating dropping funnel resulting in a color change to bright yellow. After 5} \\
\text{minutes a gelatinous off white precipitate appeared. Upon a reaction time of 17 hours} \\
\text{the white gelatinous by-product was removed by filtration and the filtrated} \\
\text{concentrated in vacuo. The crude product obtained via concentrate of the filtrates} \\
\text{was treated with aqueous potassium hydroxide (75 mL, 1.5 M) and the aqueous} \\
mixture extracted with diethyl ether (2 x 100 mL). The organic fractions were} \\
\text{combined and washed with 2% potassium carbonate before drying over MgSO₄} \\
\text{and concentrated in vacuo to give (2.4) as a colourless oil (2.83 g, 70%).} \\
\text{Rf (diethyl ether): 0.24} \\
\text{HPLC (S50D, λ=220 nm): purity 100%, retention time 2.24 minutes} \\
\text{m/z (ES+) 133.9 (100%) [M+H]⁺} \\
^1\text{H NMR (250 MHz, CD₃OD) δ (ppm): 3.78 (s, 2H), 4.93 (s, 2H), 5.22 (d, } \\
\text{J = 11.00 Hz, 1H), 5.77 (d, J = 17.60 Hz, 1H), 6.74 (dd, J = 11, 17.60 Hz, 1H), 7.31 (d,} \\
\text{J = 8.00 Hz, 2H), 7.42 (d, J = 8.00 Hz, 2H).} \\
^{13}\text{C NMR (63 MHz, CD₃OD) δ (ppm): 46.3, 113.5, 127.2, 128.5, 137.5, 137.8, 143.2.}
\end{align*}
\]
7.3.3 Synthesis of 4-vinylbenzylamine hydrochloride (2.5)$^{50}$

Vinylbenzylamine (2.4) (1.5 g, 11.26 mmol, 1eq) was dissolved in methanol (6 mL) and cooled to 0°C before isopropanol (6 mL, HCl 6N) was added drop wise over half and hour time. The resulting was white precipitate which was collected via filtration and dried in vacuo to give the desired product (2.5) as a white solid (1.56 g, 9.20 mmol, 82%).

Mp: Decompose at 202 °C

**HPLC (S50D, λ=220 nm):** purity 100%, retention time 2.30 minutes

$^1$H NMR (500 MHz, CD$_3$OD) $\delta$ (ppm): 4.10 (s, 2H), 5.29 (d, $J=11.30$ Hz, 1H), 5.84 (d, $J=18.10$ Hz, 1H), 6.76 (dd, $J=10, 90, 17.6$ Hz, 1H), 7.48 (dd, $J=8.20, 35.6$ Hz, 4H). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ (ppm): 44.10, 115.37, 127.92, 130.37, 133.82, 137.38, 139.93.

7.3.4 Synthesis of 500 nm polystyrene microspheres, (2.6)$^{116}$

PVP (Mw 29,000, 0.05g, 1.7µmol, Sigma-Aldrich) was dissolved in 92% ethanol/8% water for a final volume of 5 mL, and deoxygenated via nitrogen bubbling. AIBN (7 mg, 42.4 µmol) was dissolved in styrene (freshly washed, 0.5 mL) with VBAH (7 mg, 41.3 µmol) and DVB (freshly washed, 4.65 µL). The dispersion was deoxygenated with nitrogen bubbling before addition to the PVP/Ethanol solution. The mixture was stirred under nitrogen for 2 hours before heating to 65°C for 16 hours. Microspheres were obtained by centrifugation (8500 rpm, 5 minutes) and washed with methanol (2 x 5 mL) and water (2 x 5mL). Finally, microspheres were stored in water (5 mL) at 4°C.
Particle size distribution: mean diameter: 458.7 nm; S.D: 0.069 µm; C.V: 14.4%
Loading (Ninhydrin): 0.017 mmol/g
Nº particules per gram: 1.727 x 10^{24}
sc: 4%, 4 mg microspheres in 100 µL solution

7.3.5 Synthesis of 2µm polystyrene microspheres, (2.7)\textsuperscript{116}

\begin{center}
\text{H}_2\text{N-}
\end{center}

(2.7)

For the synthesis of 2 µm microspheres, same protocol that for 500 nm was followed with the only different that in this case the solvent was pure ethanol instead of a mixture ethanol/water. The microspheres were collected also by centrifugation and stored in water at 4°C as described above.

Particle size distribution: mean diameter: 1783.9 nm; S.D: 0.3 µm; C.V: 16.9%
Loading (Ninhydrin): 0.076 mmol/g
Nº particules per gram: 2.94 x 10^{23}
sc: 2%, 2 mg microspheres in 100 µL solution

7.3.6 Synthesis of 200 nm polystyrene microspheres, (2.8)\textsuperscript{116}

\begin{center}
\text{H}_2\text{N-}
\end{center}

(2.8)

Water (50 mL), styrene monomer (inhibitor free), DVB (inhibitor free), magnesium sulphate and VBAH were stirred together at room temperature, after nitrogen being bubbled through the solution for 30 minutes, and then heated to 80°C and stirred (350 rpm) for 20 minutes at this temperature. 2,2’-azobis(2-methylpropionamidine) dihydrochloride (initiator) in water (1 mL) was added and the mixture stirred at 80°C for 15 hours. The latex microspheres were collected by centrifugation (12,000 rpm), washed successively with methanol (2 x 50 mL) and water (2 x 50 mL) by centrifugation at 12,000 rpm for 2 hours and stored in water at 4°C.

