Scientific Report (02/05/2018 — 20/12/2018)

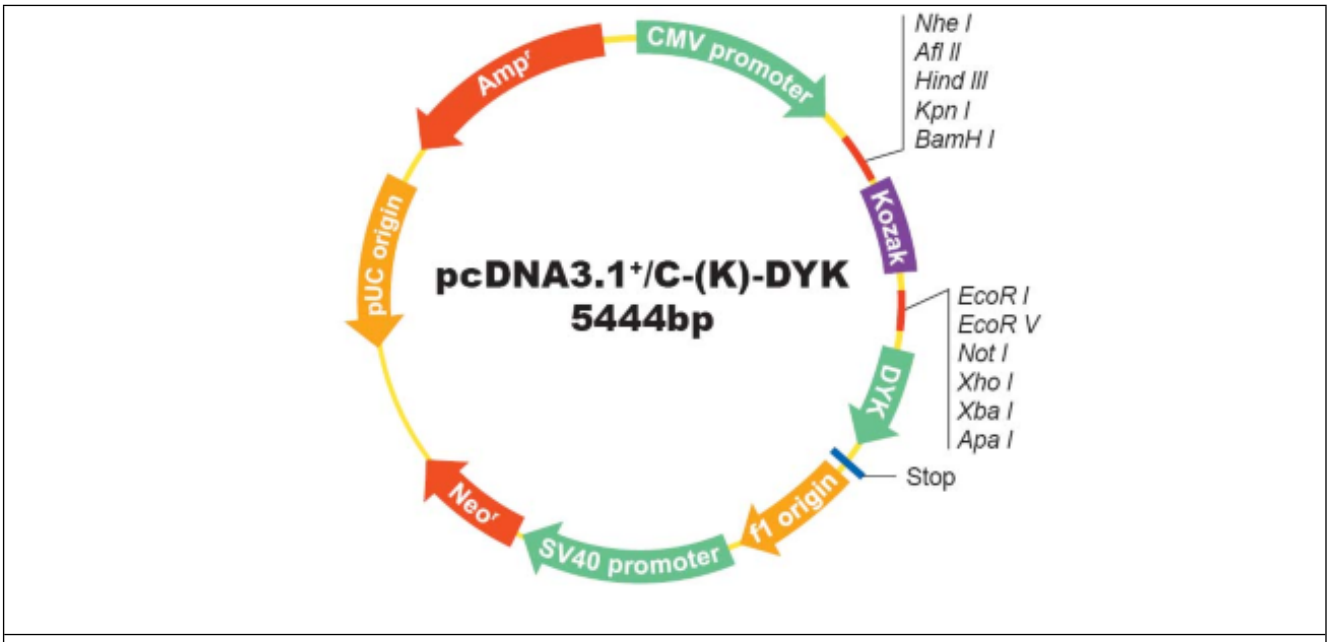
Project PN-III-P1-1.1-PD-2016-1942

"Evaluation of the therapeutic potential of non-viral apolipoprotein E gene transfer to limit progression of atherosclerosis"

Generation of non-viral transfection agents for expression of apoE3 or its fragments

1. Cloning of apoE3 sequence or its N-/C-terminal deletion fragments in a non-viral vector

The human apoE3 sequence and the N-/C-terminal regions were cloned into the non-viral vector pcDNA3.1+C-K-DYK. Driven by a CMV promoter, this expression vector for mammalian cells is equipped with a C-terminal DYK tag for easy protein detection.



