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DiOleyl-RG and related molecules as transfecting agents to improve efficiency of DNA transfection in hard - to - transfect cells

**Project in Molecular Biology,
Organic Chemistry and Pathophysiology**

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“I entered the classroom with the conviction that it was crucial for me and every other student to be an active participant, not a passive consumer... education as the practice of freedom... education that connects the will to know with the will to become. Learning is a place where paradise can be created.”

Bell Hooks

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ABSTRACT (English and Catalan)

Abstract

Cancer and Down Syndrome (DS), among others, are genetic diseases due to mutations or other alterations in cells' DNA. *PolyPurine Reverse Hoogsteen hairpins* (PPRHs) have proved to be an efficient and specific technology for silencing or editing the genes involved in cancer mutations. In this research assignment, new transfecting agents in combination with PPRHs were tested either to improve transfection into hard-to-transfect cells such as lymphoma B cells using a new cationic liposome called DO-RG (dioleil-RG); or to improve the efficiency of this process using gold nanoparticles, which already have great advantages according to literature. Apart from that, DO-RG was used as a transfecting vehicle of specific PPRH against DYRK1A, a key protein in DS main cognitive deficiencies and therefore this treatment would improve DS features. DO-RG has previously proved to efficiently transfect neuronal origin cells. Promising results are obtained from the three experiment blocks; however, more replicates should be carried out to prove statistical significance.

Resum

El DiOleil-RG i altres derivats com a agents transfectants per incrementar l'eficiència de transfecció de DNA en cèl·lules difícils de transfectar.

El càncer i la Síndrome de Down (DS), entre d'altres, són malalties genètiques a conseqüència de mutacions o altres alteracions en el DNA de les cèl·lules. Els *PolyPurine Reverse Hoogsteen hairpins* (PPRHs), pinces de polipurines, han resultat ser una tecnologia eficient pel silenciament i l'edició dels gens involucrats en les mutacions que provoquen el càncer. En aquest treball de recerca es proven diferents agents de transfecció en combinació amb els PPRHs, tant per millorar la transfecció en cèl·lules difícils de transfectar com són les de limfoma de cèl·lules B amb un liposoma catiónic anomenat DO-RG (dioleil-RG); com per millorar l'eficàcia d'aquest procés utilitzant nanopartícules d'or per les seves propietats positives que es descriuen a la literatura. A part d'això, el liposoma DO-RG s'utilitza com a vehicle de transfecció de PPRHs dirigits específicament en contra del gen DYRK1A, la proteïna del qual és clau en el desenvolupament de les deficiències cognitives característiques de la DS. Per tant, aquest tractament milloraria el desenvolupament de la malaltia. Prèviament, el DO-RG ha demostrat ser eficaç en la transfecció de cèl·lules d'origen neuronal. Els resultats obtinguts dels tres blocs principals d'estudi són prometedors; tot i això, s'haurien de fer més rèpliques per obtenir resultats significatius estadísticament.

ABBREVIATIONS

DO-RG: Dioleoyl-RG.

DOTAP: Cationic liposome N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate.

DS: Down Syndrome.

DYRK1A: Dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1A, a gene and a key protein in DS main cognitive deficiencies.

GNP: Gold nanoparticles.

HpDyrk: Different hairpins depending on the DYRK1A gene sequence they are directed against. All with reverse Hoogsteen bonds.

HpsPr-C-F: Hairpin directed against survivin gene with reverse Hoogsteen bonds.

Hps-WC-F: Hairpin directed against survivin gene with Watson and Crick bonds.

PC-3: Prostate cancer cellular line.

PPRH: PolyPurine Reverse-Hoogsteen hairpins.

RG and LP: Name given to structures synthesized in the Organic Chemistry Department of the Faculty of Pharmacy of the University of Barcelona.

SH-SY5Y: Neuroblastoma cellular line.

WSU-FSCCL: B-cell lymphoma cellular line.

FIELDS INTEGRATION

The research assignment presented below is mainly based on molecular biology, since it deals with the usage of PolyPurine Reverse Hoogsteen hairpins (PPRHs), DNA hairpin shaped molecules that, after transfection, bind the endogenous DNA and modifies the target gene transcription. Therefore, molecular cell processes such as DNA structure and transcription and translation processes are key for the understanding and development of the assignment. Moreover, different molecular methods such as PPRH design, RNA extraction or Western Blot assays, among others, were carried out.

Apart from that, organic molecules were used as possible transfecting agents. A collaboration with the Organic Chemistry section in our Faculty was done to develop the cationic liposome tested (DO-RG). They also synthesized different gold nanoparticles that have been studied both in the laboratory experiments and in the *Transmission Electron Microscopy* (TEM) presented in this assignment. Different organic properties from the molecules have been discussed to improve the transfecting agents' design.

Finally, pathophysiology has been integrated to study the disordered physiological processes associated with cancer and Down Syndrome (DS) diseases. The understanding of these pathologies is basic to identify key molecules and processes on their development and therefore to design specific target treatments.

1. INTRODUCTION

I have been developing the thesis to finish my degree (TFG) in a research group of the Molecular Biology section in the Faculty of Pharmacy and Food Science whose line of work deals with cancer research using gene therapy to accomplish gene silencing and the editing of different genes involved in these diseases.

1.1. Cancer process

Cancer is a name given to a set of related diseases which consist of an alteration of different genes involved in cells functioning control, specially the growing process and cell division. This leads to an uncontrolled growth of these cells producing a tumour and the possible spread of these carcinogenic cells to other body tissues due to its invasive capacity.

Genetic changes that contribute to the onset of cancer tend to affect three main types of genes: proto-oncogenes (oncogenes after their alteration), tumour suppressor genes and DNA repair genes. When these genes are altered, they become cancer-causing genes by allowing cells to proliferate uncontrollably (1).

There may be one or more different mutations for cancer to appear and certain mutations have been found to be shared in many types of cancer. There are two main agents that molecularly contribute to the onset of cancer: first, the presence of an initiator agent that would cause a mutation in the genetic material of a cell, and second, in different combinations, there would be the presence of a promoter that would increase the division of this damaged cell. These agents that initiate or promote cancer are called carcinogens (2).

1.2. Gene therapy

Since cancer is a genetic origin disease, nowadays the use of nucleic acids to regulate and repair different cancer-causing genes is an important explored way of cancer therapy. Some examples of anticancer gene therapy could be the antisense oligonucleotides (aODNs) or the small interfering RNAs (siRNAs). Apart from these therapies, the PolyPurine Reverse Hoogsteen hairpins (PPRH) were first described in 2009 (3).

PPRHs are unmodified DNA molecules formed by two antiparallel mirror sequences of polypurines connected by 5 thymidines conforming the shape of a hairpin. The

intramolecular bonds established within the PPRH are inverted Hoogsteen bonds between the purines. On the other hand, the intermolecular bonds, established between the hairpin and the endogenous DNA, are created by complementary of Watson and Crick. Therefore, when the PPRH molecule reaches the nucleus, it is described to form a triplex structure with one chain of the dsDNA which displaces the second strand of endogenous dsDNA and blocks transcription.

As it is shown in Figure 1, when PPRHs bind to the target sequence, gene expression is reduced by different mechanisms. On the one hand, if PPRHs bind to the template strand, they are called template-PPRHs and they interfere with the transcription process lowering mRNA and protein levels. On the other hand, if PPRHs bind to the coding strand, they are called coding-PPRHs, and they can block transcription factors. Likewise, coding-PPRHs can also bind to mRNAs and produce splicing alteration of the pre-mRNA (4).

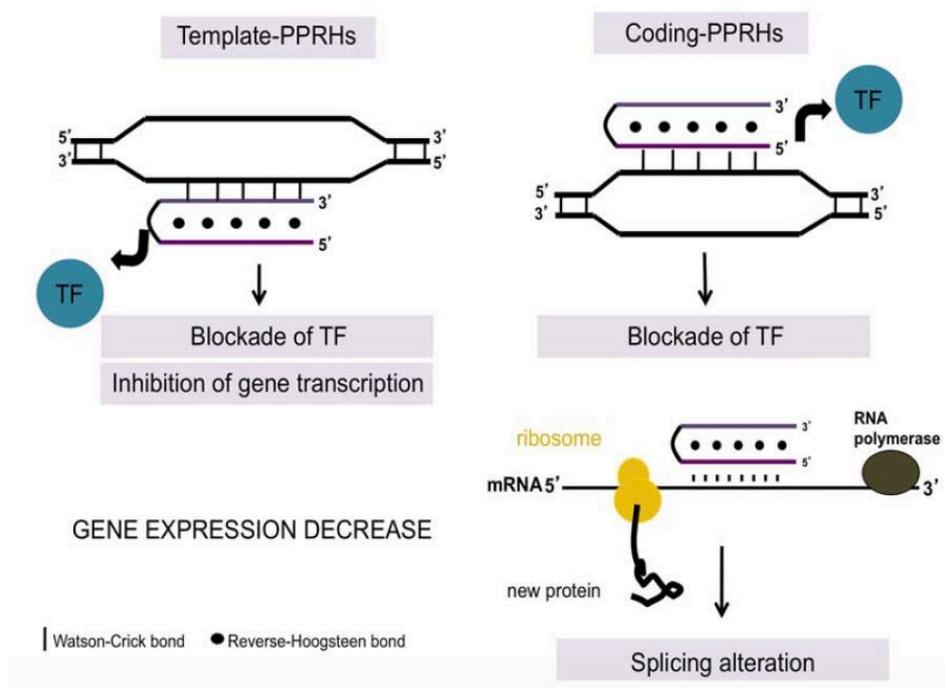


Figure 1. Template-PPRHs and coding-PPRHs mechanisms of action (5).

All these mechanisms would end up with either a reduction in protein levels or in apoptosis of the cell if the PPRH blocks essential genes for cell development or function.

