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Summary of the Dissertation of V. Sokolova

The title of my work was "Synthesis, Characterization and Application of Calcium Phosphate Nanoparticles for the Transfection of Cells". In this work chemical, biological and biochemical investigations were carried out. The main purpose was to find optimal conditions for the preparation of stable monodisperse calcium phosphate colloids and their applications in biological investigations on living cells.

Calcium phosphate nanoparticles were prepared by rapid mixing of aqueous solutions of calcium and phosphate, followed by addition of DNA to form the dispersion. This dispersion was used for cell transfection, i.e. for the non-viral introduction of DNA into living cells (Figure 1). This technique is very interesting in cell biology and the clinical medicine, because it is a tool for studying genetic regulation and protein function; it is also a medical technique that potentially allows the treatment of a wide variety of diseases of both genetic (gene therapy) and acquired origin. As many approaches for the introduction of DNA into living cells are of low efficiency and sometimes afflicted with substantial side effects, world-wide research is focused on the solutions of this problem.

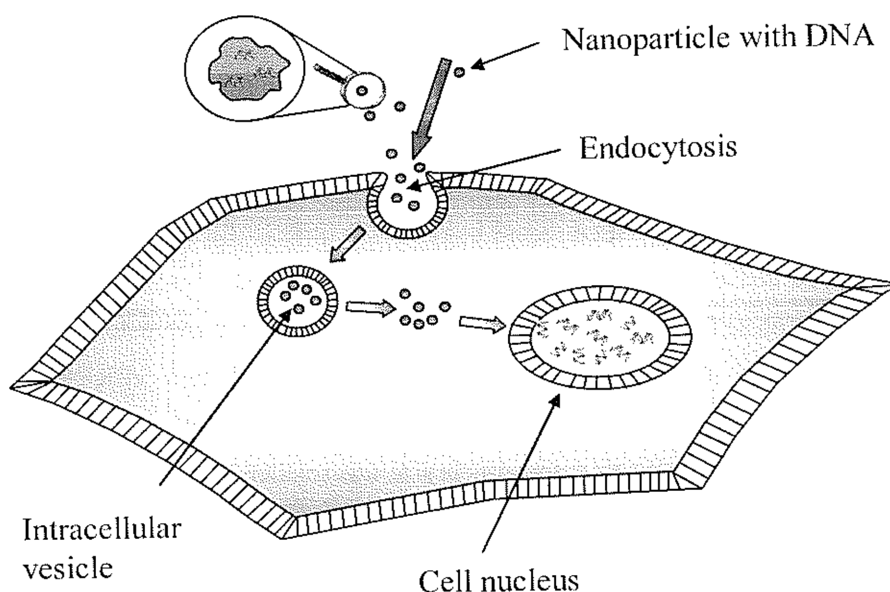

Figure 1: Schematic representation of the transfection mechanism.

Calcium phosphate nanoparticles are especially well-suited because an *in-situ* precipitation of the inorganic salt in the presence of DNA gives non-toxic and biodegradable nanoparticles which cells can immediately take up. The method is very easy and inexpensive, but the transfection efficiency is inferior to commercially available transfection agents which are based on (less biocompatible) liposomes and polymers.

The standard calcium phosphate method, introduced by Graham and van der Eb in 1973, strongly depends on the experimental parameters such as concentration, pH, precipitation time, type of DNA and also on the experimentalist. Consequently, it has to be optimised for every cell line and for every laboratory setting. The transfection solutions cannot be stored because the calcium phosphate nanocrystals grow with time into ineffective microcrystals.

We showed that calcium phosphate nanoparticles can be efficiently coated and colloidally stabilised by DNA and then used for cell transfection. The advantage of this preparation in comparison to the conventional *in-situ* precipitation method is a much better control over size and composition. The transfection efficiency could be easily quantified by fluorescence microscopy, where a plasmid DNA coding for *Enhanced Green Fluorescent Protein* (EGFP) was used. Two main directions were followed in this work: The synthesis and physico-chemical characterization of the colloids and the investigation of biological efficiency in the cell transfection.

At first, single-shell calcium phosphate nanoparticles with a size below 100 nm were prepared. In addition, a partial substitution of calcium by magnesium or aluminum was carried out because these ions have a well-known inhibitory effect on the crystallization of calcium phosphate and therefore could increase the ability of the nanoparticles to penetrate the cell. The cell experiments (T-HUVEC) showed that the transfection efficiency of the calcium phosphate/DNA nanoparticles was 3.5 %, i.e. comparable to the results of the standard calcium phosphate method (2.5 %). The transfection efficiency of calcium phosphate/DNA nanoparticles was not increased by the additions of magnesium or aluminum (2.1 % and 2.6 %), although the nanoparticle size was smaller (20-70 nm).

