Universitat de Girona

Faculty of Science | Final Degree Project Report

Developing a new method for nanoparticle characterization and cell trafficking using expansion microscopy as high-resolution fluorescent microscopy technique

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Resum

Les teràpies basades en àcids nucleics constitueixen una eina prometedora per al tractament de malalties com el càncer. Tanmateix, el principal problema que presenta aquesta disciplina consisteix en el desenvolupament de vectors segurs i eficients per l'alliberament dels gens terapèutics a les cèl·lules diana. D'aquesta manera, s'han desenvolupat els poli(beta-aminoesters) (pBAEs) modificats amb oligopèptids terminals catiònics (OM-pBAEs), facilitant l'encapsulament del material genètic i ajudant a aconseguir eficiències de transfecció altes tot mantenint la biocompatibilitat de les nanopartícules (NPs) resultants. Malgrat tot, hi ha propietats dels OM-pBAEs que encara no estan clares.

La microscòpia podria ser una bona manera de caracteritzar aquestes NPs i el seu tràfic dins la cèl·lula. Tot i això, els límits de difracció dels microscopis convencionals impedeixen la resolució de partícules més petits de 200-300 nm. Per altra banda, la microscòpia de super-resolució implica un gran cost i una gran inversió de temps. Així, s'ha desenvolupat una nova tècnica d'imatge d'alta resolució, la microscòpia d'expansió (ExM). Aquesta tècnica consisteix en l'ús de material comú de laboratori i productes químics econòmics per magnificar, de forma física, mostres biològiques, de manera que puguin ser visualitzades a la nanoescala, fent ús d'un microscopi convencional.

El principal objectiu del projecte ha sigut desenvolupar i optimitzar un protocol d'ExM per a l'estudi del tràfic de les NPs d'OM-pBAE dins la cèl·lula.

Per a obtenir el protocol desitjat, s'ha establert: la densitat cel·lular adequada per sembrar ($25 \cdot 10^4$ cèl·lules/mL), la concentració de tinció per als nuclis (0.5 µg/mL), la forma ideal de marcar la membrana citoplasmàtica (abans de gelificar) i el temps d'incubació amb ProteïnasaK (ProK) (3 hores) per garantir una expansió òptima de la mostra. Per a les NPs, s'ha modificat el protocol, reduint el tractament d'Acryloyl X i la polimerització a 30 minuts i eliminant l'ús de ProK, per mantenir les NPs formades i visualitzar-les després del procés. Finalment, la caracterització del tràfic cel·lular de les NPs ha demostrat diferències entre NPs amb diferents oligopèptids terminals, com a prova de concepte de la utilitat de la tècnica.

Així, es pot afirmar que s'han optimitzat amb èxit protocols d'ExM per caracteritzar NPs d'OM-pBAE, que podrà ser d'utilitat, per exemple, per entendre les vies endocítiques de la cèl·lula, un cop transfectada.

Resumen

Las terapias basadas en ácidos nucleicos constituyen una herramienta prometedora para el tratamiento de enfermedades como el cáncer. Sin embargo, el principal reto en esta disciplina consiste en la obtención de vectores seguros y eficientes para hacer llegar los genes terapéuticos a las células diana. Así, se han desarrollado los poli(beta-aminoesters) (pBAE) modificados con oligopéptidos terminales catiónicos (OM-pBAE) para facilitar la encapsulación del material genético y ayudando a conseguir altas eficiencias de transfección, manteniendo la biocompatibilidad de las nanopartículas (NPs) resultantes. No obstante, todavía no están claras algunas características importantes de los OM-pBAE.

La microscopía podría ser una buena forma de caracterizar estas NPs y su tráfico celular. Aun así, los límites de difracción de los microscopios convencionales dificultan la resolución de partículas más pequeñas de 200-300 nm. Por otro lado, la microscopía de super-resolución conlleva una gran inversión de costes y tiempo. Así, se ha desarrollado una nueva tecnología imagen de super-resolución, la microscopía de expansión (ExM). Esta técnica consiste en el uso de equipos de laboratorio comunes y productos químicos económicos para ampliar físicamente, muestras biológicas, de modo que puedan ser visualizadas a la nanoescala utilizando un microscopio convencional.

El objetivo principal de este proyecto ha sido desarrollar y optimizar un protocolo ExM, para el estudio del tráfico de NPs OM-pBAE en células.

Para obtener el protocolo deseado, se ha establecido: la densidad celular adecuada para sembrar ($25 \cdot 10^4$ células/mL), la concentración de la tinción de los núcleos (0.5 µg/mL), la forma ideal de teñir la membrana citoplasmática (antes de gelificar) y el tiempo de incubación con Proteinasa K (ProK)(3 horas) para garantizar una expansión óptima de la muestra. Para NPs, se ha modificado el protocolo, reduciendo el tratamiento de Acryloyl X y la polimerización a 30 minutos y eliminando la digestión con ProK, para mantener las NPs formadas y visualizarlas tras el proceso. Finalmente, la caracterización del tráfico celular de las NPs mostró diferencias entre NPs con diferentes oligopéptidos terminales, como prueba de concepto de la utilidad de la técnica.

Así, se puede afirmar que se han optimizado con éxito los protocolos proExM para caracterizar NPs de OM-pBAEs, que podrá ser útil, por ejemplo, para comprender las vías endocíticas de la célula, una vez transfectada con ellos.

Abstract

Nucleic acid-based therapies constitute a promising tool for treatment of diseases like cancer. However, the main challenge in this discipline consists in the development of safe and efficient vectors to deliver the therapeutic genes to the target cells. In this way, poli(beta-aminoesters) (pBAE)s were modified with cationic terminal oligopeptides (OM-pBAEs), to facilitate the genetic material encapsulation and, in turn, help on achieving high transfection efficacy, while maintaining the biocompatibility of the resulting nanoparticles (NPs).

Microscopy could be a good way to characterise these NPs and their cellular trafficking. Even so, the diffraction limits of conventional microscopes hinder the resolution of particle smaller than 200-300 nm. On the other hand, the acquisition of super-resolution microscopy involves a large cost and time investment. Thus, a new super-resolution imaging technology, expansion microscopy (ExM), has been developed. This technique consists on the use of common lab equipment and inexpensive chemicals to physically magnify biological specimens, so that the sample can be imaged with nanoscale resolution using a conventional microscope.

The main objective of this project has been to develop and optimise an ExM protocol, for the study of OM-pBAE NPs trafficking in cells.

To achieve the desired protocol it have been set: the appropriated cell density to seed ($25 \cdot 10^4$ cells/mL), the concentration of nuclei staining (0.5 µg/mL), the ideal way of dyeing the cytoplasmic membrane (pre-gelation) and the incubation time with Proteinase (ProK) (3 hours) to ensure an optimal and expansion of the sample. For the NPs, the protocol has been modified, reducing the Acryloyl X treatment and polymerization to 30 minutes and removing the ProK digestion, in order to keep the NPs formed and visualize them after the process. Lastly, the characterization of the cellular trafficking of NPs showed differences between NPs with different oligopeptide terminals, as a proof of concept of the utility of the technique.

Consequently, it can be stated that proExM protocols have been successfully optimized, which helped to characterize OM-pBAEs NPs, which can be useful to understand the endocytic pathways of the cell, once transfected with them.

Reflections about ethics, sustainability and gender perspective

Reflection on ethics

GEMAT is a research group formed by professors and researchers, belonging to the Industrial Engineering and Bioengineering department of IQS. With a Christian ethic, it has a Code for Conduct followed by all members of the group. In this way, looking at the group's scientific articles authorship policy, it consists in order the names according to their participation. The first name corresponds to the name of the researcher who has made the most substantial contribution to the experimental work done while last one is the director of the project.

In the scientific world, "**Publish or perish**" has led to the inclusion of authors who did not deserve to see their name in the paper , what it is known as a "**gift autorship**". On the contrary, there has been staff who have developed much of the work but have not been recognized as such.[(1)(2)] GEMAT policy, on the other hand, recognises fairly and objectively the work carried out by each of the participants, regardless of their title or status within the group.

Reflection on sustainability

The laboratory has a strict **waste management** system, which consists of the separation of each type of residue. The installations have containers for contaminated paper, plastic and glass, and also for no contaminated. Moreover, there are recipients for cytotoxic residues, non-halogenated solvents and biological waste. Waste management is of great importance in centres like this, due to laboratories are one of the main sources of biomedical waste, such us genotoxic residues, which are extremely hazardous to environment and human health because of their mutagenicity, teratogenicity and carcinogenicity.[(3)(4)]

In this way, the system followed by the centre is a good choice in place to reduce its **impact** on the environment.

Reflection on gender perspective

GEMAT has an equal number of male and female researchers. However, the highest **employment categories** (lecturer) are occupied by 3 men. It is worth noting that the three professors with this degree started their careers when, in most universities, women had more problems in accessing these positions, as they were considered to be the only ones responsible for the care of their families[(5)], which could eventually create a "**glass ceiling**" for them. At GEMAT, this situation is very different today, as evidenced by the fact that the positions immediately below them are occupied on a parity basis and that they aspire in the future to occupy leadership positions, not on the basis of gender but on the basis of merit.

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List of abbreviations

AcONA	Sodium Acetate
AcX	Acryloyl-X SE
APS	Ammonium Persulfate
ATTO	ATTO 647N NHS ester
BSA	Bovine Serum Albumin
C6CD3 or D	Aspartic acid-terminated pBAE
C6CH3 or H	Histidine-terminated pBAE
C6CK3 or K	Lysine--terminated pBAE
C6CR3 or R	Arginine acid-terminated pBAE
Cy3	Cyanine3 NHS ester
Cy5	Cyanine5 NHS ester
DAPI	4,6-diamine-2-fenilindol dihydrochloride
DMEM	Dubelcco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ET_3N	Triethylamine
ExM	Expansion Microscopy
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
NP	Nanoparticle
OM-pBAE	Oligopeptide end-modified pBAE
PaTu	PaTu 8988t

pBAE	Poly(β-aminoesters)
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
pGFP	GFP plasmid
proExM	Protein retention ExM
ProK	Proteinase K
TEMED	Tetramethyl-ethylenediamide
Tris	Tris(hydroxymethyl)aminomethan

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1. Introduction

1.1. Nucleic acid-based therapies

Nucleic acid-based therapies constitute a promising tool for treatment of diseases like cancer, cardiovascular diseases, neurological disorders and infectious diseases.[6] They could be applied as an immunotherapy, in form of mRNA vaccines, to develop targeted therapies in order to treat the disease with specificity.[7] However, the employment of naked nucleic acids presents some limitations, due to their easy removal by the kidney and the fact that they do not have a mechanism to enter the target cell cytoplasm.[8] Thus, the main challenge in this discipline consists in the development of safe and efficient vectors to deliver the therapeutic genes to the target cells.[9] Among gene therapy vectors, there are two types: viral and non-viral vectors.