To design PPRHs, polypyrimidine sequences must be found in the cancer-causing genes, more common in non-coding areas such as promoters and introns, although they can also be found in coding sequences. A range of up to 3 purine interruptions within

this polypyrimidine sequence can be accepted as it does not cause enough instability to avoid the PPRH binding to its target sequence.

1.3. Survivin target

One of the most commonly used PPRHs in the laboratory is that directed against survivin gene which decreases survivin protein levels. Survivin is an anti-apoptotic protein (does not allow cancer cells to die) and it is also linked to mitosis and angiogenesis processes, key processes for cancer development (4). This protein is over expressed in different tumours such as: prostate, lung, breast, colon, stomach, oesophagus, pancreas, spleen, uterus, ovary, non-Hodgkin's lymphoma of large cells, leukaemia, neuroblastoma, melanoma and other skin cancers that are not melanomas. However, surviving levels are undetectable in most normal tissues differentiated with some exceptions and their expression is linked to less survival, resistance to chemotherapy, worse disease progression and higher recurrence rates. For all these reasons, survivin is considered a paradigm of anti-cancer target and a good marker for diagnosis and prognosis. This gene is the target that will be used in the first part of the experiments presented in this assignment. The PPRH directed against this target will be fluorescently labelled to see its entry into the cells and the nucleus.

As the used PPRH binds to the promoter chain of gene promoter, its mechanism of action is through blocking transcription, therefore, it does not alter the mRNA splicing. PPRH sequences that target survivin gene are shown in Table 1.

Gene	Name	PPRH sequence	Location
A. Survivin	HpsPr-C WT	AGGGGAGGGATGGAGTGCAG T T T AGGGGAGGGATGGAGTGCAG T T	Promoter
B. Survivin negative control	HpsPr-C WT-WC	TCCCC TCCC TACCTCA CGTC T T T AGGGGAGGGATGGAGTGCAG T T	Promoter

Table 1. Survivin PPRH sequences. **A.** Wild-type survivin hairpin with intramolecular reverse Hoogsteen bounds. **B.** Negative control survivin hairpin with intramolecular Watson and Crick bonds that will not allow to form a triplex with the endogenous DNA. WT – Wild type refers to the fact that they have up to 3 pyrimidine nucleotides facing the purines in the endogenous DNA, and which will not prevent the formation of the intramolecular Hoogsteen within the hairpin.

1.4. Transfection process and methods

After the PPRH design, transfection must be performed. Transfection consists of introducing this PPRH into the cell so it can reach the nucleus and make its action. This transfection can be performed using different methodologies as it is explained below and each cell line has specific transfection characteristics. Generally, it has been described that postmitotic cells, those that have lost their ability to divide (neuronal cells or lymphocytes), are more difficult to transfect cells and they are commonly called **hard-to-transfect cells**. These cell types are a challenge in terms of efficiency when introducing and expressing exogenous genetic material and much care should be taken while working with them. They have been described as very sensitive to different conditions such as physical stress or temperature alterations (6).

Different mechanisms for performing nucleic acid transfection in mammalian cells have been described. They can be divided into two categories: those for transient transfection and those for stable transfection. Transient transfection is aimed at studying alterations in the expression of genes and proteins in the short term and, therefore, they are not integrated into the host cell genome. Stable transfection is aimed at generating integration of nucleic acids into the genome achieving a permanent expression in the host cell and the cells produced after its division.

Transfection can be achieved using transport and release systems that can be chemical (liposomes, with non-liposomal lipids, polyamines or dendrimers), physical (electroporation or microinjection), or virus-based methods (using retroviruses, adeno-associated virus or lentivirus).

The transfection pathway used throughout the present project is transient and chemical through liposomes. Liposomes are synthetic analogues of the phospholipid bilayer, the structural basis component of the cell membrane. As shown in Figure 2, these transfecting agents are amphipathic, what means that they consist of hydrophobic and hydrophilic parts that allow the formation of spheres in water that make electrostatic interactions with the negative charged genetic material. It leads to the formation of lipid-DNA aggregates that will be introduced into cells by endocytosis through the cell membrane (7).

Some liposome features that need to be considered are cell membrane affinity for endocytosis and its DNA or RNA affinity for binding them but with the capability of

releasing the nucleic acid into the cytoplasm. These factors vary according to particle size, lipid formulation, amount of charge and method of liposome preparation (8).

There are different advantages or disadvantages to each type of transfection that can be used with regard to the cells that are most difficult to be transfected. Liposome transfection can be said to be an easy-to-optimize technique and not too toxic to cells, although the efficiency varies greatly depending on the used agent (7).

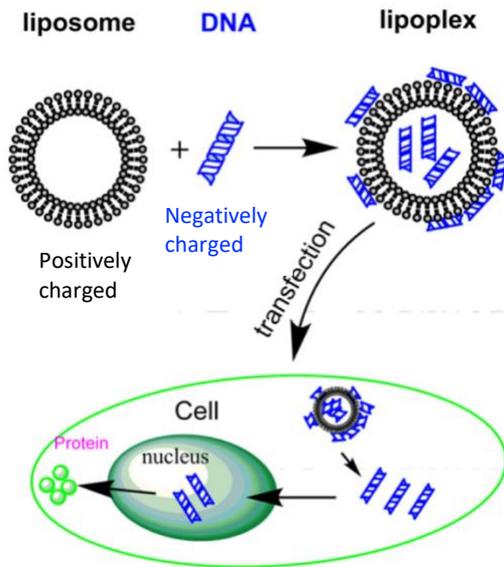


Figure 2. Transfecting mechanism of DNA molecules by liposomes (9).

1.5. DO-RG as a new transfecting agent

In a previous research assignment for *Treball Dirigit* subject entitled “DO-RG, a new transfection agent of DNA in neuronal cell”, the efficiency of DO-RG liposome was described.

DO-RG stands for dioleyl – RG (RG as an encrypted structure) and it consists of a di-cationic molecule (RG) with two oleic acids attached (Figure 3). DO-RG achieved transfection of neuronal cells efficiently without intrinsic toxicity and it meant it could be a possible transfecting vehicle for hard-to-transfect cells.

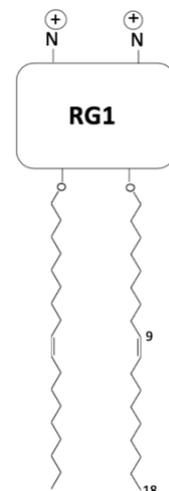


Figure 3. DO-RG molecule.

1.6. Gold nanoparticles

As described in the literature, gold nanoparticles (GNP) are useful in transfection with efficient transport and release of drugs and biomolecules into different cell types (10).

GNP have a lot of properties that make them excellent candidates for use in delivery applications. Some of them would be that the gold core is inert, non-toxic and biocompatible because it seldom reacts with other elements; the ease of fabrication taking into account size; the great results of the combination with different biomolecules such as lipids or DNA, facilitating their integration into biological systems; and finally, its big surface allowing great loading of therapeutic materials (10).

It is also stated that the organic synthesis allows better control of the fabrication of the nanoparticles, however, for the biological applications, it is needed to obtain water-soluble GNPs in order to be re-suspended in physiological fluids.

Finally, the methodology which is going to be used in this project consists of a ligand which forms a double layer around the gold (Au) core so that the formed nanoparticles have positively charged heads Figure 4. Therefore, charged heads from the first layer stabilize the gold core while the charges from the second layer are facing outwards and making the particles soluble in water and ready to bind other negative charged compounds such as DNA (11).

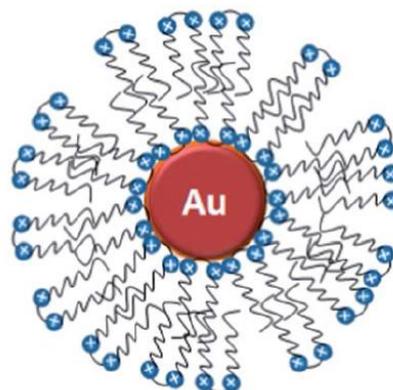


Figure 4. Schematic representation of GNP with a double layer around the gold nucleus core (water soluble).

1.7. Down syndrome disease and molecular process

Down Syndrome (DS) is a chromosomal condition (trisomy of chromosome 21) associated with intellectual impairment, a characteristic facial appearance, weak muscle tone (hypotonia), early onset of Alzheimer's disease, congenital heart disease and other development abnormalities (12,13).

The dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1A (DYRK1A) gene is located in chromosome 21 and has been directly related with DS development. The expression of this gene leads to the production of a serine/threonine protein related with some processes in cell proliferation and survival, neuronal differentiation, synaptic plasticity and neurodegeneration. Therefore, an overexpression of DYRK1A would lead to DS brain defects which makes this gene/protein a possible therapeutic target (13,14).

Recent published studies have already shown benefits treating cognitive deficiencies in mice models by inhibiting DYRK1A kinase. Some of the used molecules have been obtained from natural sources or by drug screening and medical chemistry approaches. Apart from that, some clinical trials have already been realized in young humans. All conclusions highlight that DYRK1A inhibition may well be a relevant target to improve cognition of patients with DS (13,15).

Since it is a genetic disease as well as cancer, presented before, we thought that PPRH technology could be applied as a treatment to DS and first tests are being presented in this assignment. PPRH different sequences against DYRK1A gene are shown in Table 2.

Gene	Shortening	PPRH sequence	Location
A. DYRK1A	HpDyrk-I3-T	GGGGAGGGGAAGGGAAGGGGAG T T T GGGGAGGGGAAGGGAAGGGGAG T T	Intron 3
B. DYRK1A	HpDyrk-Pr-T	AGAGGGGGAGACGGAAAGGAGGGAA T T T AGAGGGGGAGACGGAAAGGAGGGAA T T	Promoter
C. DYRK1A	HpDyrk-I3-C-Long	GGGAAGAAAGGAAATGGGAGGCAGGGAT T T T GGGAAGAAAGGAAATGGGAGGCAGGGAT T T	Intron 3
D. DYRK1A	HpDyrk-I3-C-Short	GGGATGGGGGGGACAAAAAGAAA T T T GGGATGGGGGGGACAAAAAGAAA T T	Intron 3

Table 2. DYRK1A PPRH sequences located either in the promoter region or in intron number 3.

