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Jan Majer

Příprava a charakterizace nanonosičů na bázi diamantu pro transfekci siRNA

Preparation and characterization of diamond-based nanocarriers for transfection of siRNA

Bakalářská práce

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Poděkování:

Rád bych poděkoval svému školiteli, Mgr. Petru Cíglerovi, Ph.D., za jeho rady, opravy a věcné připomínky. Zároveň bych chtěl poděkovat Mgr. Jitce Neburkové za její trpělivost a ochotu poradit během vypracovávání této práce.

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis:

Abstrakt

Ačkoliv nanodiamanty byly objeveny a připraveny před desítkami let, až nedávno se začaly využívat v medicínských a biologických oborech, zejména pro doručení léčiv a genetického materiálu do buňky a v biozobrazovacích metodách. Nanodiamanty mohou být modifikovány specifickými, pozitivně nabitými skupinami pro komplexaci s negativně nabitými nukleovými kyselinami. Tyto komplexy následně překonávají extracelulární a intracelulární bariéry a transportují nukleové kyseliny buďto do cytosolu nebo do jádra. Díky fluorescenčním centrům dusík-vakance, které mohou být v nanodiamantech vytvořeny, vykazují nanodiamanty vynikající optické vlastnosti pro sledování transfekce, protože emitují stabilní fluorescenci bez "photoblinkingu" a "photobleachingu". Tato práce shrnuje vlastnosti, syntézu, modifikace nanodiamantů a dalších vybraných nanočástic a jejich *in vitro* aplikace. Porovnává také jejich cytotoxicitu a efektivitu genového „knockdownu".

Klíčová slova: nanodiamant, nanočástice, siRNA, transfekce, PEI, DMAEMA, *in vitro*

Abstract

Although nanodiamonds were discovered and produced tens of years ago, they have been utilized in medical and biological fields just recently, particularly in drug and gene delivery into a cell and in bioimaging methods. Nanodiamonds can be modified with specific positively charged moieties for complexation with negatively charged nucleic acids. These complexes afterwards overcome extracellular and intracellular barriers and transport the nucleic acid either into cytosol or into the nucleus. Owing to fluorescence centres nitrogen-vacancy, which can be formed in the nanodiamonds, nanodiamonds exhibit excelling optical properties, as they emit stable fluorescence without "photoblinking" or "photobleaching". This thesis reviews properties, synthesis and modifications of nanodiamonds and other selected nanoparticles and their *in vitro* applications. This thesis also compares their cytotoxicity and gene knockdown efficiency.

Key words: nanodiamond, nanoparticle, siRNA, transfection, PEI, DMAEMA, *in vitro*

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Abbreviations:

ADA	adenosine deaminase
AGO	argonaute 2
ATP	adenosine triphosphate
AuNP	gold nanoparticle
CL	cationic lipid
CP	cationic polymer
CVD	chemical vapour deposition
DMAEA	dimethylaminoethyl acrylate
DMAEMA	2-(dimethylamino)ethyl methacrylate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DND	detonation nanodiamond
dsRNA	double stranded ribonucleic acid
FITC	fluorescein isothiocyanate
fND	fluorescent nanodiamond
GFP	green fluorescent
HPHT	high pressure, high temperature
HPMA	N-(hydroxypropyl)methacrylamide
IONP	iron oxide nanoparticle
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSiNP	mesoporous silica nanoparticles
NA	nucleic acid
ND	nanodiamond
NHL	neutral helper lipid
NLS	nucleus localization signal
NV	nitrogen-vacancy
ODA	octadecylamine
PAH	poly(allylamine hydrochloride)
PDMAEA	poly(dimethylaminoethyl acrylate)
PDMAEMA	poly(2-(dimethylamino)ethyl methacrylate)
pDNA	plasmid deoxyribonucleic acid
PEG	polyethylenglycol
PEI	polyethylenimine
PHPMA	poly(N-(hydroxypropyl)methacrylamide)
PLL	Poly(L-lysine)
RES	reticuloendothelial syste
RISC	RNA-induced silencing complex
RNAi	RNA interference
siRNA	small interfering ribonucleic acid
TEM	trasmission electron microscopy
TEOS	tetraethylorthosilicate
TMSPMA	3-(trimethoxysilyl)propyl methacrylate
VLP	virus-like particle