1.1.1. Viral vectors

Viral vectors present a high transduction efficiency in a wide variety of human cells, which make them a good choice for these treatments. Because of its pathogenicity, viruses must be attenuated to ensure a safe application.[10] Even so, they have shown a non-specific activation of the immune system, causing their premature elimination and preventing their repeated administration. Moreover, insertional mutagenesis has been described with the use of these vectors, so all these issues limited the clinical application of viral vectors.[11]

1.1.2. Non-viral vectors

Non-viral vectors, although traditionally described as less efficient, are a safer alternative as they do not lead to undesired immune recognition and elimination, so they can be readministered. Moreover, they are easy to produce. [10][11] In addition, these vectors are capable of delivering any type of nucleic acids, from short oligonucleotides or siRNA, to long plasmid DNA and entire mRNAs.[10] Overcoming the low efficacy limitation, as well as to study and improve their internalization and cellular trafficking, are the principal aims to develop an efficient delivering vector and the tendency in the field of nucleic acid-based therapies.[10]

Going into detail, the internalization of non-viral particles consists of various challenging steps **(Figure 1):** 1) physical contact with the plasma membrane of the cell, 2) endocytosis of the delivery complex, 3) endosome release of the genetic material, 4) trafficking through intracellular compartment and, if they are composed of DNA, 5) nuclear import in order to achieve the gene expression.[10] Additionally, the *in vivo* biodistribution of these types of delivery systems is of major importance to ensure their exclusive action on target cells.[10]

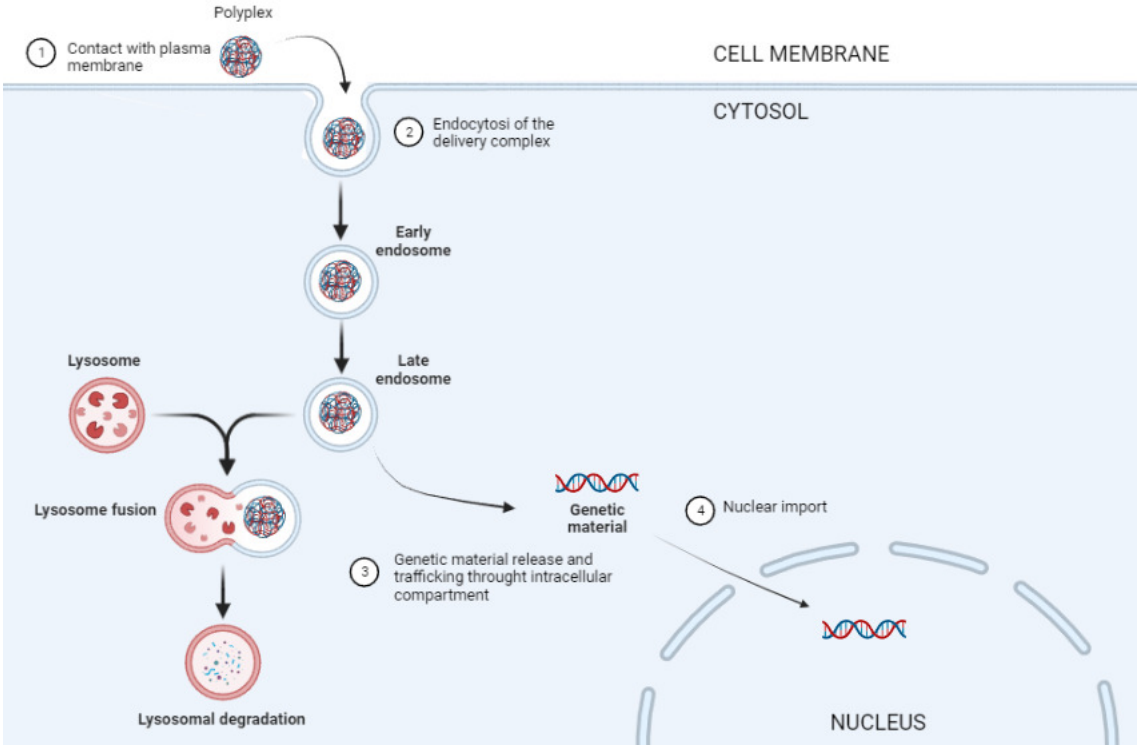

Figure 1. Non-viral particles internalization scheme.

1.1.2.1. Polymeric nanoparticles

Among non-viral vectors, polymeric nanoparticles (NPs), also known as polyplexes, have increased their relevancy during the last years. The characteristics which have made it possible include their easily administration in the body, due to their nanometric size, the fact that they can be functionalized to reach the target cells, the protection and controlled release of genetic material, the easy and cost-effective production and their biocompatibility and biodegradability.[(11)(12)]

Within the group of polymeric NPs, poly(beta-aminoester) (pBAE) polymers, a new class of cationic polymer, have demonstrated their potential to be an efficient delivery system.[(6)(13)] This is thanks to their high transfection efficiency and low toxicity, as well as biocompatibility and biodegradability thanks to the ester bonds present in their structure.[(6)(11)] They are able to self-assemble nucleic acids in aqueous media to form nanometric structures (polyplexes) in order to increase the transfection of the genetic material. Moreover, these polymers can be designed to improve the buffering capacity, with the aim to facilitate cellular internalization.[(11)] Additionally, after NPs entrance to the cell, the proton sponge effect is responsible for enhancing the endosomal escape, which is necessary for the gene expression.[(11)]

1.1.2.2. Oligopeptide end-modified pBAE (OM-pBAE)

pBAE have been used successfully for therapeutic applications like vaccination, gene-based therapy for cancer and ophthalmology, gene silencing and stem cell modification.[6]However, some authors highlighted their difficulty to efficiently encapsulate genetic material, and certain toxicity due to the presence of cationic chemical ends.[11] Thus, a new family of pBAE was developed, which are characterized by the presence of oligopeptide end modifications (OM-pBAE) **(Figure 2)**. This improvement was possible thanks to the pBAE unique structure, that allows modifications of their termini by using oligopeptides as the cationizing moieties, much more biocompatible, biodegradable and non-cytotoxic than chemical molecules.[11][13] Furthermore, pBAE polyplexes can be freeze-dried, facilitating its long-term storage, as a result of the hydrophobicity provided by hexyl monomers, which form the polymer backbone (C6).[14]

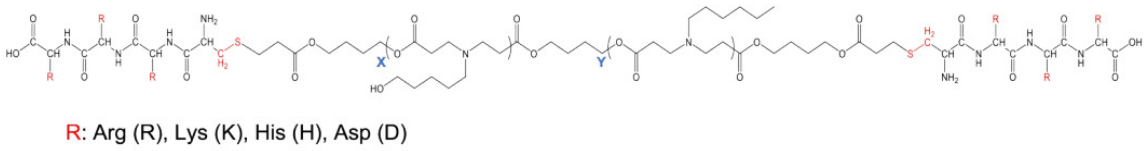

Figure 2. Oligo-modified C6pBAE structure. Reproduced from Navalón-López, Dols-Perez, Grijalvo, Fornaguera and Borrós. [14]

The chosen oligopeptides contain basic amino acids like arginine (R), lysine (K) and histidine (H) (C6CR3, C6CK3 and C6CH3, respectively). These modifications help to achieve higher transfection efficacy than other pBAE while being, in addition, safer.[9][14] C6CR3 or R ameliorates nuclear subcellular localization, C6CK3 or K enhances the plasmatic membrane crossing and C6CH3 or H promotes the endosomal escape, due to histidine buffering capacity at pH 5-6, at which this process takes place.[11][14] The only limitation of these polymers, however, is their promiscuity: cationic peptides increase the polyplex-positive surface charge, producing a non-controlled overall high transfection to all cell types and, therefore, low selectivity.[14] Aspartic acid-terminated pBAE polymer (C6CD3 or D) have been developed in order to coat the polyplexes, thereby reducing the surface charge and their promiscuity.[14] In this way, these previous results support the idea that a combination of different OM-pBAEs is key to obtain an improved delivery system.[9][14] Moreover, the addition of active targeting moieties, such as peptides, can also contribute to the selectivity to target cell lineage.

Even though this research line is really promising and has shown hints of success in preclinical studies, important features of OM-pBAEs interaction with, are still unclear.[14] Thus, in deep studies regarding the cellular and molecular mechanisms that contribute to their successful use are still required. In this context, advanced tools like microscopy could be a good way to characterise these polyplexes and their subcellular trafficking.

1.2.Expansion microscopy (ExM)

Throughout science history, optical microscopy techniques have made important contributions to the fields of biology and medicine. Even so, the diffraction limits of conventional microscopes hinder the resolution of particle smaller than 200-300 nm, therefore higher size than biomolecules and NPs.[15][16] In the last years, luckily, super-resolution microscopy has overcome this limit, allowing for the observation of single molecules and nanoscale structures.[15][16] However, the acquisition of these technologies involves a large cost and time investment due to its slow imaging speed as well as the requirement of highly trained personnel. [15][16] Thus, it is not feasible to apply super-resolution microscopy to routine experiments.

Recently, a new super-resolution imaging technology, **expansion microscopy** (ExM), has been developed to address all the above limitations. ExM consists on the use of common lab equipment and inexpensive chemicals in order to isotropically physically magnify biological specimens.[16][17] In this way, the sample can be imaged with nanoscale resolution using a conventional microscope, rather than a sophisticated and more expensive one and in a routine way, by not-so trained personnel.[17]

During the study of this technology, different protocols have been developed depending on the intended application, for the visualization of different types of objects/biologicals. Protein retention ExM (proExM) is used to expand cells and tissues labelled with fluorescent proteins and antibodies.[17][18] Expansion fluorescence in situ hybridization (ExFISH) makes possible to visualise, not only proteins, but also RNA.[17][18] Iterative expansion microscopy (iExM) consists on a repetitive expansion of the sample by performing multiple rounds of polymerization and expansion.[17][18] Lastly, expansion pathology (ExPath) is addressed to preserved human specimens.[17]

The proExM procedure, which is the one used in this project, consists of different key steps such as the seed of cells, the staining or/and immunostaining of the sample, the synthesis of a swellable polyelectrolyte gel throughout the biological specimen, the anchoring of the sample to the gel by an incubation with Acryloyl (AcX), the mechanically homogenization by the application of a proteolysis and the immersion of the gel in water to perform the expansion.[17][19]

2. Objectives

In all this context, the main **objective** of this final degree project has been to **develop and optimise an expansion microscopy protocol for the study of OM-pBAE NPs trafficking in cells.** It includes: 1) the characterization of the different OM-pBAEs nanocarriers and 2) the understanding of the endocytic pathways of the cell, once transfected with the carriers. To do it, we have based our work on the proExM technology, using various fluorophores and antibodies to label the biomolecules of interest.