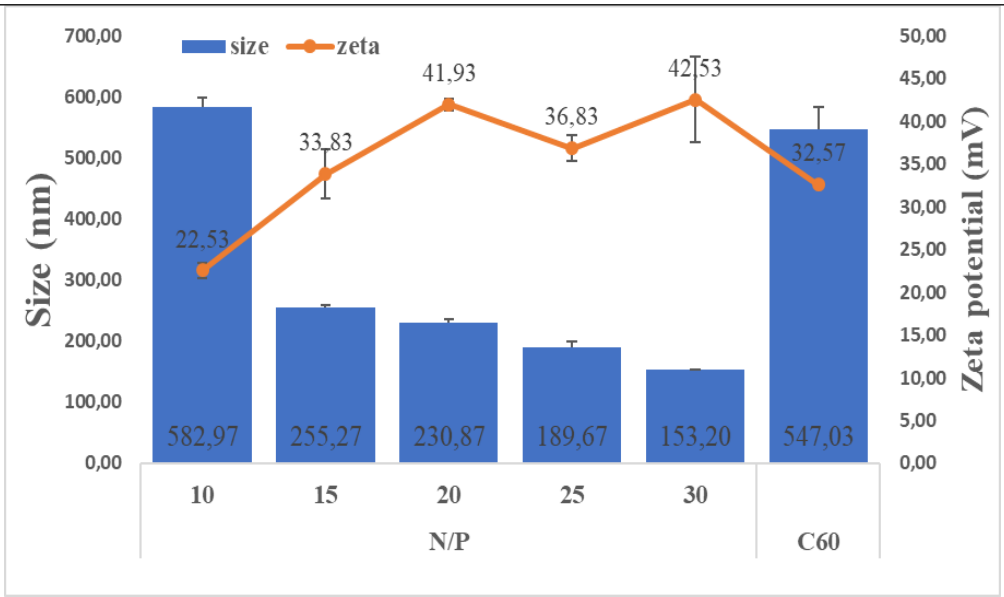
Schematic representation of the non-viral vector pcDNA3.1+C-K-DYK (5444 pb).

The entire apoE3 sequence was cloned into pcDNA3.1+C-K-DYK in EcoRI and XbaI sites. In addition, in the non-viral vector pcDNA3.1+C-K-DYK the following apoE fragments were cloned:

1. apoESSH1H2: containing the signal sequence and the first two helixes
2. apoESSH1H2RBR: containing the signal sequence, two helixes, the receptor binding region
3. apoESSH1H2RBRLBR: containing the signal sequence, two helixes, the receptor binding region and the lipid binding region
4. apoEwoRBR: containing the entire apoE sequence except for the receptor binding region
5. apoEwoLBR: containing the entire apoE sequence except for the lipid binding region

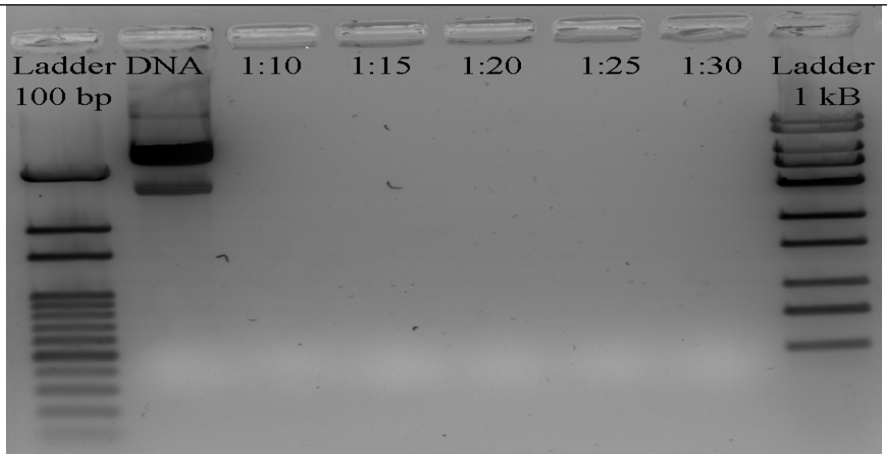
2. Coupling of the obtained plasmids to fullerene (C60) using polyethyleneimine (PEI)

To determine the optimal ratio of C60-PEI – DNA (non-viral vector) in order to obtain C60-PEI-DNA polyplexes, the C60-PEI conjugates were incubated with DNA at various N/P ratios (1:10, 1: 15, 1:20, 1:25 and 1:30), where N represents the nitrogen content of C60-PEI, and P represents the phosphorus content of the DNA. The size and zeta potential of the polyplexes were determined using a Nanosizer (Malvern). As indicated in the following figure, the C60-PEI conjugate exhibits a size of ~547 nm and a zeta potential of ~32.5 mV. In this case, the value of the hydrodynamic diameter is high and it can be attributed to expanding of the PEI chains, forming a physical network. C60-PEI-DNA polyplexes have sizes in the range 153-582 nm and zeta potential in the range of 22.5-42 mV. In the case of C60-PEI-DNA polyplexes with increasing N/P ratio, there is a decrease in size as well as an increase in zeta potential.



