Knockdown of c-myc expression by RNAi inhibits MCF-7 breast tumor cells growth in vitro

INTERNSHIP

in the field of

BIOMEDICAL ENGINEERING

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ABSTRACT

Breast cancer is the leading cause of cancer death in women worldwide. Elevated expression of c-myc is a frequent genetic abnormality seen in this malignancy. For a better understanding of its role in maintaining the malignant phenotype, we used RNA interference (RNAi) directed against c-myc in our study. RNAi provides a new, reliable method to investigate gene function and has the potential for gene therapy. The aim of the study was to knock down the c-myc expression in MCF-7 breast tumor cells.

Nanodiamonds as vectors for in vitro gene delivery via surface-immobilization with 800 Da Polyethyleneimine (PEI800) were used to deliver and express short interfering RNA (siRNA) targeting c-myc to reduce its expression in MCF-7 cells to normal. Real Time PCR method was used to measure the protein level of c-myc.

ACKNOWLEDGEMENT

An internship in a foreign country has many different benefits. These benefits can be obvious in advance, some of them do not reveal until during the internship self and some will never disclose as obvious benefits mostly in terms of soft skills. Nowadays, international experience and in particular the ability to speak English is not a qualification anymore but a prerequisite. It is a tool especially in science to enable the international collaboration, the exchange of knowledge and to maintain international relations. Only a thorough knowledge of English allows to fulfill this need efficiently and to avail oneself of international conventions and conferences. Retrospective, these last three months were very instructive in many respects. Although an internship in a foreign country is almost required to assert oneself in working environment it is still a privilege to get the possibility and the (financial) support to experience such an instructive internship. Thus, I am much obliged and I want to express my gratitude in this acknowledgement.

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Without the appropriate financial support such an internship would be unimaginable or quite simply impossible. Thus, I am indebted to the Marshall Plan Scholarship, to my aunt Rosa Leithner and to my parents Elisabeth and Hubert Stadler.

Special thanks go to the entire Ho group. They always treated me like a friend and a member of their group. At this point I want to especially point out Han Bin Man. He always had a sympathetic ear and time for all kind of requests.

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INTRODUCTION AND REVIEW

INTRODUCTION

The purpose of gene therapy is to introduce foreign genetic material into host cells to either supplement aberrant genes or endow additional biological functions. To date, however, there has been only modest progress toward this goal, mainly due to the lack of safe, broadly applicable delivery methods. There are two main approaches to gene delivery: viral and nonviral. Clinical trials have highlighted the safety risks of gene therapy via viral vectors as cancer and death have resulted in some cases. In contrast, synthetic nonviral gene delivery systems, such as liposomes and polymers, offer several advantages including ease of production and reduced risk of cytotoxicity and immunogenicity, but their use has been challenged by relatively low transfection efficiencies. This problem mainly stems from the difficulty in controlling their properties at the nanoscale, prompting the search for new modalities with better transfection efficiencies. Lately, nanocarriers have received attention in the gene therapy community including such notable examples as polyethyleneimine conjugated gold nanoparticles and multifunctional nanorods. Recent studies on nanodiamonds (NDs) have revealed NDs as attractive platform nanocarriers due to their innate biocompatibility, scalability, precise particle distribution, high surface area-to-volume ratio, near-spherical aspect ratio, and easily adaptable carbon surface for bioagent attachment. NDs have been functionalized with a range of therapeutics, proteins, antibodies, DNA, polymers, and other assorted biological agents. Furthermore, NDs are stable and dispersible in water, making them a promising and clinically important modality in improving the efficacy of the treatment of diseases and even some cancers at the molecular level. Mitochondrial function (MTT) and luminescent ATP production assays have demonstrated that NDs are not toxic to a wide variety of cell types. Compared to other carbon-based nanomaterials, which have been shown to be toxic in many studies and are naturally not water-soluble, it is envisaged that NDs can serve as enhanced, versatile nanocarriers to deliver genes of interest in biological systems. [1]

PRINCIPLES AND FUNDAMENTALS

Gene Expression

Gene expression is the process by which genes produce proteins. It is a two-step process involving production of a messenger RNA (mRNA) through a process termed transcription. The mRNA thus created undergoes translation to produce a functional protein. Some genes may express certain non-coding RNA sequences.

In essence, gene expression controls occurrence of normal or abnormal bodily functions of a living being. Favorable expression of a gene (mRNA) may equip the individual with better survival capabilities. Insufficient or over expression however may cause disease.

The oncogene c-myc

The important oncogene c-myc is believed to participate in most aspects of cellular function, including replication, growth, metabolism, differentiation and apoptosis (natural cell death). A frequent genetic abnormality seen in breast cancer is the elevated expression of c-myc. The importance of c-myc expression in breast cancer is demonstrated both by studies of transgenic mice and by clinical research. Abnormal expression of c-myc transgenes in the mouse mammary gland is associated with an increased incidence of breast carcinomas. Moreover, clinical studies have indicated that c-myc is important in the development and progression of breast cancer. [2]

It has already been shown as well as in vitro as in vivo that the knock down of the c-myc gene can decrease the breast cancer cell growth. [2] Since the NDs show very good properties in gene therapy it suggests itself to use it for gene knock down.