This main objective involves several secondary objectives:

I. Developing a cell proExM protocol.
    a. Setting up the appropriated cell density, the suitable concentration of cell nuclei staining and the ideal way to label the cytoplasmic membrane.
    b. Establishing the optimal homogenization incubation time and testing the isotropic expansion of the sample.

II. Modifying the previous procedure in order to allow the display of NPs in a expanded form, using OM-pBAE NPs as model nanosystems..
    a. Reducing all incubation times to maintain the NPs stability throughout the process.
    b. Proving fluorescence retention of different fluorophores to label polymer and GFP plasmid (pGFP).

III. Using the previously developed proExM protocols to expand transfected cells with the aim to subcellularly localize the NPs inside the cells.
    a. Checking whether fluorophores resist this protocol.
    b. Measuring the colocalization between the NPs and endosomes to study the internalization of NPs into the cells and understand how they act.

3. Materials and Methods

3.1.Materials

Several reagents have been used in this project. Paraformaldehyde (PFA), Bovine Serum Albumin (BSA), sucrose, odium acetate (AcONa), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), and Phosphate Buffer Saline (PBS) were purchased from Sigma-Aldrich®, Cyanine dyes were purchased from Lumiprobe for polymer labelling but for plasmid labeling Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit from Mirus. 4,6-diamine-2-fenilindol dihydrochloride (DAPI) and ATTO label were also acquired from Sigma-Aldrich®, while CellMask™ Orange was purchased from Invitrogen by Thermo Fisher Scientific. Dubelcco's Modified Eagle Medium (DMEM), glutamine, penicillin and streptomycin were obtained from Biowest. Lipofectamine® 2000 (Invitrogen by Life technologies from Thermo Fisher Scientific). Fetal Bovine Serum (FBS) and culture plates were acquired from Corning. The antibodies used in Confocal microscopy were obtained from Sigma Aldrich® for the polyclonal anti-Rab5 and from Thermo Fisher Scientific for the Goat anti-Mouse IgG (H+L) highly cross-adsorbed secondary antibody Alexa Fluor 488. pGFP (3486 bp) was produced and purified from *E.coli*.

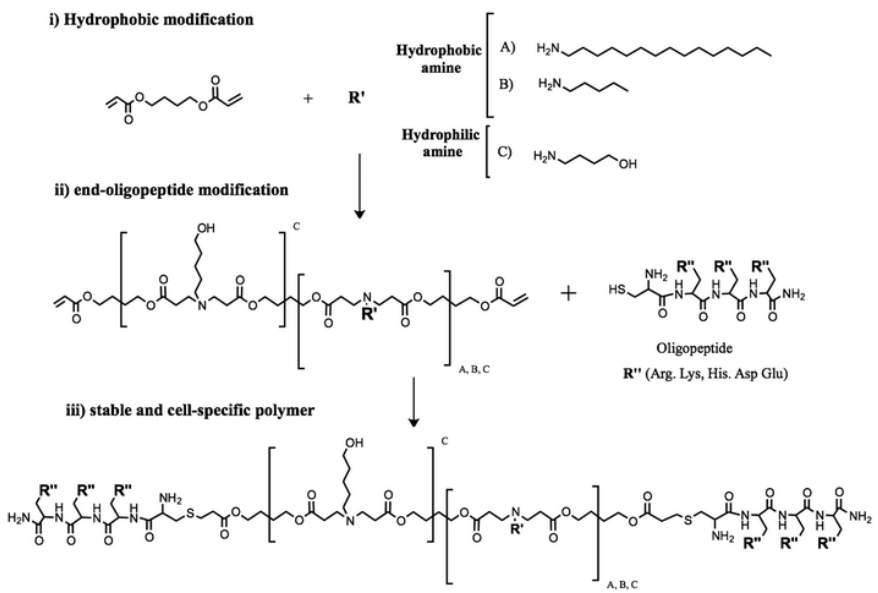

Figure 3. Synthesis of pBAE polymers. i) Combination of hydrophobic amines (A and B) with hydrophilic amine (C) for the synthesis of pBAE (R' is usually an aliphatic strand). ii) pBAES being modified by different oligopeptides moieties (R" can be Arginine, Lysisne, Histidine, Glutamic Acid or Aspartic Acid). iii) OM-pBAEs obtained. Reproduced from Dosta, Ramos and Borrós.[8]

Arginine and Histidine end-modified poly(*β*-amino ester) (pBAE) polymers (R and H, respectively), which are those used during this project, were synthesized by the Group of Materials Engineering, following a two-step procedure, shown in **Figure 3**, as described in

previous publications.[8] In brief, first, an acrylate-terminated polymer, C6 polymer, was synthesized by addition reaction of primary amines with diacrylates (at 1:1.2 M ratio of amine:diacrylate). Finally, pBAE was obtained by end-capping modification of the resulting acrylate-terminated polymer with either arginine or histidine at each end (Cys-R"-R"-R"-NH2) as shown in the figure above. In addition, to obtain RH combination, R and H polymers were mixed at a ratio of 60/40 R/H.

In terms of the proExM protocol, Proteinase K (ProK) was purchased from Thermo Fisher Scientific and the reagents used and their compositions are shown in **Table 1-5**, reproduced from Zhang, Khang, Asano, Gao and Boyden.[17]**:**

Table 1. Composition and concentration of the Acryloyl X (AcX) stock solution.

AcX stock solution	**Amount**	**Final concentration**
AcX (Thermo Fisher, cat. no. A20770)	5 mg	10 mg/mL
Anhydrous DMSO (Sigma Aldrich, cat. no. 276855	500 µl	
Total	500 µl	

Note: Stored at -20ºC in a sealed Eppendorf tube, in a dry environment to avoid hydratation.

Table 2. Preparation of ammonium persulfate (APS) stock solution with its respective concentration.

APS stock solution	**Amount**	**Final concentration (g/ 100 mL solution)**
APS (Omnipur, cat. no. 7727-54-0)	0.2 g	20
Water	0.9 mL	
Total	1 mL	

Note: Stored up to 1 month at -20ºC.

Table 3. Preparation of a tetramethyl-ethylenediamide (TEMED) stock solution with its respective concentration.

TEMED stock solution	**Amount (mL)**	**Final concentration (g/ 100 mL solution)**
TEMED (Thermo Fisher, cat. no. 17919)	0.129	10
Water	0.9	
Total	1	

Note: Stored up to 1 month at -20ºC.

Table 4. Instructions for preparing Stock X and all the solutions needed for it.

Monomer solution (Stock X)	**Stock solution concentration (g/100 mL solution)**	**Amount (mL)**	**Final concentration (g/ 100 mL solution)**
Sodium acrylate (BLDpharm, cat. no. 7446-81-3)	38 (in water)	1.13	8.6
Acrylamide (Sigma Aldrich, cat. no. 14,866-0)	50	0.25	2.5
N,N'- Methylenebis-acrylamide (Sigma Aldrich, cat. no. 14,607-2)	2	0.37	0.15
Sodium chloride (Scharlau, cat. no. 7647-14-5)	29.2 (5 M)	2	11.7 (2 M)
PBS	10X	0.5	1X
Water		0.65	
Total		4.9	

Note: Sodium acrylate, acrylamide and N,N'- Methylenebisacrylamide dissolved into water by vortexing or sonication, but not heating them, as monomers may polymerize. Acrylamide and N,N'-Methylenebisacrylamide stock solutions stored up to 6 months at 4ºC. Sodium acrylate stock solution prepared at the moment of using it. Stock X divided into aliquots of 980 µl and stored up to a month at -20ºC.[17]

Table 5. List of solutions needed to make the digestion buffer.

Digestion buffer	**Stock solution concentration**	**Amount (mL)**	**Final concentration (/ 100 mL solution)**
Tween® 20 (Sigma Aldrich, cat. no. P1379)	5% (w/v)	10	0.50 g
Ethylenediaminetetraacetic acid (EDTA) (PanReac, cat. no. 131669)	0.5 M, pH 8.0	0.2	0.2 mL (1 mM)
Tris(hydroxymethyl)amino methan (Tris) (Thermo Fisher, cat. no. 77-86-1)	1 M, pH 8.0	5	5 mL (50 mM)
Sodium chloride (Scharlau, cat. no. 7647-14-5)	(5M)	20	4.67 g (1 M)
Water		64.8	
Total		100	

Note: Divided into aliquots of 10 mL and stored up to 12 months at -20ºC.

3.2. Cell lines

Both PaTu 8988t (CLS 305133) and A549 (ATCC CRM-CCL-185^{TM}) cell lines were maintained in DMEM supplemented with 10% (v/v) FBS, 100 units/mL penicillin G, 100 µg/mL streptomycin and 2 mmol/l L-glutamine. All cells were cultured at 37ºC, under 5% CO_2/ 95% air atmosphere, to reach 70%-90% confluence. Afterwards, the maintenance cell passages were done in 100 mm x 20 mm petri dish (Corning).

3.3. Methods

3.3.1. Nanoparticles formulation

The nanoparticles were prepared at a ratio of 25:1 OM-pBAE: Nucleic acid, by mixing equal volumes of pGFP 0.5 mg/mL with the polymer (either R or a combination of R with H in a 60:40 ratio respectively) at 12.5 mg/mL in a solution of sodium acetate 12.5 mM at a pH of 5.2. The nucleic acid was added over the polymer solution, mixed by pipetting, followed by 15 minutes of incubation at room temperature (V1). The mixture (V1), after the incubation, was nanoprecipitate, for the formation of discrete structures, in a same volume (V1) of miliQ water (sample will be 1/2 diluted). Thereafter, the same volume (V1) of hepes 20 mM + 4 wt% sucrose (pH 7.4) solution was also added as a zwitterionic buffer to maintain pH at a constant value (sample will be 1/3 diluted).