Particle size distribution: mean diameter: 190 nm; S.D: 0.035 µm; C.V: 19.0%
Loading (Ninhydrin): 0.026 mmol/g


**Nº particules per gram:** $2.20 \times 10^{24}$

**sc:** 4%, 4 mg microspheres in 100 µL solution

### 7.3.7 Synthesis of Fmoc-4,7,10-trioxa-1,13-tridecanediamine succinamic acid (polyethylene glycol type spacer) (2.18)

![Chemical Structure](image)

A solution of succinic anhydride (1 g, 10 mmol, 1 eq) dissolved in acetonitrile (25 mL) was added drop wise over 1 hour under strong magnetic stirring to 4,7,10-trioxa-1,13-tridecanediamine (2.2 g, 10 mmol, 1 eq) in acetonitrile (50 mL) and stirred for a further 3 hours. The liquid phase was decanted and discarded once the waxy product had settled. The product was redissolved by the addition of an acetonitrile:H$_2$O (1:1) solution (100 mL) and the solution was chilled to 0°C prior to a drop wise addition of 9-fluorenylmethoxycarbonyl-succinimide (Fmoc-OSu) (4.38 g, 13 mmol, 1.3 eq) in acetonitrile (25 mL) under vigorous magnetic stirring during which time the pH was kept at 8/9 by addition of DIPEA. After 12 hours reaction at room temperature the solvents were removed *in vacuo* and the residue was dissolved in a 5% NaHCO$_3$ aqueous solution (100 mL) and washed with EtOAc (3 x 50 mL). The aqueous phase was then acidified to pH 2 with 37% HCl, extracted with ethyl acetate (3 x 50 mL) and the organic phases were combined, washed with brine, dried over MgSO$_4$ and concentrated *in vacuo* to afford a yellowish oil which was dissolved in hexane and purified by flash chromatography column (DCM:MeOH; 10:1) to afford the pure final compound as a sticky colorless product (3.51 g, 6.59 mmol, 65%).

**HPLC (S50D, λ=220 nm):** purity 100%, retention time 8.4 minutes.

**m/z (ES+) 565.5 (100%) [M+Na]$^+$

**$^1$H NMR** (500 MHz, CD$_3$OD) $\delta$ (ppm): 1.71-1.78 (m, 4H), 2.46 (t, $J = 6.54$, 2H), 2.62 (t, $J = 7.54$, 2H), 3.26-3.34 (m, 4H), 3.49-3.65 (m, 12H), 4.20 (m, 1H), 4.38-
4.44, (m, 2H), 5.30 (bs, 1H), 6.77 (bs, 1H), 7.29 (t, \( J = 7.41, 2H \)), 7.38 (t, \( J = 7.39, 2H \)), 7.59 (d, \( J = 7.48, 2H \)), 7.75 (d, \( J = 7.52, 2H \)). $^{13}$C NMR (500 MHz, CD$_3$OD) \( \delta \) (ppm): 28.52, 29.34, 30.40, 30.98, 38.22, 38.80, 47.25, 66.40, 69.23, 69.88, 66.92, 70.02, 70.26, 70.35, 119.90, 125.02, 126.98, 127.61, 141.25, 143.95, 156.62, 172.58, 175.21.

7.3.8 PEGylation of polystyrene microspheres, (2.9)

![PEGylation of polystyrene microspheres](image)

PEGylation of 200 nm polystyrene microspheres (2.8) was performed as shown in Section 7.2.4 forming Fmoc protected microspheres (2.9) using Fmoc-PEG-OH spacer (2.18) as carboxylic acid nature compound.

7.3.9 Fmoc deprotection on Fmoc protected polystyrene microspheres, (2.10)

![Fmoc deprotection on microspheres](image)

Fmoc deprotection of Fmoc protected microspheres (2.9) was performed as shown in Section 7.2.7 yielding amino pegylated microspheres (2.10).

7.3.10 Carboxyfuntionalisation on amino pegylated microspheres, (2.11)

![Carboxyfuntionalisation on microspheres](image)

Carboxyfunctionalisation of amino pegylated microspheres (2.10) was performed as shown in Section 7.2.5 affording carboxy pegylated microspheres (2.11).
7.3.11 Preparation of Aminohexanoic-microspheres, (2.12-214)

Coupling of aminohexanoic type spacer on 0.2, 0.5 and 2 \( \mu \)m amino functionalised microspheres were performed as shown in Section 7.2.4 where Fmoc-6-aminohexanoic acid was used as carboxylic acid nature compound, yielding 2.12-2.14. Subsequently, Fmoc removal was performed as described in Section 7.2.7.

7.3.12 Preparation of Fluorescein-microspheres, (2.15-2.17)

Labelling of 0.2, 0.5 and 2 \( \mu \)m aminohexanoic-microspheres were performed as shown in section 7.2.4 where 5(6)-carboxyfluorescein was used as carboxylic acid nature compound, yielding 2.15-2.17. In the case of microspheres labelling with any dye, microspheres washings were particularly important, which was repeated until complete clear supernatant was obtained.

7.3.13 Cellular uptake of Fluorescein-microspheres, (2.15-2.17)

Cells were plated in 24-well plates at a density of 4 x 10^4 cells per mL for HEK and B16F10 and 3 x 10^4 cells per mL for HeLa cell line. After 24 hours, the cells were beadfected according to general procedures using 86 \( \mu \)g/mL and analysis of uptake made after 3, 6, 12 and 24 hours incubation by flow cytometry in PBS supplemented with 2% FBS (General procedures).
7.3.14 Confocal microscopy of beadfected cells

HeLa cells were seeded onto a 6-well plates at a density of $1 \times 10^6$ cells/well (volume of culture media per well: 1.5 mL). After 24 hours, the cells were beadfected according to general procedures using 86 µg/mL and microscopy performed after 12 hours (general procedures). Cells were fixed and stained with DilC 18 (cell membrane) and Hoechst 33342 (cell nucleus) and imaged in 2% FBS/PBS with a confocal microscopy.

7.3.15 Synthesis of Dde-OH, (2.19)

\[
\text{HO}
\]

\[
\text{O}
\]

Dicyclohexylcarbodiimide (14.68 g, 71.3 mmol, 1 eq) was added to a solution of Dimedone (10 g, 71.3 mmol, 1 eq) and DMAP (0.87 g, 7.13 mmol, 0.1 eq) in DMF (125 mL). After that, acetic acid (8.3 mL, 142 mmol, 2 eq) was added to the solution mixture and left stirring for 60 hours at room temperature (the progress of the reaction was monitored by TLC). The reaction was finished over 36 hours. Once finished, reaction mixture was filtered to remove DCU (Dicyclohexylurea), and the organic layer was poured into water (200 mL) and extracted with diethyl ether (3 x 100 mL). The organic layer was dryness in MgSO$_4$ and then evaporated in vacuo to afford Dde-OH as orange oil (12.99 g, 58.057 mmol, 82%).

