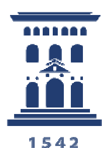

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PAMAM-based Dendrimers for Diverse Biomedical Applications

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Summary

There is a great need for new treatments in certain pathologies such as cancer or resistant infections. Among some of the most promising novel therapies is gene therapy, but it faces serious problems to overcome before using viral vectors into the clinic. To solve some of those problems *viral gene therapy vectors are recently combined with stem cells in cell therapy approaches. However, as they are complicated cells to grow with standard tissue culture methods and culture media, this approach has to be improved previously to move into manufacturing for clinical trials*.

Synthetic gene-delivery agents such as polymers, although safer than viruses, generally do not possess the required efficacy. Dendrimers are three-dimensional, highly branched, synthetic polymers with a well-defined chemical structure. In particular, PAMAM (poly(amidoamine)) dendrimers have been extensively investigated for their biological applications and they have shown clinically-relevant carrier properties that could be used for gene or cell therapy purposes. This is facilitated by controlling charge and functionality through the choice of peripheral groups, and size through generation number and PEGylation. PEG (poly(ethylene glycol)) reduces toxicity, improves water solubility, decreases enzymatic degradation, and leads to improved biodistributions characteristic.

Moreover, it has been shown that PAMAM dendrimers can facilitate antibiotic localization also inside bacteria, *and so, they can be used to enhance the antimicrobial activity. Also, they could be highly toxic to certain bacterias themselves by interacting and promoting disruption of the cytoplasmatic membrane*. By controlling the size and PEGylation degree of the PAMAM dendrimers we can modulate the interactions with the bacterial surface and their killing activity, lowering the cytotoxicity to the host cells. Therefore, PAMAM dendrimers appear as promising antimicrobial agents that are less likely to induce bacterial resistance compared to standard antibiotics.

Here we study the biomedical properties of PAMAM-derived dendrimers on their influence on the bacterial and cellular growth. *Our results show that, although the PAMAM dendrimer completely covered with PEG diminished the pseudomonas growth and it has potential to be employed as an antibacterial agent, in the case of mesechymal stem cells, the cell growth is increased without altering their pluripotenciality patterns, and it could be of great help for the future cell therapy approaches.*

PART I: INTRODUCTION

1. GENE AND CELL THERAPIES

1.1 Development and evolution

Gene therapy aims to treat both genetic and infectious diseases by restoring, modifying or enhancing cellular functions through the introduction of new genetic material into the appropriate cells in the body. While traditional drug therapies involve the administration of chemicals that have been manufactured outside the body, gene therapy takes a very different approach: directing a patient's own cells to produce and deliver a therapeutic agent. Gene therapy protocols were originally designed to correct inheritable disorders, such as cystic fibrosis, Gaucher's disease, and Duchenne muscular dystrophy (*Dekker*,3).

Of the two potential strategies for gene therapy, the *ex-vivo* and the *in- vivo*, the *ex-vivo* (cell-based delivery) strategy allows a wider range of therapies. In this type, cells (especially stem cells) would be extracted from patients, transfected with the therapeutic gene, grown in culture, and reimplanted in the patient. In the *in-vivo* (direct delivery) approach, genes are administered directly into the patient to transduce the cells in-vivo; this delivery method is fairly imprecise and limited to the specific types of human cells that the vector can reach (*Vetrini, et al*,9).

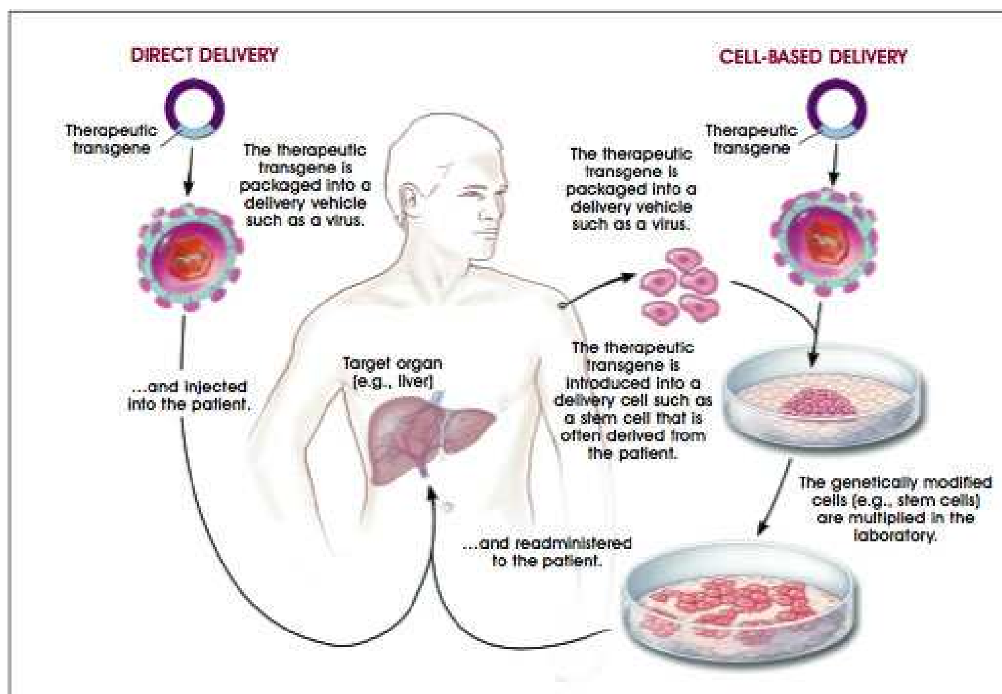


Figure 1.1. Strategies for Delivering Therapeutic Transgenes into Patients (*Dekker*,3).

The first approved gene therapy clinical protocol began in September 1990, using retroviral vectors to introduce copies of the adenosine deaminase gene into T cells from a patient with adenosine deaminase deficiency. However, clinical progress has been slow. In 2002, over 600 clinical protocols have been approved worldwide, incorporating over 3500 patients (*The Journal of Gene Medicine*). A major setback for the field occurred in September 1999, when a man died from a massive immune reaction to the Ad5 vector that was used to deliver a deficient gene. Moreover, in that same year three patients suffering from a severe combined immunodeficiency disorder (SCID-XI) developed leukemia as a result of the gene-transfer procedure. This trial reflects the path that gene therapy followed: success, but with some complications (*Dekker*,3).

Although it was conceived as a therapeutic modality for genetic disorders, over 60% of these trials have been directed to the treatment of cancer, reflecting the need for novel therapies in this field (*The Journal of Gene Medicine*). Cancer represents an enormous biomedical challenge for gene therapy and drug delivery. Tumors are caused as a consequence of mutations in the genome by addition, deletion or change in chromosomal pattern. Ideally, the goal of any cancer therapy is to selectively target and destroy diseased tissue while sparing the surrounding healthy tissue. Over the past three decades, cancer diagnosis, surgery and treatment have improved greatly, but traditional therapies (chemotherapy and radiotherapy) are still insufficient (*Agarwal, et al*,10).

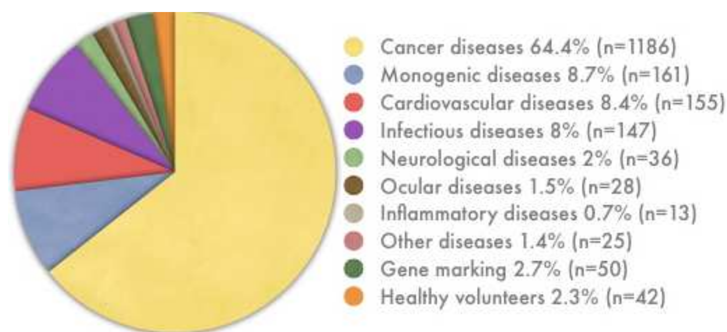


Figure 1.2. Indications addressed by Gene Therapy Clinical Trials (*The Journal of Gene Medicine*, *2012*)

In an attempt to solve this problem, new and more specific therapies exclusively targeted against cancer cells are currently being developed (*Vetrini, et al*,9; *Pandita, et al*,11; *Li, et al*,12; *Niidome, et al*,13). One of the key problems in gene therapy is the efficient delivery of therapeutic transgenes to pathological sites, upon systemic injection of the vector (*Flint*,1; *Dekker*,3). In this context, the ideal vector should show high expression levels, tissue selectivity, and ability to avoid immune response after vector re-administration or over long time periods. Amongst all the commonly used vectors,

none meet the mentioned requirements. For that reason, development of new vectors is pivotal for the development of gene therapy (*Wolinsky, et al*,6; *Vetrini, et al*,9; *Agarwal, et al*,10; *Li, et al*,12; *Niidome, et al*,13; *Chen, et al*,14).

Tumours are composed of both malignant and normal cells. The "benign" cell-types include endothelial cells forming blood vessels, infiltrating inflammatory cells, and stroma. Stromal components provide factors and structural support for malignant cells. The formation of tumour stroma closely resembles wound healing and scar formation that increase proliferation of mesenchymal stem cells (MSCs) (*Dekker*,3; *Barry*,7; *Pandita, et al*,11). Some groups have hypothesized that exogenously administrated MSCs would preferentially go to damaged areas independently of the pathology, and in the case of tumours they would engraft at the tumor site and contribute to the population of the tumour stroma (*Santos, et al*,15). *Based on that hypothesis, MSCs have been recently used as vectors to carry therapeutic genes specifically into tumoral areas.*

1.2 Vectors for Delivery

As the success of gene therapy is highly dependent on the delivery vector, research in this field has been focused on the development of a suitable delivery system. Two most important criteria for a viable gene delivery vector are safety and efficacy: it must not elicit an immune response, must not be infectious and must not cause mutagenesis due to improper gene insertion, while incorporating sufficient functionality to overcome the multitude cellular and extracellular barriers to gene transfer. The first barrier is cellular uptake, where the vector must associate with the cell surface; once inside the cell, the gene must be released from the endosomes and finally it must be able to overcome the nuclear membrane and enter the nucleus.

Currently the most common and efficient delivery systems are viruses. Major safety concerns, however, such as high toxicity and immunogenicity, have limited their general use. Investigators of non-viral delivery systems have shown marked improvements in efficiency (*Vetrini, et al*,9; *Niidome, et al*,13).

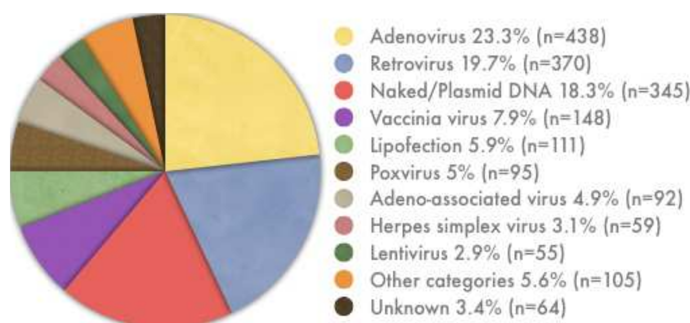


Figure 1.3. Vectors used in Gene Therapy Clinical Trials (*The Journal of Gene Medicine, 2012*)

1.2.1. Viral and cellular Vectors:

Viral gene delivery systems exploit the natural mechanisms evolved by different viruses for cellular entry and gene transfer. However, of the virus-based vector systems developed thus far, none are optimal and each system displays advantages and disadvantages characteristic of each virus (*Dekker*,3; *Marvaniya, et al*,4; *Wolinsky, et al*,6; *Agarwal, et al*,10; *Tomalia, et al*,16; *Flint*,17; *Biasco, et al*,18). In general, the genetic material of the virus is manipulated to carry a copy of the desired gene as well as a promoter. The promoter determines whether the gene functions within the cell or not. A drug usually activates the promoter, which in turn, activates the gene. The activated gene is then translated into proteins. The safety concerns (retention in the liver, immunogenicity, oncogenicity, etc.) and difficult large-scale production limits the usefulness of recombinant viral vectors (*Flint*,1; *Dekker*,3; *Biasco, et al*,18).

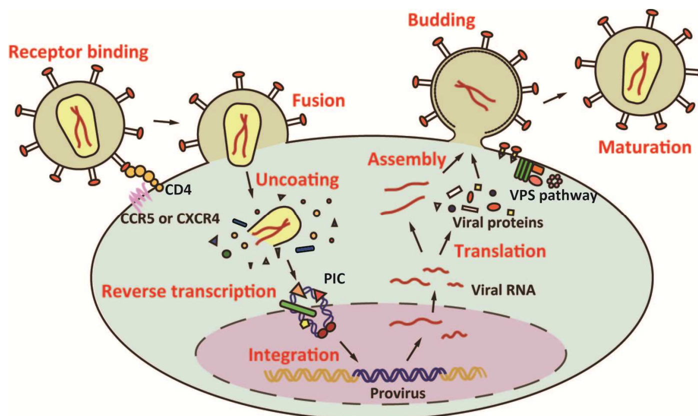


Figure 1.4. Retroviral life cycle (*Flint*,1).

Mammalian cells (fibroblasts, hepatocytes, mesothelial cells, myoblast, neuronal cells, etc) have been proposed as new vector systems, because of their properties with regard to immune recognition and toxicity. Some studies suggest that bone marrow cells could differentiate widely in different tissues and form hepatic cells, cardiomyocytes, neurones, etc (*Orkin, et al*,19; *Morrison*,20).

Mesenchymal stem cells (MSCs) have been studied as vehicles for gene and cellular therapy because they are easy to extract from bone marrow (*Flores-Figueroa, et al*,21; 22) and to recover organs with just a small population expressing the corrected gene. That is why this new field has been named “regenerative therapy” (*Pomerantz, et al*,23). There are hundreds of clinical trials ongoing using MSCs under the common hypothesis that MSCs are able to cure and regenerate the damaged areas independently on the pathology of study (*Bobis, et al*,24).

Some groups hypothesized that exogeneous administered mesenchymal stem cells (MSCs) would contribute to tumour stroma formation and thus could transport substances in a therapeutic context. Tumour stroma formation can be considered functionally analogous to the scar formation process undergone following tissue damage, both processes involving high MSC proliferation (*Mackenzie, et al*,25). Therefore, such cells could be used as a virus transporter directed specifically to different pathologies.

1.2.2. Non-Viral Vectors

Besides virus-mediated gene-delivery systems, there are several nonviral options for direct gene delivery that are attractive alternatives for improved safety, greater flexibility and ease of manufacturing. The simplest method is the *direct introduction of therapeutic DNA* into target cells. A number of barriers must be overcome: it must be protected from degradation, it must cross the cell membranes and when chemical methods are used, the complexes formed by the DNA and by the non-viral vectors must be released from the formed endosomes and decomplexation must occur. Also, it can only be done in certain tissues and requires large amounts of DNA (*Marvaniya, et al*,4; *Wang, et al*,26; *Maruyama-Tabata, et al*,27; *Fant, et al*,28; *Qi, et al*,29).