3.3.2. DNA Cy3-labelling

To label pGFP with Cy3, a 10 mg/mL Cyanine3 NHS ester (Cy3) solution, dissolved in DMF, and a 1 mg/mL pGFP solution, dissolved in AcONa pH, 8 were prepared. Then they were mixed in a 1:1 v/v ratio and let them react for 4 hours. Afterwards, the sample of Cy3 and DNA (pGFP-Cy3) is quantified by means of fluorescence and absorbance respectively with Infinite M Plex microplate reader from TECAN.

3.3.3. Labelling pBAE with fluorophores

The labelling of R was done with Cyanine5 NHS ester (Cy5) and ATTO 647N NHS ester (ATTO), at 100 mg/mL in DMSO. The solution of R, was mixed with DMSO, triethylamine (Et3N) and Cy5/ATTO at the correct concentrations showed in the **Table 6**. The solution was protected from the light and stirred in water bath with a controlled temperature 25ºC ± 2ºC. The resulting product was precipitated in a mixture 7:3 v/v of diethyl ether:acetone. Then it was dried overnight and dissolved in DMSO to obtain a solution of 100 mg/mL.

Table 6. Instructions for labelling R pBAE with Cy5 (R-Cy5) and ATTO (R-ATTO).

pBAE	Amount of polymer to label	Cy5	ET_3N	DMSO (µl)
R	35 µl (1.6 µmol)	60 µl (1.6 µmol)	3.5 µl (48 µmol)	270

pBAE	Amount of polymer to label	ATTO	ET_3N	DMSO (µl)
R	21.3 µl (0.59 µmol)	250 µl (0.59 µmol)	2.47 µl (17.7 µmol)	164

3.3.4. Cellular uptake assay

In order to assess the timings taken by the NPs to enter the cell, A549 cells were seeded on a 96-well plate, at a concentration of $15 \cdot 10^3$ cells/well. Seeded cells were incubated at 37ºC in 5% CO_2 atmosphere for different times (2, 4, 6, 24 and 48 hours) with the corresponding nanoparticles. pBAE/nucleic diluted in DMEM at a final concentration of 0.3 µg/well of nucleic acid. The 0.5% of the total of the polymer used to prepare the NPs was R-Cy5, so the uptake could be measured. Two hours after each transfection, the cells were washed with PBS (100 µl) and the medium was renewed to remove excess NPs in the medium. Once the timings were reached, the cells were washed again with PBS (100 µl) and trypsinized with trypsin-EDTA (25µl). Then after 5 minutes of incubation at 37ºC to make the trypsinization process more effective, DMEM (50 µl) was added to stop the trypsin. Last step is the cell fixation with PFA 4% (25 µl) and the Cy5 quantification by flow cytometry (NovoCyte), comparing it with a negative control of untreated cells.

3.3.5. Cells protein retention for ExM

To expand cells, A549 and PaTu 8988t (PaTu) cells were seeded in the 5 mm-diameter well of a scaffold, within a 35 mm-diameter confocal dish, at $25 \cdot 10^3$ cells/well. In case of transfection, cells were incubated at 37ºC in 5% CO_2 atmosphere for different times (2 and 4 hours) with R or RH polyplexes. pBAE/nucleic diluted in DMEM at a final concentration of 0.9 µg/well of nucleic acid and the 15% of the total polymer used to produce NPs was R-ATTO . When the transfection time was over, cells were fixed with PFA 4% (200 µl) for 10 minutes, at room temperature, before the staining begun. Then, if the endosomes labelling was required, cells were permeabilized with 0.1% Tween®20 (200 µl) for 3 minutes and blocked by 1% BSA (200 µl) for 30 minutes. Next, the sample was treated with anti-Rab5 primary antibody (200 µl), at 5 µg/mL for 1 hour, followed by three times wash with PBS 1X and a 30-minutes incubation with

Alexa fluor 488 secondary antibody (200 µl), at 5 µg/mL. Once the endosomes labelling was completed, cells were stained with DAPI (200 µl) and CellMask (200 µl) for 20 minutes each one, at 0.5 and 25 µg/mL, respectively, at room temperature in the dark. Between these steps, a wash with PBS 1X was performed. After the staining, the sample was washed with PBS 1X and consecutively treated with AcX (200 µl), at 0.1 mg/mL, overnight, in the dark and at room temperature. To form the gelation solution, Stock X, TEMED and APS stock solutions were mixed in a 98:1:1 volumetric ratio. This solution was vortexed for 10 seconds and immediately placed in ice to avoid the premature polymerization. The AcX solution was then aspirated and the gelation solution was applied to the sample (200 µl). It was incubated on ice for 5 minutes, before transferring to the incubator at 37ºC in 5% CO_2 atmosphere for 1 hour in the dark. For the digestion step, the sample was taken out of the incubator and cooled for 5 minutes at room temperature. Meanwhile a ProK:digestion buffer solution was prepared in a 1:100 ratio, which was, subsequently, poured on top of the formed gel (400 µl). The process of digestion had a duration of 3 hours, at room temperature in the dark and on an orbital shaker at 60 rpm. Finally, to perform gel expansion, the ProK solution was aspired, the scaffold was removed and the gel was unhooked from the dish surface, with a spatule, to allow good expansion. Then, the gel was immersed in milliQ water for 2 hours, at room temperature in the dark and on an orbital shaker at 60 rpm, but the water was changed every 30 minutes. The expanded samples were imaged with a Leica TCS SP8 laser-25 scanning confocal spectra microscope. the nuclei size and the overlap coefficients were measured with ImageJ software. In the case of the overlap coefficient the JACoP plugin was used.

3.3.6. Nanoparticles protein retention for ExM

The nanoparticles protocol was adaptation of the previous one. In this case, pBAE/nucleic, at a final concentration of 4.15 µg/well of nucleic acid, were plated in the confocal dish. The 12.5% of the total polymer used to prepare the NPs was R-ATTO and DNA was pGFP-Cy3. Next, the AcX solution (100 µl), at 0.1 mg/mL, was poured and incubated for 30 minutes at room temperature in the dark, followed by the addition of the gelation solution (200 µl). It was also incubated on ice for 5 minutes, before transferring to the incubator at 37ºC in 5% CO_2 atmosphere for 30 minutes in the dark. Once the gel formed, the sample was taken out of the incubator and cooled for 5 minutes at room temperature. The scaffold was then removed and the gel was unhooked by the same way as above and it was immersed in milliQ water for 2 hours, to perform the expansion. The water was also changed every 30 minutes.

3.3.7. Statistical analysis and software

Statistical differences were evaluated using Microsoft Excel, by performing an F-test to compare the equality of variances between the samples and a T-test to compare their means. P-values lower than 0.05 were considered to be significantly different. All the plots were designed using GraphPad Prism® and the schemes where made with Biorender. Finally, the nuclei size and the overlap coefficients were measured with ImageJ software. In the case of the overlap coefficient the JACoP plugin was used.

4. Results and Discussion

The ExM procedure **(Figure 4)** is based on the synthesis of a swellable polyelectrolyte gel throughout a biological specimen using sodium acrylate to produce a superabsorbent material, together with the comonomer acrylamide and the cross-linker N-N'-methylenebisacrylamide.[17][19] Target biomolecules are anchored to the network employing Acryloyl (AcX).[15] After the polymerization, triggered by the initiator ammonium persulfate (APS) and the accelerator tetramethyl-ethylenediamide (TEMED), the sample is mechanical homogenized by the application of a proteolysis, using a non-specific Proteinase K (ProK).[15][17][19] Finally, the polymer is immersed in water to perform the expansion, allowing the embedded sample to swell. Consequently, biomolecules are separated from each other, which improves the effective resolution of the conventional microscope and enables nanoscale features to be imaged (~50 nm).[15][17][19]

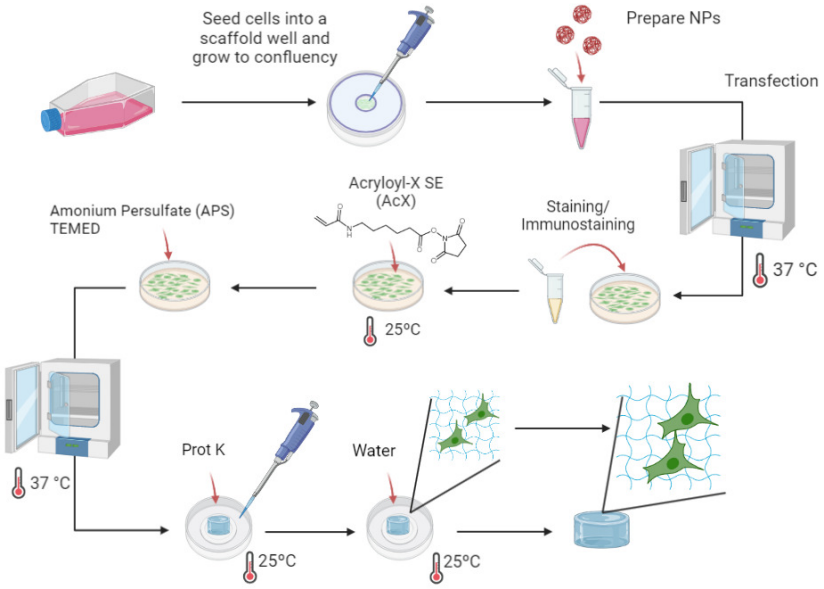

Figure 4. Expansion Microscopy (ExM) procedure scheme.

4.1. Cells expansion

Although the expansion microscopy (ExM) general procedure was already developed by F. Chen, P.W. Tillberg and E.S. Boyden, some aspects such as the culture density, cell dye concentration and their application method and homogenization incubation time had to be optimized. These are important procedures which affect to the final analysis of the sample. In this way, in this project, work has been done to improve all this steps and, consequently, the visualization and examination of the cells, defining a final protocol. In the following, each topic is described in detail.

4.1.1. Cell density

When seeding a cell line, the number of plated cells must be taken into account in order to promote their growth and obtain a confluent culture without having a too dense culture that would inhibit its growing by contact. Furthermore, if the protein retention ExM (proExM) procedure is applied to the sample, this parameter becomes more important to ensure the observation of cells after the expansion. For this purpose, the density of the PaTu 8988t (PaTu) cell culture, a model pancreatic adenocarcinoma cell line, was modified during the study.

As it can be seen in **Figure 5**, the culture seeded with 150,000 cells/mL showed low density, while in the culture seeded at 250,000 cells/mL the cells filled around 80%. If it is taken into account that in the AcX step, not all the cells join the polyelectrolyte gel and, also, in the expansion process all biomolecules separates from each other, losing the visibility of the cells, using the higher cell density tested is the best option. As a result, it is more probable to find visible cells to study. In addition, excess density, which could be detrimental for the cells, is avoided too. Thus, in the following, 250,000 cells/mL will be used.