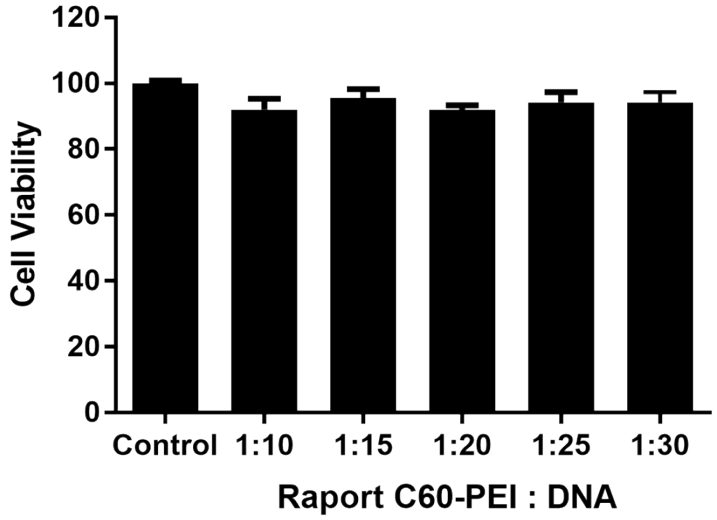
Size and zeta potential of C60-PEI-DNA polyplexes at the following N/P ratios: 1:10, 1:15, 1:20, 1:25, 1:30.

The ability of the C60-PEI conjugate to bind DNA was tested agarose gel retardation assays. The C60-PEI conjugates were incubated with DNA at various N/P ratios (1:10, 1:15, 1:20, 1:25, 1:30). The DNA concentration was maintained constant (1 μg/well) and the C60-PEI concentration increased according to the N/P ratio used (1:10, 1:15, 1:20, 1:25, 1:30). As shown in the figure below, the C60-PEI polyplexes have an excellent DNA binding capability since for all N/P ratios tested (including low N/P ratios, such as 1: 1 and 1:10) the DNA was complexed by C60-PEI. These results demonstrate that C60-PEI can be used in the generation of C60-PEI-DNA polyplexes at low N/P ratios for cell transfection purposes.



Testing the C60-PEI capacity to bind DNA by agarose gel retardation assays.