Other approaches use other vectors to transport DNA to the cell: *synthetic nonviral vectors*. Synthetic vectors are more versatile: their chemical and physical properties, for instance, allow the packing of a large variety of gene lengths, in contrast to the capsid restricted capacity of viruses. Although synthetic vectors are now routinely used in laboratories, their very low delivery efficiency remains prohibitive for their use in gene delivery (*Marvaniya, et al*,4; *Li, et al*,12; *Niidome, et al*,13; *Voulgarakis, et al*,30).

One major approach in nonviral gene therapy is based on *cationic polymers*. In the following pages we will focus on an example of this type of polymer, a *modified polyamidoamine (PAMAM) dendrimer.*

2. MICROBIAL RESISTANCE

Genes for resistance to antibiotics, like the antibiotics themselves, are ancient. However, the increasing prevalence of antibiotic-resistant bacterial infections seen in clinical practice stems from antibiotic use both within human medicine and veterinary medicine. In some countries, antibiotics are sold over the counter without a prescription, which also leads to the creation of resistant strains (*R. Murray*,31). In medicine, the major problem of the emergence of resistant bacteria is due to misuse and overuse of antibiotics. *Pseudomonas aeruginosa* is a highly prevalent opportunistic pathogen. One of the most worrisome characteristics of *P. aeruginosa* is its low antibiotic susceptibility, which is attributable to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes (for example, mexAB-oprM, mexXY, etc.) and the low permeability of the bacterial cellular envelopes.

As resistance towards antibiotics becomes more common, a greater need for alternative treatments arises. However, despite a push for new antibiotic therapies there has been a continued decline in the number of newly approved drugs. Antibiotic resistance therefore poses a significant problem. Novel therapies combining standard antibiotics and synthetic vectors are being developed (*Calabretta, et al*,32; *Lopez, et al*,33; *Tang, et al*,34; *Wang, et al*,35; *Hong, et al*,36; *Cheng, et al*,37; *Feliu, et al*,38; *Tyssen, et al*,39; *Rasines, et al*,40).

3. DENDRIMER-BASED VECTORS

3.1 Cationic Polymers

Polymers are very versatile molecules that can be tuned to act as gene carriers. Variations in polymer molecular weight, 3-D architecture, size, chemical composition, number of repeating units, degree of branching, side chain length, density, etc., may be used in vector’s design (*Majoros*,2). Cationic polymers have been used since the last three decades as a material for gene delivery (*Majoros*,2; *Tomalia, et al*,16). They possess high density of positively charged primary amines which can interact with the negatively charged phosphate groups of DNA to form condensed structures (polyplexes) which enter cells by endocytosis. Several kinds of cationic polymers have been reported to mediate efficient gene transfection of different cells lines, such as Poly-L-lysine (PLL), which was the first cationic polymer based gene delivery vehicle,

Polyethylenimine (PEI) and *PAMAM dendrimers (Bai, et al,8; Chen, et al,14; Fant, et al,28; Hong, et al,36; Cheng, et al,37; Dufes, et al,41; Hadidi, et al,42).*

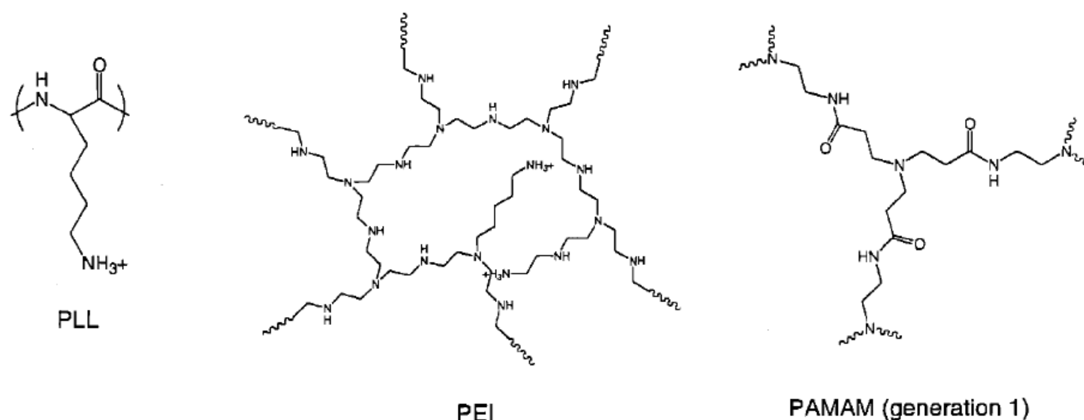
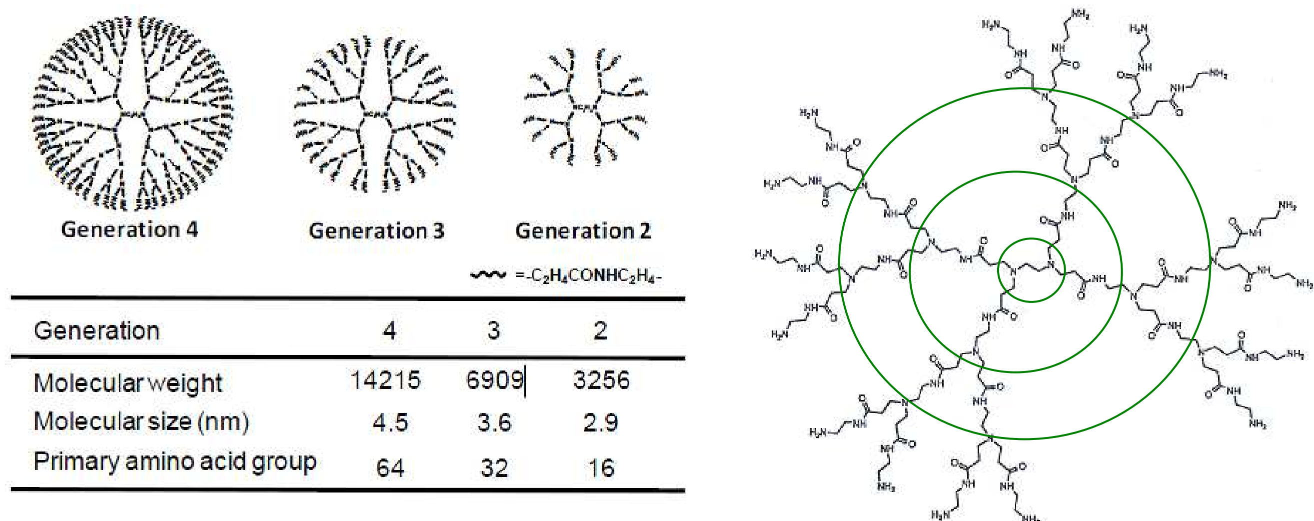


Figure 1.5. Chemical structures of selected cationic polymers (*Majoros*,2).

3.2 Dendritic architectures

Since the discovery of dendrimers in the late 70's, the research interest for developing new synthetic routes as well as finding new applications for dendrimers are ever increasing (*Tomalia, et al*,43). Dendrimers are three-dimensional macromolecules that can be prepared with a level of control not attainable with most linear polymers, leading to nearly monodisperse, globular macromolecules with a high surface area and large number of peripheral groups and reproducible formulations (*Majoros*,2).

A dendrimer is built up of layers of repeating units around a central core. Each layer of monomers builds up one generation, starting with the central branched core molecule as generation 0 (G_0) and increasing with each successive addition of branching points (i.e., G_1, G_2, etc.). Dendritic macromolecules tend to linearly increase in diameter and adopt a more globular shape with increasing dendrimer generation, while the number of end-groups increases exponentially with each generation, resulting in a large number of terminal units at high generations (*Majoros*,2; *Navarro, et al*,44).



Generation	4	3	2
Molecular weight	14215	6909	3256
Molecular size (nm)	4.5	3.6	2.9
Primary amino acid group	64	32	16

Figure 1.6. Chemical structures of PAMAM Dendrimers (G4, G3, G2) and dendrimer generation (adapted from (*Majoros*,2)).

Dendrimers offer the precise control and predictability of modifications that is required for modern drug delivery and targeting. Almost all aspects of the dendrimer can be modified through the different parts of the dendrimers to fit different needs, including its chemical nature, molecular weight, size, structure, and dimensions. In addition, the molecular dimensions of higher generation dendrimers are comparable to the dimensions of medium-sized proteins (*Marvaniya, et al*,4; *Tomalia, et al*,16).

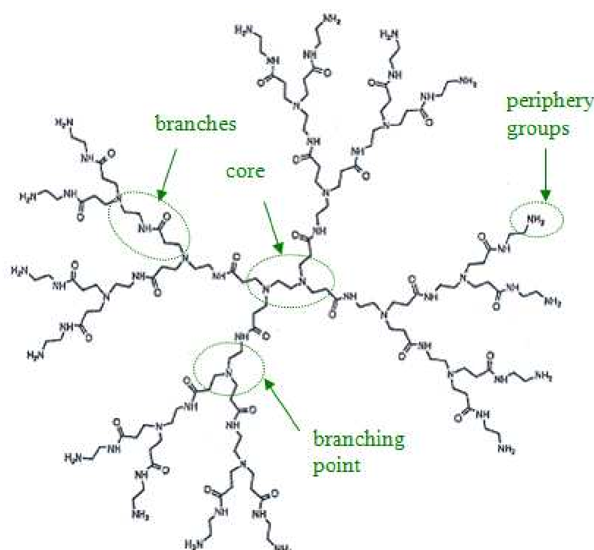


Figure 1.7. Parts of a PAMAM dendrimer (adapted from (*Wolinsky, et al*,6)).

3.3 Synthesis and Features

In the early eighties, the development of dendrimers progressed until Tomalia et al. synthesised branched "Starburst" polyamidoamine (PAMAM) molecules and coined the name *dendrimers*, which is derived from the greek words dendron (tree) and meros (part). *These dendrimers were the first ones to become commercially available.*

The dendrimer can be grown outwards from a central core, a process known as the divergent method pioneered by Tomalia and Newkome, or it can be prepared by Fréchet's convergent method.

In the divergent methods, dendrimer grows outwards from a multifunctional core molecule. The core molecule reacts with monomer molecules containing one reactive and two dormant groups giving the first generation dendrimer. Then the new periphery of the molecule is activated for reactions with more monomers. The process is repeated for several generations and a dendrimer is built layer after layer (Fig. 1A). The divergent approach is successful for the production of large quantities of dendrimers.

The convergent methods were developed as a response to the weaknesses of the divergent synthesis. In the convergent approach, the dendrimer is constructed stepwise, starting from the end groups and progressing inwards. When the growing branched polymeric arms, called dendrons, are large enough, they are attached to a multifunctional core molecule (Fig. 1B). The convergent growth method has several advantages. It is relatively easy to purify the desired product and the occurrence of defects in the final structure is minimized.

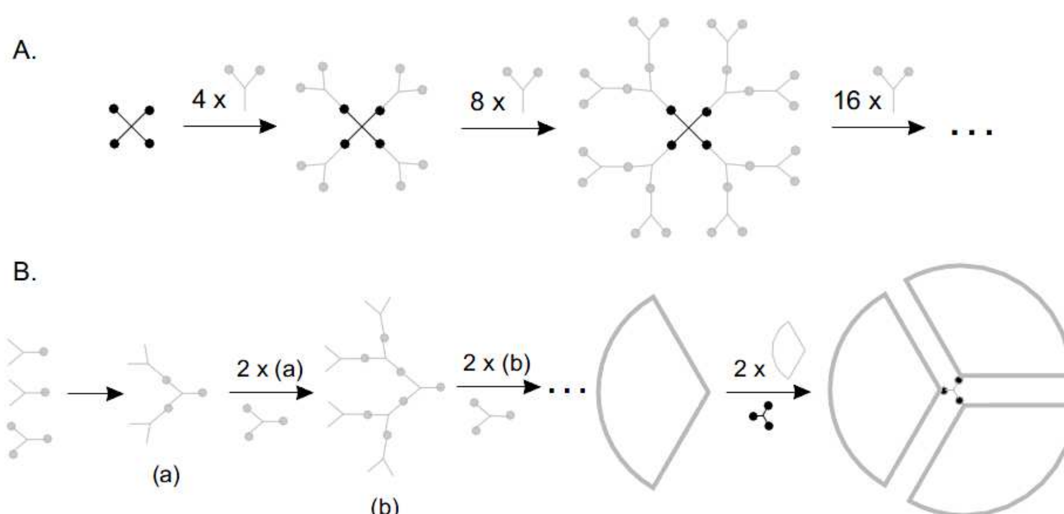


Figure 1.8. A) The divergent growth method; B) The convergent growth method (*Marvaniya, et al*,4).

3.4 Physicochemical Properties

Because of their molecular architecture, dendrimers show some unique physical and chemical properties, which make them particularly interesting for drug and gene delivery applications. In contrast with linear polymers, the intrinsic viscosity of dendrimer solutions does not increase linearly with mass but shows a maximum at a specific generation (*Nandy, et al*,5). This is likely to be because of the way in which dendrimer shape changes with generation (ie, lower generations adopt a more open planar-elliptical shape with transition to a more compact spherical shape for higher generations). The compact shape also reduces the likelihood of entanglement, which affects larger classic polymers. The actual conformation for dendrimers, however, depends on the nature of the solvent. The presence of numerous terminal groups facilitates multiple simultaneous interactions with solvents, surfaces, or other molecules and, as a consequence, dendrimers tend to show high solubility, reactivity, and binding.

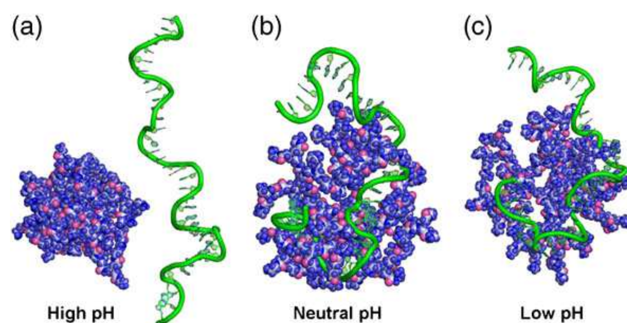


Figure 9. Equilibrium snapshot of ssDNA-dendrimer complexes at (a) high, (b) neutral and (c) low pH. The dendrimer has been shown in sphere representation in different shades of blue and pink, while the DNA is shown in the cartoon representation (*Majoros*,2; *Nandy, et al*,5).