1. Introduction

Gene therapy is an experimental technique that uses exogenous nucleic acids (NAs) to treat or to prevent diseases by replacing defective genes, inactivating undesirable genes and by introducing entirely new gene into the cell. The cell membrane and the nucleus are difficult to penetrate through for nucleic acids. To transport the nucleic acid into the cell, a large variety of gene transfer methods have been introduced. Currently these methods consist of three groups: viral, physical and chemical methods. Gene delivery is a promising tool for delivering exogenous genetic material into a cell by introducing therapeutic agents for disease treatment and it is used both to treat genetic diseases and to produce proteins for direct therapeutic application. Ideally, the delivery methods should show high transfection efficiency and low cytotoxicity. Overall, the gene therapy is still being problematic, mostly because of lack of acceptable vector systems to deliver the nucleic acids.

Recently, nanodiamonds, carbon-based nanomaterials, have emerged as an interesting tool in medical application and bioapplication. Their optical properties surpass any other fluorescent nanoparticles currently available, owing to their high photostability resulting in absence of photoblinking and photobleaching. Nanodiamonds are also favourable for their biocompatibility and the possibility for further modifications of their surfaces. Nanodiamonds were already modified with polymers and used for transfection of nucleic acids.

Nucleic acids used for gene delivery can be divided into two groups, depending on their mechanism of interaction. First group consists of plasmid DNA or other large DNA molecules, which are transported into the nucleus for gene expression. Second group is responsible for inhibition of expression and consists of two other sub-groups. First sub-group consists antisense oligonucleotides (~15-30 bps nucleotides), which enter the nucleus, where they inhibit the expression of specific genes by hybridization with complementary mRNA molecules. The second sub-group is composed of siRNA, shRNA, piRNA and miRNA, nucleotides with expression interfering activity in cytoplasm through hybridization with homologous mRNA. This thesis focuses on siRNA and DNA transfection.

2. Transfection *in vitro*

Eukaryotic cells can uptake exogenous genetic material (siRNA, DNA, etc.), if specific conditions are met. This process is called transfection (Fig. 1). Transfection is used in molecular biology for studying gene functions, protein expression via gene silencing or incorporation into the chromosome itself (depending on nucleic acid's nature), which alters phenotype of the cell and is also essential for gene therapy (Jones *et al*., 2013; Kim and Eberwine, 2010).

To reach its destination, nucleic acid must overcome restricting and degrading barriers, both extracellular and intracellular. The research aims for methods with low toxicity to the cells/tissue and for high transfection efficacy (Jin *et al*., 2014).

The stability of the foreign nucleic acid in the cell depends on its chemical and physical properties (Recillas-Targa, 2006). The stable foreign genetic material is usually integrated into the genome of the host cell. The new transgene is then expressed even after the cell replicates. On the other hand, transient form is being expressed for a specific amount of time and there is no process of genome integration (Kim and Eberwine, 2010).

2.1. DNA Transfection

To guide DNA into the nucleus, NLS (nuclear localization signal) is usually introduced. NLS are peptidic molecules which bind to *importins* – nuclear envelope binding proteins. Importins then transfer the cargo past the nuclear envelope using Ran-GTPase transfer system. NLS can be attached to the transfected DNA either covalently or electrostatically. The process of translocation of the foreign DNA follows two steps: ATP-independent binding to the suface of nucleolemma and ATP-dependent translocation of the DNA through the nucleolemma (Collas and Aleström, 1996).

2.2. RNA Transfection and RNA Interference

Transfecting RNA, whether it is messengerRNA (mRNA) or small interfering (siRNA), indisputably holds multiple advantages (for instance a lack of integration into the host genome).

The requirement for DNA transcription is bypassed, when using the mRNA, since the mRNA is not needed in the nucleus for its proper expression. Thus the foreign mRNA introduced to the cell is expressed in the cytoplasm in minutes after the transfection(Kim and Eberwine, 2010). Other major advantage is the possibility to transfect the entire population

of mRNA molecules of the cell – *transcriptome*. Sul *et al*. managed to transport the transcriptome of a differentiated rat astrocyte into a differentiated rat neuron cell. Using phototransfection for its high efficiency, he successfully changed the target cell's phenotype (Sul *et al*., 2009).