In summary, PPRH have been described as efficient therapeutic technologies in genetic diseases and different PPRH vehicles and applications have already been tested.

2. OBJECTIVES

The main aim of the present work was to improve the transfection vehicles of hard-to-transfect cells using new liposome agents such as DO-RG or GNP.

Different strategies were followed to reach this objective:

- I. To test the efficiency of different transfecting agents as vehicles for PPRHs in different cellular lines.
 - i. Testing the DO-RG – HpsPr-C-F complex entrance, nucleus uptake and cytotoxicity in a cellular line of lymphoma of B cells.
 - ii. Testing the efficiency of newly synthesized gold nanoparticles in prostate cancer cells and in neuroblastoma cells.
- II. To apply the DO-RG properties of transfecting neuroblastoma cells as a possible therapy in Down Syndrome.

3. MATERIAL AND METHODS

3.1. Cell culture

Cellular lines of prostate cancer (PC-3 cells), neuroblastoma (SH-SY5Y cells) and B-cell lymphoma (WSU-FSCCL cells) were grown in Ham's F12 medium supplemented with 10% of fetal bovine serum and incubated at 37 °C in 5% of CO₂. Trypsinization of cancer cells was carried out to detach them using 0.05% Trypsin in PBS (phosphate buffered saline) 1X (154 mM NaCl, 3.88 mM NaH₂PO₄ and 6.1 mM NaHPO₄, pH 7.4). WSU-FSCCL cells grew on suspension.

3.2. Design of PPRHs

PPRHs were designed using the Triplex-Forming Oligonucleotide Target Sequence Search software. PPRHs were synthesized as non-modified desalted oligodeoxydenucleotides from Sygma-Aldrich and, once lyophilized, they were resuspended in sterile Tris-EDTA (ethylene diamine tetraacetic acid) buffer (1 mM EDTA and 10 mM Tris, pH 8.0) and frozen at -20 °C until their use.

A Watson-Crick hairpin (Hp-WC) against survivin was used as a negative control since it forms intramolecular Watson-Crick bonds instead of reverse Hoogsteen bonds. Therefore, the PPRH polypurine domain cannot bind to the polypyrimidine target sequence in the DNA. Specific PPRH sequences and the one for the negative control and their respective abbreviations are described in Table 1.

Moreover, specific PPRH sequences against DYRK1A gene are described in Table 2.

3.3. Transfection of PPRHs

Cells were plated in 6-well dishes. Transfection mixtures were prepared including 100 nM PPRH, 1.05-2.1 μM DO-RG / 1-2 μL GNP -Gold-DO-RG or Gold-ODIM- (different liposomes and different concentrations depending on the experiment) and completed with culture medium up to 200 μL. The mixture was incubated for 20 min at room temperature and finally, the PPRH-liposome complex already aggregated was added to the wells obtaining a final volume of 1 mL/well. Referring to the transfecting agent concentration and quantity units expressed in the results point:

- DO-RG molecular weight equate to 956 g/mol and, therefore, 1 μg is equal to 1.05 μM and 2 μg is equal to 2.10 μM when they are incubated in a volume of culture medium of 1 mL.
- Since we do not know the precise concentration of GNPs the quantity during the assignment will be expressed in μL.

3.4. Structures of the transfecting agents

DOTAP is the main liposome used during the last years in our lab. It has used to transfect most of the cancer cell lines with the exception of neuronal cancer cells and lymphoma cells. Because of this, and with a collaboration with the section of Organic Chemistry in our Faculty, different compounds were synthesized to allow the transfection of this hard-to-transfect cells. These different compounds shared a common structure with two positive charges instead of one, as DOTAP has, and we called it RG as an encrypted name (since a patent is expected to be filed). Different chains were included to this RG structure to achieve the different molecules tested in a previous assignment I wrote for the *Treball Dirigit* subject. The best molecule achieved resulted to be Dioleil-RG (DO-RG) presented in Figure 5.

Apart from that, the collaboration went on and gold nanoparticles (GNP) were synthesized since nanoparticles are known to facilitate the entrance of DNA to the cells. Throughout this assignment three gold nanoparticles are presented: Gold-DO-RG (Gold-dioleil-RG), Gold-ODIM (Gold-Protofano-LP) and Gold-ABIM (Gold-Azobenceno-LP); their schematic structures are also shown in Figure 5.

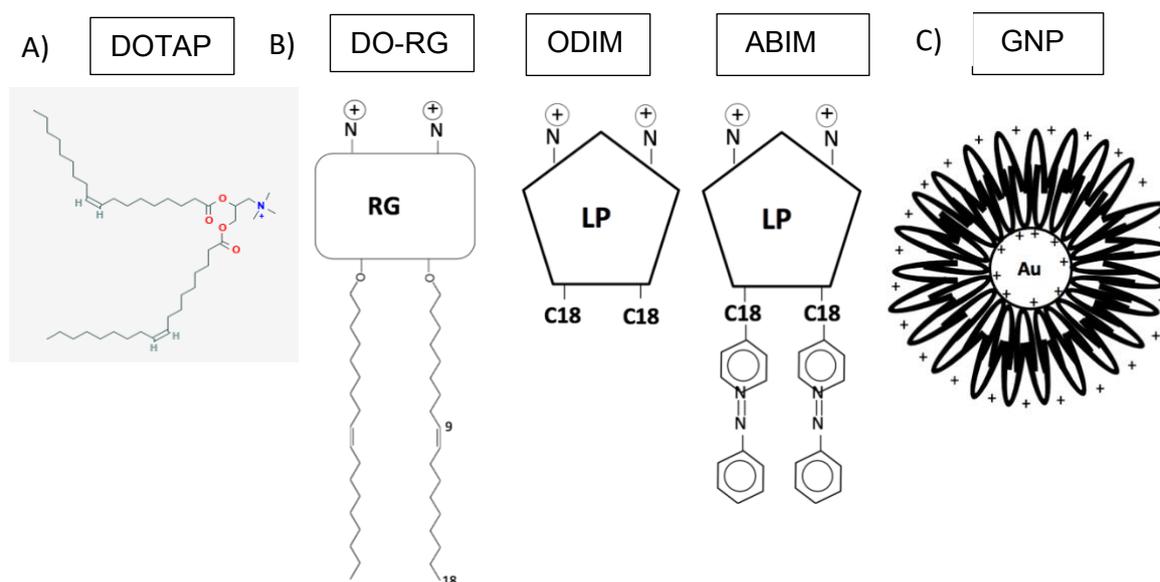


Figure 5. Representation of (A) DOTAP structure; (B) DO-RG, ODIM and ABIM structures and (C) liposome attachment to Gold nanoparticle with positive charges in both sides of the liposome molecule to bind to both gold and DNA.

DO-RG was synthesized by Rosa Griera, ODIM was synthesized by David Limón Magaña and all Gold nanoparticles were synthesized by the research group of Lluïsa Pérez García in The University of Nottingham (Akhil Jahin and Jordan Potts).

3.5. Nucleus extraction

Cells (90,000) were plated in 6-well dishes and transfected the day after. Twenty-four hours after transfection cells were collected in an Eppendorf®. Centrifugation was carried out (800 x g at 4 °C for 5 min) and cells were resuspended gradually in 1 mL hypotonic buffer (HB – NaCl 15 mM, KCl 60 mM, EDTA 0.5 mM, Tris-HCl 15 mM, pH 7.4) and let it settle 5 minutes in ice. Triton X-100 0.1% was added to break the cell membranes and afterwards centrifuged at 1,200 x g at 4 °C for 10 min. Finally, cells were resuspended in 300 µL of HB and observed in a fluorescence microscope with a transparent surface.

3.6. Cytotoxicity assays

Cells (10,000) were plated in 6-well dishes and transfected the day after. After 3 days 500 µg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-tetrazolium Bromide (MTT) and 100 µM of sodium succinate were added to the culture medium in each well. After 2.5 hours of incubation at 37 °C to allow the reaction, the medium was removed and a solubilization reagent (0.57% acetic acid and 10% sodium dodecyl sulfate in dimethyl sulfoxide (DMSO)) was added. Absorbance was measured at 570 nm using a Modulus Microplate luminometer. The results were expressed as the percentage of cell survival taking as a reference the control well where no transfection mixture was added, only culture medium.

3.7. RNA extraction

Cells (30,000) were plated in 6-well dishes and transfected the day after. Total RNA was extracted from SH-SY5Y cells 48 h after transfection using TRIzol reagent (guanidinium thiocyanate brand name). The manufacturer's specifications were followed to obtain a high-quality isolated RNA which was quantified by measuring its absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer.

3.8. Reverse transcription (RT-PCR)

Reverse transcription was performed to obtain cDNA molecules. A reaction mixture was prepared up to a final volume of 20 µL containing 1 µg of total RNA, 125 ng of random hexamers, 500 µM of each dNTP, 2 µL of 10X buffer, 20 units of RNAase inhibitor and 200 units of Moloney murine leukemia virus reverse transcriptase (M-MLV reverse transcriptase). The reaction was carried out at 42 °C for 1 h.