3.5 PAMAM and PAMAM-modified dendrimers

In recent years, PAMAM dendrimers and modification of their surface functional groups with targeting compounds, fluorescent functional groups and drugs have been investigated as carriers for gene therapy, magnetic resonance, development of vaccines, antivirals, antibacterials and anticancer therapies (*Maruyama-Tabata, et al*,27; *Dufes, et al*,41; *Kim, et al*,45). The advantage of PAMAM dendrimers as a drug carrier is that they are highly branched macromolecules, small in size with a low polydispersity (*Wolinsky, et al*,6). Ideal PAMAM dendrimers used as a drug delivery system should be non-toxic, no-immunogenic and biodegradable.

Their synthesis is a divergent process starting from an ethylenediamine core, the repetitive steps are a Michael addition of amines to the double bond of methylacrylate and the formation of mide bonds between the terminal esters and ethylenediamine (*Majoros*,2).

This dendrimers are considered a proton-sponge, which contain a large number of secondary and tertiary amines, exhibiting pKa values between physiological and lysosomal pH. That means that in a physiological environment (pH 7.4), most primary amines of the PAMAM dendrimer are protonated, while tertiary amines inside the dendrimer are not protonated until the pH drops to 4 (*Marvaniya, et al*,4; *Wolinsky, et al*,6). Because of this, they have an increase overall ionic interaction with DNA and other negatively-charged molecules (such as Adenoviruses) and produce very stable and highly soluble DNA complexes (*Hadidi, et al*,42).

However, as observed with other cationic macromolecules, including liposomes and polymeric micelles, PAMAM dendrimers with positively charged surface groups are prone to destabilize cell membranes and cause cell lysis (*Wolinsky, et al*,6; *Qi, et al*,29). Many functionalization strategies have been employed to mask the terminal amino groups and thereby reduce the inherently associated positive charge and resultant cytotoxicity. One way to reduce the cytotoxicity of cationic dendrimers involves partial surface derivatization with chemically inert functionalities such as polyethylene glycol (PEG). This observation can be explained by the reduced overall positive charge on these surface-modified dendrimers (*Wolinsky, et al*,6; *Fant, et al*,28; *Qi, et al*,29; *Hadidi, et al*,42; *Navarro, et al*,44; *Huang, et al*,46).

All the studies performed on this work were carried out using a modified PAMAM dendrimer. We report the use of a *poly(ethylene glycol) methyl ether (mPEG)-G3-polyamidoamine (PAMAM) dendrimer.* This was in part because PAMAM generations 0 through 10 (G0–G10) are commercially available featuring a wide number of peripheral groups (4 to 4096), end-group functionality (e.g. amine, carboxylic acid, hydroxyl) and molecular weights (657 to 935,000 g/mol).

Here we will show some possible applications different to any described up to the moment by using this dendrimeric form. The growth of mesenchymal stem cells and bacterias (such as Pseudomonas) were opposite but with potential clinical application

4. OBJECTIVES IN THIS PROJECT

The *specific objectives* to reach will be:

1. Characterize the formation of complexes and stability in cell culture conditions of the dendrimeric form.

2. To test the *toxicity of the dendrimer* through *in vitro* experiments to be complexed on a near future to therapeutic DNA or adenoviruses to suffer of EPR effect and will target specifically tumors.

3. To *study the growth changes* on the MSCs culture conditions when incubated with PAMAM-based dendrimeric forms and how they could make an influence on the surface markers or pluripotency status of the cell

4. To test the *potential antimicrobial efficiency* of the dendrimer.

PART II: MATERIALS AND METHODS

1. SYNTHESIS OF mPEG$_{2000}$-G3-PAMAM

The commercial amine-terminated G3 PAMAM dendrimer with an ethylenediamine core (20 wt% in methanol, Sigma-Adlrich, Spain) was vacuum-dried to remove traces of methanol. Poly (ethylene glycol) methyl ether (PEG, Mn=2000) was first thoroughly dried and then dissolved in tetrahydrofuran (THF) and triethylamine (TEA) (both distilled and dried). PEG was firstly activated via a reaction with p-nitrophenyl chloroformate chloride, which was slowly added to the mixture and stirred for 24h at room temperature (a calcium chloride tube is used to prevent any moisture from getting into the reaction vessel). The hydroxyl groups of methylated PEG reacted with the reagent and eliminated a molecule of HCl, as shown in Figure 2.1. The salt was filtered off and the filtrate was concentrated and then added dropwise to diethyl ether to precipitate as activated mPEG p-nitrophenyl chloroformate. The precipitate was dissolved in THF again and it was purified by reprecipitation from THF into diethyl ether. Alternatively, the purification could be carried out in a Sephadex-LH20 column using MeOH as eluent.

Next, pegylation was performed by linking activated p-nitrophenyl chloroformate PEG (4-NPC-PEG) with G3 PAMAM dendrimer. A solution of the dendrimer was dissolved in dimethyl sulfoxide and mPEG p-nitrophenyl carbonate was added to the solution at 4ºC and then stirred for 72h at room temperature. The reaction mixture was evaporated using a rotatory evaporator. The obtained product was dissolved in distilled water and filtrated in order to eliminate the low molecular weight reagents. The solid product was characterize by ^{1}H MNR and kept protected from moisture and light.

The synthesis was carried out with the collaboration of Ramón Cervera, from the Liquid Crystals and Polymers Group (University of Zaragoza).

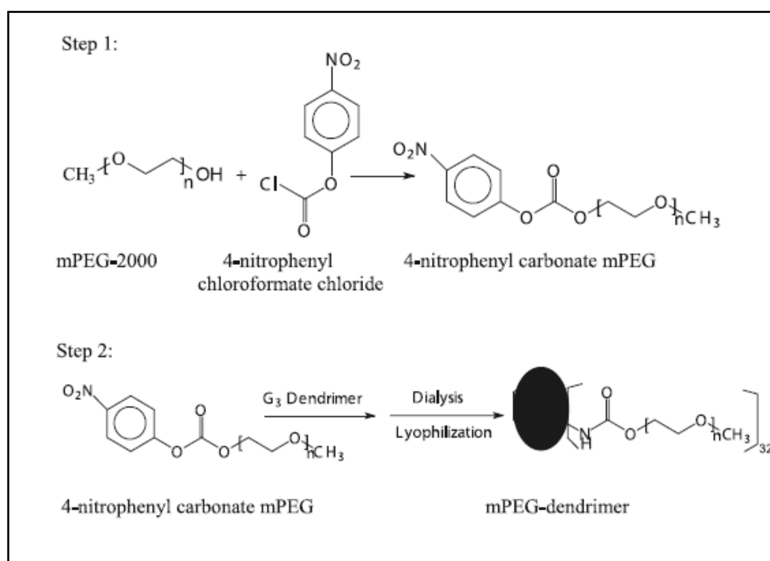


Fig 2.1. Diagram showing the synthesis of mPEG-G3-PAMAM dendrimer (*Bai, et al*,8).

2. STRUCTURAL ANALYSIS OF mPEG$_{2000}$-G3-PAMAM

2.1 Material Characterization

The stoichiometry of mPEG$_{2000}$-G3-PAMAM was established by 1H NMR. Since each PAMAM dendrimer conjugate was not unimolecular, characterization represented the average value from its polymeric distribution. Spectra were recorded on a Bruker AVANCE 400 spectrometer operating at 400 or 300 MHz and using DMSO and d6/D2O as solvents. Chemical shifts are given in ppm relative to TMS, and the solvent residual peak was used as internal standard. Ten milligrams of the dendrimer were dissolved in DMSO or deuterated solvent to perform the analysis.

MALDI mass spectra were obtained using 2,5-dihydroxybenzoic acid (DHB) and 2,4,6-trihydroxyacetophene (THAP) as matrices on a AUTOSPEC Vacuum General. Average molecular weights were calculated from the mass range encompassing the desired major peak.

Fourier transform infrared (FTIR) spectra were recorded on a Mattson Genesis II FTIR. Samples were scanned between 4,000 and 700 cm^{-1} at a resolution of 1cm^{-1}.

2.2 Assessment of dendrimer degradation

mPEG$_{200}$0-G3-PAMAM was dissolved in DMSO-*d6*/D$_2$O (10mg/ml) or left solid. Some of the aliquots were kept protected from light but others were exposed to light at 4ºC, room temperature and 37ºC for periods of time ranging from 24-72h. The solid samples were dissolved in DMSO-*d6*/D$_2$O (10mg/ml) right before the analysis.

The integrity of mPEG$_{2000}$-G3-PAMAM was established by ^{1}H *NMR* using the same equipment and conditions as previously described (Bruker AVANCE 400).

3. DNA-DENDRIMER COMPLEX FORMATION

3.1 Plasmid DNA Preparation

The pEGFP plasmid (Addgene, USA) was amplified in chemically modified *E.coli DH5α* (competent bacteria) according to standard procedures and it was purified using a NucleoBond Xtra Midi extraction kit (Macherey-Nagel, Germany) according to the manufacturer's instructions.

Briefly, *E.coli* competent bacteria (they are more likely to incorporate foreign DNA as their cell walls are altered, so that DNA can pass through more easily) were thaw on ice, an then a volume of pEGFP was added and everything was incubated on ice for 30

minutes. The mixture was heat-shocked by incubating it at 42°C for 30 seconds in a thermo-shaker TS-100 (Biosan, Latvia) and then they were placed on ice immediately (to reduce damage to the cells). Luria Broth (LB) medium was added to the cells, and incubated at 37°C for 1 hour with agitation in a thermo-shaker. Several dilutions of the transformation mixture were plated onto separate LB-amp agar plate and grown overnight at 37ºC in a bacteriological incubator (Incubat, JPSelecta, Spain). Inoculated plates were incubated inverted to prevent the condensation of water from dropping onto the agar surface, which can result in the colonies coalescing.

Colonies were picked and placed in LB-kanamycin (25µg/ml), and then incubated at 37ºC in a shaking incubator KS 4000i control (IKA, Germany) overnight. The culture was transferred to centrifuge tubes and the pellet resuspended in different lysis buffers that contain Tris-EDTA, RNase, sodium hydroxide and SDS. These compounds lead to the rupture of the cell wall and membrane, and also inhibit nucleases and degrade RNA and chromosomal DNA (but not the covalently closed circular plasmid DNA). Plasmid DNA was precipitated with 60% isopropanol, washed with 70% ethanol, air-dried and finally resuspended in dH_2O.

DNA concentration and purity of pEGFP were determined by using a NanoDrop spectrophotometer (Thermo Scientific, Spain). The intactness and identity of the plasmid were confirmed by agarose gel electrophoresis. For linearization, the plasmid was digested with *PStI* (New England Biolabs, UK).

To prepare LB-broth, the premix LB powder was dissolved (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl (w/v)) (Scharlau, Scharlab, Spain) in dH_2O while heating and then the mixture was autoclaved (20 mins, 121ºC, 2atm). The medium was kept at 4ºC. To make the LB-agar plates, the premix LB powder was dissolved (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar (w/v)) (Scharlau, Scharlab, Spain) in dH_2O while heating and then autoclave the bottle (20 mins, 121ºC, 2atm). Agar was cooled to ~55ºC and additives added when necessary, such as ampicillin (100 µg/ml) or kanamycin (25 µg/ml). A thin layer of LB Agar (~10ml) was poured into each plate and cooled until it was solid. Plates were flipped so as to avoid condensation on the agar and stored at 4ºC until usage.

All the procedures were carried out at the flame (Bunsen), and all the materials (30 mins, 121ºC, 2atm) and solutions (20 mins, 121ºC, 2atm) were autoclaved or filtered (0.2 µm syringe filter) and sprayed with 70% ethanol to ensure sterile conditions so as to prevent contamination.

3.2 Gel Retardation Assay

This assay is also known as electrophoretic mobility assay and it is used to study the formation of DNA-particle complexes. The technique of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbone. For this reason, when an electrical potential is placed on the DNA it will move towards the positive pole. The rate at which the DNA will move is slowed by making the DNA move through an agarose gel. The agarose forms a porous lattice and the DNA slips through the holes.

In summary, the basis of the assay is that, as the size of the DNA-particle complexes is larger than DNA alone, they should migrate more slowly than the free DNA fragments.

The gel was done by mixing the agarose (Lonza, Spain) with 1X TAE (Tris-acetate-EDTA) buffer and heating until the agarose was dissolved. The agarose was cooled and ethidium bromide (a DNA intercalating agent used as a fluorescent dye) (Fermentas, Thermo Fisher Scientific, Spain) was added. The mixture was poured into a gel electrophoresis tray with a comb -to make the wells- and let dry for 45 minutes.

$mPEG_{2000}$-G3-PAMAM and pDNA were diluted in dH_2O and Vector/pDNA complexes were prepared at various weight ratios ranging from 0.1:1 to 400:1 and incubated for 30 minutes at room temperature. The complexes were mixed with appropriate amounts of 6X loading buffer (which contains a dye to assess how fast the gel is running and a reagent to render the samples denser than the running buffer) (Takara, UK) and then electrophoresed on a 0.5% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml) for 45 minutes at 100V. All the gel electrophoresis equipment used was supplied from Biorad (Spain). The location of pDNA in the gel was analyzed on a G:Box UV transilluminator (Syngene, UK).

4. EVALUATION OF BACTERIAL GROWTH

4.1 Bacterial Preparations

The bacteria used in the analysis were *Escherichia coli* and *E.coli*-EGFP (Gram-positive, G+), *Pseudomonas aeruginosa* (Gram-negative, G-), *Enterobacter aerogenes(Guo Qing-Song, et al,47)*, *Salmonella typhimurium (Guo Qing-Song, et al,47)* and *Staphylococus.aureus* (G+) (kindly donated by the University Francisco de Vitoria, Madrid). Gram-positive bacteria retain the color of the crystal violet stain in the

Gram stain because their cell wall is composed of a thick layer of peptidologlycan; Gram-negative bacteria lose the crystal violet stain because their layer of peptidologlycan is very thin. All the microorganisms were grown at 37ºC and maintained in LB-agar plates at 4ºC; *E.coli*-EGFP was maintained in LB-kanamycin. Inoculated plates are incubated inverted to prevent the condensation of water from dropping onto the agar surface.