**Rf** (2:1, EtOAc/Hexane): 0.55

**HPLC (SODNEW, λ=254):** purity 98%, retention time 3.321 min

$m/z$ (ES+) 183.0 (100%) [M+H]$^+$

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ (ppm): 1.06 (s, 6H), 2.34 (s, 2H$_2$), 2.52 (s, 2H$_2$), 2.59 (s, 3H).$^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ (ppm): 28.15, 28.50, 30.60, 46.85, 52.42, 112.31, 195.14, 197.86, 202.41.
7.3.16 Synthesis of Fmoc-Lys(Dde)-OH (2.20)

![Chemical Structure](image)

Trifluoroacetic acid (84 µL, 1.1 mmol) was added to a stirred suspension of Fmoc-Lys-OH (4.04 g, 10.7 mmol, 1 eq) and Dde-OH (2.19) (5.84 g, 32.1 mmol, 3 eq) in ethanol (95 ml) at room temperature. The mixture was then refluxed for 60 hours (the progress of the reaction was monitored by TLC). After 60 hours, the solvent was evaporated and the orange residue dissolved in ethyl acetate (75 mL). The organic solution was washed with 1M aqueous KHSO\(_4\) (3 x 100 mL). After drying and concentrated in vacuo. A chromatography column in 8:1, DCM/MeOH was runned to removed unreacted Dde-OH to give, after evaporation of the solvent, a brown-yellow solid (3 g, 54.4%).

**Rf (2:1, DCM:MeOH):** 0.20

**HPLC (SODNEW, λ=254):** purity 100%, retention time 3.7 min.

**m/z** (ES+) 533.3 (100%) [M+H]\(^+\)

**\(^1\)H NMR** (500 MHz, CDCl\(_3\)) \(\delta\) (ppm): 1.00 (s, 6H), 1.49-1.76 (m, 6H), 2.32 (s, 4H), 2.50 (s, 2H), 3.33 (d, \(J = 4.90\) Hz, 2H), 4.17 (t, \(J = 7.06\) Hz, 1H), 4.33 (t, \(J = 7.51\) Hz, 3H), 7.27 (t, \(J = 7.48\) Hz, 2H), 7.36 (t, \(J = 7.38\) Hz, 2H), 7.57 (t, \(J = 8.11\) Hz, 2H), 7.73 (d, \(J = 7.50\) Hz, 2H), 13.26 (s, 1H)

**\(^13\)C NMR** (125 MHz, CDCl\(_3\)) \(\delta\) (ppm): 18.04, 22.52, 28.14, 30.03, 31.82, 36.64, 43.17, 50.59, 52.44, 54.03, 67.00, 107.81, 119.95, 125.08, 127.01, 127.68, 141.19, 143.67, 156.35, 173.88, 175.83, 198.08.

### 7.4 Experimental for Chapter 3

#### 7.4.1 Synthesis of neuropeptides

Design synthesis and characterisation of the neuropeptides (hexa and nonapeptide) as well as the tripeptide fragment were carried out by Dr Perez-Lopez
from Gomez-Vidal’s research group at the Organic and Medicinal Chemistry department of the University of Granada in Spain.

7.4.2 Cellular uptake on neuroblastoma cells

SH-SY5Y neuroblastoma cells were plated in 24-well plates at a density of 4 x 10^4 cells per mL. After 24 hours, the cells were beadfected according to general procedures using 2.15-2.17 samples at two different concentrations (43 and 86 µg/mL) and analysis of fluorescence intensity and uptake made after 12 hours incubation by flow cytometry in PBS supplemented with 2% FBS (General procedures).

7.4.3 Cell viability on neuroblastoma cells

Cells were seeded onto a 96-well plates at a density of 1 x 10^4 cells/well. Cells were incubated for 24 hours prior to beadfection with different samples (Section 7.2.9.3) at concentrations of 43 and 86 µg/mL. After 24 hours, the old media was removed and replaced with fresh phenol red-free culture media (90 µL) and MTT (10 µL). Cells were incubated for 5 hours. After this time, MTT solubilisation solution was added and the 96-well plates was gently shaken for 1 hour (General procedures).

7.4.4 Toxicity induction on neuroblastoma cells

Cells were seeded onto a 96-well plates at a density of 1 x 10^4 cells/well. Cells were incubated for 24 hours prior to addition of different cytotoxic cocktails (NMDA/Glycine). After different neurotoxic condition time exposures, the cytotoxic cocktail was removed and cells returned to their cell culture medium for 24 hours. After this time, the old media was removed and replaced with fresh phenol red-free culture media (90 µL) and MTT (10 µL). Cells were incubated for 5 hours. Next, MTT solubilisation solution was added and the 96-wells plate was gently shaken for 1 hour, cell viability was calculated as shown in section 7.2.9.6 (General procedures).
7.4.5 Spacer couplings on microspheres, (3.5-3.6)

Coupling of either aminohexanoic or PEG type spacer on 0.5 µm amino functionalised microspheres were performed as shown in Section 7.2.4, where either Fmoc-6-aminohexanoic acid or Fmoc-PEG-OH spacers were used as carboxylic acid nature compounds, yielding 3.5 and 3.6. Subsequently, Fmoc removal was performed as described in Section 7.2.7.

7.4.6 Linker couplings on 3.5 and 3.6, (JMC 330 AN, AC, PN and PC)

Linker couplings on 3.5 and 3.6 were performed as shown in Section 7.2.5 affording JMC 330 AN, AC, PN and PC (General procedures).

7.4.7 Neuropeptide-microsphere conjugation

Carboxy functionalised microspheres (JMC 330 AN, AC, PN and PC) (0.1 mL; 1eq) were washed in DMF (3 x 0.1 mL) and suspended in DMF (0.1 mL). Next, DIC (5 eq) was added and the suspension mixed for at least 2 hours at rt. After this time beads were collected by centrifugation and a previously prepared solution of HA, HP, HPP, NA, NP, NPP, APL-HP1, APL-HP2, APL-HP3, APL-HP4, APL-HP5, APL-HP6, APL-HP8 or APL-HP-9 (3 eq) in DMF (0.1 mL) was added, subsequently DIPEA (0.1 eq) was also added. The suspension was mixed on the Thermomixer at 1400 rpm for 18 hours at room temperature to yield the library of peptide-microsphere conjugates (General procedure).

7.4.8 Neuroprotection assay on neuroblastoma cells

SH-SY5Y neuroblastoma cells were seeded onto a 96-wells plate at a density of 1 x 10⁴ cells/well. Cells were incubated for 24 hours prior to beadfection according to general procedures using the library of peptide-microsphere at two different concentrations (1.24 and 2.47 µM). After 24 hours, media containing beads was removed and cytotoxic cocktail (0.1 µM NMDA/1 mM Glycine) applied for 5 hours. After this time cells were returned to their cell culture medium and incubated for 24 hours. Next, the old media was removed and replaced with fresh phenol red-
free culture media (90 µL) and MTT (10 µL). Cells were incubated for 5 hours. After this time, MTT solubilisation solution was added and the 96-well plates was gently shaken for 1 hour, cell viability was calculated as shown in section 7.2.9.6 (General procedures).