3.9. Real-time PCR

The StepOnePlus Real-Time PCR System was used to perform the quantitative PCR. The reaction mixtures were prepared with a final volume of 20 μ L containing 1X SYBR Universal PCR Master mix, 0.25 μ M of reverse and forward primers, 5 μ L of cDNA and H₂O mQ. The primer sequences to determine DYRK1A mRNA levels were 5'-GTTCTTGTGTCAGTTGGGGCAG-3' and 5'-TCCTGGTGGTTTGTACTCCC-3'; cyclophilin primers were used to normalize (or better: were used as an endogenous control) and their sequences were 5'-GGAGATGGCACAGGAGGAAA-3' and 5'-CGTAGTGCTTCAGTTTGAAGTTCTCA-3'.

The reaction was performed by QuantStudio 3 Real-Time PCR System and the PCR cycling conditions were 10 min denaturation at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The mRNA quantification was calculated using the $\Delta\Delta$ Ct method, where Ct is the threshold cycle that corresponds to the cycle where the amount of amplified mRNA reaches the threshold of fluorescence.

3.10. Gel electrophoresis

3.10.1. Agarose gel electrophoresis for binding experiments

On the one hand a gel was prepared at 0.8% agarose in TAE buffer 1X (40 mM Tris-acetate and 1 mM EDTA) up to a final volume of 33 mL. On the other hand, 140 ng of fluorescent PPRH (Hps-WC-F), different concentrations of the different liposomes and deionized water up to 10 μ L were mixed. After 20 minutes incubation at room temperature 2 μ L of loading buffer 6X were added and the 12 μ L samples were load into the gel. The gel ran at 10 V/cm and the marked PPRH with fluorescence was revealed in a UV lamp.

3.10.2. Denaturing acrylamide gel electrophoresis (7%/4%) for protein

Crystals and separators were assembled and sealed using 1% agarose. A 7% resolving solution mixture was prepared in an Erlenmeyer (7 mL acrylamide 30%, 7.5 mL resolving buffer Tris HCl 1.5 M, pH 8.8, 15.2 mL deionized water, a tip of spatula of persulfate, degasification, 300 μ L SDS (sodium dodecyl sulfate) 10% and 24 μ L TEMED 100% up to a final volume of 30 mL) and poured between the two crystals until polymerization. Next, a 4% stacking solution mixture was prepared in another Erlenmeyer (1.34 mL acrylamide 30%, 2.5 mL stacking buffer pH 6.8 Tris HCl 0.5 M, 6 mL deionized water, a tip of spatula of persulfate, degasification, 100 μ L SDS 10 % and 8 μ L TEMED 100 % up to a final volume of 10 mL) and poured between the two crystals until polymerization

including the comb to create the wells. TANK 1X buffer (Tris-Glycine SDS running buffer 1X) was used to carry out the electrophoresis.

3.11. Western blot analyses

Total protein extracts from SH-SY5Y cells (90,000) were obtained 72 h after transfection. Cells were washed once with PBS 1X and collected by scrapping in 150 μ L of lysis buffer (150 mM NaCl, 1 mM EDTA, pH 8.0, 1.0% Igepal CA-630 (NP-40), 0.5 % sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM PMSF, 10 mM NAF, Protease inhibitor cocktail and 50 mM Tris-HCl, pH 8.0). Centrifugation was carried out to remove cell remains (12,000 x g at 4°C for 10 min).

Whole cell extracts were resolved in 7%/4% SDS -polyacrylamide gels and transferred to PVDF membranes. The blocking solution was 5% Blotto. Membranes were incubated overnight at 4 °C with a primary antibody against DYRK-1A (1:100 dilution; sc-100376, Santa Cruz Biotechnology, Heidelberg, Germany). Detection was achieved by secondary HRP-conjugated anti-mouse antibody (1:2500 dilution; sc-516102, Santa Cruz Biotechnology, Heidelberg, Germany). Chemiluminescence was detected with Image Quant LAS 4000 mini technology.

3.12. Transmission Electron Microscopy method

Copper grids with a carbon coating were used. An incandescent discharge treatment with air was applied for 30 s at 4 milliamps in a CTA 005 Glow discharge.

5 mL of nanoparticle sample were placed on Parafilm and the copper grid was placed on the sample for 1 minute, followed by a drop of water for 1 minute and finally on the 2% uranyl acetate staining agent for 1 minute, excess staining agent was removed with filter paper and allowed to dry at 24 °C for at least 2 h.

Samples were observed in a Tecnai Spirit microscope (EM) (FEI, The Netherlands) equipped with a LaB6 cathode. Images were acquired at 120 kV and at room temperature with a 1376 x 1024-pixel Megaview CCD camera. Where samples were entered, the acentric height, magnifications and desired focus were adjusted.

This last method was performed by David Limón Magaña.

4. RESULTS

My research project has been divided into two different assignments, the first part has been delivered in the “*Treball Dirigit*” subject and the second part is going to be my degree final project “*Treball de Fi de Grau*”, presented here. Before going on with the development of this point, I will sum up the results obtained on the first part of the research project.

The research project started as a consequence that the most used liposome in our lab, DOTAP, did not have the capacity to transfect the known hard-to-transfect cells such as neuronal and lymphoma cells. Thanks to a collaboration with the section of Organic Chemistry in our Faculty of Pharmacy and Food Sciences, different new synthesized liposomes or cationic derivatives were tested and one of them, DO-RG (dioleoyl-RG, RG as an encrypted structure), resulted to be efficient in neuronal cells transfection showing a great apoptotic effect in combination with PPRHs against cancer-causing genes such as survivin and with absence of intrinsic toxicity. Some entrance and cytotoxicity experiments among others were carried out to support this hypothesis and it was concluded that DO-RG could beat the hard-to-transfect cells membrane and successfully transfect them.

Henceforth, some of this assignment aims were on the one hand, to investigate whether this DO-RG liposome worked in lymphoma cells since they are also hard-to-transfect cells; and on the other hand, to use the DO-RG liposome as the vehicle to carry the DYRK1A PPRH inside neuronal cells to investigate if it could have a therapeutic effect for down syndrome.

The results will be presented in two main parts. One part consists on the research of different liposome transfection methods. First, we tested the DO-RG in lymphoma cells since it had proved to work in other hard-to-transfect cells, such as neuroblastoma. Next, gold nanoparticles in combination with the previous liposomes were tested since they are known to increase the transfection efficiency.

The second part consists of an application DO-RG could have in the Down Syndrome treatment with specific PPRHs since this disease affects neuronal cells and DO-RG liposome has proved to transfect this type of cells.

4.1. Transfecting agents

4.1.1. DO-RG – *HpsPr-C-F* effect in WSU-FSCCL cells

Since DO-RG had already been effective transfecting neuroblastoma cells there was a possibility that it would work in B cell lymphoma cellular line (WSU-FSCCL). Both cellular lines are described as hard-to-transfect cells and did not show effect using DOTAP, the best transfecting agent used in the lab previously.

To start testing DO-RG as a transfecting agent in WSU-FSCCL cells, uptake and cytotoxicity experiments were carried out. In addition, nuclear extraction was performed to corroborate that PPRH was entering the nucleus. This experiment could be carried out because the PPRH used (against survivin gene, *HpsPr-C-F*) was marked with a fluorescent molecule so it could be observed in a fluorescence microscope.

As shown below in Figure 6, different DO-RG concentration treated cells were observed with a fluorescence microscope to know if the uptake was being successful and at which concentration.

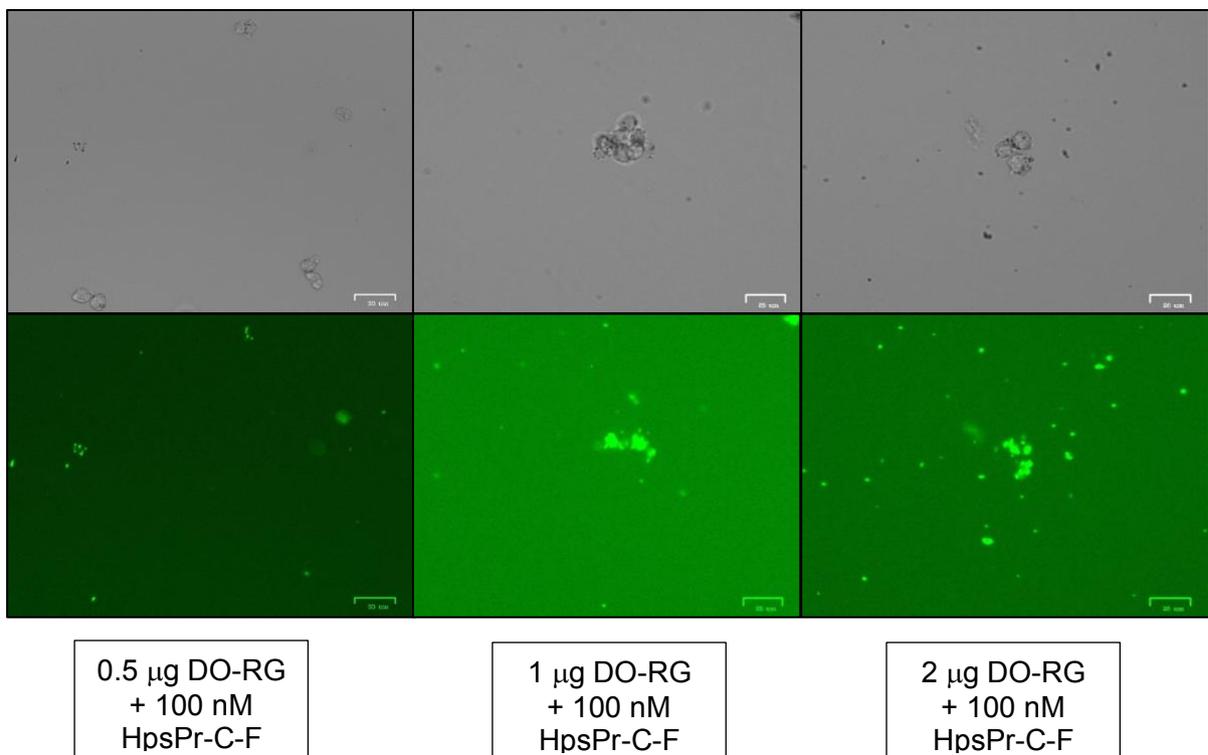


Figure 6. Fluorescent PPRH against survivin (*HpsPr-C-F*) entrance in WSU-FSCCL cells using different DO-RG liposome concentration.