To freeze the bacteria, one colony from each plate was inoculated in LB-broth and grown at 37ºC O/N while shaking. Each bacterial suspension was diluted with sterile glycerol solution to a final 15% v/v glycerol concentration. The samples were kept in ice during the process and at the end quickly transferred to a -80°C freezer for long-term storage. For thawing, bacteria were left on ice for 30 minutes and the suspension directly plated. Bacteria were incubated O/N at 37ºC.

All the procedures were carried out at the flame (Bunsen), and all the materials (30 mins, 121ºC, 2atm) and solutions (20 mins, 121ºC, 2atm) were autoclaved or filtered (0.2 μm syringe filter) and sprayed with 70% ethanol to ensure sterile conditions so as to prevent contamination.

4.2 Bacterial Assays

4.2.1. LB plate assay

Ampicillin (Sigma, Spain), kanamycin (Invitrogen by Life Technologies, Thermo Fisher Scientific, Spain) and $mPEG_{2000}$-G3-PAMAM were added to LB-agar plates at different concentrations ranging from 0.5-500 μg/ml and let air-dry. Aliquots of each culture were left O/N on LB in agitation at 140 rpm and were then plated. Both antibiotics and dendrimer solutions were previously diluted in dH_2O and filtered with a 0.2 μm syringe filter besides the flame. Plates were incubated at 37°C to allow colony formation and observed after 24h.

4.2.2. LB broth assay

Ampicillin, kanamycin and $mPEG_{2000}$-G3-PAMAM (diluted in dH_2O and filtered with a 0.2 μm syringe filter) were added to LB-broth at 50, 100 and 500 μg/ml and then a colony from each bacteria strain was resuspended in each broth. Bacteria suspensions were checked for turbidity after 24h incubation at 37ºC while shaking at 140 rpm and the positive suspensions with the higher antibiotic or dendrimer concentration were plated in LB-agar and incubated overnight.

4.2.3. LB microplate assay

The minimum inhibitory concentration (MIC) of the PAMAM derivatives, that is, the lowest concentration of the compounds that inhibits the visible growth of *P.aeruginosa* and *S.aureus* after 18-24 h of incubation, was determined using a broth microdilution technique. A pre-inocule of *P. aeruginosa* and *S.aureus* were grown O/N in agitation in LB-broth. Serial dilutions of the bacterial suspensions were prepared in a 24-well plate (MW24) and absorbance was measured to adjust the bacteria concentration to $2x10^5$ CFU/ml (Abs~0.20 at 630 nm) (stock solution). Dilutions of $mPEG_{2000}$-G3-PAMAM were prepared in dH_2O (50-4000 μg/ml).

The stock bacterial solutions were distributed into a 96-well sterile plate (MW96) and each of the prepared dendrimer solutions was added to the wells. Control wells were composed of bacterial solutions without dendrimers (positive controls) and LB medium without bacteria but with dendrimer (negative controls). To reduce evaporation during incubation (which is critical to the assay), each of the empty outer wells of the microtiter plate was filled with sterile water to increase the water vapor pressure in the incubator. The microtiter plates were incubated at 37 °C and 140 rpm O/N. At the end of the incubation, the pellets formed were resuspended prior to scanning the optical densities using a Synergy HT (BioTek, USA) at 630 nm, which is proportional to the number of bacteria in suspension.

5. EVALUATION OF CELL GROWTH

5.1 Culture of Cell Lines

Human Neuronal Gliobastoma U251MG cells (Cancer Research UK, Cell Lines Services) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l D-Glucose, 4 mM L-Glutamine and 1.0 mM sodium pyruvate (Gibco, Invitrogen by Life Technologies, Thermo Fisher Scientific, Spain). Also, 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin (Lonza) and 1% amphotericin B (Lonza) were added to complement the media. Cells were maintained at 37ºC in normoxic conditions (21% O_2; 5% CO_2) in an Excella ECO-170 CO_2 incubator (New Brunswick Scientific, UK).

When subculturing, media was removed and cells were washed with DPBS (Gibco, Invitrogen by Life Technologies, Thermo Fisher Scientific, Spain). Then, 1% trypsin solution (Trypsin-Versene 10X, Lonza) was added and cells were incubated at 37ºC for

5 minutes, allowing trypsin to break down the proteins which enable the cells to adhere to the vessel. Trypsin action was stopped by adding fresh cell culture media and a part of the cells was resuspended and dispensed into a new flask or plate. Cells were passaged every 4-5 days (80-90% confluence).

Murine MSCs (bone marrow of C57BL/6 mouse at ≤ 8 weeks after gestation, Gibco, Invitrogen by Life Technologies, Thermo Fisher Scientific, Spain by Life Technologies, Spain) were cultured in Dulbecco's modified Eagle's medium (DMEM-LG) containing 1 g/l glucose, 2 mM L-glutamine and 1.0 mM sodium pyruvate (Gibco, Invitrogen by Life Technologies, Thermo Fisher Scientific, Spain). Also, 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% amphotericin B were added to complement the media.

Alternatively, mMSCs were cultured in MesenCul MSC Basal Medium (Mouse), adding 10% MesenCult Mesenchymal Stem Cell Stimulatory Supplements (Mouse) (StemCell, Miltenyi Biotec, Spain), 1% penicillin/streptomycin and 1% amphotericin B.

In both cases, mMSCs were maintained at 37ºC under hypoxic conditions (1% Oxygen and 5% CO_2) in a Forma Series II Water jacketed CO_2 Incubator, HEPA class 100 (Thermo Fisher Scientific, Spain).

For subculturing, the same procedure as previously described for U251MG cells was followed, but using 2.5% trypsin (Trypsin-Versene 10X, Lonza, Spain) (as in this case cells showed a higher adherence). Cells were passaged every 6-7 days when using DMEM-LG and every 3-4 days when using MesenCult (in both cases, when cells reached 80-90% confluence).

For freezing, cells were first trypsinize by using the same procedure as for passaging them; then the pellet was resuspended with freezing media (80% FBS, sterile 20% DMSO) and the cryovial (Simport, USA) was left at -80°C for a minimum of 24h; then the vial was transferred to the liquid N_2 tank for indefinite storage.

When thawing, the vial was removed from the liquid N_2 tank and it was held it in the 37°C water bath until the freezing media was thawed. Gently pour cells into a falcon tube with PBS, centrifuge and resuspend in media. Media needs to be changed the next day to prevent cell damage with possible remaining DMSO.

All the cultures were tested for mycoplasma and all the cell culture plastic labware was supplied by Nunc (Thermo Fisher Scientific, Spain).

A vertical laminar flow hood Safemate 1.2 (BioAir, Italy) was used for all the culturing procedures. The tray was swab with 70% ethanol before and after each use

and UV was irradiate for 10 minutes. Full protective clothing and nitrile gloves must be worn at all times when using the hood and the incubators.

Cells were observed under an AE31 Research Grade Inverted Microscope (Motic, Spain) and manually counted using a Neubauer hemocytometer chamber (Blaubrand, Germany).

5.2 Viability Assays

5.2.1. Crystal Violet method

Crystal violet (CV) is a triphenylmethane dye (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline) that stains DNA. CV is a simple assay that is useful for obtaining quantitative information about the relative density of cells adhering to multi-well cluster dishes. **Upon solubilization, the amount of dye taken-up by the monolayer and the intensity of the color produced are proportional to cell number.**

U251MG and mMSCs cells were seeded at a density of $2x10^3$ cells/well in a MW96, or alternatively at $4x10^4$ cells/well in a MW24. A stock solution of 50mg/ml was made on the moment by diluting the dendrimer in dH_2O. Then, the solution was filtered with a 0.2µm syringe filter (25mm diameter, Nalgene, Labclinics, Spain) in the laminar flow hood and kept protected from light at 4ºC.

Upon 24h incubation and without removing the media, fresh media was added to the control wells and $mPEG_{2000}$-G3-PAMAM diluted in media at different concentrations (0.5, 1 and 5 mg/ml) was added to the rest of the wells (in triplicate). Media was removed after 24, 48 and 72h of incubation with the dendrimer and cells were fixed with 1% Glutaraldehyde for 10 mins; then, PBS was added and the plate was kept in the incubator until the day of the analysis. Then, PBS was removed and 0.1% crystal violet (dissolved in ethanol) (Roth, Spain) was added for 30 mins at room temperature. After the staining, the dye was removed washing with tap water and the plate air-dried for a couple of days at room temperature. 10% acetic acid was added to the wells to extract the dye from the cells, transferred to a new MW96 and absorbance read on a Synergy HT (BioTek, USA) plate reader at 590nm. Citotoxicity was expressed as relative viability of cells (% of the control cells incubated with medium only). All the experiments were repeated at least three times.

5.2.2. Alamar Blue method

Resazurin (7-hydroxy-10-oxido-phenoxazin-10-ium-3-one) is a redox dye that is commercially available as Alamar Blue, which exhibits both colorimetric and fluorometric change that relates to cellular metabolic activity. The assay is based on the ability of viable, metabolically active cells to reduce resazurin to resorufin and dihydro-resorufin. This conversion occurs intracellularly, where the oxidized form of the resazurin enters the cytosol and is converted to the reduced form by mitochondrial enzyme activity by accepting electrons from NADPH, FADH, FMNH, NADH as well as from numerous cytochromes. The reduction related to growth causes the resazurin to be converted from the oxidized (or non-fluorescent) blue form to the reduced (fluorescent) red form. Since Resazurin is not-toxic to cells and is stable in culture media, continuous measurement of cell proliferation in vitro can be achieved. **Toxic compounds that impair cell viability and proliferation also affect the capacity to reduce resazurin, and the rate of dye reduction is directly proportional to the number of viable cells present.**

U251MG and *mMSCs* cells were seeded at a density of 1-3x10^3 cells/well in a MW96. Upon 24h incubation, media was removed and fresh media was added to the control wells and mPEG$_{2000}$-G3-PAMAM diluted in media at different concentrations (0.5, 1 and 5 mg/ml) was added to the rest of the wells (in triplicate). The dendrimer stock solution at 50mg/ml in dH_2O was prepared as previously described.

Media was removed after 24, 48 and 72h of incubation with the dendrimer and fresh media was added to all the wells (control and sample wells); then a 10% of the media volume of Alamar Blue solution (Invitrogen by Life Technologies, Thermo Fisher Scientific, Spain) was added. The reading of the plates was done on a daily basis to prevent the cells from contaminating, so they were seeded in different plates, and each plate was discarded after measuring the fluorescence. After 3h incubation at 37ºC, fluorescence was read at 530/590 (excitation/emission) on a Synergy HT (BioTek, USA) plate reader. Citotoxicity was expressed as relative viability of cells (% compared to the control cells incubated only with medium).

To set up the best conditions, different combinations were assayed (seeding concentration, culturing media, incubation time with assay reagent, etc), but in all cases the experiments were repeated at least three times.

5.2.3. MTT method

The MTT assay is a sensitive, quantitative and reliable colorimetric assay that measure viability, proliferation and activation of cells. The assay is based on the capacity of the cellular mitochondrial dehydrogenase enzyme in living cells to reduce the yellow water-soluble substrate 3-(4,5-dimethylthiazol- 2yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue/purple formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the cell number. The MTT assay has greater applicability in the detection of cells which are not dividing but are still metabolically active. It can, therefore be used to measure the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. Unfortunately, during conduct of the test, cells die making it impossible to conduct follow-up cell culture assessments.

mMSCs cells were seeded at a density of 1-3x10^3 cells/well in a MW96 and allowed to attach overnight. Media was removed and fresh media was added to the control wells and $mPEG_{2000}$-G3-PAMAM was diluted in media at different concentrations (0.5, 1 and 5 mg/ml) was added to the rest of the wells (five replicates). The stock solution of dendrimer at 50mg/ml in dH_2O was prepared as previously described. A stock solution of 5mg/ml MTT (Serva electrophoresis, Labclinics, Spain) in PBS (pH 7.4) was made and filtered with 0.2µm syringe filter; aliquots were kept protected from light at -20ºC.

Media was removed after 24, 48 and 72h of incubation with the dendrimer and fresh media was added to all the wells (control and sample wells); then MTT solution was added to a final concentration of 0.5mg/ml. As in the case of Alamar Blue, the reading of the plates was done on a daily basis to prevent the cells from contaminating, so they were seeded in different plates, and each plate was discarded after measuring the fluorescence. After 4h incubation at 37ºC, MTT solution was removed and dimethylsulfoxide (DMSO) was added to dissolve the insoluble formazan crystals. Finally, absorbance was read at 570nm on a Synergy HT (BioTek, USA) plate reader. The viability of mMSCs cells exposed to the dendrimers was expressed as a percentage of the viability of cells grown in the absence of the dendrimers.

Different conditions were assayed (seeding concentration, culturing media, incubation time with assay reagent …), but in all cases the experiments were repeated at least three times.

5.2.4. Interference Tests

To eliminate possible interferences between the dendrimer assay readings, media (complete DMEM) alone or with different concentrations of sterile $mPEG_{2000}$-G3-PAMAM were added to a MW96 in triplicate and incubated at 37ºC for 24h. After the incubation period, wells were washed with PBS and then the assay reagent was added, or alternatively the assay reagent was directly added without washing. The assay reagents were crystal violet, alamar blue and MTT, and the procedures followed were the ones described above.

In a different assay, cells were seeded at 1-3x10^3 cells/well in a MW96, incubated for 24h at 37ºC and treated with the same concentrations of $mPEG_{2000}$-G3-PAMAM. The procedures described previously for crystal violet, alamar blue and MTT were followed, but without the addition of the assay reagents.

5.2.5. Trypan Blue method

Trypan blue is a diazo dye used to selectively color dead cells in blue. Since cells are very selective in the compounds that pass through the membrane, in a viable cell trypan blue is not absorbed; however, it goes through the membrane in dead cells. Hence, dead cells are shown as a distinctive blue color under a microscope. However, trypan blue staining cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell functions.

To reassure, mMSCs were seeded at 1-3x10^3 cells/well in a MW96 and incubated for 24 h at 37ºC. Cells were washed with DPBS and trypsinized. 0.4% trypan blue in PBS was added to the cell suspension in a 1:1 to 1:5 ratios (depending on the cell concentration) and incubated at room temperature for 3-5 min. Viable and dead cells were manually counted using a Neubauer hemocytometer chamber. Viability values of treated cells were expressed as a percentage of that from corresponding control cells.

	CV	AB	MTT	TB
Direct cell counting technique	x			x
Metabolic active cells		x	x	
Cell proliferation rate		x	x	
Living-cell exclusion (vital stain)	x			x
Non-toxic to cells		x		

Figure 2.2. Summary of the principal characteristics of the different viability methods.