7.4.9 Cytotoxicity assay on neuroblastoma cells

Cells (SH-SY5Y and ARN8) were seeded onto a 96-well plates at a density of 1 x 10^4 cells/well according to general procedures and incubated for 24 hours. After this time, cells were treated with samples ACNA and ANHP at concentrations ranging from 2.8 to 13 µM. After 24 hours of samples treatment, old media was removed and replaced with fresh phenol red-free culture media (90 µL) and MTT (10 µL). Cells were incubated for 5 hours. After this time, MTT solubilisation solution was added and the 96-well plates was gently shaken for 1 hour, cell viability was calculated as shown in section 7.2.9.6 (General procedures).

7.4.10 Confocal microscopy on neuroblastoma cells

SH-SY5Y cells were seeded onto a 6-well plates at a density of 1 x 10^6 cells/well (volume of culture media per well: 1.5 mL). After 24 hours, the cells were beadfected according to general procedures using ACNA and ANHP at a concentration of 1.24 µM and microscopy performed after 24 hours (General procedures). Cells were fixed and stained with Cell Tracker Red (cytoplasm) and Hoechst 33342 (cell nucleus) and imaged in 2% FBS/PBS with a confocal microscopy.

7.5 Experimental for Chapter 4

Knocking down on Stem Cells

7.5.1 Double functionalised fluorescent microspheres preparation, (4.2.1.2-4.2.1.6)

For the preparation of double functionalised microspheres the protocol previously described in section 7.2.4 (for Fmoc-PEG-OH and Fmoc-Lys(Dde)-OH couplings) was followed. Fmoc deprotections were performed according to the
Next, Dde deprotection on the amino side chain was performed according to protocol described in section 7.2.8.2, after Dde deprotection, the labelling of the microspheres was performed according to the protocol previously described in section 7.3.12.

7.5.2 siRNA coupling to microspheres, (4.2.1.7-4.2.1.11)

Carboxy functionalisation of the microspheres was performed following the protocol described in section 7.2.6 (General procedures). Once carboxy functionalised microspheres (150 µL) were isolated by centrifugation and washed with MES buffer (pH 5.5, 3 x 150 µL) before activation with 1M EDC solution in MES (150 µL) for 4 hours. Microspheres were isolated by centrifugation and treated with RNase Zap™ solution (150 µL, Ambion) before being transferred to an RNase-free eppendorf and washed with RNAse-free deionised water (2 x 150 µL). siRNA (Oct3/4, Sox2 or scramble; 1.5 nmol) was added to microspheres in RNase-free PBS (150 µL) and shaken at rt for 18 hours. After this time siRNA-microspheres were isolated by centrifugation, yielding 4.2.1.7-4.2.1.11 and stored in RNase-free deionised water at 4°C if experiment was performed on the day or frozen (please see section 7.5.3 for frozen protocol) if siRNA-microspheres stock was desired.

7.5.3 Freezing of microspheres

After siRNA coupling into microspheres, microspheres were washed with 10% DMSO/H₂O (biological grade DMSO/RNAse-free deionised water; 5 x 100 µL) and samples stored at -80°C.* Before being used for transfection, frozen beads were washed with RNAse-free deionised water (5 x 100 µL) and transferred into RNAse-free deionised water to perform the required experiment.

* Up to 6 months of siRNA-microspheres stability and activity has been tested.

7.5.4 Cellular uptake analysis

Hela and ES-E14tg2a cells were seeded in the appropriate growth media to 24-well plates (ES-E14tg2a cells required plates gelatinised with 0.1% gelatine/PBS) at a density of 3 x 10^4 cell/well (well volume: 350 µL). After 24 hours, the old media
was removed and replaced with fresh serum-free and antibiotic-free media containing samples \((2.6 \text{ and } 4.2.1.2-5)\) at a concentration of \(43 \mu\text{g/mL}\). After 48 hours incubation cells were washed, harvested by trypsination and analysis of cellular uptake was made by flow cytometry (general procedures) and compared to untreated cells.

### 7.5.5 Gene silencing on Oct4-GiP cells

Oct4-GiP and ES-E14tg2a cells were seeded in the appropriate growth media to 24-well plates at a density of \(3 \times 10^4\) cells/well. After 24 hours, the old media was removed and replaced with fresh serum-free and antibiotic-free media containing samples \((2.6 \text{ and } 4.2.1.6-10)\) at a concentration of \(86 \mu\text{g/mL}\) or siRNA lipofected with Lipofectamine\textsuperscript{TM} 2000*. After 24-72 hours incubation cells were washed, harvested by trypsination and analysis of GFP expression was made by flow cytometry (general procedures) and compared to untreated cells.

*For each well, Lipofectamine\textsuperscript{TM} 2000 (1 µL) was added to PBS (50 µL) and incubated at rt for 5 min. It was then added to siRNA (20 pmol) in PBS (50 µL) and incubated at rt for 20 min.

### 7.5.6 Pseudoconfocal microscopy on Oct4-GiP cells

Pseudoconfocal and RT-PCR studies were carried out in collaboration with Dr. Alessandra Livigni from Institute of Stem Cell Research (ISCR), School of Biological Sciences in Edinburgh University.

Oct4-GiP cells were seeded in GMEM media onto a 24-well plates at a density of \(3 \times 10^4\) cells/well. After 24 hours, the old media was removed and replaced with fresh serum and antibiotic-free media containing samples \((4.2.1.7\text{-}4.2.1.9)\) at a concentration of \(86 \mu\text{g/mL}\). After 48 hours incubation cells were washed with PBS (Sigma-Aldrich; 2 x 100 µL) and fresh medium was added. Cells were imaged by pseudoconfocal microscopy according to general procedures.
7.5.7 Cells sorting

Oct4-GiP cells were plated on gelatine-coated 24-well plates in the appropriate medium at a density of $3 \times 10^4$ cells/well. After 24 hours, the old media was removed and replaced with fresh serum and antibiotic-free media containing samples (4.2.1.7-4.2.1.9) at a concentration of 86 µg/mL. After 48 hours, pseudoconfocal microscopy study was performed (section 7.5.6), and cells incubated for another 48 hours before being washed with PBS (100 µL) and trypsinised. The pellet was resuspended in 0.5 mL FACS buffer (PBS + 1% FBS + 0.25 mM EDTA). Cells were then sorted on a MoFlo MLS high-speed sorting flow cytometer (Beckman Coulter) and replated afterwards.