Fluorescent cells were observed; therefore, we could make the hypothesis that the complex PPRH – DO-RG liposome was achieving transfection and that the PPRH would be able to produce its effect in the cell nucleus.

To go deeply, nucleus extraction experiments were performed to make sure the PPRH was achieving the nucleus. And, as shown in Figure 7, the results seemed to be satisfactory since nuclei were presented fluorescent which meant that the PPRHs were reaching their target location.

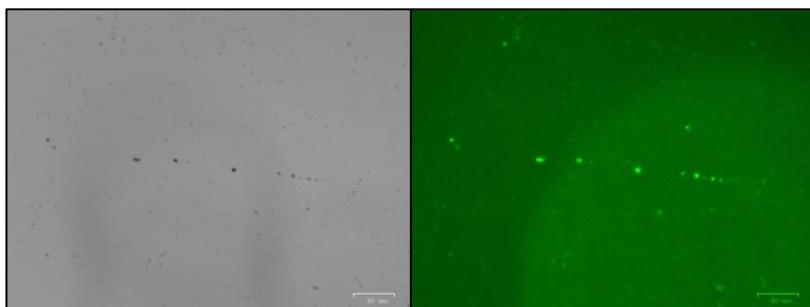


Figure 7. Fluorescent PPRH against survivin (*HpsPr-C-F*) entrance in WSU-FSCCL cell nucleus using 2 µg of DO-RG liposome.

Afterwards, cytotoxicity assays were carried out to observe and quantify the effect on the lymphoma cells viability. Different DO-RG concentration in combination with different PPRHs were tested and results are presented in Figure 8.

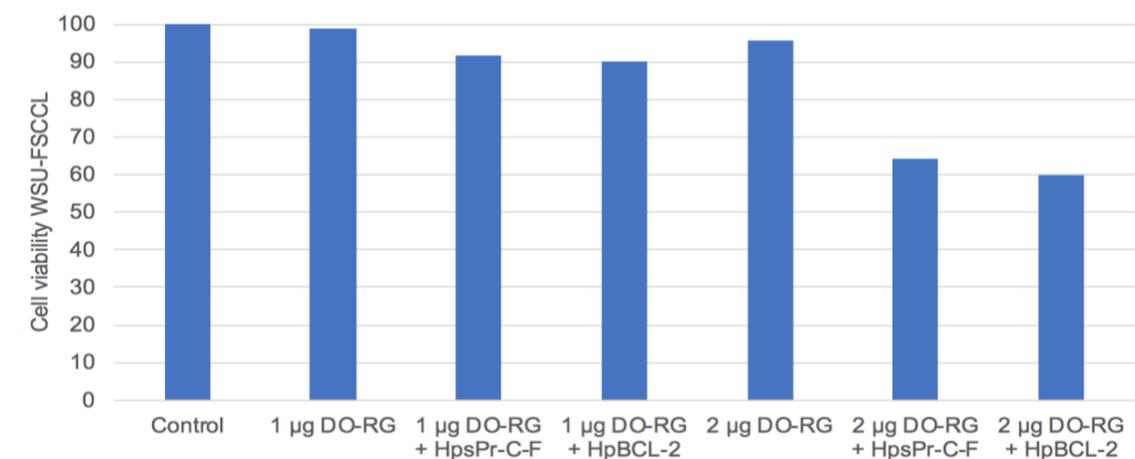


Figure 8. Cellular viability of WSU-FSCCL cells depending on the transfection mixture added. [*HpBCL-2* PPRH binds to a gene encoding for B cell lymphoma 2, a protein that regulates cell death (apoptosis). Like survivin, it is an antiapoptotic protein] (16).

Although uptake experiments seemed promising, when cytotoxicity tests were carried out not much effect was observed. There is a chance that the effect obtained using 2 µg

of DO-RG could be optimized. Also, it must be taken into account that this cell line is very sensitive and intrinsic toxicity of the liposome is easily reached. Experiments carried out using 3 μg of DO-RG to transfect showed high toxicity.

To proceed with this investigation, cell and nucleus fluorescence should be quantified in a flux cytometer to make sure the observed fluorescence is due to the PPRH entrance and not to other spread PPRH molecules which interfere in the microscope and are located outside of the cell or just attached to the membrane. Additionally, more cytotoxicity tests should be carried out to determine the most effective liposome concentration.

4.1.2. Gold nanoparticles

As explained in the introduction, gold nanoparticles (GNP) had been described in the literature as efficient molecules in the delivery with low inherent toxicity. Therefore, and once more in collaboration with the Organic Chemistry section of our Faculty it was decided to go a step forward and combine the DO-RG liposome, which already showed efficiency in transfection, with gold forming nanoparticles. Three new molecules were produced: Gold-DO-RG, Gold-ODIM and Gold-ABIM; and all of them were tested in the experimental part.

To begin with, as shown in Figure 9, binding assays were performed to test whether the different gold nanoparticles combined with lipidic cationic molecules had the capacity to bind the DNA or not.

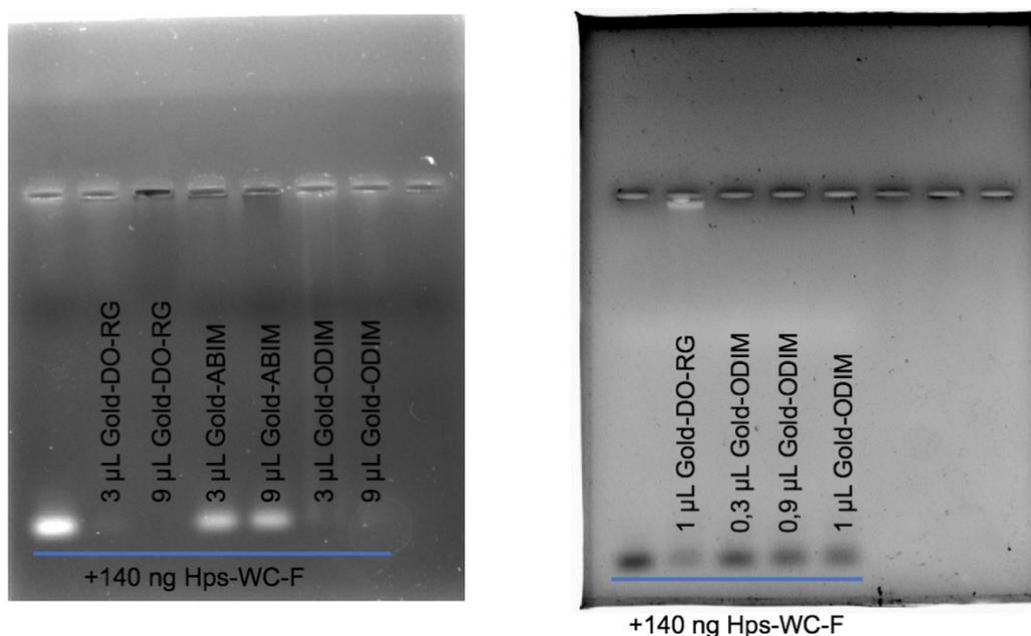


Figure 9. Agarose gels revealed after electrophoresis. The volume of the liposome used to form the complex with the PPRH is indicated in each well.

Both Gold-DO-RG and Gold-ODIM presented efficacy in binding the DNA while Gold-ABIM did not. Therefore, Gold-ABIM was no longer used. Furthermore, taking into account the revealed gels results it was decided that 1 μ L would be the appropriate volume to start testing these molecules in cell internalization assays.

Following on, prostate cancer cells (PC-3) and neuroblastoma cells (SH-SY5Y) were cultured in 6-well dishes and transfected using different liposomes – GNP combined molecules with the presence or absence of PPRH so the liposome-PPRH effect could be compared with the liposome intrinsic toxicity.

Before cytotoxicity assays were performed some pictures of transfection process were obtained with the fluorescence microscope. It can be observed in Figure 10-13 that the liposome-PPRH complex reaches the cells since the fluorescence is the result of a marker attached to the hairpin (PPRH) and therefore the uptake process is being successful in both cellular lines and using either Gold-DO-RG or Gold-ODIM.

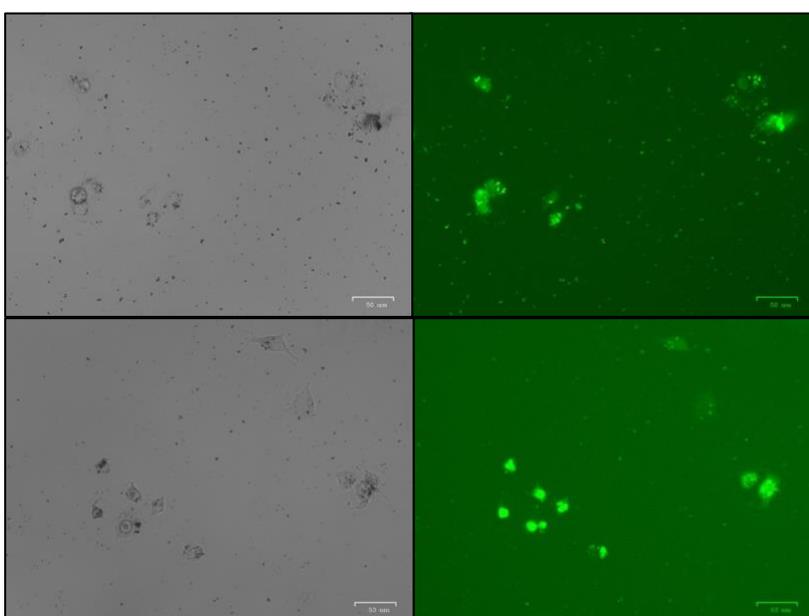


Figure 10. Fluorescent PPRH against survivin (*HpsPr-C-F*) entrance in **PC-3 cells** using **Gold-DO-RG liposome**.