5.3 Gelatin Assay

Sterile mPEG$_{2000}$-G3-PAMAM (previously filtered) was diluted in PBS-0.1% gelatin solution (Type B, 2% in H_2O, Sigma, Spain) to different concentrations (0.5, 1 and 5 mg/ml), added to the wells of a MW96 plate and incubated 1h at room temperature. Then, surplus dendrimer-PBS-gelatin solution was removed and a fine layer of the gelatin-dendrimer complex remained at the bottom of the wells.

U251MG and mMSCs cells were seeded over the gelatin at a density of $3x10^3$cells/well in a MW96. Media was removed after 24, 48, 72 and 96h of incubation with the dendrimer and fresh media was added to all the wells (control and sample wells); then a 10% of the media volume of Alamar Blue solution (Invitrogen by Life Technologies, Thermo Fisher Scientific, Spain) was added. From this point, the procedure was carried out the same as the Alamar Blue protocol. Citotoxicity was expressed as relative viability of cells (% of control cells incubated with medium only).

5.4 Evaluation of Cellular Alterations

5.4.6. Analysis of Cell Cycle and Immunophenotyping

Flow cytometry is a technology that allows a single cell to be measured for a variety of characteristics, determined by looking at how they flow in liquid and measuring visible and fluorescent light emissions, allowing cell sorting based on physical, biochemical and antigenic traits.

Analysis of a population of cells' replication state can be achieved by fluorescence labeling of the nuclei of cells in suspension and then analyzing the fluorescence properties of each cell in the population. This approach reveals distribution of cells in three major phases of the cycle (G1 vs S vs G2/M) and makes it possible to detect apoptotic cells with fractional DNA content.

For the *cell cycle analysis*, mMSCs cells were seeded into P60 plates at $1x10^6$cells/plate and incubated at 37ºC for 24h. Then, cells were washed with DPBS and fresh media was added to the control plate and a dilution of dendrimer (1mg/ml) was added to the sample plate. Cells were incubated another 72 hours. This concentration of dendrimer was chosen based on the results obtained in the citotoxicity assays (see Results section). After the incubation, cells were washed with DPBS, trypsinized and centrifuged. The pellet was resuspended in media, $1x10^6$ cells were counted and separated into a new tube and centrifuged again. The pellet was washed by resuspending cells in DPBS and centrifuging. Finally, cells were fixed (for the

permeabilisation of the cell plasma membranes) by resuspending them in DPBS and then ice-cold 70% ethanol was slowly added to the side of the tube. Cells can be kept at -20°C for a maximum of a week. At the day of analysis, the cells were washed from ethanol, stained with 5 µg/ml propidium iodide (PI, Becton Dickinson, Spain) at the presence of RNase A (Invitrogen by Life Technologies, Thermo Fisher Scientific, Spain) for 30 min at 37°C.

Antibodies are powerful tools for studying cell surface protein expression signatures. For the *immunophenotyping analysis*, a fluorescent dye is attached to antibodies and these cells can then be subjected to flow cytometry and the amount of the receptor on their surface detected as a level of fluorescence. Antibodies used for these experiments were anti-CD73 (Phycoerythrin (PE)-conjugated), anti-CD105 (FITC-conjugated) as positive controls and anti-CD34 (PE-conjugated) (Becton Dickinson, Spain) as negative control. mMSCs cells were seeded into P100 plates at $2x10^6$cells/well and incubated at 37ºC for 24h. Then, cells were washed with DPBS and fresh media was added to the control plate and a dilution of dendrimer (1mg/ml) was added to the sample plate. Cells were incubated for 72 hours. After the incubation, cells were trypsinized. $2x10^6$ cells were separated into a new tube, washed with DPBS and finally resuspended in PBS-EDTA. Cell suspensions were separated in different tubes and tested separately for the three markers by staining with 5 µg/ml of each antibody.

The cycle analysis and immunofluorometric analysis were performed using FACSAria (Becton Dickinson, Spain). Data were collected at the wavelength of 580 nm for PI and PE and 520 nm for FITC.

5.4.7. mMSCs Adipogenic Differentiation

Mesenchymal stem cells are multipotent in nature and have the unique ability to differentiate into various cell types. Therefore, to confirm the identity of cells as MSCs, their adipogenic differentiation potential was evaluated.

mMSCs cells were seeded into MW12 at $1.2x10^5$ cells/well ($3x10^4$ cells/cm^2) with MesenCult Basal Medium (Mouse) and incubated at 37ºC for 24h. Then, cells were washed with DPBS and fresh media (Basal MesenCult) was added to the positive control wells (+ctr) (Sample A, Figure 2.2) and a dilution of dendrimer (1mg/ml in MesenCult Basal Medium) was added to the sample wells (+D) (Sample C, Figure 2.2). Cells were incubated for 72 hours. This concentration of dendrimer was chosen based

on the results obtained in the citotoxicity assays (see Results section). *Adipogenesis* was induced by culturing confluent mMSCs (+ctr and +D wells) in Complete MesenCult Adipogenic Medium (Mouse) (StemCell, Miltenyi Biotec, Spain). This media was prepared by diluting Adipogenic Stimulatory Supplement (Mouse) (StemCell, Miltenyi Biotec, Spain) 1/5 with MesenCult MSC Basal Medium (Mouse); 1% peniciline/streptomycine was added. Negative control cells (-) were maintained in Basal MesenCult Medium. Cells were checked every day and media was changed every 2-3 days.

Alternatively, cells were seeded into MW24 at $1.5x10^5$ cells/well; after 24h of incubation, they were washed with DPBS and adipogenesis was induced by adding Complete MesenCult Adipogenic Medium (Mouse) with a dilution of the dendrimer (1mg/ml in MesenCult Adipogenic Medium) to the sample wells (+D) (Sample D, Figure 2.2). Cells were incubated for 24-48 hours. The positive control wells (+ctr) (Sample B, Figure 2.2) were incubated 72h in Basal Medium previous to differentiation. After the incubation period, the differentiation process followed as previously explained (cells were checked every day and media was changed every 2-3 days).

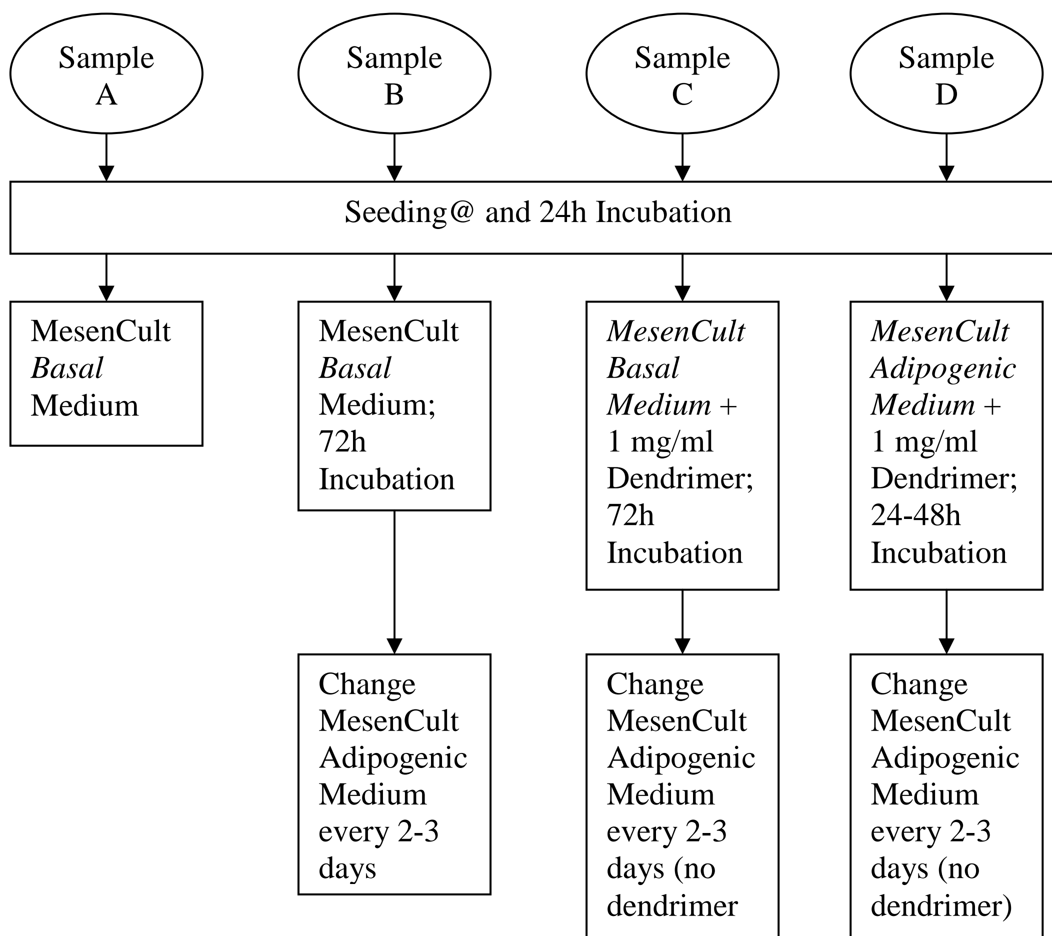


Fig 2.3. Diagram showing the strategy followed for the mMSCs differentiation.

In both cases, adipogenic differentiation was confirmed after 14 days by Oil-Red staining, which stains lipid droplets within the adipocytes. Oil-Red was prepared by mixing Oil-Red powder (Sigma, Spain) with 99% isopropanol (stock solution, one year stability). Then, 3 parts of the stock were mixed with 2 parts of dH_2O; the mixture was left to settle 10 minutes and slowly filter using a funnel and filter paper (stable only for 2 hours). Cells were observed under an AE31 Research Grade Inverted Microscope (Motic) and pictures were taken with a Moticam 5000 cooled Digital Camera.

PART III: RESULTS AND DISCUSSION

1. STRUCTURAL ANALYSIS OF mPEG$_{2000}$-G3-PAMAM

The integrity of mPEG$_{2000}$-G3-PAMAM was tested right after being synthesized (^{1}H MNR carried out by R. Cervera). After several weeks the integrity of the dendrimer was checked.

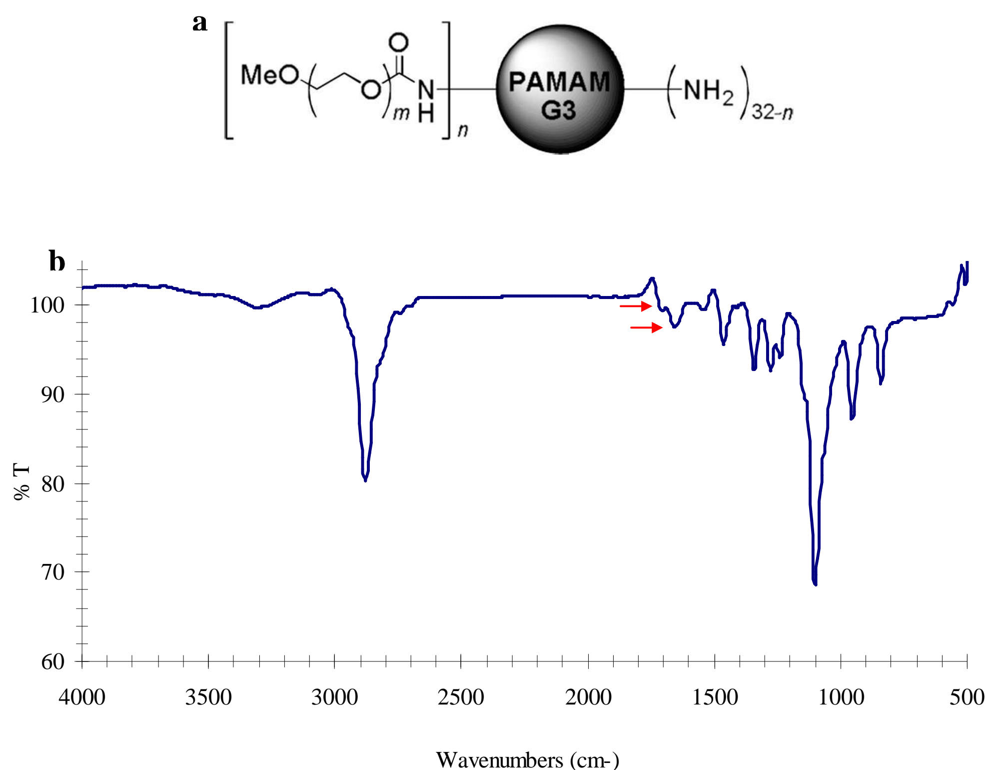


Figure 3.1. (a) General structure of mPEG-G3-PAMAM and (b)FTIR spectrum of mPEG$_{2000}$-G3-PAMAM dendrimer. The red arrows points at the peaks related to a change in length of –C=O. Approximate peaks at 3300cm^{-1}, 2800 cm^{-1}, 1450 cm^{-1}, 1300 cm^{-1} and 1100 cm^{-1} correspond to H-O, C-H, N-H (amide II), C-O and C-O-C, respectively.

Emergence of peaks at 1705cm-1 and 1655cm-1 shows the change in length of –C=O (stretching) due to the binding between the carboxyl of mPEG and the amide of PAMAM.

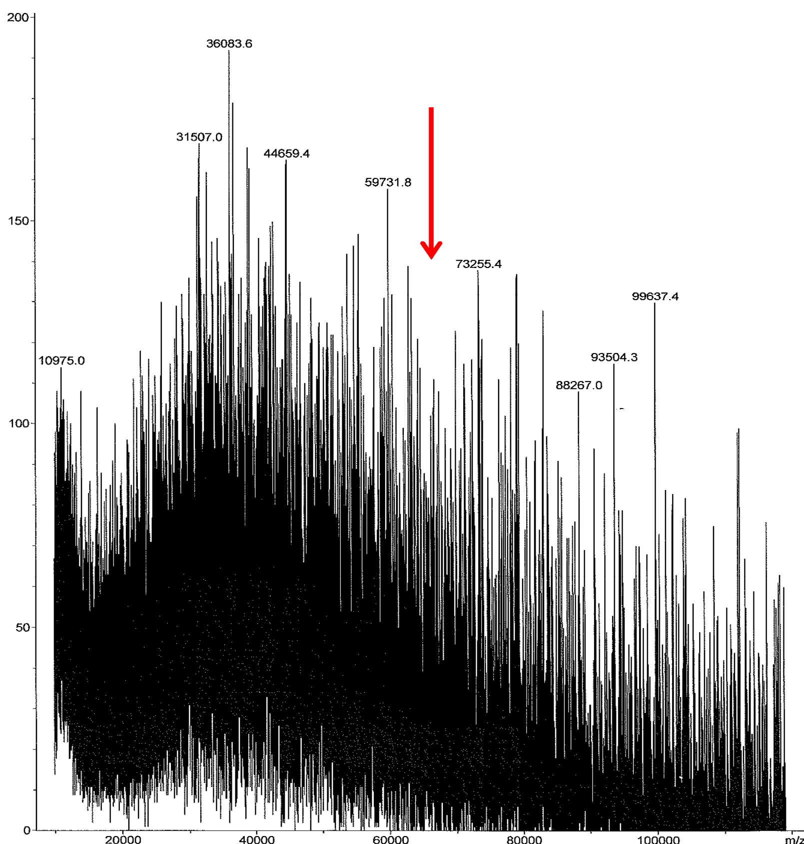


Figure 3.2. MALDI-LP spectrum of $mPEG_{2000}$-G3-PAMAM using DHB as a matrix. The red arrow points at the calculated mass.