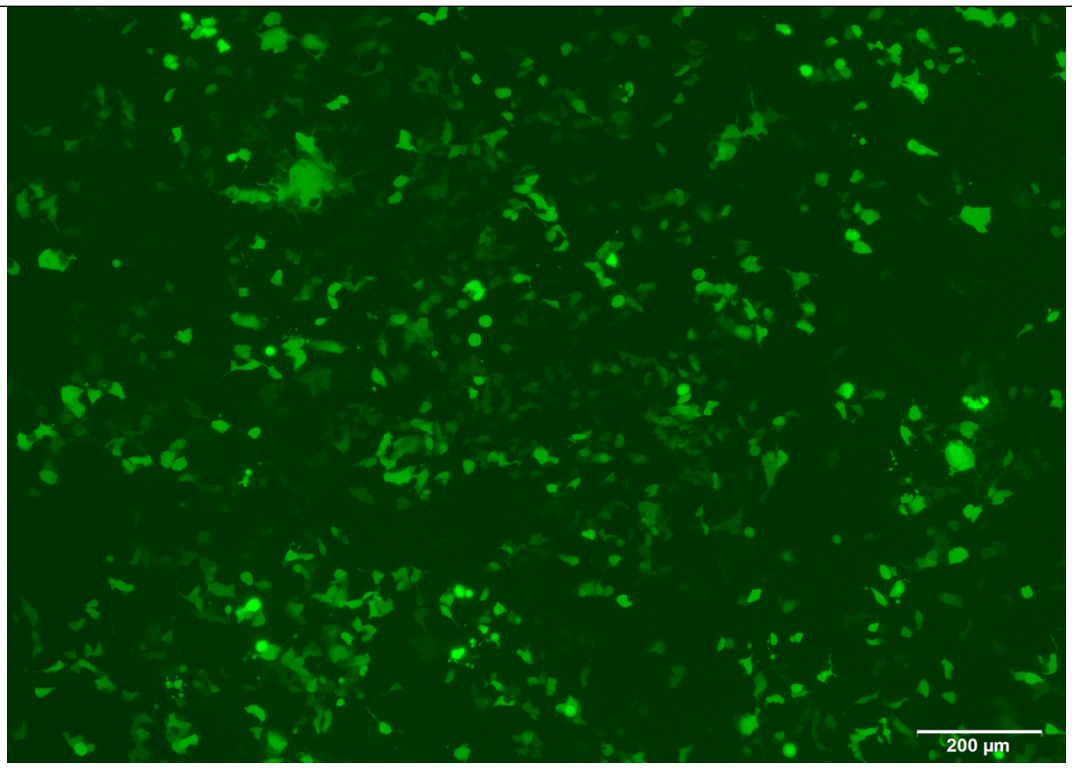
The viability of AD293 cells in the presence of C60-PEI-DNA polyplexes with various N/P ratios was determined by XTT technique. Cells were seeded in 96-well plates at a density of 10,000 cells/well in quadruplicate. One the day after seeding, C60-PEI-DNA polyplexes with various N/P ratios (1:10, 1:15, 1:20, 1:25 and 1:30) were added to the adherent cells. After 48 hours of incubation, cell viability in the presence of C60-PEI-DNA polyplexes was determined by XTT technique. As shown in the following figure, the C60-PEI-DNA polyplexes (irrespective of the N/P ratio used) have not significantly influenced cell viability, suggesting that C60-PEI-DNA polyplexes can be used in cell transfection processes.



The effect of C60PEI DNA polyplexes on cell viability.

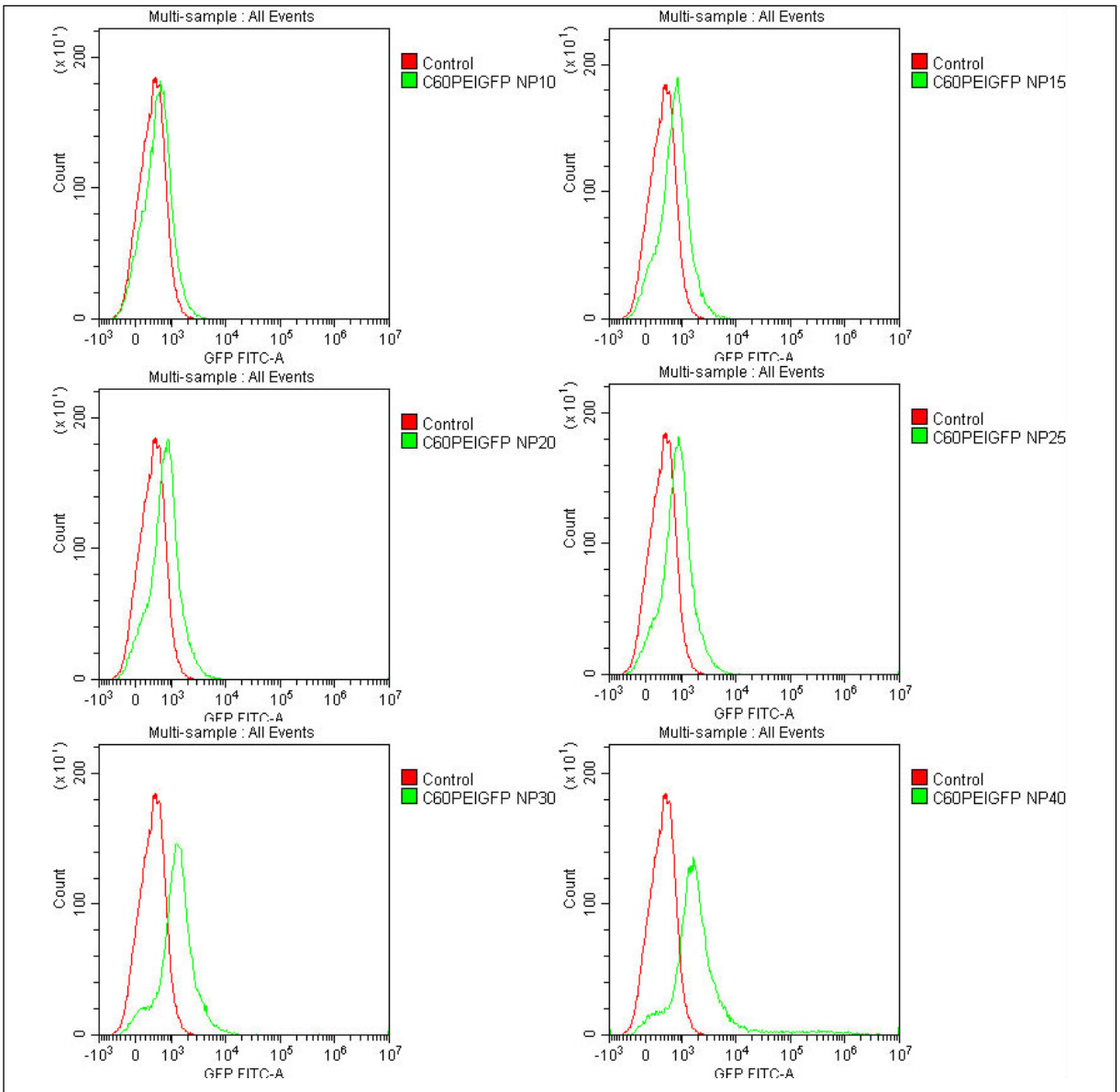
3. Testing the functionality of C60-apoE/GFP polyplexes as non-viral transfection agents