Nanodiamonds

Nanodiamonds where discovered in 1963 when a brilliant Ukrainian physicist named Vladimir Danilenko found that soot from the explosion of well-known military explosive contained nanodiamond in high concentration. It took more than forty years until the diamond agglutinates were distintegrated into primary particles by stirred-media milling. This final product was called single-nano buckydiamond (SNBD). SNBD particles, having a remarkably small average size and

narrow distribution, 4.8 ± 0.7 nm, represent complex core-shell structure with thin graphitic surface layer(s) around core diamond. It is not a simple diamond crystal but a novel nanocarbon with mixed structure, very likely the most stable among nanocarbons of this size range. [3]

Small interfering RNA

RNA stands for ribonucleic acids. siRNA stands for small interfering RNA, which is also known as silencing RNA. dsRNA is a long, double-stranded nucleotide of RNA in molecules that perform various functions of biological systems in cells. siRNA transfection is the "transference", even sometimes known as infection, involved gene silencing experiments. In order to successfully optimize siRNA transfection, the correct transfection agent as well as method will enhance and increase efficiency and results.

Scientists have discovered that small interfering RNA (siRNA) may be extremely valuable in silencing gene expression, as well as studying gene functions in a multitude of cells. The success of such experiments often relies on the method of delivery of siRNA. siRNA can be temporarily transfected using transfection reagents. In many cases, cell types may make such transfection difficult, limited, or even impossible.

Determining adequate transfection parameters can result in success or failure of RNAi effects in various cell cultures. Conditions in parameters may include culture conditions, the type as well as the amount of transfection agent used, the amount of time transfection agents are exposed to cells, as well as the purity, quality and quantity of siRNA used in particular experiments.

Other considerations which enable siRNA transfection include, but are not limited to the health of the cultured cells conditions under which transfection occurs, the method of transfection that is introduced. Cells must be healthy in order to ensure maximum cell viability. Healthy cells are also easier to transfect than damaged or poor quality cells. Various methods that will help ensure the health as well as adequate number and quantity of cells offer guidelines to enhance success in various experiments. Also, cell density, as well as transfection reaction volume, and exposure time play an important role in the success or failure of siRNA transfection experiments.

Nanodiamond-based siRNA transfection reagents are an emerging trend to ensure high efficiency transfection systems. Transfection, the process of introducing foreign nucleic acids into host cells, is one of the most powerful Molecular Biology tools currently in use. To rationally design a therapeutic gene delivery vector for examine transfection, important lessons may be learned from examining the mechanisms of gene delivery via proven vectors such as PEI. PEI, a polycation introduced for transfection, is able to bind to DNA via electrostatic interactions, protect the DNA from degradation, and is hypothesized to disrupt endosomal intake through the proton sponge effect thereby enhancing DNA delivery efficiency. The transfection efficiency and toxicity of PEI correlate strongly with its molecular weight. For example, low molecular weight (LMW) PEI is some 2 orders of magnitude less efficient than its 25 kDa counterpart at the same concentration, presumably because of its inability to condense DNA effectively. Additionally, LMW PEI exhibits much lower cytotoxicity compared to its high molecular weight (HMW) analogues. Moreover, previous studies have shown that cross-linked LMW PEI could enhance gene transfer without compromising its low cytotoxicity. [1]

Gene silencing (RNAi)

Gene silencing, also termed RNA interference (RNAi), is the technique through which the expression of an otherwise "switched on" gene is suppressed. Though a gene may be silenced at transcriptional or post-transcriptional stages, RNAi is specifically post-transcriptional gene silencing mechanism achieved by introducing a double stranded RNA (dsRNA) and using siRNA or microRNA.

Gene silencing is thought of as an inherent process in the overall regulation of gene expression in many organisms giving them protection from viral infections and other undesirable genetic elements. Recent advances in gene silencing (RNAi) basic research lead to concept or RNAi therapeutics against many incurable diseases including cancer, hepatitis, viral infections and HIV by suppressing expression of the genes responsible for promoting these diseases.

Gene silencing takes effect by degrading the mRNA required for protein synthesis. In absence of necessary mRNA, the cell is rendered incapable of producing the protein thus suppressing expression of the protein producing gene. Mammalian cells however exhibit a strong antiviral

response if transfected with dsRNA larger than 30 base pairs (bp). Researchers circumvent this issue by introducing smaller dsRNA containing 21- 25 bp.

Gene silencing in mammalian cells is very complicated. It involves transfecting recipient cells with dsRNAs. In the host cell, the introduced dsRNA is fragmented into smaller double stranded RNA sequences each containing 21-25 nucleotides. These segments called small interfering RNA (siRNA) form a part of the silencing machinery used to suppress gene expression. The guide antisense strand of siRNA slices the complementary mRNA degrading it before its translation.

EXPERIMENTAL METHODS

CELL LINES AND CELL CULTURE

The breast cancer adenocarcinoma cell line MCF-7 was obtained from the American Type Culture Collection. The cells were grown in Eagle's MEM (modified eagle's medium) supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin. The MCF-7 cells were maintained in a humidified 37°C incubator with 5% CO2, fed every three days with complete medium, and subcultured when confluence was reached. To control the growth and healthiness of the cells they were daily observed under the microscope (Figure 1). Reduced serum transfection media was used for all positive control experiments.