7.5.8 Viability of ES cells

ES cells were seeded onto a 96-well plates at a density of $1 \times 10^4$ cells/well. Cells were incubated for 24 hours prior to beadfection with different samples (Section 7.2.5.3) at concentration of 86 µg/mL. After 24 hours, the old media was removed and replaced with fresh phenol red-free culture media (90 µL) and MTT (10 µL). Cells were incubated for 5 hours. After this time, MTT solubilisation solution was added and the 96-well plates were gently shaken for 1 hour (General procedures).

Thiol-mediated siRNA delivery

7.5.9 Synthesis of 5(6)-(N-thioethyl)tetramethylrhodaminamide, (4.2.2.1)

![Chemical structure](image_url)
Cysteamine (11.75 mmol) was dissolved in DMF (15 ml) and added to the previously preactivated 2-chlorotriyl chloride beads (2.25 mmol). Just before mixing, Diisopropylethylamine (DIPEA) (11.75 mmol) was added. The solution mixture was allowed to stir overnight at room temperature. After the coupling a positive ninhydrin test was obtained, confirming that the coupling had been successfully performed. Then, the resin (0.23 mmol) was suspended in DCM. In a separate vial, 5(6) Carboxytetramethylrhodamine (0.23 mmol) was dissolved in DMF/DCM (1.35/0.15mL), then Oxyma (0.23 mmol) was added and the solution mixture mixed for 4 minutes at rt before the addition of DIC (0.23 mmol) and mixed for 8-10 minutes at rt. The solution mixture was allowed to stir overnight, after overnight stirring the reaction mixture was stopped and washed subsequently in DMF (3 x 2 mL), DCM (3 x 2 mL), MeOH (3 x 2 mL), DMF (2 x 2 mL), DCM (2 x 2 mL), MeOH (1 time), DMF (1 time), DCM (1 time) and Et$_2$O (1 time) to ensure that any unreactive dye has been removed before proceeding to the cleavage. The resin cleavage was performed in a cocktail solution (90%TFA/5%TIS/5%DCM) which was applied for 2 hours in stirring, then the cocktail solution was filtered off, again 1 mL of cocktail solution was applied and filtered off subsequently. Finally, the resin was washed with 1 mL of DCM. The solvent was evaporated and the remaining product was precipitated in cold Et$_2$O (2 x 15 mL), the centrifugation afforded a brown/orange powder which were characterised as detailed below.

**Mp:** 175-180 °C.

**IR (cm$^{-1}$):** 2851 (w), 1669 (m), 1173 (s).

**ELSD (S$^{50DNEW.M}$):** 3.2minutes (97%purity).

**HRMS (ES$^+$), C$_{27}$H$_{26}$O$_4$N$_3$S$_1$ (M-H):** calcd 488.16495, found 488.16507.

$^1$H-NMR (500 MHz, CD$_3$OD) $\delta$ 8.40-8.35 (m, 1H), 8.33-8.29 (m, 1H), 7.07-7.04 (m, 3H), 6.98 (dd, 2H, $J$= 2.24, 9.52 Hz), 6.88 (d, 2H, $J$= 2.13 Hz), 3.42 (t, 2H, $J$= 6.79 Hz), 3.34 (t, 2H, $J$= 6.82 Hz), 3.22 (s, 12H).  $^{13}$C-NMR (125 MHz, CD$_3$OD) $\delta$ 191.21, 166.94, 163.02, 159.88, 159.00, 140.42, 139.200, 137.09, 135.91, 131.84, 119.31, 116.98, 115.69, 114.88, 114.57, 97.55, 41.00, 39.27, 34.93.
7.5.10 Spacer couplings on microspheres, (4.2.2.4-4.2.2.5)

Coupling of either aminohexanoic or PEG type spacer on 0.5 amino functionalised microspheres were performed as shown in Section 7.2.4, where either Fmoc-6-aminohexanoic acid or Fmoc-PEG-OH spacers were used as carboxylic acid nature compounds, yielding 4.2.2.4 and 4.2.2.5. Subsequently, Fmoc removal was performed as described in Section 7.2.7.

7.5.11 Preparation of spaced-cleavable microspheres, (4.2.2.6-4.2.2.7)

\[
\text{4.2.2.6}=\text{Ahx spacer} \\
\text{4.2.2.7}=\text{PEG spacer}
\]

In a typical reaction, 3(2-Pyridyldithio)propionic acid (10 eq) and DIPEA (15 eq) were added to either 4.2.2.4 or 4.2.2.5 suspended in DMF (0.5 mL). The resulting suspension was mixed on a rotary wheel at rt for 18 hours. After this time, the microspheres were washed with DMF (3 x 0.5 mL), methanol (3 x 0.5 mL) and deionised water (3 x 0.5 mL), yielding 4.2.2.6 (Ahx spacer) and 4.2.2.7 (PEG spacer) cleavable microspheres. Microspheres were stored in deionised water at 4°C.

7.5.12 Preparation of spaced-non cleavable microspheres, (4.2.2.8-4.2.2.9)

\[
\text{4.2.2.8}=\text{Ahx spacer} \\
\text{4.2.2.9}=\text{PEG spacer}
\]

In a typical reaction, 3-(Maleimido)propionic acid (4.2.2.3, 10 eq) and DIPEA (15 eq) were added to either 4.2.2.4 or 4.2.2.5 suspended in DMF (0.5 mL). The resulting suspension was mixed on a rotary wheel at rt for 18 hours. After this time, the microspheres were washed with DMF (3 x 0.5 mL), methanol (3 x 0.5 mL) and deionised water (3 x 0.5 mL), yielding 4.2.2.8 (Ahx spacer) and 4.2.2.9 (PEG spacer) cleavable microspheres. Microspheres were stored in deionised water at 4°C.
7.5.13 Labelling of spaced microspheres with 5(6)-carboxytetramethylrhodamine, (4.2.2.14-4.2.2.15)

![Chemical structure of labelling reagent](image)

4.2.2.14 = Ahx spacer  
4.2.2.15 = PEG spacer

Labelling of spaced microspheres were performed as shown in section 7.3.12 where 5(6)-carboxytetramethylrhodamine was used as carboxylic acid nature compound, yielding 4.2.2.14-4.2.2.15 (General procedures).