To test whether C60-PEI-DNA polyplexes can be used as transfection agents, the C60-PEI conjugates have been complexed with a specific DNA able to induce green fluorescence protein (GFP) expression that can be monitored by fluorescence microscopy. As illustrated in the following figure, AD293 cells were successfully transfected with C60-PEI-pGFP polyplexes.



AD293 cells transfected with C60-PEI-pGFP (this image was taken using a fluorescence microscope).

GFP expression in AD293 cells transfected with C60-PEI-pGFP polyplexes was also evaluated by flow cytometry. For this, AD293 cells were transfected with C60-PEI-pGFP polyplexes at various N/P ratios (1:15, 1:20, 1:25, 1:30, 1:40) and GFP expression was analyzed using a flow cytometer (Cytoflex). As shown in the figure below, GFP expression in cells transfected with C60-PEI-pGFP polyplexes increases as the N/P ratio increases.



Flow cytometry analysis of cells transfected with C60-PEI-pGFP polyplexes using increasing N/P ratios: 1:10, 1:15, 1:20, 1:25, 1:30, 1:40.

In conclusion, the results obtained this year in this project are summarized below:

1. The total sequence of apoE3 and five fragments of apoE were cloned into the non-viral vector pcDNA3.1+C-K-DYK
2. The obtained plasmids were coupled to fullerene (C60)-polyethyleneimine (PEI)
3. Functionality of C60-PEI-GFP polyplexes as non-viral transfected agents has been demonstrated *in vitro:* AD293 cells were successfully transfected with C60-PEI-pGFP (as shown by fluorescence microscopy, immunoblotting and flow cytometry experiments)

Dissemination of results:

1. Bisphenol A down-regulates hepatic apolipoprotein A1 expression, VG. Trusca, M. Dumitrescu, IM. Fenyo, IF. Tudorache, AV. Gafencu, poster presented at „ The 10th National Congress with International Participation and the 36th Annual Scientific Session of the Romanian Society of Cell Biology" Craiova, Romania, 6-9 June 2018
2. Bisphenol A down-regulates apolipoprotein A1 expression and exerts pro-atherogenic effects, VG. Trusca, M. Dumitrescu, IM. Fenyo, IF. Tudorache, AV. Gafencu, poster presented at "The 12th Central and Eastern European Proteomic Conference", Bucharest, Romania, 24 - 26 October, 2018

The results obtained in the project are part of a manuscript in preparation.