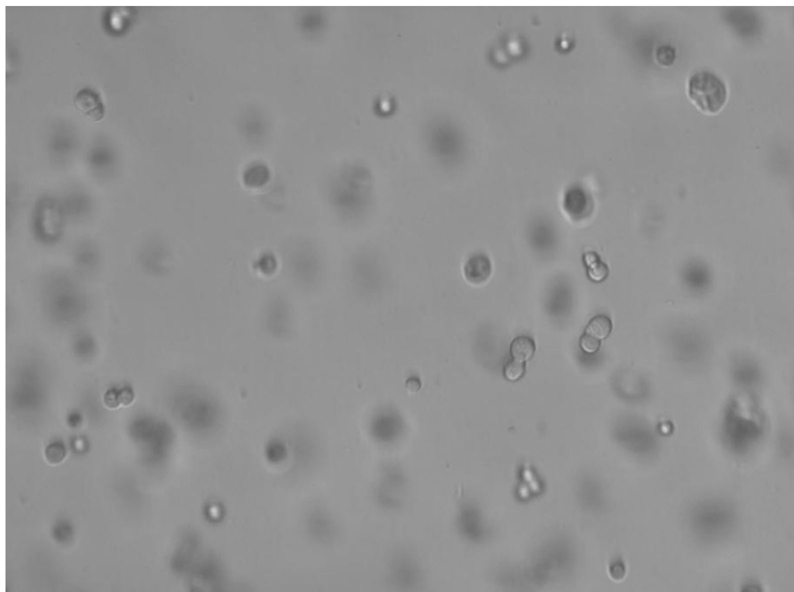


Figure 1: MCF-7 cells after subculturing

After about one to two weeks the cells were confluent (Figure 2) and we were able to plate them for the experiment. We only used cell lines with no more than 10 passages, although the MCF-7 cells are very resistant and can be split about 30 times (due to ATCC).

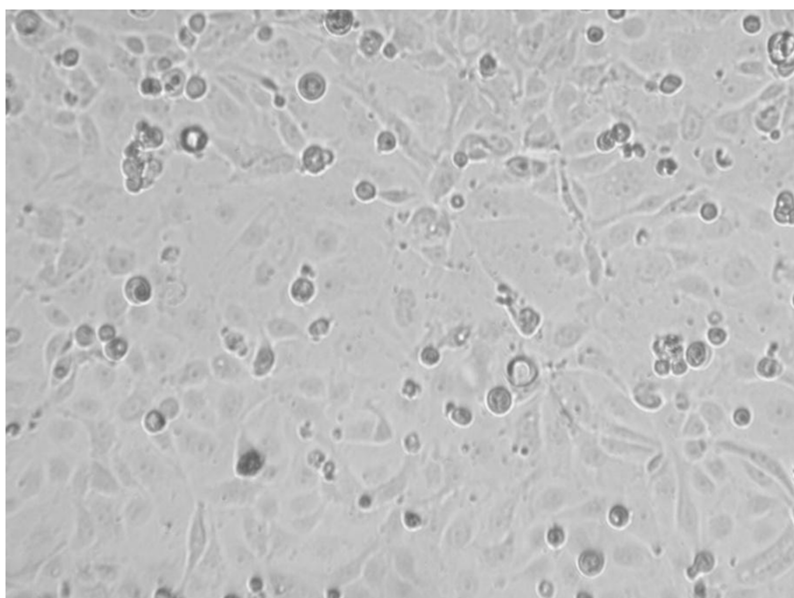


Figure 2: MCF-7 cells confluent and ready to plate

ND-PEI COMPLEX PREPARATION

ND gel (15% w/v in water) was obtained from the NanoCarbon Research Institute, Ltd., Japan. C-myc siRNA was obtained from Santa Cruz Biotechnology Inc.. All commercial reagents were used without purification. ND gel was diluted in pure water at the desired concentration and sonicated with a Branson 2510 sonicator for 2 hours. To prepare ND-PEI, the ND solution was mixed with 800 Da PEI (Sigma-Aldrich) at a 1:20 ratio of excess 800 Da PEI. Following one minute of vortexing, the sample was centrifuged for 2 hours at 14000 rpm to pellet ND-PEI complexes. The excess PEI was removed with the supernatant, and the pellet was resuspended in pure water. Three washes in total were performed to remove excess PEI. The final product was an optically clear solution. Prior to usage, siRNA was incubated with ND-PEI and added directly to the experiment within 10 min.

PARTICLE SIZE AND POTENTIAL MEASUREMENT

The preparation of ND-PEI siRNA complexes was accomplished by coating NDs with 800 Da PEI, and incubating with siRNA directly before transfection (Figure 3). Several washes were performed to remove excess PEI. While 800 Da PEI is a less efficient transfection reagent due to its low molecular weight (LMW), high molecular weight (HMW) PEI sacrifices biocompatibility for better transfection efficiency. Additionally, research has shown cross-linked LMW PEI increases its transfection efficiency. Our approach utilizes the carboxylated ND surface as a platform for LMW PEI. Therefore, we hypothesized this combination of materials would not only possess the innate biocompatibility inherent to both 800 Da PEI and ND, but also act as a platform for larger molecular weight complexes to improve transfection efficiency.

Essentially, the interactions are electrostatic owing to the opposing surface charge of NDs and PEI, and PEI and siRNA. These differences in charge are what require the combination of ND and PEI to reconcile and allow successful complexing between ND, PEI, and siRNA. Without PEI, siRNA

loading on NDs would not be significant. Additionally, without NDs, LMW PEI transfection efficiency remains poor. Therefore, we hypothesized that this combination of materials would not only possess the innate biocompatibility inherent to both 800 Da PEI and ND, but also act as a platform for larger molecular weight complexes to improve transfection efficiency.