7.5.14 Labelling of spaced-cleavable or non cleavable microspheres with a thiol functionalised dye, (4.2.10-4.2.2.13)

![Chemical structure of labelling reagent](image)

4.2.2.10 = Ahx spacer  
4.2.2.11 = PEG spacer

Microspheres were washed with DMF (3 x 200 µL) and resuspended in DMF (100 µL). In a separate vial, the thiol functionalised dye (4.2.2.1, 1 µmol) was
dissolved in slightly acid PBS pH 6.9 (150 µL) and mixed for 4 minutes before addition to microspheres 4.2.2.6-4.2.2.9. The solution mixture was allowed to stir overnight at rt, yielding 4.2.2.10-4.2.2.13.

7.5.15 Cellular uptake analysis of fluorescence thiolated microspheres

HELA cells were plated in 24-well plates at a density of 3 x 10⁴ cells per mL. The following day, the old media was removed and fresh medium containing samples 4.2.2.10, 4.2.2.12 and 4.2.2.14 at a concentration of 43 µg/mL and incubated for 3, 6, 12 and 24 hours. The cells were then washed with PBS, harvested with trypsin/EDTA and centrifuged for 4 minutes at 1000 rpm. The pellet was resuspended in PBS supplemented with 2% FBS and analytical flow cytometry was performed according to general protocol.

7.5.16 Deprotection of disulfide-modified siRNA, (4.2.2.16)

Commercial thiolated siRNA (Qiagen, 50 µL) was mixed with a 100 mM DTT solution (50 µL, pH 8.3) and the solution thermomixed at 1400 rpm at 20°C for 30 minutes. After being reduced, the siRNA was desalted on a desalting column previously equilibrated with PBS pH 6.9.

7.5.17 Bioconjugation of free thiol derivative into microspheres, (4.2.2.16-4.2.2.19)

Thiol functionalised microspheres (150 µL) were isolated by centrifugation and washed with PBS buffer pH 6.9. Microspheres were isolated by centrifugation and treated with RNase Zap™ solution (150 µL) before being transferred to an RNase-free eppendorf and washed with RNAse-free deionised water (2 x 150 µL). Free thiol derivative was added to microspheres in RNAse-free PBS (150 µL) and shaken at rt for 18 hours. After this time siRNA-microspheres were isolated by centrifugation, yielding 4.2.2.16-4.2.1.19 and stored in RNAse-free deionised water at 4°C.
7.5.18 GFP silencing on HeLa-GFP cells

HeLa-GFP cells were seeded in the appropriate growth media to 24-well plates at a density of $3 \times 10^4$ cells/well. After 24 hours, the old media was removed and replaced with fresh serum and antibiotic-free media containing samples (4.2.2.16-4.2.2.19) at two different concentrations (5.7 µM and 1.4 µM) or siRNA lipofected with Lipofectamine™ 2000*. After 48-72 hours incubation cells were washed, harvested by trypsination and analysis of GFP expression was made by flow cytometry (general procedures) and compared to untreated cells.

*For each well, Lipofectamine™ 2000 (1 µL) was incubated in PBS (50 µL) and incubated at rt for 5 min. It was then added to siRNA (20 pmol) in PBS (50 µL) and incubated at rt for 20 minutes.

7.5.19 Microscopy study on silenced HeLa-GFP

HeLa-GFP cells were seeded onto a 6-well plates at a density of $1 \times 10^6$ cells/well (volume of culture media per well: 1.5 mL). After 24 h, the cells were beadfectioned according to general procedures using 4.2.2.9 conjugated with a TAMRA-labelled siRNA according with the protocol described in section 7.5.17 at a concentration of 1.4 µM and microscopy performed after 24 h (General procedures). Cells were stained with Hoechst 33342 (cell nucleus) and imaged in 2% FBS/PBS with a fluorescence microscopy.

7.5.20 Cell viability

HeLa-GFP cells were seeded onto a 96-well plates at a density of $1 \times 10^4$ cells/well. Cells were incubated for 24 hours prior to beadfection with different samples (Section 7.2.5.3) at concentration of 86 µg/mL. After 24 hours, the old media was removed and replaced with fresh phenol red-free culture media (90 µL) and MTT (10 µL). Cells were incubated for 5 hours. After this time, MTT solubilisation solution was added and the 96-well plates were gently shaken for 1 h (General procedures).
Microplexes-based gene silencing using spermine functionalised microspheres

7.5.21 PEG spacer coupling on microspheres

Coupling of PEG type spacer on 0.5 µm amino functionalised microspheres were performed as shown in Section 7.2.4, where Fmoc-PEG-OH spacers were used as carboxylic acid, yielding PEGylated microspheres. Subsequently, Fmoc removal was performed as described in Section 7.2.7.

7.5.22 100% Spermine coupling, (4.2.3.1, 3 and 5)

Microspheres were preactivated with DSC as followed: DSC (2.8 mg, 10.7 µmol, 4 eq) was dissolved in 1 mL of dry DMF and allow to stir for 10 minutes before the addition of DIPEA (1.6 µL) and then added to the PEGylated microspheres (1 mL, 1 eq). The suspension was mixed at 40°C for 3 hours. Then the suspension was centrifuged and the supernatant discarded before adding the spermine solution. Spermine (5.4mg, 26.8µmol, 10 eq) was dissolved in 1mL of Dry DMF and allow to stir for 10 minutes before the addition of DIPEA (1.6 µL) and then added to the microspheres (1 mL, 1eq). The suspension was mixed at room temperature overnight yielding 4.2.3.1, 3 and 5.

7.5.23 50% Spermine coupling, (4.2.3.2, 4 and 6)

Microspheres were preactivated according with the protocol described in section 7.5.22. Then the suspension was centrifuged and the supernatant discarded before adding the spermine solution. Spermine (2.7 mg, 13.4 µmol, 5 eq) and Ethanol Amine (0.8 µL, 13.4 µmol, 5 eq) were dissolved in 1mL of Dry DMF and allow to stir for 10 minutes before the addition of DIPEA (1.6 µL) and then added to the microspheres (1 mL, 1eq). The suspension was mixed at room temperature overnight yielding 4.2.3.2, 4 and 6.
7.5.24 Guanidilation of spermine functionalised microspheres, (4.2.3.1G-6G)

Sodium carbonate (2.8 mg, 26.8 µmol, 10 eq) and Pyrazole-1-carboxamide (3.9 mg, 26.8 µmol, 10 eq) were dissolved in 1 mL of water and then added to the microspheres (4.2.3.1-4.2.3.6; 1 mL, 1 eq). The suspension was mixed at 50°C overnight before washing, yielding 4.2.3.1G-6G.