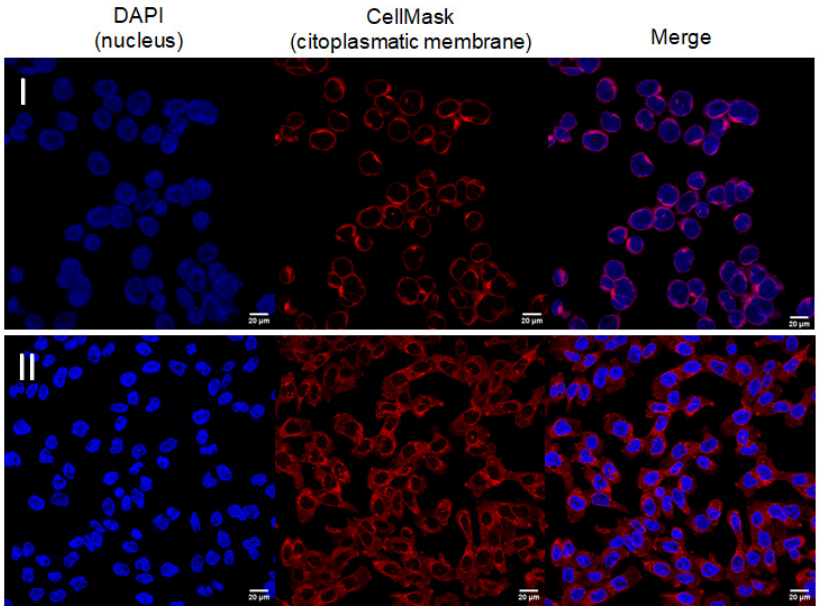

Figure 5: Pre-expanded PaTu cells, stained with DAPI and CellMask. Images obtained with a 40X confocal microscope objective. Scale bar: 20 µm. I) Cells plated at 150,000 cells/mL. II) Cells plated at 250,000 cells/mL. Different staining channels in where DAPI channel is shown in blue, CellMask in red and last channel merge the previous ones.

4.1.2. Nucleus staining

During the observation of cells by microscopy, being able to localize them and differentiate their subcellular compartments, such as the nucleus, is a key issue. The nucleus corresponds to one of the most important structures of the cell and a good way to stain it is the use of 4,6-

diamine-2-fenilindol dihydrochloride (DAPI). DAPI is an ultraviolet-fluorescent DNA-binding fluorochrome, which is used to stain the nuclear DNA content, being excited at 358 nm wavelength and emitting at 461 nm wavelenght. However, in live cells, DAPI is not very efficient due to its cell impermeability, so it should be applied on fixed cells,[20] but this will not represent a problem, since the ExM protocol requires cell fixation. In this way, DAPI was the chosen fluorophore to mark the nucleus. Thus, two different DAPI concentrations (0.1 µg/mL and 0.5 µg/mL) were tested with the objective of visualising well defined PaTu cell nuclei, pre and post-expansion. Both samples were treated with DAPI for 20 minutes, ProK for 3 hours and were expanded for 2 hours with water.

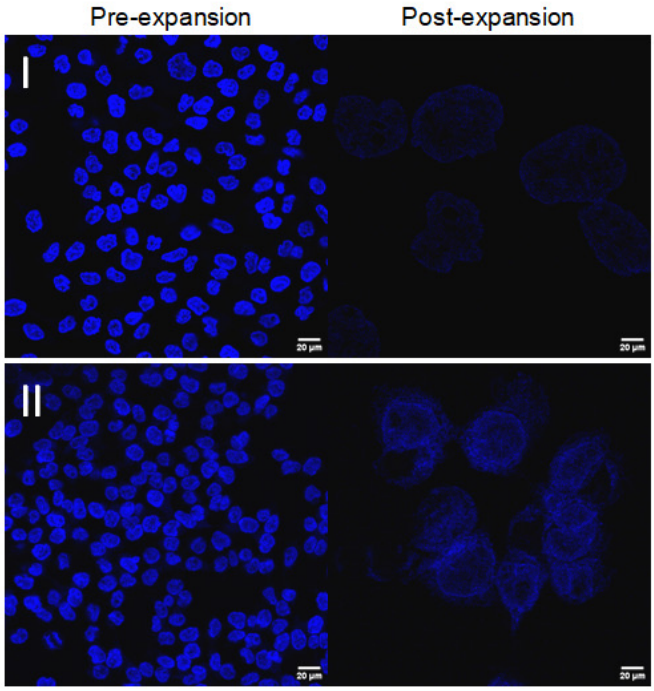

Figure 6. PaTu cell nuclei stained with DAPI. Images obtained with a 40X confocal microscope objective. Scale bar: 20 µm. I) Cell nuclei treated with DAPI 0.1 µg/mL. II) Cell nuclei treated with DAPI 0.5 µg/mL. Both display the pre (left) and the post (right) expansion images.

It can be observed on the **Figure 6** that, with the same time of DAPI, ProK and water incubation, the cell nucleus treated with DAPI 0.5 µg/mL **(Figure 6II)** are more defined than those treated with DAPI 0.1 µg/mL **(Figure 6I)**, which, once expanded, lose practically all fluorescence. With the proExM technology, biomolecules labelled with fluorophores spatially separated from each other[17], which makes more difficult to maintain its brightness and, consequently, to identify the shape of the nucleus. Therefore, the 0.5 µg/mL solution is appropriate, rather than the manufacturer's recommended application dilution (0.1 µg/mL), which is not enough concentrated in this case. It should be noted that this concentration has been used to expand the cells up to 3-4-fold **(Figure 8B)**, so it is possible that the more the cells are expanded, more concentration will be needed.

4.1.3. Cytoplasmic membrane staining

As well as nucleus, the cytoplasmic membrane is also a key part of the cell to visualise, as it helps to delimit it and enables to differentiate between internalized vs surface-retained structures. In the specific case of expanding nanoparticles (NPs)-transfected cells, staining the cytoplasmic membrane would make it easier to determine whether the polymers and nucleic acids are inside or outside the cell. In this study, orange CellMask was the chosen plasma membrane staining, which is a specific fluorescent commercial marker indicated to this purpose, being excited at 555 nm wavelength and emitting at 572 nm wavelength. It was selected taking into account the further labelling of the NPs with ATTO 647N NHS ester (ATTO), to avoid overlapping of the excitation/emission wavelengths.

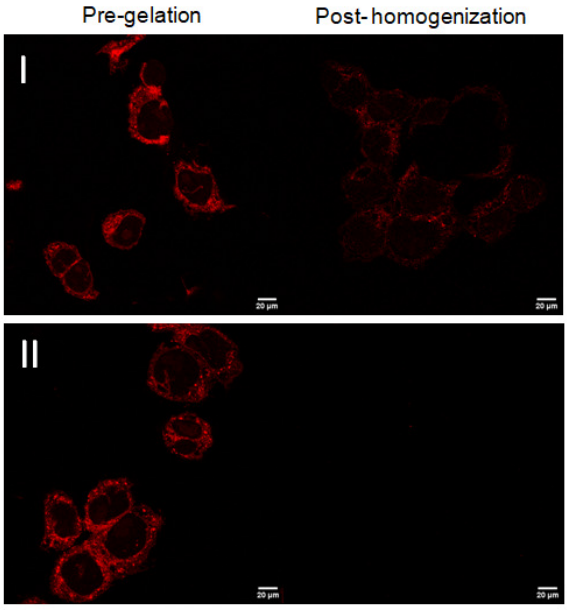

Figure 7. Images of PaTu cell cytoplasmic membranes stained with CellMask, taken with a 40X confocal microscope objective. Scale bar: 20 µm. I) Cells after one hour of expansion. II) Cells after two hours of expansion, once it finished. The images on the left show the membranes stained before the gel formation, while those on the right show the membranes after the homogenization step.

Two proExM procedures have been developed to stain cells. The first one involves the pre-gelation staining, while in the second one the dye is applied after the homogenization step.[16] As a result, these different protocols have been tested in this study, using a 25 µg/mL CellMask concentration, in order to ensure its visibility after the expansion process. In both cases the PaTu samples were treated with CellMask for 20 minutes, ProK for 3 hours and were expanded for 2 hours with water.

By the results of the cytoplasmic membrane staining, which are shown on **Figure 7**, it can be determined that, after one hour of expansion **(Figure 7I)**, the CellMask staining is more evident in the pre-gelation treated cells than in those treated post-homogenization. It is confirmed when

the expansion is finished, since there is no trace of the dye in the image on the right of **Figure 7II,** while it can be seen in the image on the left. The post-homogenization staining protocol is not always effective, probably due to the ProK used in that step degrades the proteins which provide the structural integrity to cells[16]. Consequently, the cytoplasmic membrane is degraded, impeding CellMask anchoring to much of the membrane lipids. Furthermore, in the post-homogenization staining protocol, the dye has to be applied above the polyelectrolyte gel, which could intercede in the diffusion through the gel to the cells. In conclusion, the pre-gelation staining has been set as the appropriated way to proceed.

4.1.4. Mechanical homogenization of the sample

The mechanical homogenization involves the digestion of a large part of the biomolecules in the sample and softening its structures so that they expand. Thus, the term of homogenization refers to equalizing the way in which the whole sample can be expanded. There are different ways to carry out this step, such as a high temperature treatment (e.g., via autoclave) to denature the specimen or by a proteolysis. [16] In this project, ProK was chosen to handle this process. Some other proteases can be used for the proteolysis (e.g., Endoproteinase Lys-C), but they are too mild, leading to an incomplete homogenization and anisotropic expansion.[21] Thus, one sample was treated with ProK for 1 hour, while another one was treated for 3 hours to test the ProK effect. Each was expanded for 3 and 2 hours, respectively, as excessive swelling leads to the loss of fluorescence.