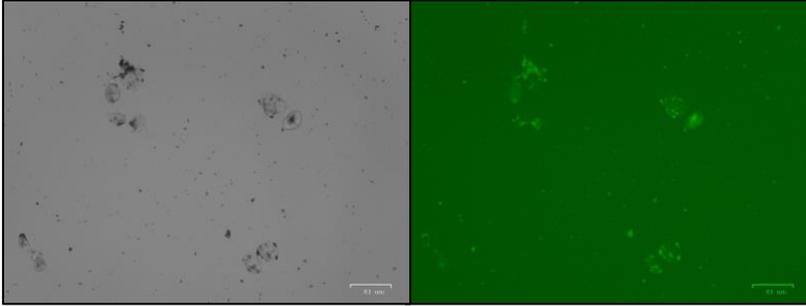


Figure 11. Fluorescent PPRH against survivin (HpsPr-C-F) entrance in **PC-3 cells** using **Gold-ODIM liposome**.

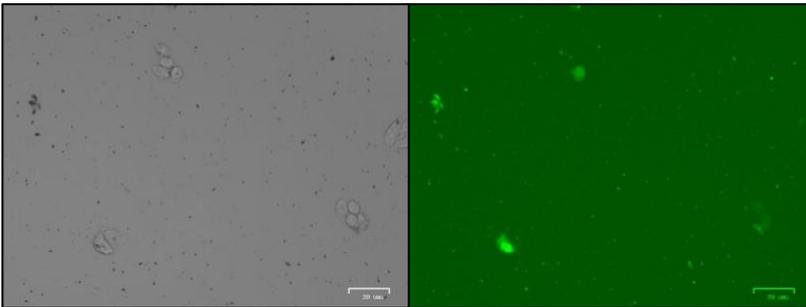


Figure 12. Fluorescent PPRH against survivin (HpsPr-C-F) entrance in **SH-SY5Y cells** using **Gold-DO-RG liposome**.

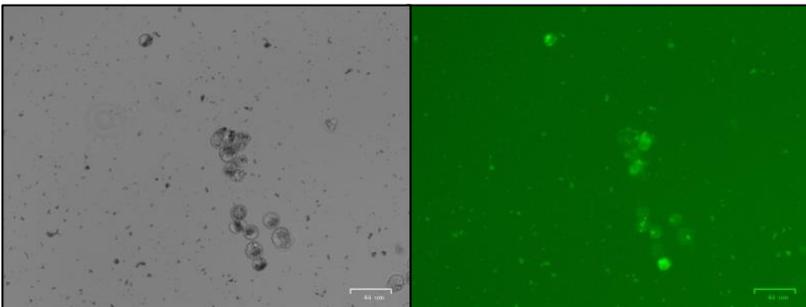


Figure 13. Fluorescent PPRH against survivin (HpsPr-C-F) entrance in **SH-SY5Y cells** using **Gold-ODIM liposome**.

Once fluorescence was observed, cytotoxicity assays with different cancer cellular lines were carried out to determine if GNP were efficient candidates as transfecting agents. GNP were both tested alone and in combination with the PPRH against survivin and results are presented in Figure 14.

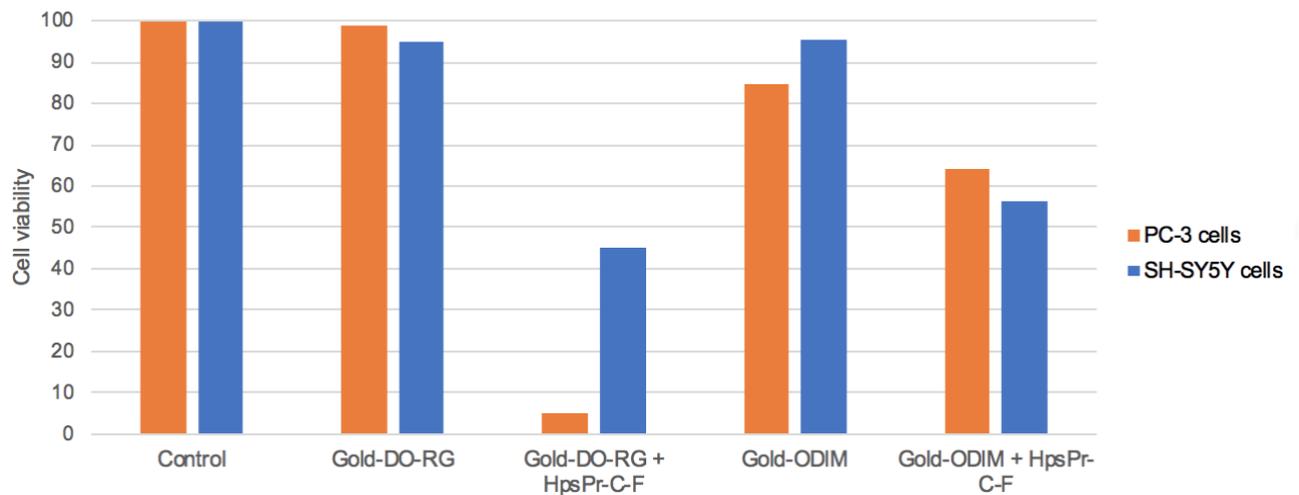


Figure 14. Cellular viability of PC-3 and SH-SY5Y cells depending on the transfection mixture added (Gold-DO-RG or Gold-ODIM + HpsPr-C-F). It was added 1 μ L of each liposome and 140 ng of PPRH (HpsPr-C-F).

Observing Figure 14 it can be stated that Gold-DO-RG has a high effect in PC-3 cells and also a considerable effect in SH-SY5Y cells with no intrinsic toxicity showed in either of these cell lines. As far as Gold-ODIM, it was observed that it has some effect in both cell lines, surprisingly better in SH-SY5Y cells, known as hard-to transfect cells, but it is not as good as the effect of Gold-DO-RG. Apart from that, Gold-ODIM did not show much intrinsic cytotoxicity either.

In addition to these results, Gold-DO-RG and Gold-ODIM were observed with a Transmission Electron Microscopy (TEM) and the photos of the different aggregates are presented in annex I. We can see how, in super concentrate conditions, PPRHs are conjugated with the liposome to form the complex. We can also observe how PPRHs and liposomes look like when they are alone in the sample.

4.2. DO-RG applied in Down Syndrome

As previously mentioned, the results of another assignment presented before clearly showed that the new molecule DO-RG had a great efficiency in transfecting neuroblastoma cells (SH-SY5Y). It was proved that the PPRH-liposome (DO-RG) complex could enter the cells by endocytosis and achieve the nucleus, the target location where the PPRH will have its effect.

The best transfecting agent used in the lab had been, until now, the DOTAP molecule that has the drawback of not lipofecting hard-to-transfect cells as neuronal or lymphoma cells. Therefore, the synthesis of DO-RG was a great hope for neuronal diseases as neuroblastoma or DS as it is going to be explained below.

Since overexpression of DYRK1A protein was related with DS main features and cognitive deficiencies, there was the possibility that PPRHs could decrease the expression of this protein. Therefore, given that the DYRK1A gene is not essential for the cell survival the aim was to reduce the DYRK1A mRNA and protein levels and avoid the disease symptoms while maintaining the neurone alive.

To investigate this possibility, neuroblastoma cells (SH-SY5Y) were cultured in 6-well dishes and treated with PPRHs directed against different polypyrimidine sequences found in the DYRK1A gene. PPRHs sequences are presented in Table 2.

Ideally, the experimentation should had been performed using a Down Syndrome cell line, that we did not have available. Instead, the research work was done with neuroblastoma cells and the results can be a guide for later on investigation in this disease treatment.

The experimental part started with some cytotoxicity tests, different PPRH against DYRK1A gene in combination with DO-RG were applied to the wells. As shown in Figure 15, cells did not die when they were treated and the lowest cell viability observed was more than 80%, therefore, RNA extraction experiments were interesting to carry out.

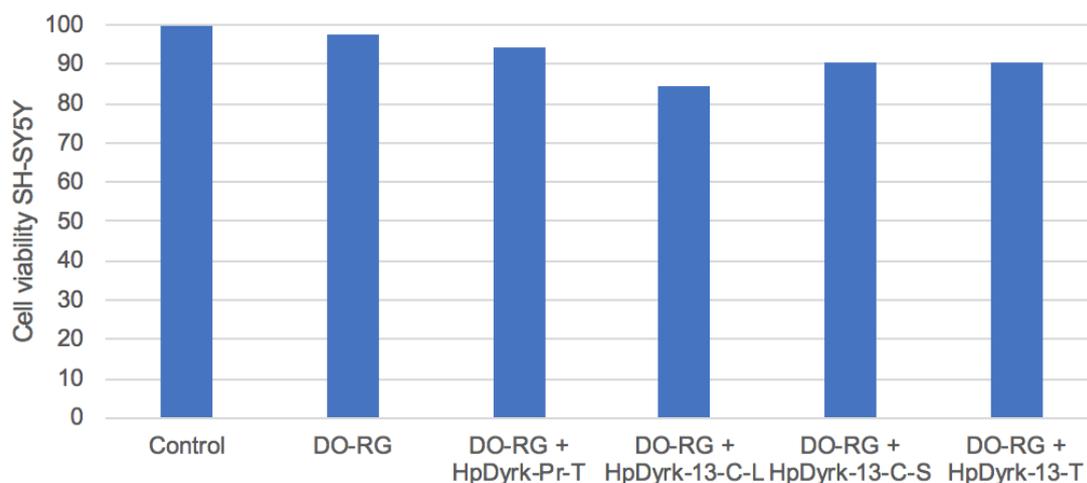


Figure 15. Cellular viability of SH-SY5Y cells depending on the transfection mixture added.