As happens in higher dendrimer generation (in this case a lower generation but completely covered with long PEG chains), the spectral lines became weak and, as a result, we did not obtain a line structure, but a general molecular weight distribution can be observed (Fig.3.2). From that spectrum we calculate a theoretical mass of ~ 70000 Da.

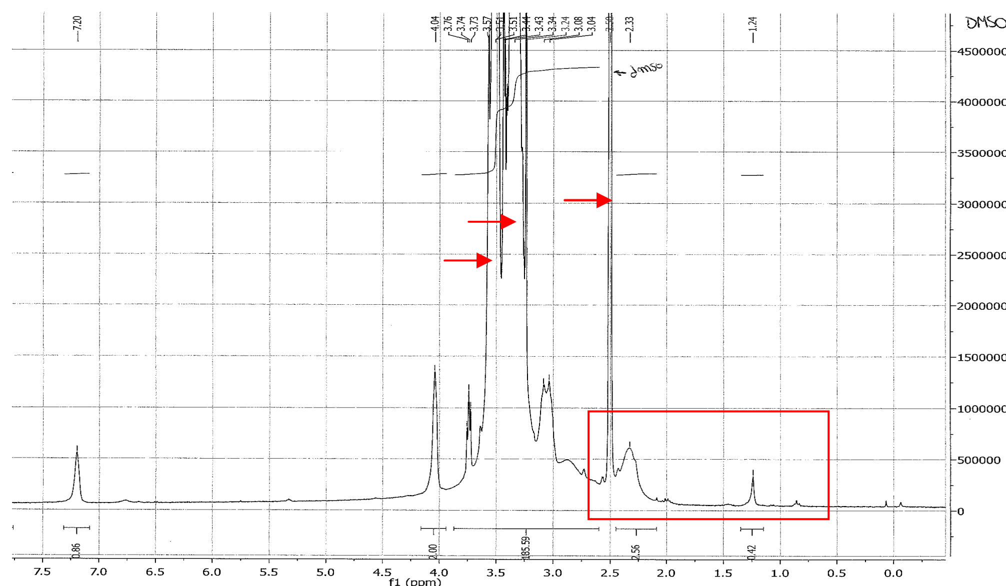


Figure 3.3. ^{1}H NMR spectrum of $mPEG_{2000}$-G3-PAMAM in DMSO-d6. Red arrows point to CH_2-PEG, CH_3-PEG and DMSO (from left to right); red box locates PAMAM-dendrimer-CH_2.

The results suggested that a mPEG-G3-PAMAM dendrimer complex was formed. All the data presented here agrees with previous published results (*Kim, et al*,48). Finally, the integrity of mPEG$_{2000}$-G3-PAMAM was established by ^{1}H NMR after 18 weeks up to 8 months of keeping it in *darkness and room temperature*. The dendrimer was further dissolved in deuterated water and kept protected from light, exposed to light at 4ºC, room temperature and 37ºC for periods 72h. Any of the spectra show differences compared to the one obtained before (data not shown) concluding that the dendrimer is stable at room temperature for long periods of time.

2. DNA-DENDRIMER COMPLEX FORMATION

Currently the practical use of unmodified PAMAM dendrimers has been questioned by reports that show relatively high citotoxicity as a result of their high surface charge density. Pegylation increases bioavailability of polymer-DNA complex by enhancing solubility and increasing the circulation time in the bloodstream; also facilitates dissociation of DNA inside the cell (*Fant, et al*,28).

Before trying the transfection efficiency of the dendrimer, the amount of plasmid DNA that binds the dendrimer is optimized so neither vector nor therapeutic agent is wasted.

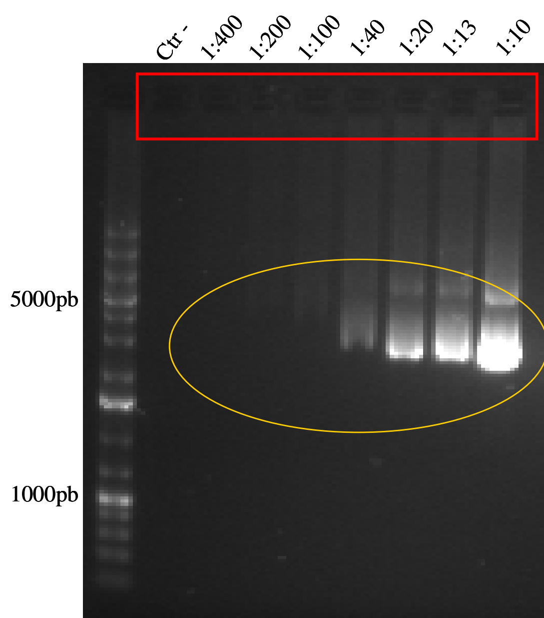


Figure 3.4. Gel Retardation Assay. Ratio Plasmid:Dendrimer (increasing amounts of plasmid). Lane 1: DNA marker (with known bands for estimating the molecular weight of the sample bands). Lane 2: negative control with dendrimer alone. Lanes 3-9: mixtures of pEGFP (concentrations of pEGFP ranging from 0.125-5 µg) and dendrimer (50 µg). There is no retention of Dendrimer-Plasmid complexes in the wells (red box). The intensity of the bands related to pEGFP increases with increasing plasmid concentration (yellow circle).

When incubation of the dendrimer with the plasmid DNA, if the ratio is optimal, the vector incorporates all the plasmid and the complex formed is retained in the well. When the ratio dendrimer:plasmid is higher than the optimal one, extra-not bound plasmid runs along the gel.

The gel retardation assay demonstrated that *DNA would not package mPEG$_{2000}$-G3-PAMAM dendrimer* at D/P ratios up to 400 (Fig. 1). Because it is completely covered with mPEG, G3-PAMAM has no external positive charge to bind the plasmid DNA (negatively charged) and then it will not be useful for plasmid transfections into the cells.

3. EVALUATION OF BACTERIAL GROWTH

We have evaluated the antibacterial activity of mPEG$_{2000}$-G3-PAMAM against *E.coli* and *E.coli-EGFP* (Gram-positive, G+), *P. aeruginosa* (Gram-negative, G-), *E. aerogenes(Guo Qing-Song, et al,47)*, *S. typhimurium* (*Guo Qing-Song, et al*,47) and *S. aureus* (G+). The cytoplasmatic cell membrane is the main target for many antibacterial agents, and PAMAM dendrimers have shown great potential interacting and disrupting them (*Cheng, et al*,37).

In a first attempt, bacteria were incubated in plates containing LB agar with a dilution of the dendrimer at a specific concentration. In order to compare the activity of the dendrimer with effective antibiotics commonly used in the lab, similar concentrations were tested (from 0.5-100 µg/ml). As it is shown in Figure 3.5, grow inhibition is observed in Ampicilin and Kanamycin plates (used as positive controls), but no effect can be observed for any of the bacteria with the dendrimer (plates with antibiotics and dendrimer at lower concentrations are not shown).

In a second attempt, bacteria were incubated with the dendrimer in solution, so as to favor interactions between both. Again, all the bacteria were found to be resistant to the dendrimer at concentrations of 100-500 µg/ml (Fig. 3.6) (lower dendrimer concentrations are not shown).

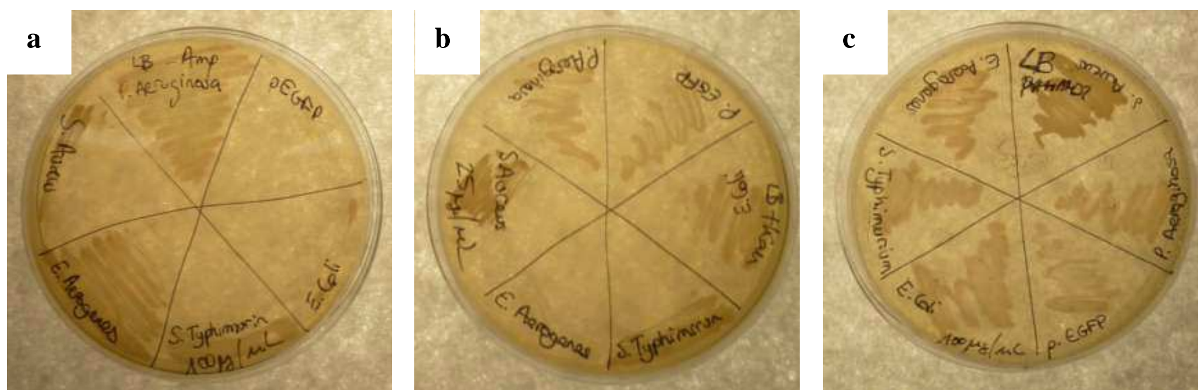


Figure 3.5. Antimicrobial activity using a plate assay using (a) ampicilin (100 µg/ml), (b) kanamycin (25 µg/ml) and (c) mPEG2000-G3-PAMAM (100 µg/ml).

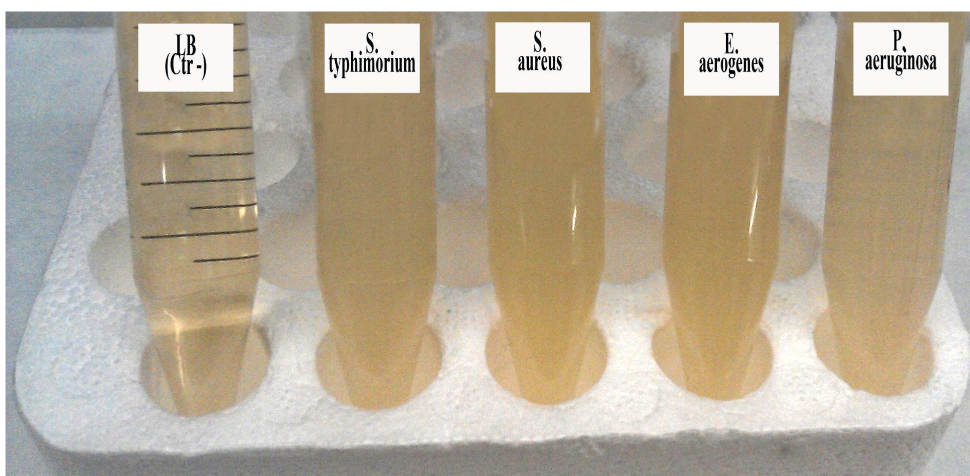


Figure 3.6. Antimicrobial activity of mPEG2000-G3-PAMAM using a broth assay. All the bacteria shown here were incubated with 500 µg/ml. LB alone was used as the negative control (no turbidity). The increased turbidity is a sign of bacterial growth.

Eventually, a more accurate and reproducible assay was used to asses the activity of the dendrimer. In this case, instead of incubating the dendrimer with an unknown number of microorganisms, we adjusted the number of bacteria to a certain value of cfu/ml prior to incubation with $mPEG_{2000}$-G3PAMAM. Then, the optical density at 635 nm was measured, which is proportional to the number of bacteria. In this case we only used *P. aeruginosa* and *S. aureus*, since both are common pathogens in ocular and vaginal infections and have been previously tested with PAMAM-derivates successfully (*Lopez, et al*,33).

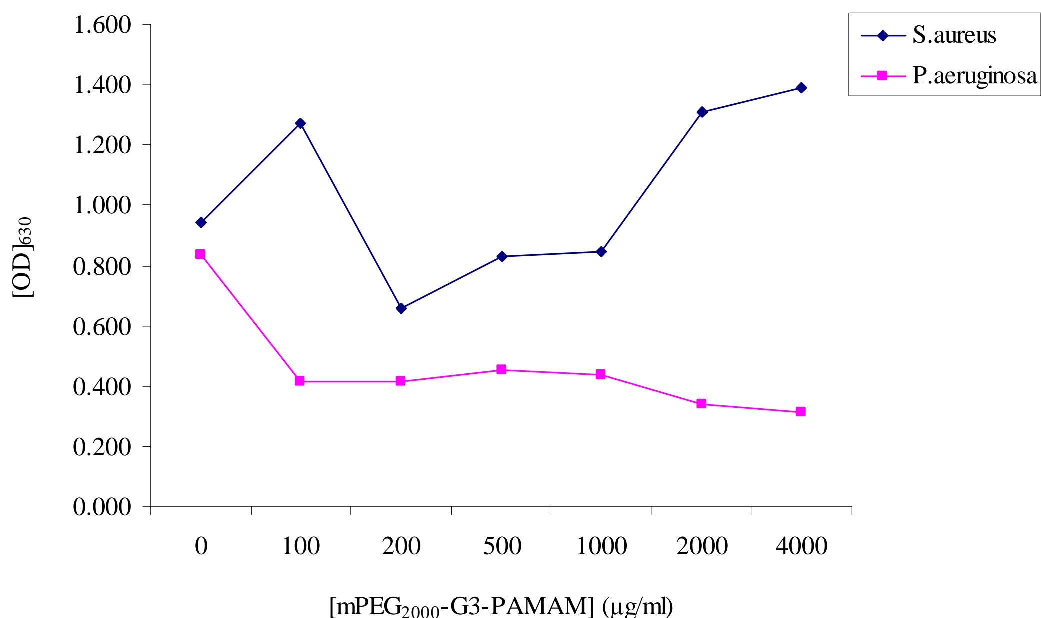


Figure 3.7. Antimicrobial activity of mPEG$_{2000}$-G3-PAMAM using a modified broth microdilution assay. Gram+ bacteria (blue) and Gram- (pink).