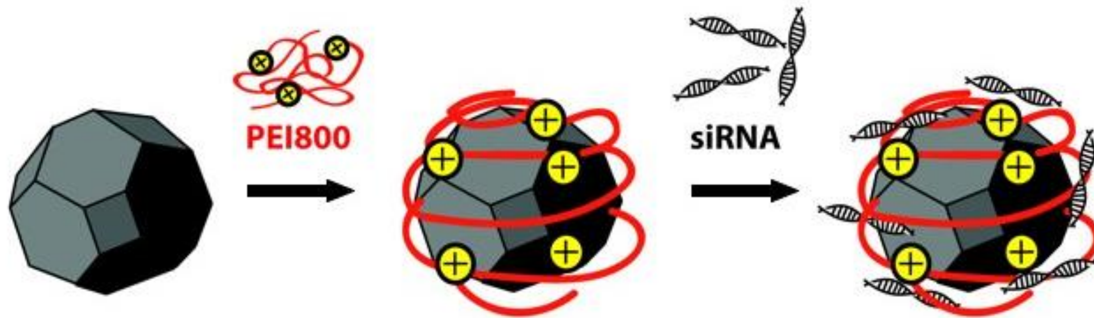
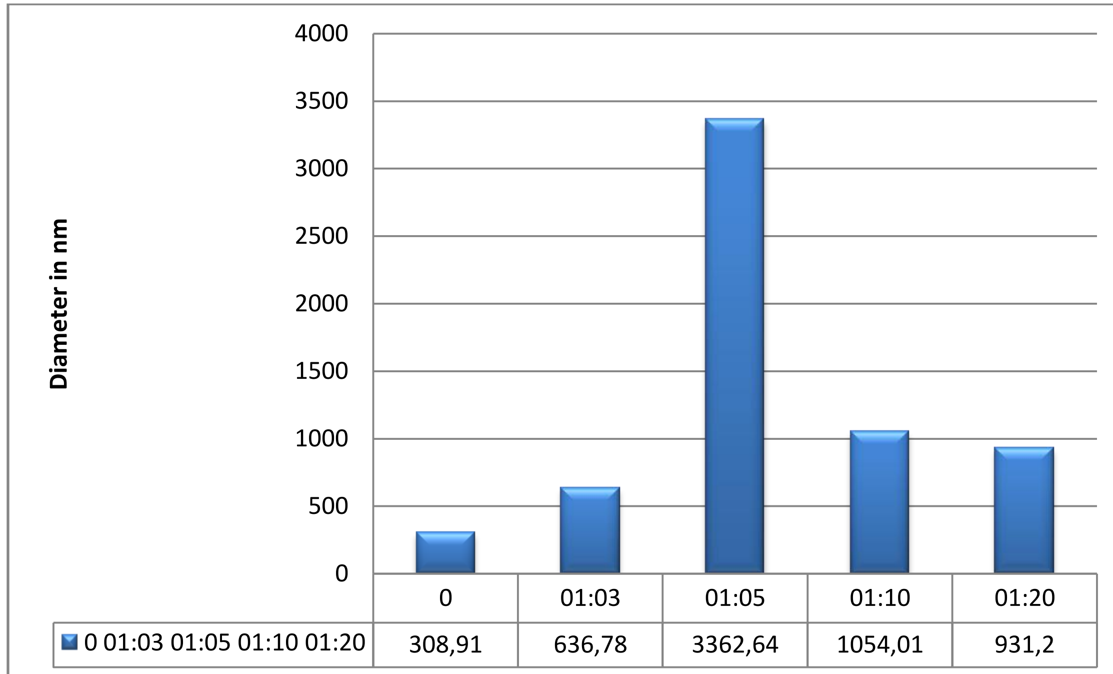


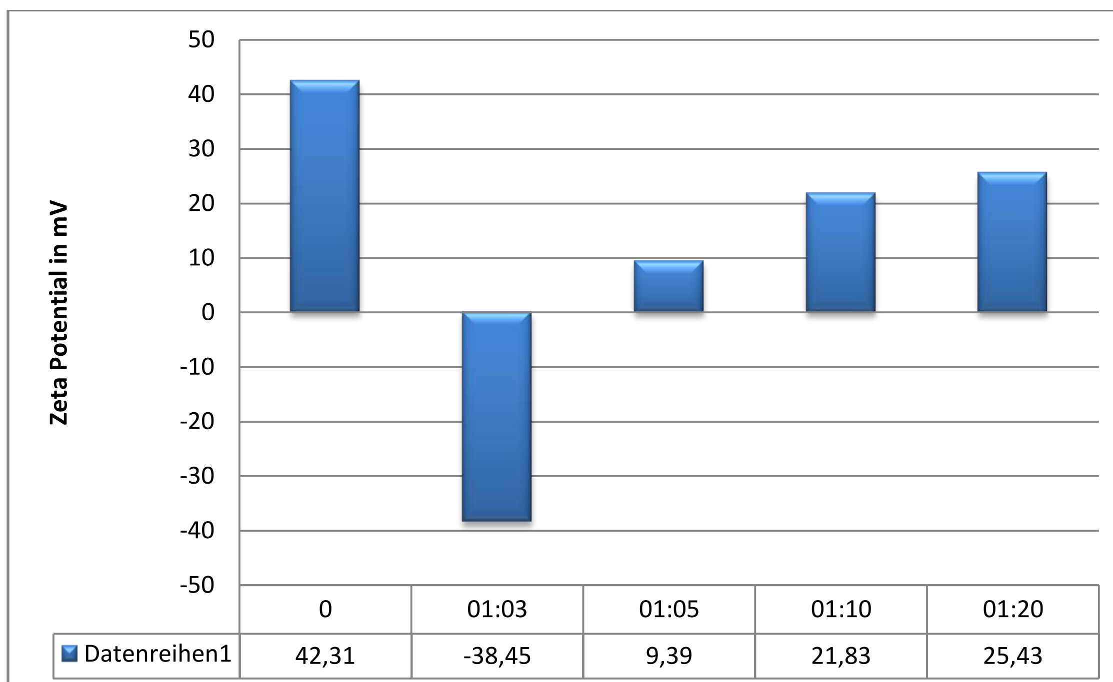
Figure 3: Illustration of Nanodiamonds coated by PEI, siRNA attached

The characterization of ND-PEI complexes was performed via dynamic light scattering (DLS). The ND-PEI complexes with siRNA were prepared in 1mL of pure water at various siRNA : ND-PEI weight ratios ranging from 0 to 1:20. siRNA and ND-PEI were allowed to complex for 10 minutes. The measurements were performed with a Zetasizer Nano ZS (Malvern). Size measurements were performed at 25 °C at a 173° scattering angle. Mean diameter was determined via cumulative analysis. ζ potential measurements were performed using folded capillary cells at 25 °C. The results are shown in Figure 4 and Figure 5.