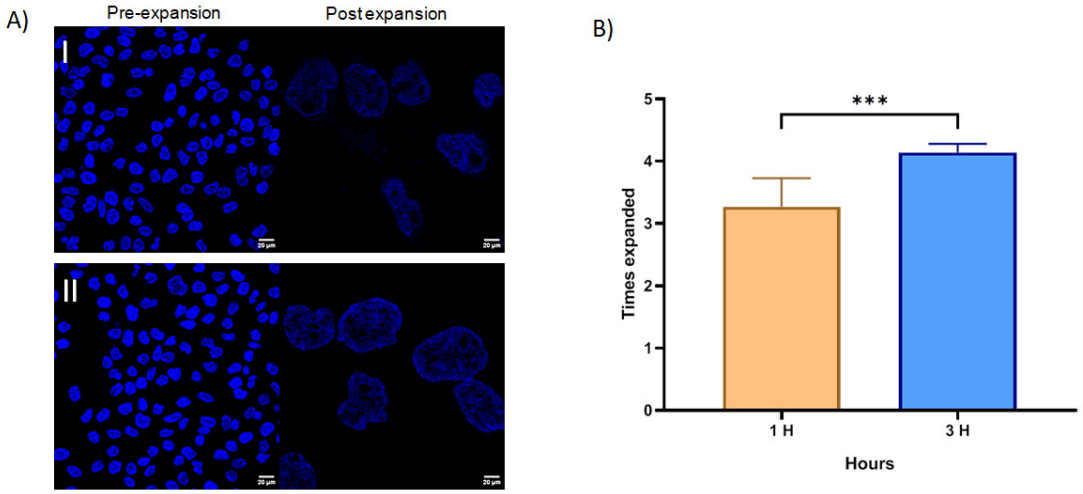

Figure 8. PaTu cells expansion according to the ProK incubation time. A) Cell nuclei pre and post expansion images, taken with a 40X confocal microscope objective. Scale bar: 20 µm. I) Sample treated with ProK for 1 hour. II) Sample treated with ProK for 3 hours. The images on the left display the cells before the expansion, while those on the right show them after the expansion. B) Graphic representation of both sample expansion. In orange is shown the sample treated for 1 hour with ProK, while in blue the sample treated for 3 hours. P-value < 0.0001 (****), p-value < 0.001 (***), p-value < 0.01 (**), p-value < 0.05 (*) and p-value > 0.05 (ns).

The results presented in the **Figure 8B** evince that the cells only reach a little more than 3-fold expansion when they are subjected to 1 hour of ProK and expanded 3 hours, whereas with a 3 hours treatment of ProK they achieve a 4-fold expansion in only 2 hours of expansion. The main reason for this may be consequence of the lack of specimen deterioration, as a result of the shorter working time of ProK. As this proteinase is the responsible for digesting many of the molecules in the sample, insufficient incubation time could lead to incomplete homogenization, preventing optimal expansion[15]. In conclusion, 3 hours of ProK incubation was established as the appropriated option to perform this protocol.

4.1.5. Isotropic expansion

In case a sample observed by microscopy is not analysed directly, but has to be done through a polyelectrolyte gel, additional issues have to be considered. In this way, having a negligible deformation of the sample, during the homogenization and expansion, is a key aspect to ensure its reliable visualization. So as to confirm an isotropic expansion of the sample, the same area was localized and examined pre and post-expansion. A549 cells of the sample were stained with DAPI and CellMask, treated with ProK for 3 hours and expanded in water for 2 hours.

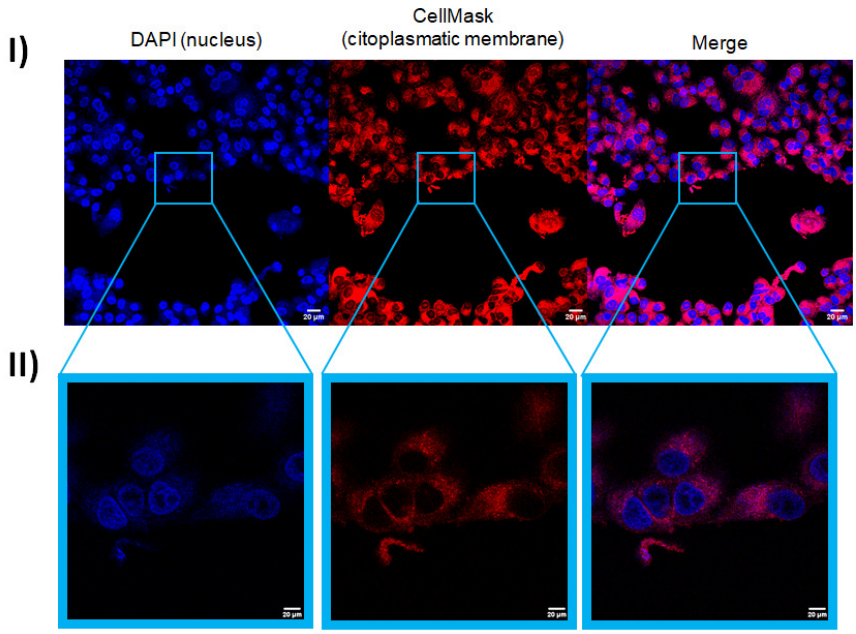

Figure 9. Images of the same area of the sample before and after the expansion step. All were obtained with a 40X confocal microscope objective. Scale bar: 20 µm. I) Area before the expansion. II) Area within the blue square after expansion. The blue channel shows DAPI, the red channel is for CellMask and the last channel is a merger of the previous channels.

The comparison between the pre and post expansion area is presented in the **Figure 9** and, as can it be seen at first sight, there is no obvious evidence of any deformation in the sample. With these results it can be affirmed that the homogenization step is properly performed, promoting

an isotropic expansion and allowing the specimen to be observed without any additional difficulty other than the small loss of fluorescence. Furthermore, taking into account that all images were taken with the same objective and zoom, it is demonstrated that this process considerably increases the resolution of the image, which was the principal aim.

In conclusion, a protocol to expand cells has been successfully developed. With all the experiments done during the study, it has been set: **250,000 cells/mL** as the appropriated cell density to seed, **0.5 µg/mL DAPI** as the correct concentration of nuclei staining, **pre-gelation** staining as the ideal way of dyeing the cytoplasmic membrane and a **3 hours** treatment with ProK as the best to ensure an optimal and isotropic expansion of the sample. All these modifications have helped to achieve a high-resolution technique to examine cells, in a less expensive way than sophisticated technologies.

4.2. OM-pBAE nanoparticles expansion

When working with oligopeptide end-modified poly(beta-aminoester)s (OM-pBAE) NPs some aspects can affect their stability, leading to disassembly. As a result, the proExM protocol used above, with cells, is not really appropriated if the main objective is the expansion of NPs. For this purpose, it had to be modified. Furthermore, new different fluorophores were used, so it had to be tested whether they were suitable for the process.

4.2.1. Protocol modifications

It has been demonstrated that OM-pBAE NPs are sensitive to both room temperature (25ºC) and culture incubation temperature (37ºC).[14] This NPs characteristic was a limitation that had to be overcome in this project, due to the long time incubations performed in the cells expansion protocol. For this reason, some of the protocol steps were shortened. First of all, the ProK step was removed due to NPs are formed by electrostatic interactions, so no amide bounds have to be broken to homogenize the sample.[11][22] Then, incubation with AcX, which in the case of the cells is overnight, was readjusted to 30 minutes, just as the polymerization step. Should it be mentioned that 15 minutes of AcX were also tested but, later, the sample did not polymerize, so images could not be taken. The reason could be that the lack of acrylamide groups, supplied by AcX, hinder the polymerization of the sample.

4.2.2. Crosstalk

Before examining the images of thre NPs, one aspect to check was the crosstalk between the two fluorophore channels. This is a good way to affirm that what is seen in the images are either formed NPs or, on the contrary, a fluorophore is being excited and emitted in both channels.

Controls for each fluorophore were made, using specifically RH polyplexes and labelling only R in one sample, while the other had only GFP plasmid (pGFP) labelled. RH refers to the terminal end-oligopeptides of the OM-pBAE, mixed at a ratio of 60/40 R/H, as a demonstrated before in the group, being one of the most efficacious peptide combination in transfecting cells.[14][23] ATTO and Cyanine3 NHS ester (Cy3) were the chosen fluorophores used to label R and pGFP. Considering that only crosstalk was to be analysed, samples were not subjected to the proExM protocol.

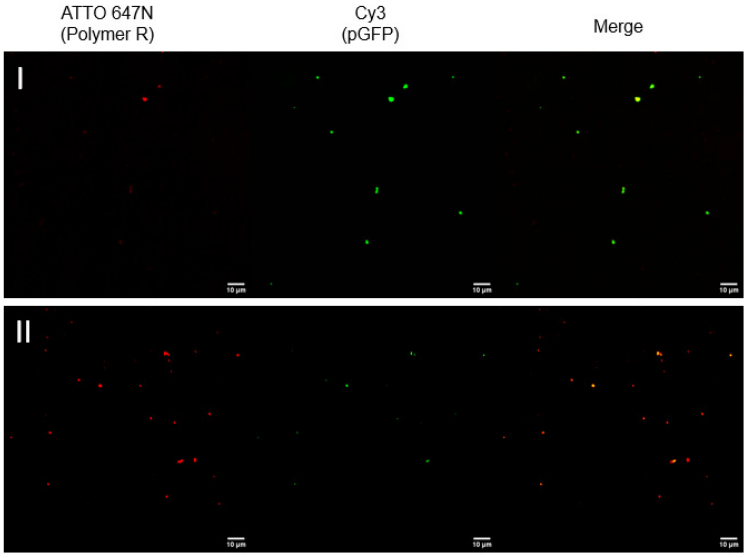

Figure 10. RH polyplexes images, taken by a 40X confocal microscope objective. Scale bar: 10 µm. I) NPs with pGFP labelled. II) NPs with R labelled. Red channel shows R, green channel is for pGFP and the last channel is a merger of the previous channels.

Results in the F**igure 10** display some weak fluorescence in the red channel in **Figure 10 I** and in the green channel in **Figure 10 II**. However, this signal is very low, compared to that in the opposite channels. Having fluorescence in channels where no signal is expected, due to the lack of its respective fluorophore, indicates a slight crosstalk between the channels. Even so, this signal can be eliminated through image analysis and thus, not much colocalization is detected in the merge channel, so it can be said that there is reliable specificity when observing labelled NPs. Consequently, colocalization in the merge channel of the following experiments should be considered as formed NPs. Therefore, this confirms both fluorophores can be used together in proExM experiments.

4.2.3. Fluorophores tolerance

In the same way as in the case of cells, fluorophores are also needed to identify NPs in the sample. The presence of this labelling allows to see whether the polyplex is formed or not. That is why it is so important that the used fluorophores withstand each of the steps. ATTO exhibits a

high retained fluorescence throughout the process.[16] Meanwhile, Cy3 is one of the fluorophores that is not compatible with the standard protocol due to it is a cyanine dye, which are destroyed during the polymerization[15][16]. Its resistance to this step was tested with the shorter AcX incubation.