As expected, *mPEG2000-G3-PAMAM is more efficient in inhibiting the growth of P.aeruginosa (Gram-) (concentrations of 100 µg/ml and above) compared to S.aureus (Gram+).* This can be explained taking into account that the antibacterial action of the dendrimers involves disruption of the cytoplasmic membrane of the bacteria, and in the case of Gram- bacteria, the top of the plasma membrane is covered by negatively-charged proteins that facilitate membrane permeation and hence killing of the bacteria. We will check in the near future another 2 Gram – bacterias, *E. aerogenes* (*Guo Qing-Song, et al*,47) and *S. typhimurium* (*Guo Qing-Song, et al*,47) to test if is a general effect in all the Gram - bacterias.

In the initial assays the bacteria appear to be resistant to the dendrimer at concentrations up to 500 µg/ml (Fig. 3.7). In that case we looked at the turbidity to determine the result, and in this case, although we measure the OD to obtain more specific data, we could also see growth of the bacteria in all the wells, meaning that the bacteriostatic effects could be identical in both assays but we did not have enough resolution.

It is also possible that the character of the PEG chains to some extent enhances the permeation through the cell membrane. Moreover, the fact that the dendrimer retains antibacterial activity at high concentrations (4 mg/ml) could be an effect of the detachment of some of the PEG chains from the PAMAM and the dendrimer. Higher

concentrations of dendrimer could be tested, but they would be substantially higher than those of common antibiotics, and in general terms, more expensive, and then some of the advantages would be lost.

Although PAMAM dendrimers strongly bind to bacteria and third generation has proved to be more effective because they easily penetrate towards the bacterial membrane (*Lopez, et al*,33), we can conclude that complete PEGylation deactivates the dendrimer against bacteria but our dendrimer still retains some of the antibacterial effect against Gram - bacterias.

4. EVALUATION OF CELLULAR GROWTH AND ALTERATIONS

The citotoxicity of PAMAM dendrimers in mammalian cells lines due to the nature of the surface charge has been noted. Before using this type of vectors in any *in vivo* application, a citotoxicity profile must be done *in vitro*.

In our study, the citotoxicity of $mPEG_{2000}$-G3-PAMAM dendrimer was evaluated using four commonly used methods: Crystal Violet (CV), Alamar Blue (AB), MTT and Trypan Blue (TB). At least, two different cell types must be tested, our cells of interest (MSCs) and a tumoral cell line (U251MG).

An interesting phenomenon was observed when measuring the cell viability of both U251MG (Fig. 3.8a) and mMSCs (Fig. 3.8b) cells after incubation with increasing concentrations of the dendrimer at different times (*Hoskins, et al*,49). The presence of the dendrimer resulted in greater absorption readings compared to the control. This phenomenon could be explained by the adherence of the sticky polymers to the well surface. The value of cell viability that appeared was still larger compared to the visual inspection of viable cells under microscope (Fig. 3.9A, 3.9B). Until 42h, cells show more proliferation at lower dendrimer concentrations; at 72h and concentrations between 1-5 mg/ml, the dendrimer shows citotoxicity effects.

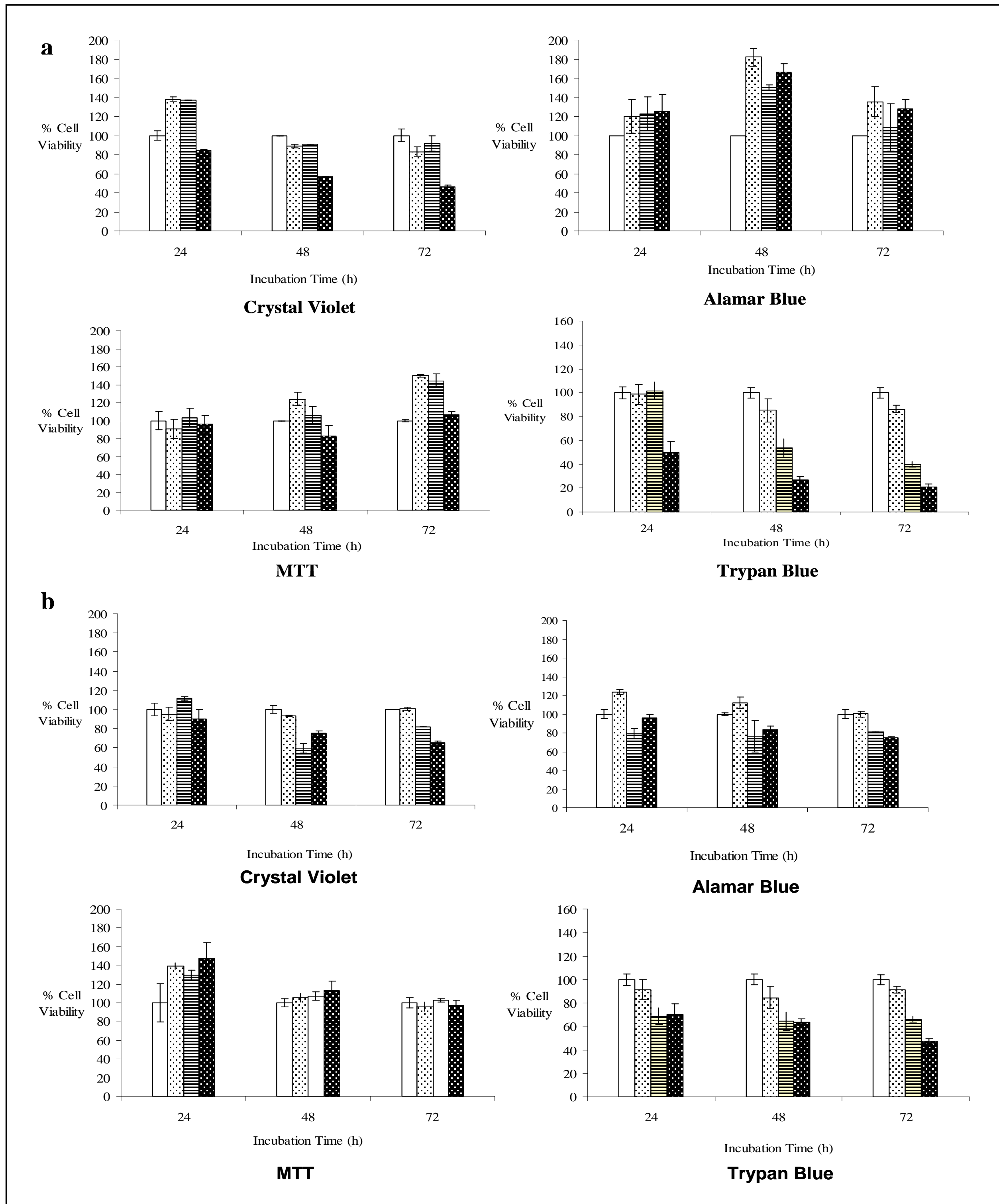


Figure 3.8. Cell Viability assessed by the four methods. (a) U251MG, (b) mMSCs.

□ Ctr, 0.5 mg/ml, 1 mg/ml, 5 mg/ml

Based on the above observations, trypan blue exclusion was used as the standard method to validate the cell viability data obtained by the above assays (Fig. 1 and 1). This method involved direct counting of viable cells and hence eliminated the possibility of interference from occurring. The results demonstrated a large difference between the cell viability data from trypan blue counting and all other three enzyme activity-based assays. These values correlated well with visual estimations when observing under the microscope.

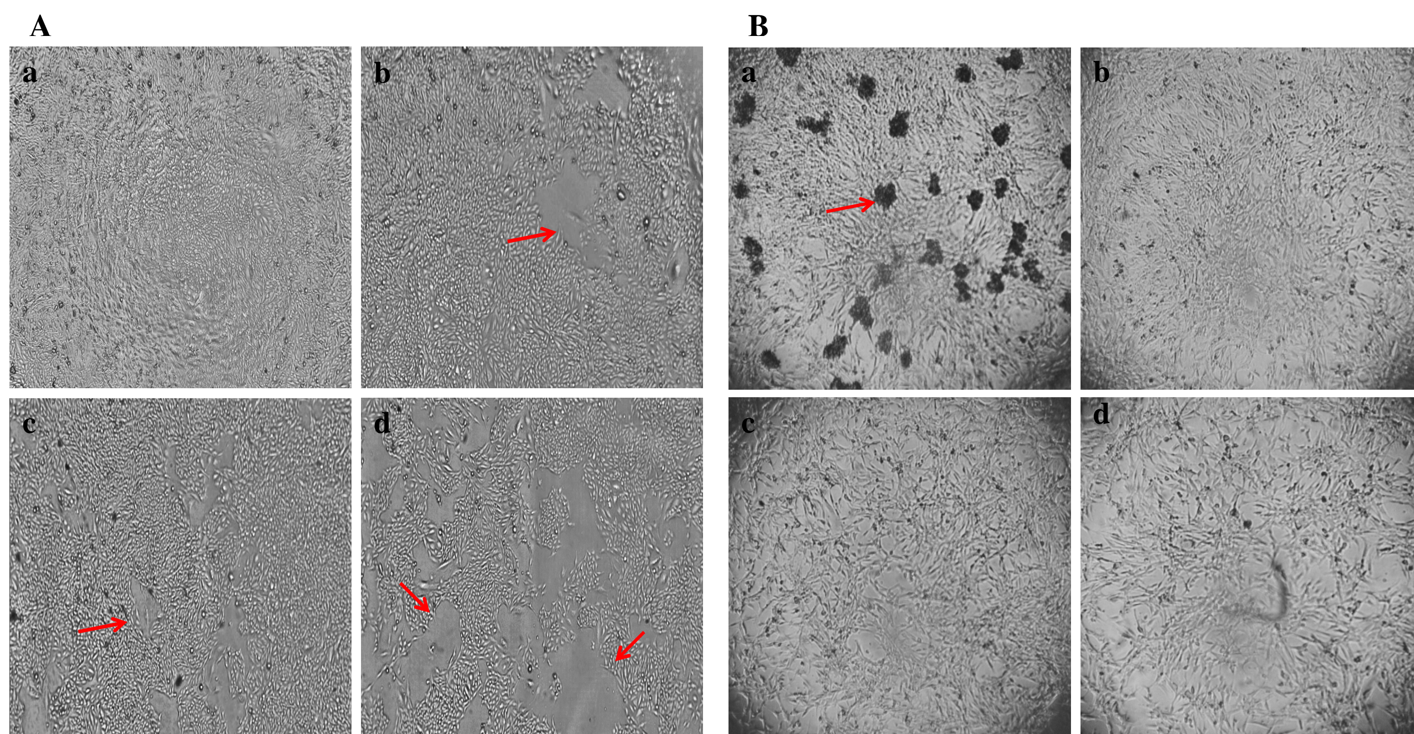


Figure 3.9. Inverted microscope images (4X) of both cell lines after 72h incubation with and without the dendrimer. A) U251MG (a, control with no dendrimer; b, 0.5 mg/ml; c, 1 mg/ml; d, 5 mg/ml); **B) mMSCs** (a, control with no dendrimer; b, 0.5 mg/ml; c, 1 mg/ml; d, 5 mg/ml). Red arrows indicate the presence of cell accumulations (mMSCs-a) or holes in the monolayer.

Images of both cell lines at 72h of incubation show less cellular growth with higher dendrimer concentrations (Fig. 3.9Ac, 3.9Ad, 3.9Bc, 3.9Bd). In the case of mMSCs, cells that were incubated only with medium without dendrimer tend to form agglomerations (Fig. 3.9Ba), which is a sign of uncontrolled growth that ends in the cells differentiation and so, of their lost in multipotency.

In order to measure the level of interference with CV, AB and MTT, the same assays were carried out, but incubating the cells with dendrimer and without reagents. Furthermore, dendrimer with assay reagents in the absence of cells were also analyze. No significant effect on absorbance or fluorescence readout was observed (data not shown). Therefore, the increased absorbance and fluorescence could appear due to a combination of cells, dendrimer and assay reagents. Also, the changes in the metabolic state of the cells could be implicated. There is evidence that for the AB assay, there is accumulation of colorless products with time of incubation, which can also affect the final results.

To analyze whether culturing the cells with a different formulation of the dendrimer worked better in terms of citotoxicity and rate of proliferation, the dendrimer was incubated in gelatin in the bottom of the wells prior to seeding them, so they could grow on top of the monolayer gelatin-dendrimer. This way the dendrimer was intended to be more accessible, or for longer times to the cells, as it would be trapped in the gelatin.

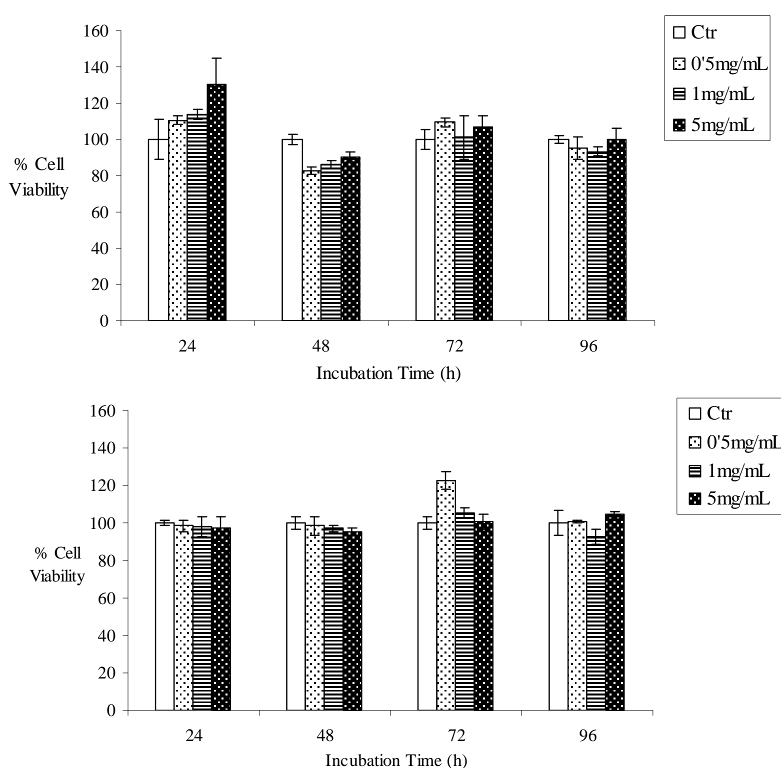


Figure 3.10. Cell Viability by AB, culturing over a monolayer of dendrimer-gelatin. On top, U251MG cells; on the bottom, mMSCs.