	0	01:03	01:05	01:10	01:20
0 01:03 01:05 01:10 01:20	308,91	636,78	3362,64	1054,01	931,2

Figure 4: measurement of the diameter



	0	01:03	01:05	01:10	01:20
Datenreihen1	42,31	-38,45	9,39	21,83	25,43

Figure 5: Measurement of the zeta potential

Although the optimal binding ratio of siRNA : ND-PEI was achieved at 1:5, this does not demonstrate that it is the optimal ratio for transfection. Thus, we chose w/w ratios from 1:3 to 1:20 to determine the best loading for transfection. [4]

RETARDATION GEL

Gel electrophoresis was performed to confirm siRNA binding with ND-PEI, which revealed complete loading at a w/w ratio of 1:5 siRNA to ND (Figure 6). The binding gel was run as a standard experiment to determine the weight ratio where ND could load siRNA with no excess.

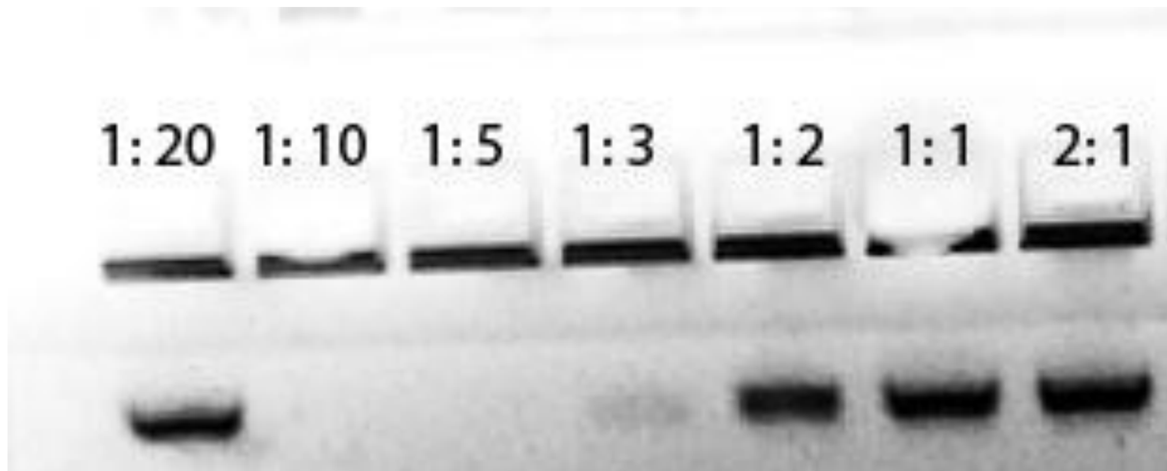


Figure 6: Gel electrophoresis, result with different binding ratios

FlashGel (Lonza) was used for electrophoresis of siRNA-nanoparticle complexes. siRNA:ND-PEI weight ratios ranging from1:20 to 20:1 were run on the gel at 200 V for 2 minutes. Gels were imaged with the FlashGel system. The experiment was repeated in duplicate.

REAL TIME POLYMERASE CHAIN REACTION (RT PCR)

Theory

Cells in all organisms regulate gene expression and turnover of gene transcripts (messenger RNA), and the number of copies of an mRNA transcript of a gene in a cell or tissue is determined by the rates of its expression and degradation.

In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is necessary. The polymerase chain reaction is a common method for amplifying DNA. For mRNA-based PCR the RNA sample is first reverse transcribed to cDNA with reverse transcriptase.

Development of PCR technologies based on reverse transcription permits measurement of DNA amplification during PCR in real time, i.e., the amplified product is measured at each PCR cycle. The data thus generated can be analyzed by computer software to calculate relative gene expression in several samples.

To make the amplified product visible a DNA-binding dye binds to all double-stranded (ds)DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified. The reaction is run in a Real-time PCR instrument, and after each cycle, the levels of fluorescence are measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product). With reference to a standard dilution, the dsDNA concentration in the PCR can be determined.

As described above, a comparison of a measured DNA/RNA sample to a standard dilution will only give a fraction or ratio of the sample relative to the standard, allowing only relative comparisons between different tissues or experimental conditions. To ensure accuracy in the quantification, it is usually necessary to normalize expression of a target gene to a stably expressed gene. This can correct possible differences in RNA quantity or quality across experimental samples.

Fluorescent reporter probes detect only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and enables quantification even in the presence of non-specific DNA amplification. Fluorescent probes can be used in multiplex assays – for detection of several genes in the same reaction – based on specific probes with different-colored labels, provided that all targeted genes are amplified with similar efficiency. The specificity of fluorescent reporter probes also prevents interference of measurements caused by primer dimers, which are undesirable potential by-products in PCR.

Preparation for PCR

There exists a lot of theory how to prepare the PCR, but in general one has to find his own protocol and rules. Cells are living subjects and every step depends on the quality and quantity of the cell lines and how precise the scientists work. Since we were working with RNA we had to be very careful to protect the samples. One single mistake can destroy the whole experiment and because the preparation for the PCR takes several weeks it also would destroy a lot of hard work.