7.5.25 Spermine-functionalised microspheres-siRNA complexes formation

Spermine functionalised microspheres (4.2.3.1-6) were washed in OPTI-MEM medium (3 x 100 µL). Then, siRNA in OPTI-MEM medium (100 µL, 1 nmol) was added to the microspheres and solution mixture mixed at rt for 30 minutes yielding 4.2.3.7-4.2.3.12.

7.5.26 Gel Retardation Assay

Agarose Type I-B (245mg) was dissolved in TEB Buffer (35mL) before the addition of EtBr (2.5µL) and kept at 60°C for 10 minutes. The solution was then poured into the tray and kept at room temperature for 1 hour to allow solidification. Several samples were prepared using siRNA (0.5 µg/µL), Lipofectamine™ 2000 commercially available transfecting agent and microspheres (20µg/µL). LIP: 2µL of lipofectamine™ 2000 + 2µg of siRNA, 1:5; 0.4 µg of microspheres + 2 µg of siRNA, 1:1; 2 µg of microspheres + 2 µg of siRNA, 5:1; 10 µg of microspheres + 2 µg of siRNA, 10:1; 20 µg of microspheres + 2 µg of siRNA, 20:1; 40 µg of microspheres + 2 µg of siRNA, 50:1; 100 µg of microspheres + 2 µg of siRNA and siRNA: 2µg of siRNA. The complex was formed in OPTI MEM medium for 30 minutes (section 7.5.25), by adding fixed amount of siRNA to different amount of microspheres to obtain polycation:siRNA weight ratio ranging from 0.5:1 to 50:1. The gel was run at 80 V for 45 minutes.
7.5.26 DLS & Zeta Potential Measurements

Size and colloidal stability of microplexes were measured according with the protocol described in sections 7.2.3.3 and 7.2.3.4.

7.5.27 GFP silencing study by flow cytometry

HeLa-GFP cells were plated in 48-well plates at a density of 2 x 10^4 cells per well. The following day, the old media was removed and fresh serum- and antibiotic-free medium containing microplexes with different siRNA amounts, with different siRNA: microspheres ratios and functionalised differently were incubated for 72 hours. After 72 hours, cells were washed in PBS, harvested by trypsination and analytical flow cytometry performed according to general procedures.

7.5.28 GFP silencing study by cytometry

HeLa-GFP cells were plated in 48-well plates at a density of 2 x 10^4 cells per well. The following day, the old media was removed and fresh serum- and antibiotic-free medium containing microplexes with different siRNA amounts, with different siRNA: microspheres ratios and functionalised differently were incubated for 72 hours. After 72 hours, cells were washed in PBS and a microscopy study performed according to general procedures.

7.5.29 Microscopy study on silenced HeLa-GFP

HeLa-GFP cells were seeded onto a 6-well plates at a density of 1 x 10^6 cells/well (volume of culture media per well: 1.5 mL). After 24 hours, the cells were bead-fected according to general procedures using a sample microplexing 4 µg of TAMRA-labelled siRNA according with the protocol described in section 7.5.25 in a microspheres: siRNA ratio of 10:1 and microscopy performed after 24 hours (General procedures). Cells were stained with Hoechst 33342 (cell nucleus) and imaged in 2% FBS/PBS with a fluorescence microscopy.
7.5.30 Cell viability

HeLa-GFP cells were seeded onto a 96-well plates at a density of $1 \times 10^4$ cells/well. Cells were incubated for 24 hours prior to beadfection with different samples (Section 7.2.9.3) at concentration of 86 µg/mL. After 24 hours, the old media was removed and replaced with fresh phenol red-free culture media (90 µL) and MTT (10 µL). Cells were incubated for 5 hours. After this time, MTT solubilisation solution was added and the 96-well plates were gently shaken for 1 hour (General procedures).

7.6 Experimental for Chapter 5

7.6.1 General information

All experiments using the F5.BW hybridoma cell line (including flow cytometry and confocal microscopy) were performed by Dr. Jess Borger from Institute of Immunology and Infection Research, School of Biological Sciences in Edinburgh University.

7.6.2 Preparation of Fluorescein-microspheres, (2.15 and 2.17)

Preparation of fluorescein labelled microspheres of 200 nm (2.15) and 500 nm (2.17) were performed according to the protocol described in Section 7.3.12.

7.6.3 Plasmid DNA linearisation

A cocktail containing 10x Buffer B (10 µL), 10x BSA (10 µL) and 10 units (5 µL) of the restriction enzyme (Sph I or PciI) in molecular biology grade water (Sigma; 35 µL) was prepared. Next, 0.5 µg of plasmid DNA (GFP, Lck-YFP, Csk-YFP or PEP-YFP) (40 µL) was added to the previously prepared cocktail to get a final solution mixture of 100 µL which was incubated in a water bath incubator at 37°C for 18 hours. Digestion was stopped by heating up to 70 °C for 10 minutes.
7.6.4 Analytical electrophoresis gel

Agarose electrophoresis gel was performed on 1x Tris-Acetate-EDTA buffer. Samples were loaded at 0.5 µg in 1x Blue juice loading dye where a 1 kb molecular weight DNA was used as ladder. Gel was visualised by UV.

7.6.5 Ethanol precipitation

To precipitate DNA, 10 µL of 3 M sodium acetate plus 250 µL of cold ethanol was added to the solution containing DNA (20 µg; 100 µL). The solution was then placed at -20°C for 18 hours. Then, samples were centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant (Ethanol) was removed and the pellet was rinsed in 70% ethanol in water. The washed pellet was centrifuged again for 10 minutes at 13,000 rpm at 4°C. The ethanol was removed and the pellet was air-dried yielding 6 µg of DNA.

7.6.6 TdT addition of aa-dUTP, (5.2)

1 µg of DNA (20 µL) was added to a solution containing 10x TdT buffer (5 µL), CoCl$_2$ (5 µL), 10 mM aa-dUTP (10 µL) and 20U of TdT and the solution mixture (50 µL) was allowed to incubate at 37°C for 20 minutes. After 20 minutes the enzyme was inactivated by heating up to 70 °C for 10 minutes. After inactivation, the 50 µL reactions were pooled to get a final volume of 300 µL and precipitate overnight. The resulting DNA pellet was resuspended in 50 µL of molecular biology grade water.