Following on, RNA extraction and specific quantification of DYRK1A mRNA levels were performed to observe the mRNA levels variation. Results are shown below in Figure 16.

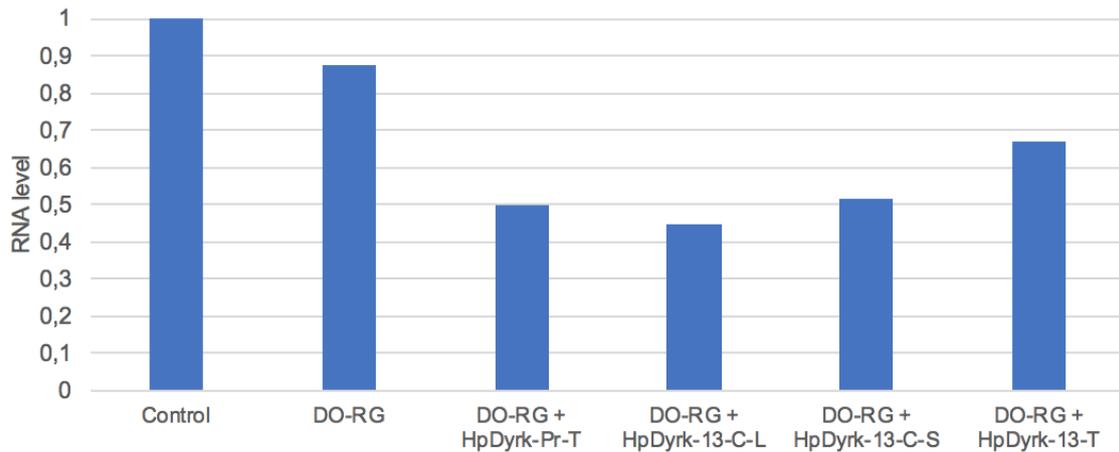


Figure 16. mRNA levels depending on the effect the PPRH against DYRK gene has achieved.

DYRK1A mRNA levels were decreased between 30 and 60 % depending on the PPRH used. The lowest levels were achieved with HpDyrk-Pr-T and HpDyrk-13-C-L. With these results and according to the papers referred to in the introduction it could be stated that PPRH gene therapy could be a possible treatment for DS. However, more experiments and optimization of the therapy should be investigated to support this hypothesis. A following step could be the quantification of protein levels to compare it with mRNA levels and see if there is a concordance between them.

According to this planning, a first western blot was made but the protein extraction was not satisfactory, and therefore, the results are not going to be presented. Despite this, the new primary antibody against DYRK1A protein was proved to work and showed good signal at 90 kDa level (Figure 17), which is in accordance with the molecular weight of the protein. Therefore, new experiments should be done to make progress in the project.

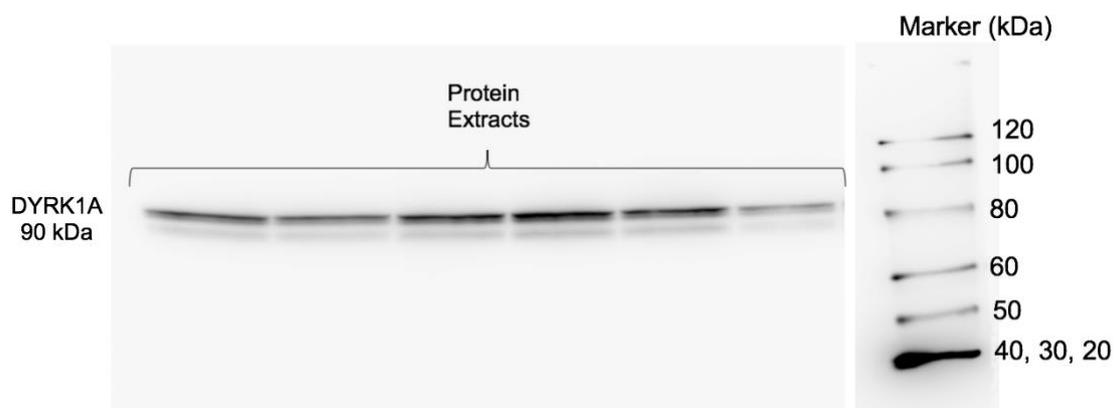


Figure 17. Protein levels detected in a first western blot carried out using an antibody against DYRK1A protein. Left columns are referred to different combinations of control and treated SH-SY5Y cells protein while the right column is the marker to know how the electrophoresis is going and where your protein is according to its size.

Results are not decisive since protein extraction did not perfectly work but we decided to go on with the western blot to test the antibody since it was the first time it was being used and we did not really know whether it would work or not and at which concentration.

5. DISCUSSION

The research experiments I have been conducting in the laboratory have a broad spectrum, with different long-term aims as it is exposed previously. First steps are being performed to make an approach of how these aims look like and it concludes opening doors for future projects.

As presented in the aims section, the development of the research consisted of two main parts. In the first one, related to transfecting agents, DO-RG capability was tested in other hard-to-transfect cells while new gold nanoparticles were tested as an innovation in the laboratory. The second one dealt with the application of DO-RG-PPRH as a possible Down Syndrome therapy.

First of all, lymphoma B cells were transfected using DO-RG, a new cationic liposome that had proved to efficiently work in neuronal cells, both hard-to-transfect cells. Transfection seems to have succeeded as fluorescent cells and nucleus were observed. However, cytotoxicity tests showed a maximum effect of 40%. More cytotoxicity tests should be carried out and try to optimize the best dose observed. Achieving transfection in hard-to-transfect cells is a really ambitious aim for scientist community and it would mean an interesting discovery.

Apart from going on testing DO-RG, new forecasts related to gold nanoparticles seemed that could work. Some meetings and analyses of the aim were done together with the scientists from the Organic Chemistry section of the Faculty, and as a result, three new cationic liposomes combined with gold were synthesized. As it was described in the literature, they could increase the efficacy in transfecting DNA or other molecules.

These three molecules started to be tested in our laboratory, in the Molecular Biology section, and some studies such as the PPRH-GNP entrance succeeded as we could see fluorescent cells in the microscope due to the fluorescence mark in the PPRH. Cytotoxicity tests showed effect as well, the combination of GNP with PPRH reduced cell viability levels being Gold-DO-RG the most efficient transfecting agent out of the three.

The results achieved seem to be very positive although more tests should be performed to make sure the level of efficiency when combined with the PPRH, excluding the intrinsic toxicity of GNP. Furthermore, these achievements should be compared to the effect

exerted by DO-RG-PPRH complexes to determine if GNP really increase the transfecting capacity of these liposomes.

The last aim consisted of applying the DO-RG capacity of transfecting neuronal cells to study if it could be a possible treatment for Down Syndrome patients. Since overexpression of DYRK1A protein was described in the literature as a main feature for cognitive disorders, we aimed at using PPRH to decrease the expression of these gene and therefore decrease the disease manifestations. Different PPRH against DYRK1A gene were designed and tested in neuroblastoma cells, since we could not obtain a DS cell line. Positive results were obtained from cytotoxicity tests since cells did not die with the DO-RG-PPRH treatment while RNA levels were observed to decrease in RNA extraction experiments. In a near future, further experiments as quantification of protein levels should be carried out to show more evidence and in this assignment the antibody which binds to DYRK1A protein already is proved to work.

As a global analysis, all the three points of the study have positive results and seem a possible field to explore. However, although different experiment replicas have been carried out more tests should be performed to validate them with statistical significance.

After drug discovery processes, further steps such as preclinical trials in animals should be carried out. Finally, clinical trials including all genders and ethnicities should be performed according to preclinical results.

6. CONCLUSIONS

In this study different experiments have been carried out to contribute to cancer and Down Syndrome gene therapy research in mammalian cells. Different transfecting strategies have been developed and it can be concluded that:

- DO-RG – HpsPr-C-F complex seems to achieve entrance and nucleus release in hard-to-transfect cells such as lymphoma B cells and neurons. The effect can be observed already with 2 µg of the liposome in cytotoxicity assays without presenting intrinsic toxicity.
- Two out of the three new Gold nanoparticles synthesised and tested proved to be efficient as transfecting agents (Gold-DO-RG more than Gold-ODIM) without intrinsic toxicity in combination with HpsPr-C-F.

As far as the application of DO-RG transfecting properties in neuronal cells in Down Syndrome:

- According to the experiments performed in neuroblastoma cells, DO-RG together with a PPRH against DYRK1A gene seem to reduce mRNA levels without cytotoxic effects in cells. If the protein levels were also reduced, this approach could very well be highly positive for DS therapy, as DYRK1A is responsible of a lot of cognitive deficiencies in the disease.

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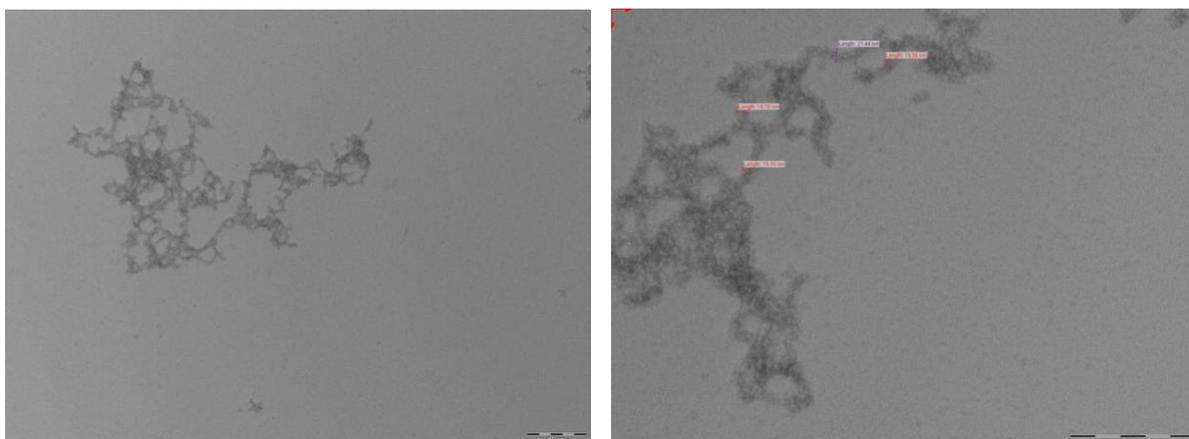
ANNEX I (TEM images)

Transmission Electronic Microscopy (TEM) images obtained in the Parc Científic de Barcelona in collaboration with David Limón from the Organic Chemistry section of the Faculty.