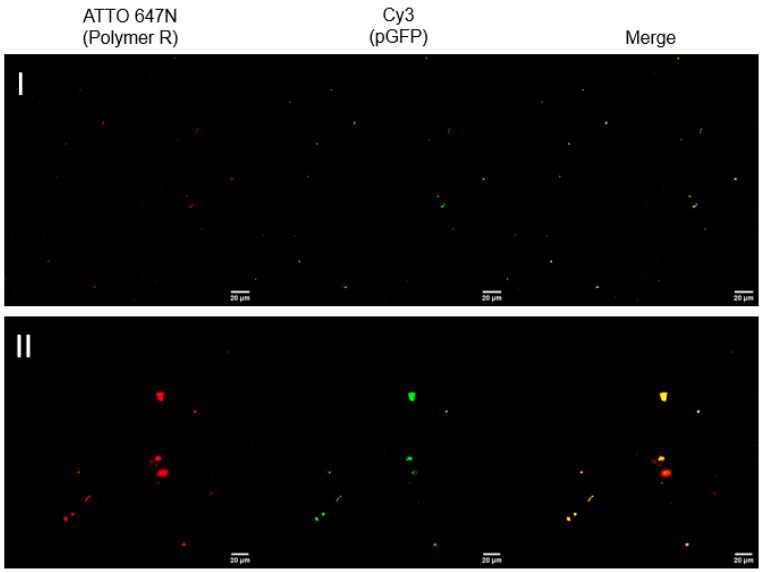

Figure 11. R polyplexes presents in the polymerized sample. Images obtained with a 40X confocal microscope objective. Scale bar: 20 µm. I) NPs before the expansion step. II) NPs after 1 hour of expansion. Red channel shows R, green channel is for pGFP and the last channel is a merger of the previous channels.

The results in the **Figure 11** confirm that both fluorophores resist the entire process and they can be used to label NPs to be expanded by this protocol. Halving the polymerization time has been a key modification to ensure the visualization of Cy3 once the gel is formed.

In **Figure 11II** it can be seen a high colocalization between both fluorophores, which indicates the presence of formed NPs. The enlargement of the NPs in 1 hour of expansion is also noticeable, although it is not possible to measure it, due to the low resolution of the pre-expanded image **(Figure 11 I)**. Should it be mentioned that NPs could form aggregates, thereby increasing the size of the eventually observed particles.

To terminate the NPs expansion chapter, **it has been concluded that the modification of the protocol defined in the cells chapter, resulted on a successfully expansion of NPs.** The main changes applied have been: the reduction of the time of polymerization and the incubation with AcX to 30 minutes and the removal of the ProK step. In addition, the tested fluorophores (ATTO and Cy3) withstand this specific process and help to visualize NPs with a higher resolution, once finished its expansion.

4.3. Transfected cells expansion

Once the cell and NPs proExM protocols were optimized, it was the moment to work with cells transfected with OM-pBAE polyplexes. Achieving a considerable expansion of this samples could allow the group to characterize the NPs and its cell trafficking. To do it, the NPs proExM protocol cannot be used, since the sample contains cells, which must be homogenized to achieve sufficient magnification. Thus, the cells proExM protocol was recovered.

4.3.1. Crosstalk and fluorophores

In order to see both cells and NPs all at once, all their respective fluorophores must be used in the same sample. In addition, a fluorophore that labels the endosomes, to observe their colocalization with NPs, have to be considered. Immunostaining with Alexa fluor 488, labelling a secondary antibody, together with the appropriated primary antibody, could be a good option, as it also shows a high retained fluorescence during the protocol.[16]

On the contrary, the limitation of employing so many fluorophores in a sample, is that the visible light spectrum remains full and one emission may overlap with another as displayed in **Figure 12**.

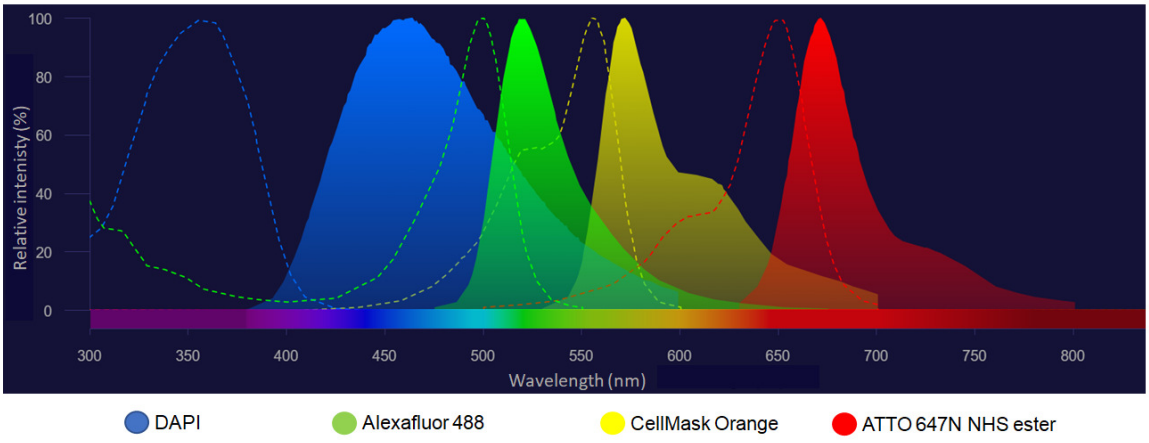

Figure 12. Image (taken from Fluorescence SpectraViewer - Thermo Fisher Scientific) of the visible light spectrum filled by the fluorophores used in the study. Broken line plots are the excitation plots of each fluorophore, while those filled with different colours are the emission plots. DAPI spectrum is shown in blue, Alexa fluor 488 in green, CellMask in yellow and ATTO in red.

Moreover, fluorophores that were not used in the cells proExM protocol, such as ATTO and Alexa fluor 488, must be tested. In those cases, Alexa fluor 488 is bound to an anti-RAB5 antibody, which in turn is bound to RAB5 of early endosomes. Controls of each fluorophore were performed to verify the absence of crosstalk and their tolerance to the treatment. A549 cells were transfected using R polyplexes. In this case, pGFP was not labelled, since Cy3 did not resist 1 hour of polymerization, required for the cell expansion.

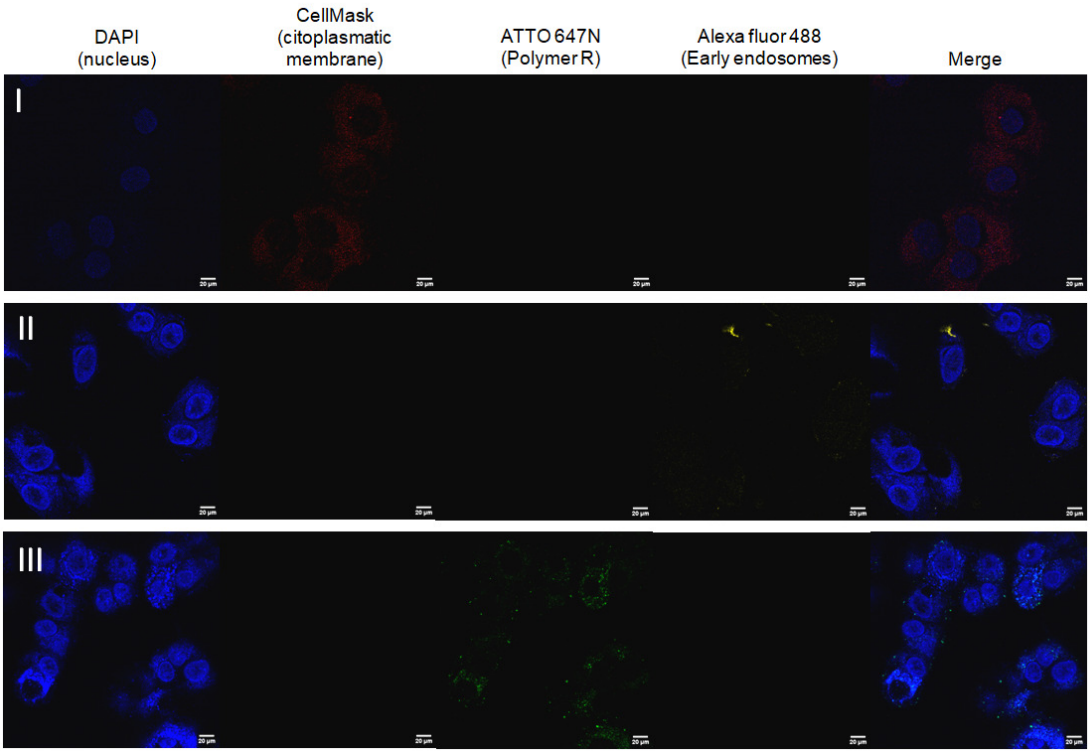

Figure 13. A549 cells transfected, using R constructs, after 2 hours of expansion. Images taken by a 40X confocal microscope objective. Scale bar: 20 µm. I) Cell mask control. II) Alexa fluor 488 controls. III) ATTO control. DAPI channel is shown in blue, CellMask in red, ATTO in green, Alexa fluor 488 in yellow and the last channel merge the previous channels.

As it can be appreciated in the **Figure 13,** CellMask, ATTO and Alexa fluor 488 give a signal within its respective controls. However, no additional fluorescence is detected in any sample. These results confirmed that ATTO and Alexa fluor 488 can retain their fluorescence throughout all the process, albeit in a reduced form, so they can be used to label NPs and endosomes, respectively. The NPs may withstand the entire process, as they are inside the cells, which protect them from ProK effect. Moreover, no crosstalk is detected between the different channels, thereby ensuring the presence of all the biomolecules labelled. Once these requirements are met, the protocol is ready to be used to analyse transfected cells.

4.3.2. Cellular uptake and transfection

If the main objective is to see how NPs enter the cells, it is necessary to know how long they need to do it, so that as many NPs as possible can be observed. In this way, it is also a good way to know, quantitatively, the differences between the timings tested with the protocol, in terms of uptake. For this purpose, A549 cells were transfected with R and RH NPs, 2, 4, 6, 24 and 48 hours before their fixation. R was labelled with Cyanine5 NHS ester (Cy5) for detection by flow cytometry.