To get ready for the PCR the cells needed to be prepared. When they were confluent they needed to be subcultured and plated in 6-well-plates. Before that they got counted. We used 150 000 cells per plate (Figure 7). Now different samples were made (Figure 8). They were put back in the incubator for 24 hours.

The following day the cells were harvested. After taking the transfection media out of the well plates they were washed with Trizol. This is a method of total RNA isolation by a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture. The method provides a pure preparation of undegraded RNA in high yield and can be completed within four hours. It is particularly useful for processing large numbers of samples and for isolation of RNA from minute quantities of cells or tissue samples. [4] After harvesting the cells the pure RNA immediately needed to be stored in -80° freezer. After leaving it in the freezer over night we got the cDNA synthesis started (reverse transcriptase). For the cDNA synthesis we used a kit. As attachment I added the RT-PCR protocol to get a picture of the work.

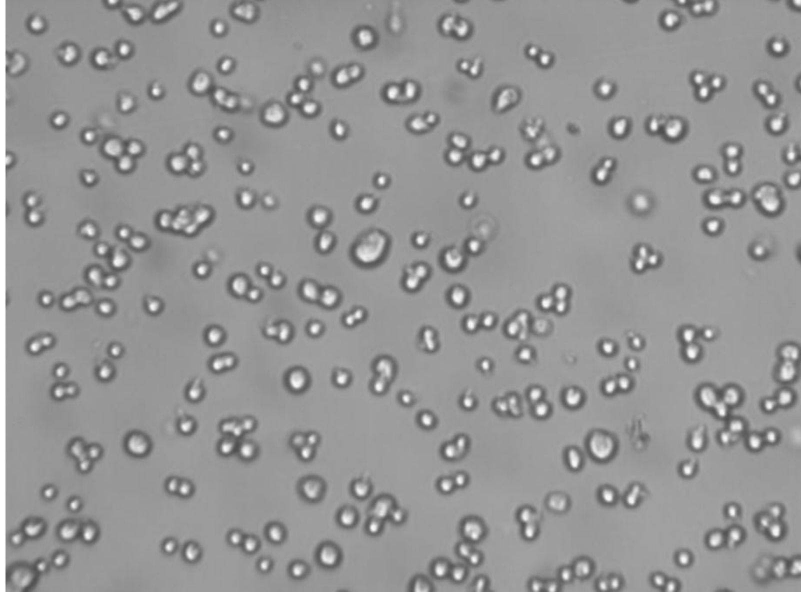


Figure 7: Cells plated in 6-well plate

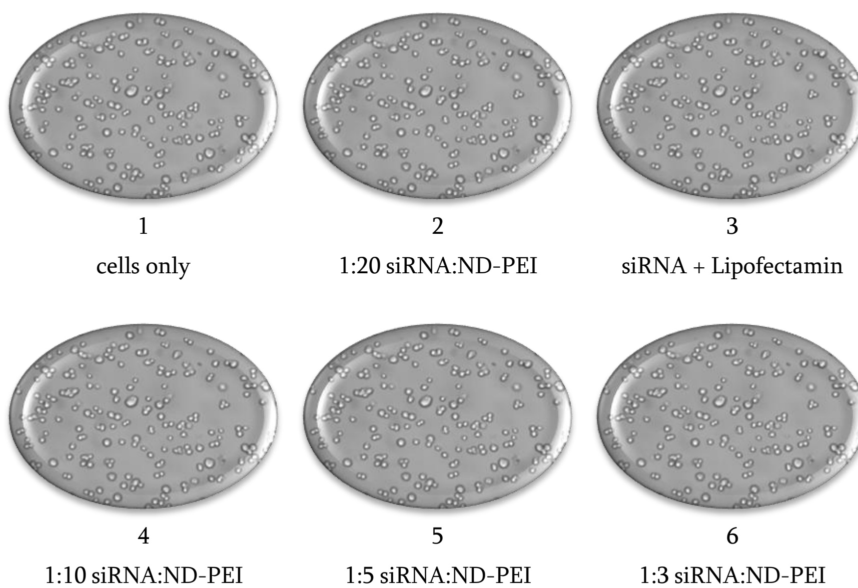


Figure 8: 6 well plate

RESULTS

The results of my work were very positive. We were able to knockdown the overexpressed c-myc, one of the most dangerous oncogenes that exist. This is another proof of the outstanding properties of nanodiamonds.

The following graphs show how well the gene silencing worked.