The photos show different samples including isolated PPRH and the different GNP alone and in combination with the PPRH. Furthermore, a more distant photo is presented on the left part while on the right part it can be appreciated that sample from closer.

PPRH

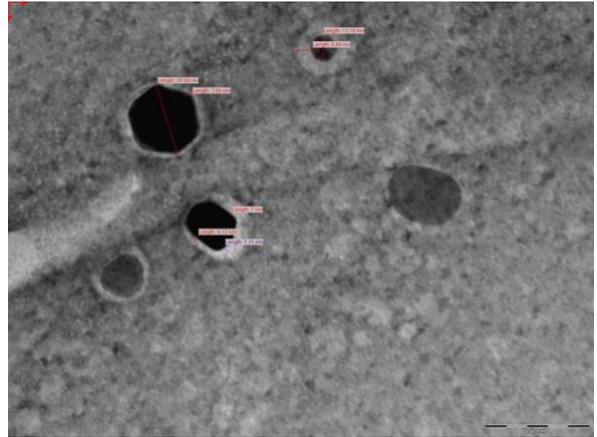
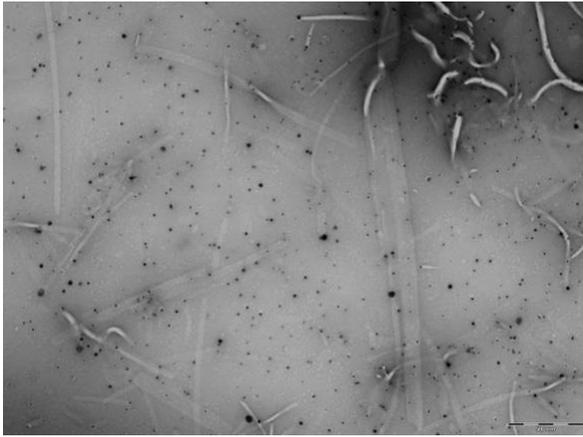
PPRHs were observed without nanoparticles as a control. The only kind of material that can be observed in both samples is curled fibres, which can be stained either negatively (dark surroundings, clear organic material) or positively (dark material).



Sample composition: PPRH 5 μ M in miliQ water.

GNP_ODIM

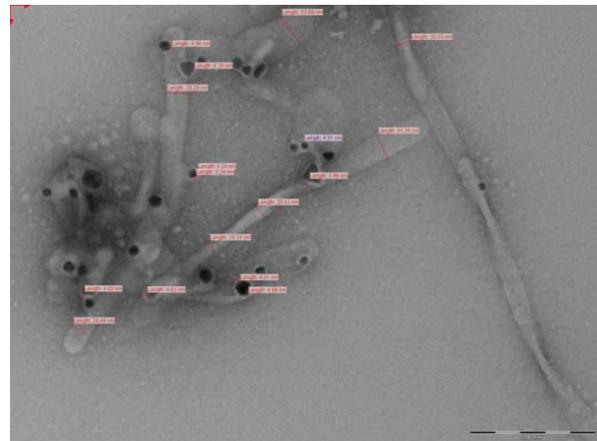
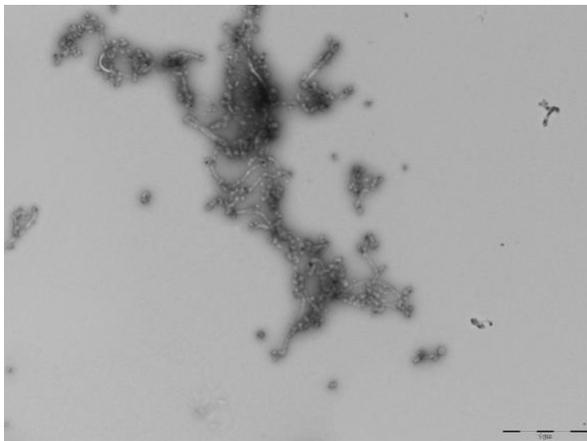
It is possible that the observed fibres are composed of ODIM, where ODIM self-assembles in presence of water to form them. Nanoparticles (dark points) vary in shape, observing circular, oval, triangular and polyhedral forms, and vary in size. Moreover, the organic coating thickness is in accordance with a bilayer model (light coating).



Sample composition: GNP.ODIM 1.3 nM in miliQ water.

GNP.ODIM – PPRH

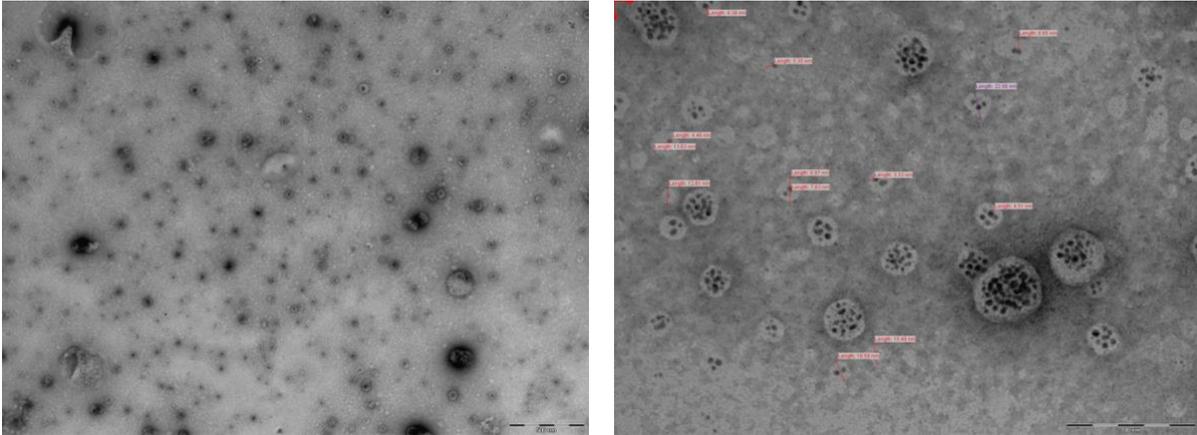
These observations suggest that the fibres in GNP.ODIM are composed of ODIM, and when the sample is in contact with the PPRH, both ODIM fibres and the nanoparticles (covered with ODIM as well) strongly interact with the PPRH, adopting the arrangement of PPRH. PPRH is difficult to observe in GNP.ODIM-PPRH, as probably ODIM fibres are covering it.



Sample composition: GNP.ODIM 1.3 nM, PPRH 5 μ M, Tris·HCl-EDTA (TE) 500 μ M in miliQ water.

GNP.DO-RG

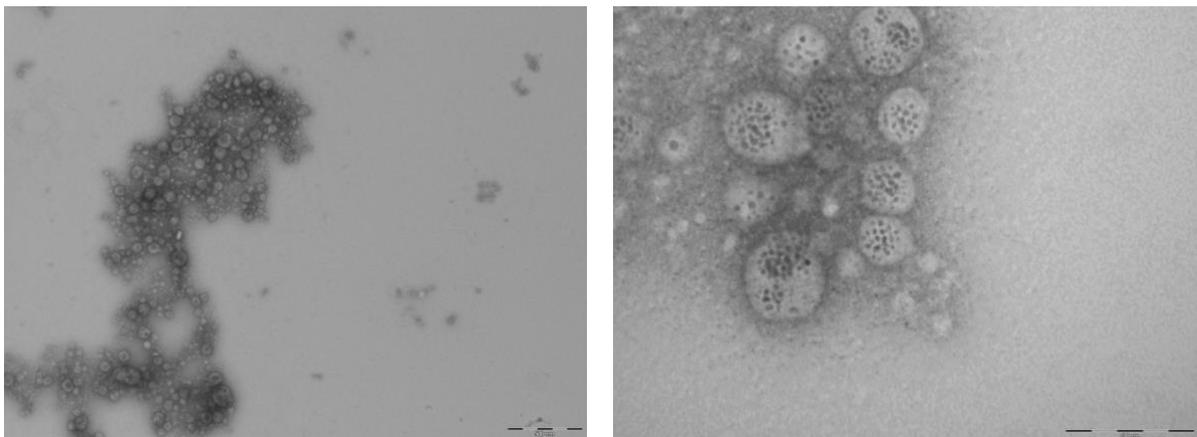
GNP.DOPI shows nanoparticles arranged in clusters (1 to more than 20 nanoparticles per cluster). Clusters of nanoparticles are formed by spheres of DO-RG containing the gold nanoparticles. These clusters of nanoparticles are not interacting with each other, as they are completely dispersed along the sample.



Sample composition: GNP.DO-RG 359 nM in miliQ water.

GNP.DO-RG – PPRH

Nanoparticles are grouped in clusters recovered of negatively stained material, which is most probably DO-RG. These clusters attach to PPRH, adopting its arrangement (curled/bended structure).



Sample composition: GNP.DO-RG 359 nM, PPRH 5 μ M, Tris·HCl-EDTA (TE) 500 μ M in miliQ water.