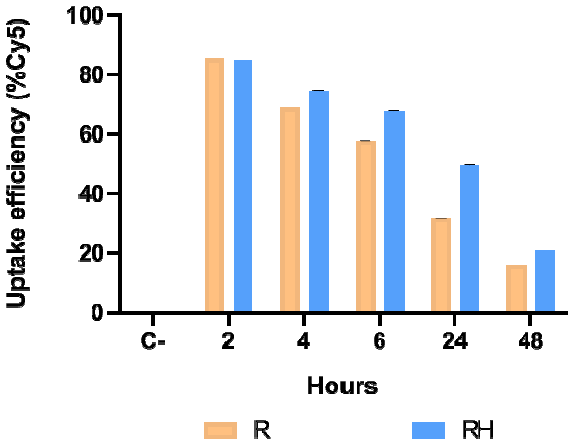

Figure 14. Uptake efficiency at short and large timings of some OM-pBAEs polyplexes in A549 cells. Uptake efficiency of R NPs is represented in orange while that of RH NPs is shown in blue.

As the results in **Figure 14** show, the maximum uptake of both NPs takes place at short timings, reaching more than 80%. Then, the presence of labelled polymer gradually decreases over the time, until 48 hours after the transfection, when values are around 20%. Thus, approximately 60% of the polymer degrades, probably thanks to the ester bonds present in their structure, which make them biocompatible and biodegradable.[9][11] Both constructs follow a similar pattern, as RH NPs contain 60% of R. It also can be seen that, in general, the uptake values of RH polyplexes are slightly higher than those of R. The main reason could be that R, which is more positive than RH, remains attached to the membrane, while RH enters the cell more easily. Moreover, as H helps to the endosomal escape[14] its levels of degradation in the lysosome may be lower than those of R.[24]

4.3.3. Transfected cells visualization and subcellular colocalization of the polyplexes: validation of the established proExM protocol

Once the uptake efficiency of the NPs was known, 2 and 4 hours after the transfection were the established timings to be used for the proExM protocol. This ensures a high number of NPs to be visualized after expansion. In addition, the images obtained could be compared with the results shown in the **Figure 14** and confirm them. Then, A549 cells were transfected with R and RH NPs containing CD6C3 labelled with ATTO, 2 and 4 hours before fixation.

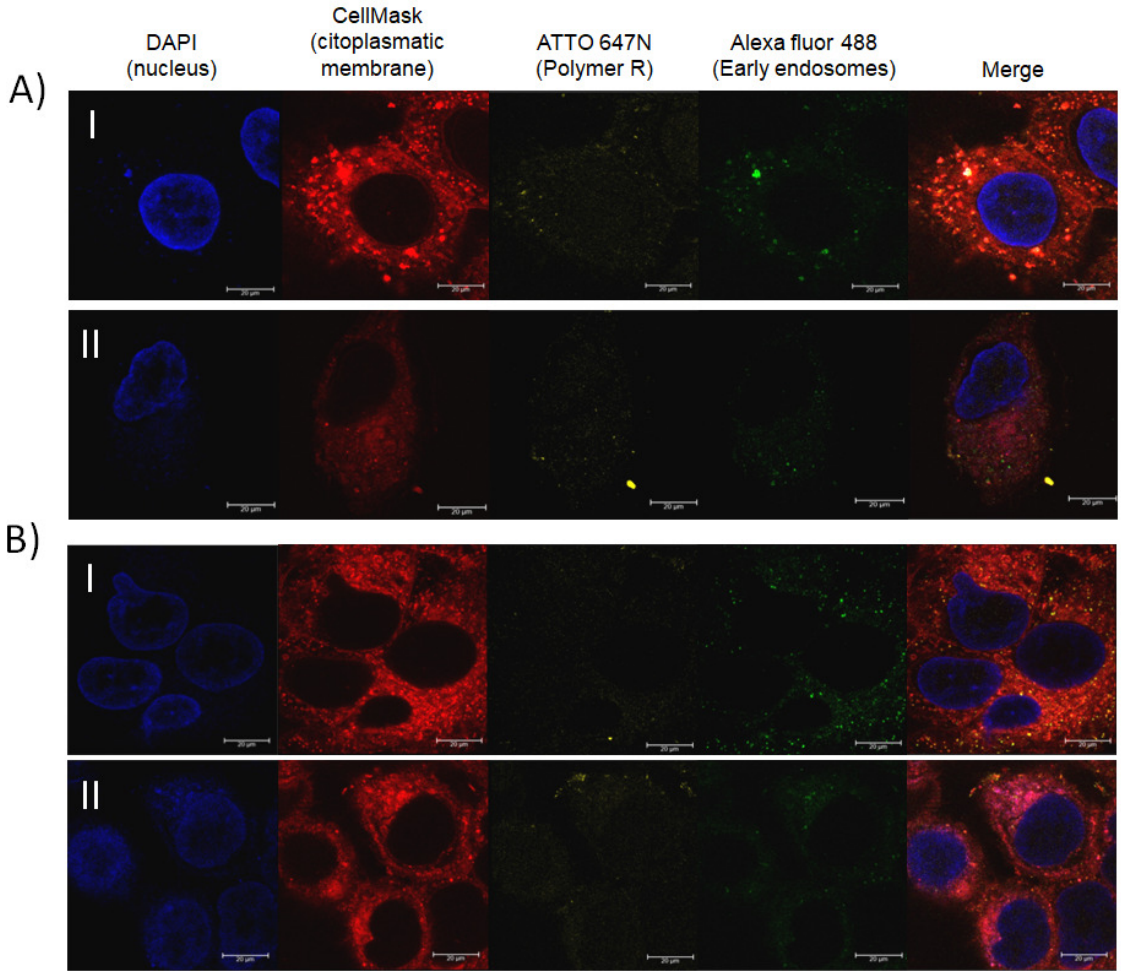

Figure 15. Confocal microscopy of the different polyplexes on different timings in A549 cells. All the images were taken with 63X objective and zoom 2. Scale bar: 20 µm. A) Cells after 2 hours of transfection. I) R NPs. II) RH NPs. B) Cells after 4 hours of transfection, following the same order. DAPI channel is displayed in blue, CellMask in red, Alexa fluor 488 in yellow, ATTO in green and the last channel merge the previous channels.

Looking the images in **Figure 15**, both cells and NPs are visualized with a high resolution and NPs can be accurately located within the cell cytoplasm. Early endosomes can also be found throughout the cytoplasmic membrane. In addition, red fluorescent spots in the cytoplasm, which colocalize with the labelled polymer, can be detected in the CellMask channel. They may be late endosomes, since they are not labelled by Alexa fluor 488 but with CellMask, which also stains cytoplasmic membranes that form endosomes. For this reason, a further step could be the use of anti-RAB7 antibodies, rather than anti-RAB5, in order to label the late endosomes and see if they actually colocalize with the polymer. It could also be done to label lysosomes. This could be a good way to track the NPs and realize whether or not they achieve the endosomal escape, at different timings and whether the polymer is being degraded.

All this analysis is only a qualitative examination. To really confirm the expected results **(Figure 14)**, colocalization between NPs and early endosomes and cytoplasmic membranes was calculated with the ImageJ software.

Table 7. Quantitative analysis of the colocalization between NPs and early endosomes and cytoplasmic membranes. Overlap coefficients are presented in values between 0 and 1.

		Early endosomes - NPs	**Cytoplasmic membranes - NPs**
NPs type	Timing (hours)	Overlap coefficient	Overlap coefficient
R	2	0.24	0.48
RH	2	0.34	0.4
R	4	0.43	0.7
RH	4	0.28	0.48

The values in the **Table 7** show that the colocalization of early endosomes and NPs increases with the time (0.24 to 0.43) for R polyplexes, while is maintained for those of RH (~0.3). These results confirm that R polyplexes remain attached to the membrane the first 2 hours, although the flow cytometry detect them as internalized NPs. At 4 hours they enter the cell, as the colocalization with early endosomes increases. On the other hand, the RH internalization at 2 hours is higher than that for R, since it is not as positive charged as R. Equally, between cytoplasmic membranes and NPs the same pattern is followed but with higher values (0.48 to 0.7 for R and 0.4 - 0.5 for RH). However, this indicates that most of R, at 4 hours, remains within cytoplasmic membranes, which probably are early or late endosomes. With this information it is realized that it has not escaped to the cytoplasm yet. Per contra, RH also preserve its colocalization with membranes and shows a lower value than R at 4 hours. Thus, as stated above, H may help the polyplexes to escape from the endocytic pathway.

To conclude this section, it has been determined that transfected cells could be expanded, allowing the NPs to be sees within the cytoplasm and endosomes at higher resolution both ATTO and Alexa fluor 488 are suitable fluorophores to withstand all steps, including 1 hour of polymerization and 3 hours of ProK. This helped to confirm and understand that R remains attached to the membrane for the first 2 hours, due to its higher positive charge, and enters later than RH. RH, in turn, escapes from the endosomal pathway more easily than R, thanks to the action of H.

5. Conclusions

In this project, a proExM protocol has been optimized for the first time, in order to be applied simultaneously for the visualization of cells allowing to characterize the NPs trafficking in cells. In this way, the following conclusions have been drawn from this study:

- Regarding cells, a method has been developed to expand and observe them with high resolution, using a conventional microscope.
    - The appropriated cell density for seeding has been established at 250,000 cells/mL.
    - Confirmed that DAPI 0.5 μg/mL is the suitable concentration for staining nuclei and that CellMask staining prior to the formation of the gel has been determined is the best procedure for labelling the cytoplasmic membrane.
    - 3 hours of ProK has been chosen as the ideal incubation time for optimal and isotropic expansion, reaching 4-fold cell enlargement.
- The previous protocol has also been adapted, with success, to take into account the requirements of the NPs to maintain their stability. Moreover, other fluorophores have been used, in this modified protocol, to test their tolerance to the entire process.
    - Incubating the NPs with AcX for 30 minutes, rather than overnight, helps to their stability and makes their resistance to the process more likely.
    - ATTO and Cy3 withstand the entire protocol, retaining their fluorescence, so they can be used to label polymer and pGFP, respectively. Reducing the polymerization time to 30 minutes has been a key modification to ensure the visualization of Cy3.
- Finally, the proExM protocol for cells was recovered, achieving the expansion of transfected cells.
    - ATTO and Alexa fluor 488 withstand 1 hour of polymerization and 3 hours of ProK, making them suitable for labelling polymer and endosomes, respectively. Consequently, NPs could be seen inside the cytoplasm and endosomes, at higher resolution after expansion.
    - In terms of characterizing the cellular trafficking of NPs, R remains attached to the membrane for the first 2 hours, due to its higher positive charge, and enters later than RH, at 4 hours. Meanwhile, RH escapes from the endosomal pathway more easily than R, thanks to the action of H.

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