There is no effect, neither positive nor negative, over any of the cell types when they grow on top of a dendrimer-gelatin monolayer (Fig. 3.10).

Lastly, as the results indicate an increase in cell growth and cells grow "healthier" (Fig. 3.11) and with a better morphology (Fig. 3.9B) in the presence of the dendrimer, a comparison was made between the maximum absolute values of cellular growth achieved by culturing the cells in DMEM-Low Glucose and MesenCult both with Alamar Blue and MTT. The reason for this was to see whether the levels reached by cells growing in DMEM-LG in the presence of mPEG$_{2000}$-G3-PAMAM matched or were similar to those reached by cells in MesenCult. If so, the expensive MesenCult medium could be substitute by normal DMEM with dendrimer. *The analysis showed that the absolute values for cells in MesenCult (2.500 in MTT and 1200 in AB) were always well above those for cells in the presence of the dendrimer but when dendrimer is added only once. Further studies about the formulation should be done (1.000 in MTT and 600 in AB).*

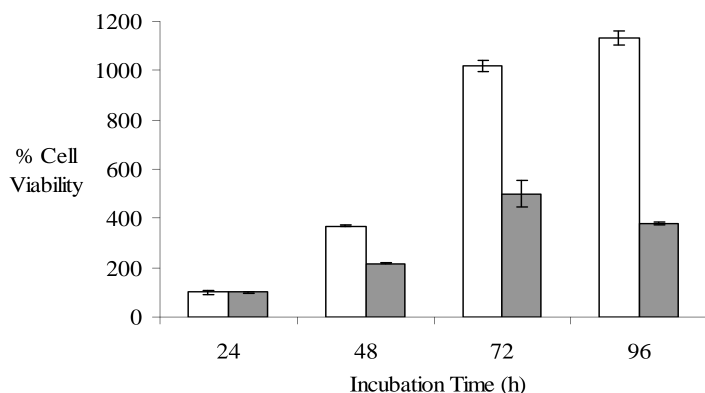


Figure 3.11. Cell Viability comparison. mMSCs growing in a basic DMEM-LG media (white) and in a enriched media (grey) analyzed by the AB method.

In summary, our results suggest that the method of analysis for the level of citotoxicity matters. Taking together the results obtained with trypan blue, the interference assays and the images, we can conclude that, as expected, *the level of biocompatibility of the PEG$_{2000}$-G3-PAMAM dendrimer is high, showing citotoxicity for periods higher than 72 hours and concentrations above 5 mg/ml.* Also, at lower incubation times (<24h) and low concentracions (0.5 mg/ml), the dendrimer could have a proliferation boosting effect that needs to be further investigated.

To get an insight into the cells replication state in the presence of mPEG$_{2000}$-G3-PAMAM, cell cycle analyses were performed. This approach reveals distribution of cells in three major phases of the cycle (Fig. 1), where quiescent and G_1 cells will have one copy of DNA and will therefore have 1X fluorescence intensity. Cells in G_2/M phase of the cell cycle will have two copies of DNA and accordingly will have 2X intensity. Since the cells in S phase are synthesizing DNA they will have fluorescence values between the 1X and 2X populations.

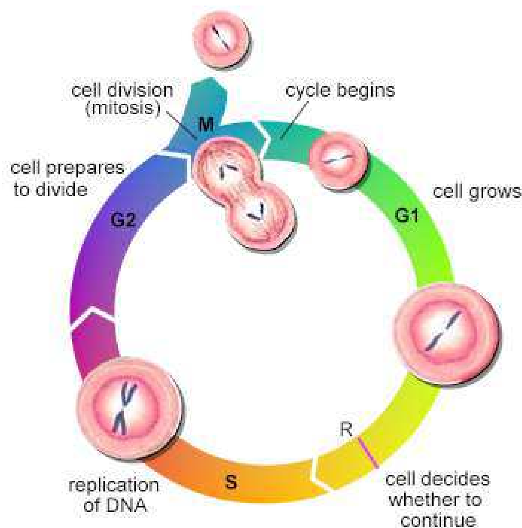


Figure 3.12. Cell cycle. When not in the process of preparing for cell division, cells remain in the G1 portion of the cell cycle (synthetic growth phase). The G2 phase is a time for repair of any DNA damage which has occurred during the preceding cell cycle phases, and for the reorganization of the DNA structure which must take place before the DNA can be divided equally between daughters during Mitosis.In the S phase they have a DNA content just barely above their starting G1 content and increases progressively until they complete the S phase with the G_2 DNA content (*Barry*,7).

The dendrimer at concentrations up to 1 mg/ml has a minor impact in the growth and proliferation of mMSCs. As compared with the control, when cells were exposed to 1 mg/ml of the dendrimer for 72h the number of cells following the normal cell cycle is practically the same (Fig. 3.13). Most of the cells remain in the G_1 phase (96.05% and 91.32%), which indicates cell growth and proliferation. There is a small variation in the number of cells that are in S phase, which increases from 1.20% (control) to 3.21% (mMSCs incubated with the dendrimer) and also on the number of cells in phase G_2/M from 2.74% to 5.46%. These results may suggest a slighter stimulation of division of the cells in the presence of the dendrimer which can explain the enhancement on the cellular growth.

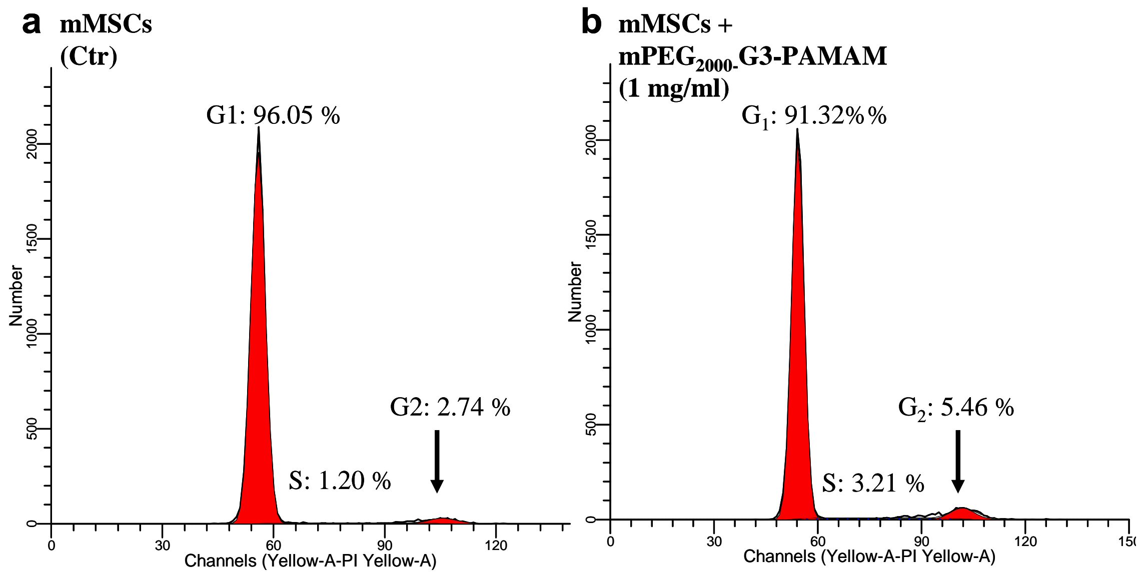


Figure 3.13. Histogram showing the cell cycle phase distribution of mMSCs cultured for 72h with 1 mg/ml of mPEG$_{2000}$-G3-PAMAM in comparison with the control group. The X-axis shows the intensity of the detected signal and the Y-axis measures the number of events (cells) counted.

Mesenchymal stem cells are multipotent cells in nature and have the unique ability to differentiate into various cell types among which osteogenic and adipogenic differentiation are considered as hallmarks. Therefore, to confirm that the dendrimer doesn't affect the identity of the cells, CD34, CD73 and CD105 cell surface markers, and adipogenic differentiation potential were evaluated.

The majority of the cells both in the presence and absence of dendrimer expressed high levels of CD73 and CD105 (Fig, 3.14A and 3.14B), although the marker CD34 displayed low or very little expression (Fig, 3.14A and 3.14B). The lower intensity coming from the markers of the cells incubated with the dendrimer is due to the lower amount of total cells compare with the control cells.

mMSCs were incubated in adipogenic differentiation medium to identify the multipotency character. One week after adipogenic induction, formation of intracellular lipid vacuoles were detected in these cells. Thereafter, lipid accumulation increased along with the inductive periods, which was chemically stained by Oil Red O, a specific staining, which identifies lipid droplets in adipocytes, thereby, confirming adipocytic nature of these cells after 14 days (Fig. 3.14C).

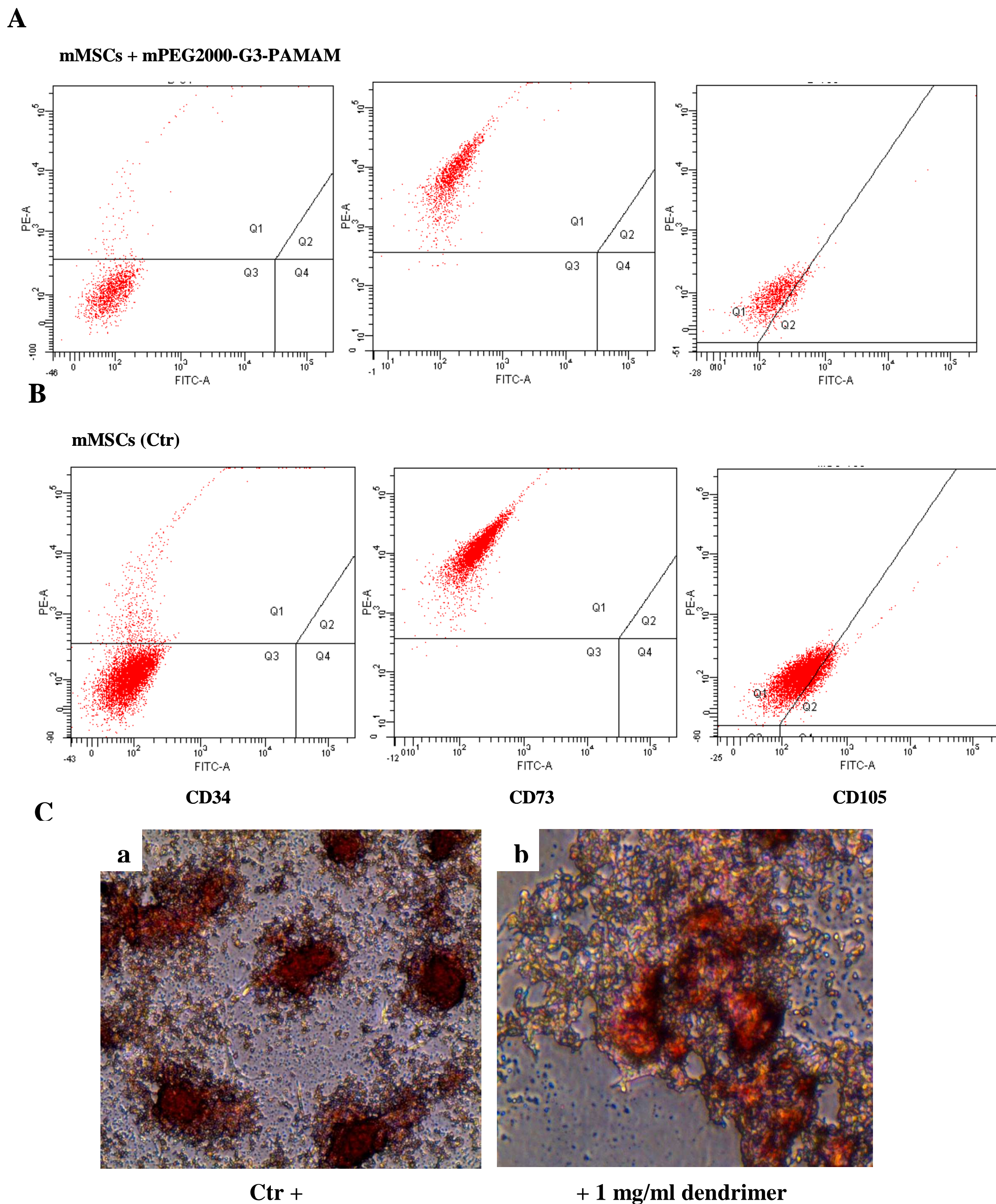


Figure 3.14. Effect of mPEG$_{2000}$-G3-PAMAM dendrimer on the multipotent characteristics of mMSCs. (A) and (B) Characterization of MSCs by flow cytometry. Antibodies used for these experiments were anti-CD73 (PE), anti-CD105 (FITC) as positive controls and anti-CD34 (PE) as negative control. The X and Y-axes measure the different emissions, displaying a dot for each of the cells that show that particular emission. Analysis with flow cytometer showed that over 95% cells of the mMSCs populations were positive for both CD73 and CD105 (specific surface markers of MSCs) and negative for CD34, thus confirming their identity as MSCs. **(C) Adipogenic differentiation of cultured MSCs was confirmed by Oil-red O staining.** MSCs were cultured in appropriate induction media in the absence (a) or presence (b) of 1 mg/ml of dendrimer added at different times of the process, differentiate normally (more time is required for fully differentiation of the monolayer)

CONCLUSIONS

In this study we work with a $mPEG_{2000}$ modified G3-PAMAM dendrimer. This dendrimer was selected on an attempt to find equilibrium between high toxicity to the host cells and more efficiency towards delivery and killing pathogens. We use a PAMAM dendrimer because it has been previously demonstrated that they are widely used for different biomedical applications and they are readily commercially available. There is evidence that lower generations (as ours) of this PAMAM dendrimers are more efficient towards killing bacteria and delivering DNA than higher generations and there are less citotoxic. We though of this specific dendrimer as it could have further applications in the near future as the delivery of viral vectors as well. Lastly, modification of the external groups with PEG_{2000} makes the dendrimer more biocompatible (compared to longer PEG chains).

In this work we have given good proof that the PAMAM dendrimer completely covered with PEG is highly biocompatible (~1mg/ml) and doesn't affect the phenotypic characteristics or the differentiation potential of the cell types tested. On the other hand, this modification shields the positive charges of the dendrimer and so its ability to attach molecules such as DNA working as therapeutic agents is very limited. In this line, although some activity is retained, the dendrimer has a lowered potential as antimicrobial.

For future works, a more effective vector should be used that contains a lower degree of PEGylation, being more toxic to bacteria, but that still could be used at high concentrations with cells and would have enough external charge for attachment of molecules for gene delivery.

Acknowledgments

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