The reason of the low transfection efficiency is the degradation of DNA on the way to the nucleus by enzymes in the cells; therefore multi-shell calcium phosphate/DNA-nanoparticles were developed to protect DNA (Figure 2).

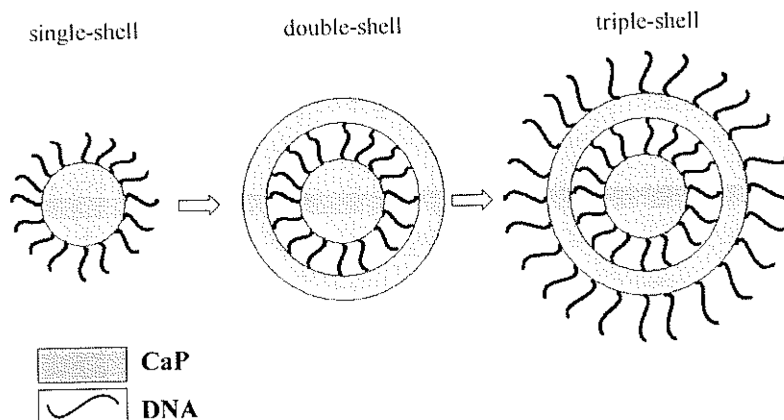

Figure 2: Schematic representation of the three types of calcium phosphate/DNA nanoparticles, developed to protect DNA.

We observed a continuous increase of the transfection efficiency when going from single-shell to triple-shell nanoparticles from 3.5 % up to 10 %. We achieved about 10 % of transfected cells with the commercial transfection agent Polyfect® (Figure 3). Such shells help to protect DNA from enzymes (nucleases) inside the cytoplasm in the cell, reaching the efficiency of Polyfect®. In principle, different layers can subsequently deliver different agents. Note that cationic polymers and dendrimers like Polyfect® are not permitted for in-vivo applications due to their high toxicity.

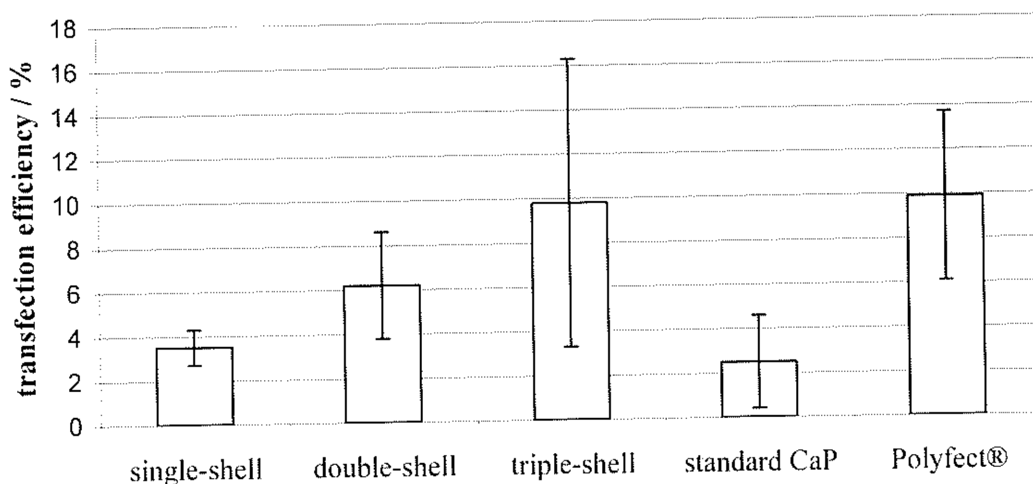

Figure 3: Comparison of the transfection efficiency of T-HUVEC in RPMI 1640 medium without FCS by different methods. The error bars represent the standard deviation (*N*=3).

Calcium phosphates should be advantageous due to their high biocompatibility and good biodegradability compared to other types of nanoparticles used for cell transfection such as iron oxide (magnetite), silica or gold. In contrast to the classical calcium phosphate method, the particle/DNA dispersions can be stored for weeks without loss of their transfection efficiency. It was shown by the high transfection efficiency that the DNA remained intact in the multi-shell particles. Protamine, a protein which contains nuclear localization sequences, was used to improve the efficiency of DNA delivery into cells. Calcium phosphate/DNA/protamine nanoparticles had a higher transfection efficiency compared to calcium phosphate/DNA nanoparticles.