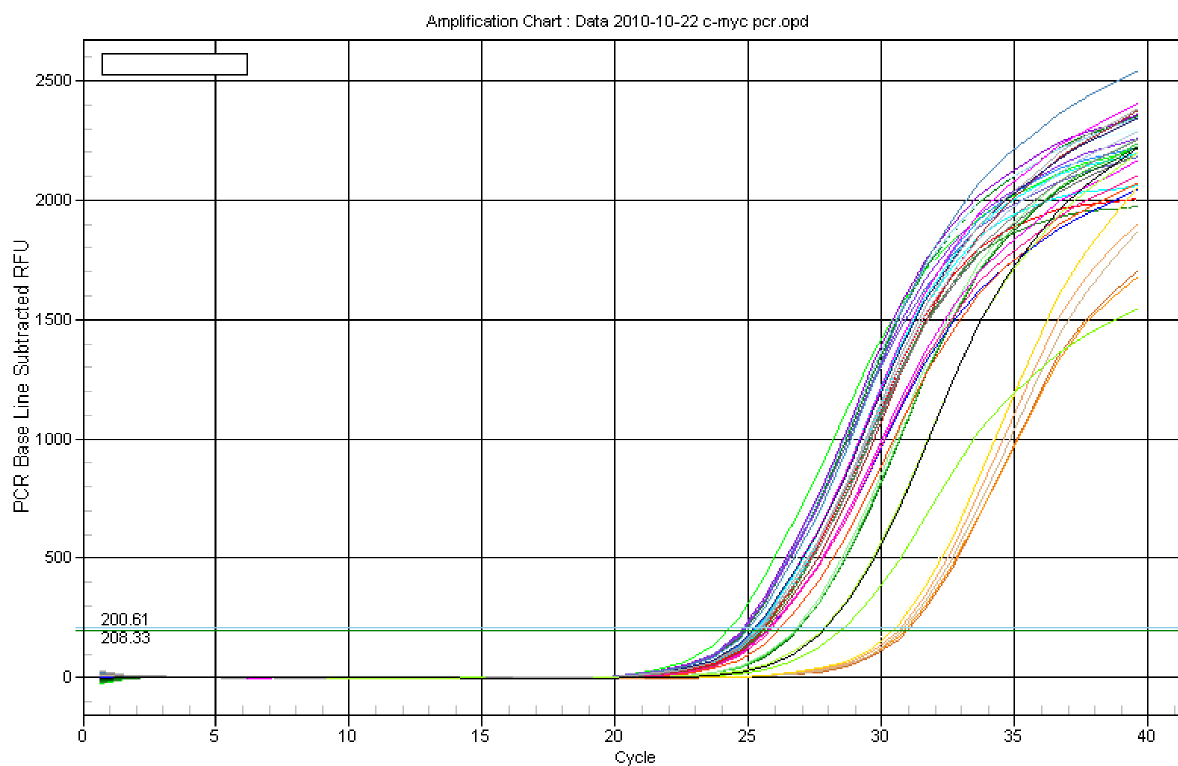


Figure 9: Amplicitation Chart of cDNA

The amplification chart shows the amplification of the RNA. Each cycle is saved and the final exponential curve is shown in the chart. The different colors show the different samples. All in all we had 12 samples put on a 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	x	x	x	x	x	x	x	x	x	x	x	x
B	x	1	1	1	7	7	7					x
C	x	2	2	2	8	8	8					x
D	x	3	3	3	9	9	9					x
E	x	4	4	4	10	10	10					x
F	x	5	5	5	11	11	11					x
G	x	6	6	6	12	12	12					x
H	x	x	x	x	x	x	x	x	x	x	x	x

x … To put a sample on the edge would be inaccurate, so we kept them free.

1 – 6 … Control samples. The used control gene was Actin.

7 – 12 … Knock down samples (c-myc)

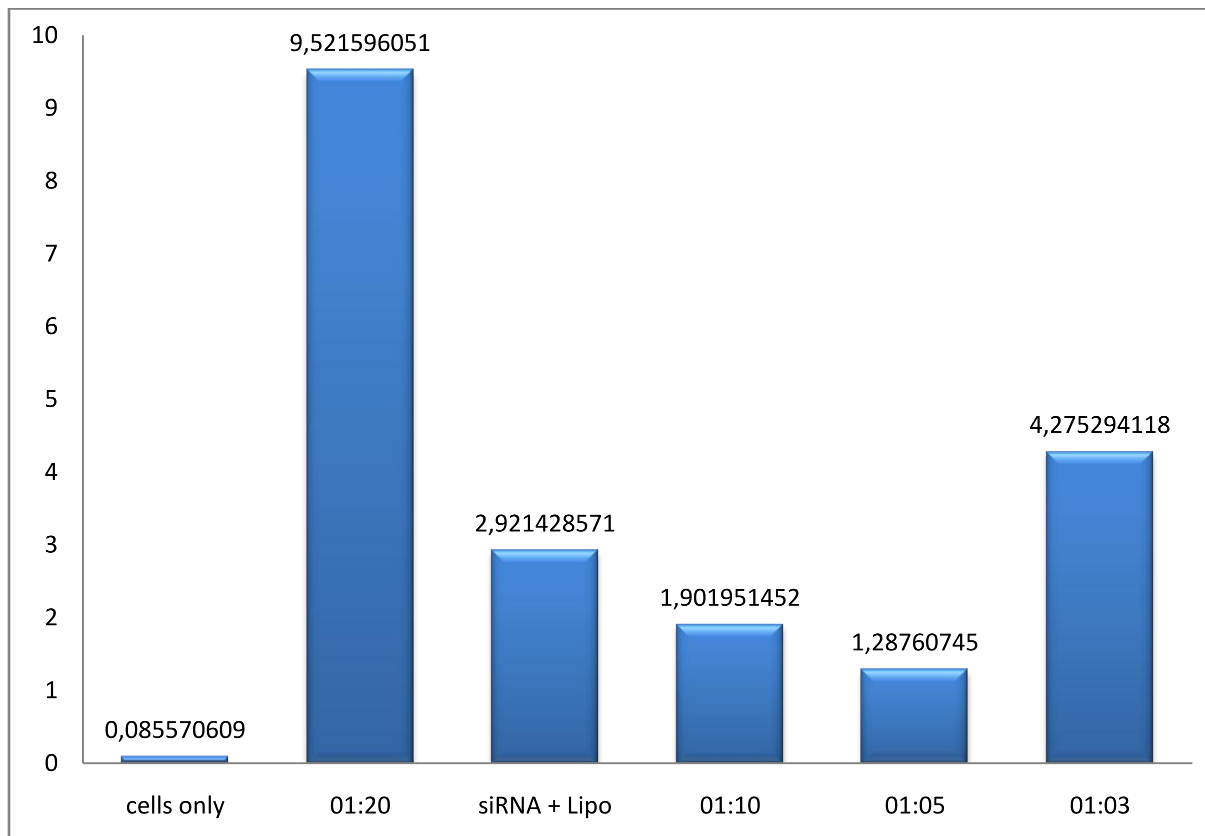


Figure 10: Normalized gene expression

This figure (Figure 11) shows the normalized gene expression. The best ratio siRNA:ND-PEI was 1:5.