7.6.7 DNA quantitation

Using a 1/10 dilution of the DNA quantitative the concentration in µg/µL using the Nanodrop to yield:

<table>
<thead>
<tr>
<th>DNA</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP DNA</td>
<td>105 µg</td>
</tr>
<tr>
<td>Lck DNA</td>
<td>112 µg</td>
</tr>
<tr>
<td>PEP DNA</td>
<td>97 µg</td>
</tr>
<tr>
<td>CsK DNA</td>
<td>95 µg</td>
</tr>
</tbody>
</table>
7.6.8 Preparation of carboxy functionalised microspheres, (5.1)

Carboxy functionalised microspheres (5.1) were prepared according with the protocol described in section 7.2.4 for double PEGylation and section 7.2.7 for Fmoc deprotections. Next, the carboxylic group was coupled following the protocol described in section 7.2.5 (General procedures).

7.6.9 IDNA-microspheres conjugation, (5.3-5.6)

Carboxy functionalised microspheres (5.1) (0.1 mL; 1eq) were washed in molecular biology grade water (0.1 mL x 3 times) and suspended in 0.1 M solution of EDC in water (0.1 mL) and solution mixed for 4 hours at rt. After mixing, microspheres were collected by centrifugation and microspheres resuspended in PBS pH 7.4, then DNA solution (0.1 mL) was added and the solution mixture mixed at room temperature for 18 hours to yield 5.3-5.6. After mixing, it was found quite important not to perform any washing to the IDNA-microspheres conjugates.

7.6.10 pDNA and IDNA lipofection on adherent cells

Cells (HeLa and HEK-293T) were seeded in 24-well plates at a density of 4 x 10^4 cells per mL. (350 µL per well)

For each well, Lipofectamine™ 2000 (2.5 µL) was incubated in OPTI-MEM® Serum-free medium (62.5 µL) at 25°C for 5 minutes. It was then added to DNA (either IDNA or pDNA; 1.25 µL) in OPTI-MEM® Serum-free medium (63.75 µL) and incubated at 25°C for 20 minutes.

After 24 hours, the old media was removed from the cells and replaced with fresh serum- and antibiotic-free media (285 µL) containing DNA’s treated with Lipofectamine™ 2000 in OPTI-MEM® (65 µL). After 24 hours, cells were washed, harvested by trypsination and prepared for flow cytometric analysis as described in the general procedures.
7.6.11 pDNA beadfection on HEK-293T cells

HEK-293T cells were seeded onto a 24-well plates at a density of $4 \times 10^4$ cells per mL. After 24 hours, the old media was removed from the cells and replaced with fresh serum- and antibiotic-free media containing different samples at a concentration of $1 \mu g$ DNA/well. After 48 hours, cells were washed and confocal microscopy performed, subsequently cells were harvested by trypsination and prepared for flow cytometric analysis as described in the general procedures.

7.7 Experimental for Chapter 6

7.7.1 Double functionalised microspheres preparation, (6.1)

For the preparation of double functionalised microspheres the protocol previously described in section 7.2.4 (for Fmoc-PEG-OH and Fmoc-Lys(Dde)-OH couplings) was followed, Fmoc deprotections were performed according to the protocol described in section 7.2.7 (General procedures).

7.7.2 Labelling of double functionalised microspheres, (6.2-3)

Following the protocol described in section 7.2.8.2 Dde deprotection on the amino side chain was performed. After Dde deprotection, the labelling of the microspheres was performed according to the protocol previously described in section 7.3.12 (General procedures).

7.7.3 Fluorogenic substrate-microspheres conjugation, (6.5-7)

Double functionalised microspheres (0.5 mL; 1 eq) were washed in DMF (0.5 mL x 3 times). Separately, the fluorogenic substrate (DEVD-AFC, 1 eq, Alexis) was dissolved in DMF/DMSO 3:1 (0.5 mL), then EDC (3 eq) was added and the solution mixture mixed for 8-10 minutes at rt. The solution mixture was then added to microspheres and suspension mixed on the Thermomixer at 1400 rpm for 1 hour at rt.
7.7.4 Beadfection-apoptosis induction into HeLa cells

HeLa cells were seeded onto a 48-well plates at a density of $1.5-2 \times 10^4$ cells per mL. After 24 hours, the old media was removed from the cells and replaced with fresh media containing samples (6.2-6.7) at two different concentrations (43 and 86 µg/mL). After 24 hours, selected wells were treated with Staurosporine (0.5 µM) for 3 hours. After 3 hours cells were washed, harvested by trypsination and prepared for flow cytometric analysis as described in the general procedures.

NOTE: After apoptosis being induced, the cell washing has to be performed with extremely careful as apoptotic cells tend to detach easier from well surface.

7.7.5 Fluorescence microscopy of apoptotic cells

HeLa cells were seeded onto a 48-well plates at a density of $1.5-2 \times 10^4$ cells per mL. After 24 hours, the old media was removed from the cells and replaced with fresh media containing samples (6.2-6.7) at a concentration of 43 µg/mL. After 24 hours, selected wells were treated with Staurosporine (0.5 µM) for 3 hours. After 3 hours the old media was removed, washed with PBS and fluorescence microscopy performed in 2% FBS/PBS

NOTE: After apoptosis being induced, the cell washing has to be performed with extremely careful as apoptotic cells tend to detach easier from well surface.
References


83. Wang, D. Q.; Robinson, D. R.; Kwon, G. S.; Samuel, J., Encapsulation of plasmid DNA in biodegradable poly(D,L-lactic-co-glycolic acid) microspheres as a


101. Bangs Laboratories, I. Polymer Microspheres for Research and Diagnostic Use.


159. Riefler, G. M.; Firestein, B. L., Binding of neuronal nitric-oxide synthase (nNOS) to carboxyl-terminal-binding protein (CtBP) changes the localization of CtBP from the nucleus to the cytosol - A novel function for targeting by the PDZ domain of nNOS. *Journal of Biological Chemistry* 2001, 276, (51), 48262-48268.


211. Villa, A.; Marziliano, N.; Dina, G.; Biffi, A.; Conese, M., Comparison between Cationic Polymer and Lipid in Plasmidic DNA.