General questions remain regarding the way of nanoparticles into the cell and their route within the cell. To address this question, we used the red-fluorescencing marker tetramethyl-rhodamine isothiocyanate (TRITC) bound to bovine serum albumine (BSA) as marker. Nanoparticles functionalized with pcDNA3-EGFP for transfection and TRITC-BSA for labelling were prepared (Figure 4). As control, we prepared calcium phosphate nanoparticles functionalized with TRITC-BSA only, and also nanoparticles (aggregates) of pcDNA3-EGFP and TRITC-BSA, i.e. without a calcium phosphate core.

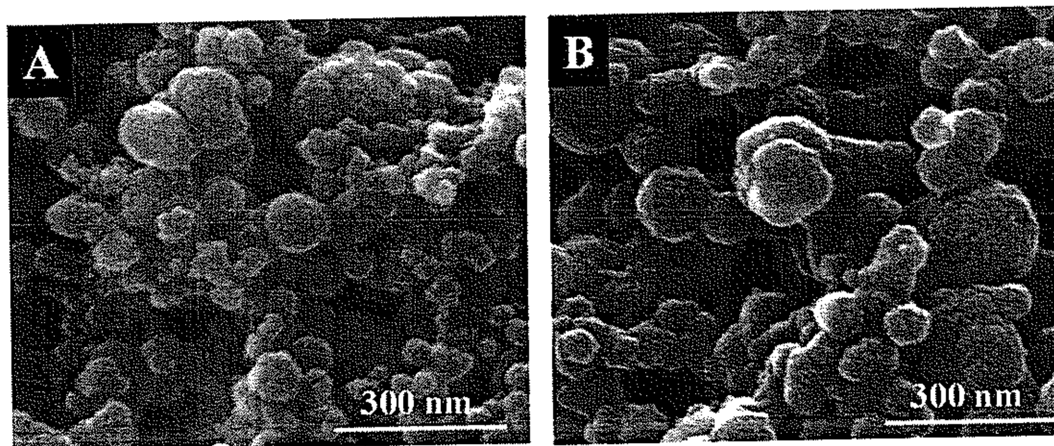

Figure 4: Scanning electron micrographs of CaP/BSA (A) and CaP/DNA/BSA nanoparticles (B).

By fluorescence microscopy and laser confocal microscopy we followed the way of the nanoparticles in contact with T-HUVEC cells. They adsorbed on cellular membrane after 2 h and could be found in the cytoplasm after 8 h where they accumulated near the nuclear membrane (Figure 5). The transfection efficiency correlated with the distribution of the nanoparticles in the cells. DNA/BSA aggregates alone were not able to penetrate the cell

membrane, i.e. the inorganic nanoparticles (calcium phosphate) are necessary as carriers to enter the cell.

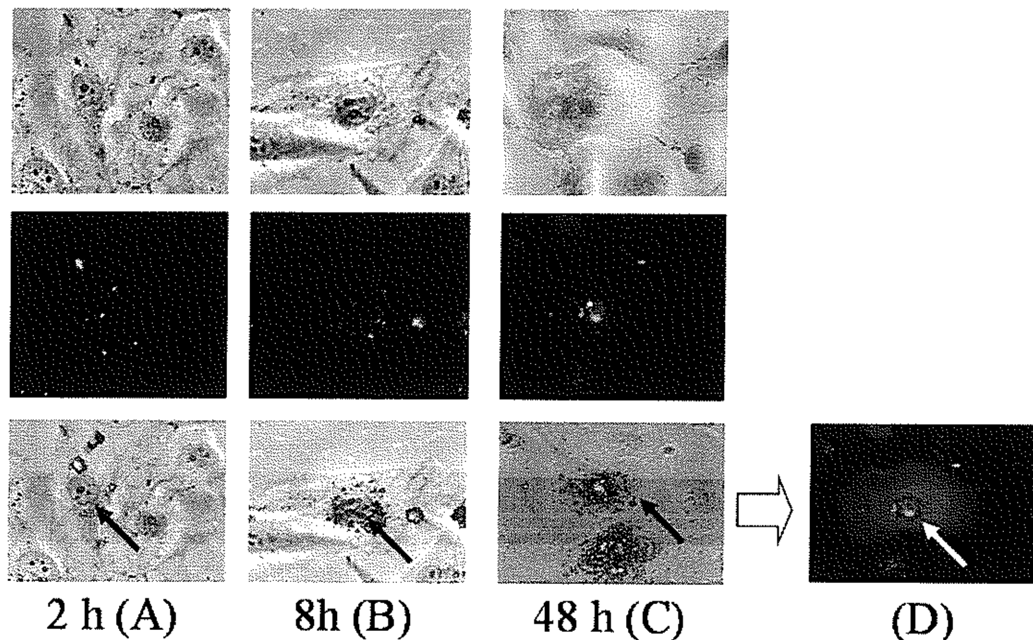

Figure 5: Transmission light microscopy (top row), fluorescence microscopy (center row) and overlay of both pictures (bottom row) of T-HUVEC transfection experiments. In light microscopy, all cells and their nuclei are visible. In the central row, the CaP/DNA/BSA nanoparticles appear as bright red dots. Arrows indicate binding of nanoparticles to the cell surface after 2 h (A), penetration into the cytoplasm after 8 h (B), and accumulation on the nuclear membrane after 48 h (C). After 48 hours, the transfected cells appear green with incorporated red-fluorescing nanoparticles (D).