Nanodiamonds (NDs) represent promising strategies for efficient siRNA delivery given the multitude of beneficial properties integrated into one platform that include uniform particle sizes, material scalability, the ability to carry nearly any type of therapeutic, and preserved biocompatibility, among others. With this results that have been done in vitro the next step will be to try the nanodiamond vectors in vivo. Even if it was only a little success it still was a step in the right direction and brings us closer to the improvement of cancer therapy.

RT-PCR PROTOCOL

PART 1: CELL HARVEST/RNA PURIFICATION

1. Turn on temperature centrifuge, set to 4°C
2. Get 2 RNase-free tubes per sample and label the tubes
3. Fill 1 of the tubes with 550 µl Isopropanol, fill the other one with 200 µl chloroform for each sample
4. Aspirate and harvest samples. Take out the media. Try not to touch the bottom, be careful. Then add 1ml of Trizol to the cells. Wash the well with the Trizol.
5. Cell lysis with 1 ml Trizol, add to chloroform tube. Vortex well, 5 – 10 seconds, until chloroform and Trizol are mixed (pink and creamy)
6. Centrifuge 15 minutes with 14 000 rpm at 4°C
7. Remove 450 µl supernatant (only the clear liquid, be very careful. 450 µl is an approximate value). Add to isopropanol tube. Vortex well, but low speed (4-5) for 5-10 seconds
8. Store samples overnight in -80°C freezer (or at least 2 hours)

PART 2: CDNA SYNTHESIS

1. Turn on UV/Vis spec and centrifuge. Set the temperature to 4°C. Check water bath, set temperature to 37°C, it should be half full.
2. Bring a Styrofoam box and get ice. Ice has RNase so be careful not to touch it.
3. Thaw samples.
4. Centrifuge at 4 °C for 30 minutes at 14 000 rpm, if there's no pellet, centrifuge longer (about 60 min, depending on the amount of cells).
5. Get 3 0.5 ml tubes for each sample. Label tubes, fill 2 sets of tubes with 98 µl DEPC water.

KEEP SAMPLES ON ICE AS MUCH AS POSSIBLE

6. Put samples on ice, remove supernatant → bubblefuge → remove more supernatant
7. Add 500 µl 70% EtOH to each sample. Gently break pellet off tube wall by pipetting up and down.
8. Centrifuge for 5 minutes. 14 000 rpm, 4°C.
9. Thaw: 5x Buffer, Oligo DT Primer, and Nuclease Free Water from cDNA synthesis kit.
10. Put samples on ice, remove supernatant → bubblefuge → remove more supernatant, as much as possible! There should be no EtOH left!
11. Airdry for 5 minutes. Keep tubes on ice, open it and put tissues on top so nothing can get into the tube! They should be totally dry, so 5 minutes could not be enough

12. Add 75 µl DEPC water (±µl based on pellet size) to each sample to dissolve the pellet. If you're not sure add rather less than too much! You can always add more water, but you can't put any out once you added it!
13. Gently pipette to break up pellet → vortex gently → 10 minutes sit → vortex gently
14. Add 2 µl of each RNA sample into the respective 0.5 ml microtubes (filled before with DEPC water) and vortex
15. Perform nucleic acid analysis with UV-Vis spec
16. Enter λ= 260 values into computer spreadsheet and print it
17. Add water to each tube → add RNA to each tube → keep on ice (the 3rd set of tubes that is still empty)
18. Vortex thawed reagents. Do not vortex enzyme. Spin down all reagents
19. MAKE MASTER MIX: S x 4 µl 5x Buffer, S x 2 µl Oligo DT, S x 1 µl enzyme (S = Sample). Make a little bit more since the volume is very little!
20. Vortex low speed → add 7 µl to each sample → vortex samples
21. Put sample in water bath at 37°C for 1.5h
22. After 1.5h take out the cDNA, store in -20°C freezer.

PART 3: RT-PCR PLATING AND RUN

1. Make a plate coding grid for samples vs. genes + turn on PCR machine + lamp
2. Make serial dilutions: $\frac{1}{10}, \frac{1}{100}, \frac{1}{1000}$ and $\frac{1}{10000}$. 2 µl sample + 18 µl DEPC water
3. Make your MASTER MIXES + standard mix: enter sample number into calculator
   a. The basic mix: 88 µl total
      i. 39.4 µl DEPC
      ii. 45.0 µl super mix (In -20°C fridge)
      iii. 01.8 µl forward primer
      iv. 01.8 µl reverse primer
4. Get out as many tubes as samples X genes (see grid) + 4 extra tubes for standards
5. Add 88 µl of the respective MASTER MIX into each of the tubes
6. Add 2 µl of the respective cDNA samples into each of the tubes
7. Add the $\frac{1}{10}, \frac{1}{100}, \frac{1}{1000}$ and $\frac{1}{10000}$ cDNA dilutions to tubes
8. Vortex al the sample tubes
9. Diagram well grid and fill in wells with sample codes. Each sample takes up 3 wells
10. Get out a well plate, add 25 µl of each sample into appropriate wells
11. Cap the wells with the cap strips
12. Spin down plate for 3 minutes, 500 rpm, SX4750 rotor. If an error occurs, press CE and retry.
13. Put in plate → choose protocol → choose/design plate

14. Click “RUN” → “collect well factors” → begin run → click “yes” for both windows
15. Run about 1.5 hours → gene exp tab → recalculate → copy data to MS Excel → analyse

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