Another part of this work was to develop a delivery system for gene silencing in which these nanoparticles are used for down-regulation of gene expression by targeting the mRNA resulting in effective gene silencing. Stable calcium phosphate/oligonucleotide dispersions were prepared and their successful biological application in vitro to HeLa-EGFP cells was shown.

We used single- and double-stranded oligonucleotides for the functionalization and characterization of calcium phosphate nanoparticles. To obtain a stable colloid, both the oligonucleotide concentration and the nature of oligonucleotides must be considered. Monodisperse colloids could be prepared with single-stranded oligonucleotides at a concentration of 9 μM and with double-stranded oligonucleotides at a concentration of 45

μM. The size of the stabilized nanoparticles is in the range of 100 nm, i.e. well-suited for cellular uptake.

The efficiency of gene silencing using single- and triple-shell was 27 and 48 %, respectively. This transfection efficiency was the same in both cases after two weeks of storage at 4 °C, indicating a high stability of the colloidal dispersions. siRNA alone (without calcium phosphate as a carrier) showed almost no gene silencing (4±22 %). Single-shell nanoparticles, the standard calcium phosphate method and Polyfect® did not show statistically significant differences in the transfection efficiency (Figure 6). In contrast, the triple-shell nanoparticles were much more efficient for transfection.

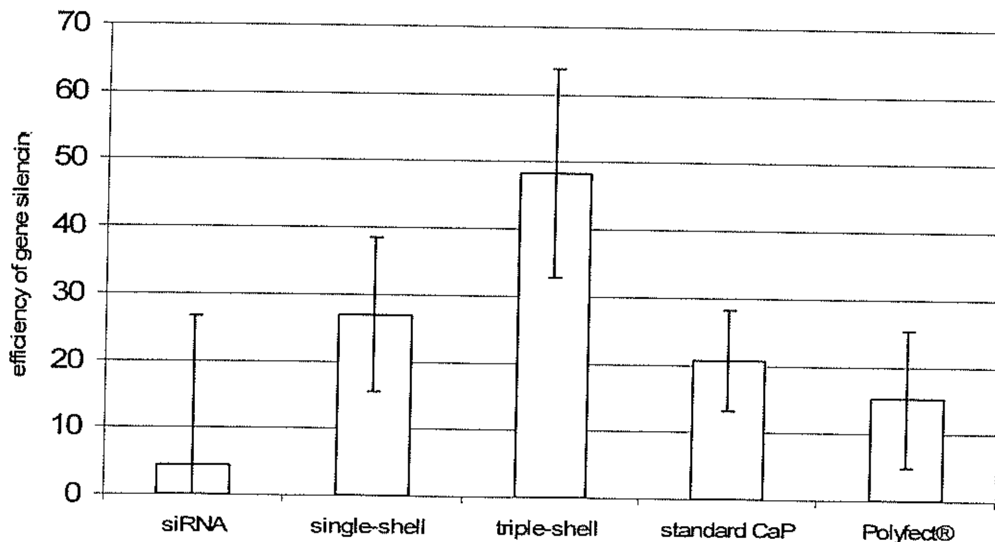

Figure 6: Relative EGFP gene silencing efficiency on HeLa-EGFP cells. The error bars represent the standard deviation. There were statistically significant differences between single-shell/triple-shell ($P<0.001$), triple-shell/standard calcium phosphate method ($P<0.001$), triple-shell/Polyfect® ($P<0.001$) and single-shell/siRNA ($P<0.01$).

We also performed the preparation of calcium phosphate samples with ubiquitin (as a model protein) by the co-precipitation method. It was shown that ubiquitin can be adsorbed as well as incorporated into the calcium phosphate particles. This method shows opportunities in the preparation of calcium phosphate ceramics with bioactive proteins, e.g. proteins of the BMP family. An implantation of such biofunctionalized calcium phosphate ceramics (e.g. cold-isostatically pressed into an appropriate shape) in bone defects should stimulate the development of new bone at the implantation site.