When transfecting siRNA, the introduced nucleic acid is in a double-stranded form (dsRNA) with a homologous sequence to the mRNA. The homologous mRNA is then silenced by RNA interfering (RNAi) mechanisms. First ever siRNA transfection in mammalian cells was achieved by using 21-nucleotide long siRNA duplexes (Elbashir *et al*., 2001), although 19 to 24-nucleotide variations have been introduced as well (Liang *et al*., 2013; Wei *et al*., 2009). To silence the specific mRNA, a RNA-Induced Silencing Complex (RISC) triggers the mRNA cleavage. RISC is activated by either siRNA or miRNA. The Argonaute protein subfamily, *AGO*, consisting of AGO 1-4 has the ability to repress the mRNA's activity, though only AGO2 is able to cleave the target nucleic acid (slicer activity). AGO2 protein is also the only protein (in the Argonaute family) needed for RISC proper function, since specific knock-down of Ago1, 3, 4 had no effect on RISC's function (Meister *et al*., 2004). RISC associated RNase III enzymes (Drosha, Dicer) cleave the long dsRNA molecule into approx. 21 nucleotide long dsRNA with 1 nucleotide overhangs on the 3' ends. Afterwards, the passenger strand is degraded by the RISC. The remaining siRNA's antisense (guide) strand with two 3' one nucleotide overhangs is then used by the RISC's AGO2 for guidance to the complementary mRNA. Whether the AGO2 incorporates the first or the second strand is determined by the strand's relative thermodynamic stability of the first 1-4 bases at 5' end – the less stable strand is used as the guide strand. The entire process of RISC assembly consists of: a) RISC-loading, an intake of the dsRNA into the AGO2 (RISC); b) RISC-wedging, an opening the 5' end of the duplex; c) RISC-unwinding, a dissociation and removal of the second RNA strand (Kwak and Tomari, 2012; Schwarz *et al*., 2003).

2.3. Extracellular Barriers

In vitro, several factors must be acknowledged for a successful transfection. Optimal cell density for transfection varies, depending on the cell type and the application methods. For example, the lipids are great transfection agents because of their good membrane permeability. Application of a vector, which is not colloidal, but aggregated, may result in false-positive results, thus meaning the vectors may successfully be transfected into the adherent cells, though cells in suspension will not manifest any result of gene delivery *in vivo*.

Vector delivery *in vivo* has been challenging. Except the reticuloendothelial system (RES), the vectors must overcome other hurdles like enzymatic degradation, serum deactivation, complement-mediated clearance, etc. To prevent these interactions, vector surfaces are sterically protected by protective moieties (PEG, HPMA), which will be discussed later on in the text.

Endocytosis poses as the most common mechanism of cellular entry for lipoplexes, polyplexes and nanocarriers, using several uptake pathway like macropinocytosis, clathrin mediated and caveolae mediated endocytosis, etc. (Rehman *et al*., 2013).

2.4. Intracellular Barriers

Upon the cellular entry, the genetic payload is located inside of early endosomes, subsequently late endosomes and lysosomes. The escape of exogenous cargo, *the* endosomal escape, is a limiting step of successful transfection with only 1-2% efficiency and the process itself runs in a limited period of time.

The research shows that the nucleic acid release appears to occur in mildly acidic environment, in early endosomes. The cargo escape may also occur in leaky macropinosomes (Gilleron *et al*., 2013).

In lysosomal compartments, nanopolymers are presumably leaving the endosome by "proton sponge effect", where unprotonated amines of polymers are able to bind protons, which leads to an influx of protons into the lysosome and Cl^- anions consequently, due to the electro-chemical gradient. The lysosome then swells, until the membrane ruptures. However, the proton sponge hypothesis has not yet been proven, on the contrary, it seems as an incorrect hypothesis (Benjaminsen *et al*., 2013), leaving the endosomal escape unexplained.

Unlike RNA, exogenous, plasmid DNA's (pDNA) destination is the cell's nucleus. Due to its size and shape, pDNA cannot pass through the nucleus pore. This means that the only time suitable for pDNA to enter the nucleus is during the cell division, mitosis. Size of pDNA also significantly slows it's mobility in the cell, leaving the exogenous DNA vulnerable to the host cell's defence mechanisms for a longer period of time (Jones *et al*., 2013; Zhang *et al*., 2012).

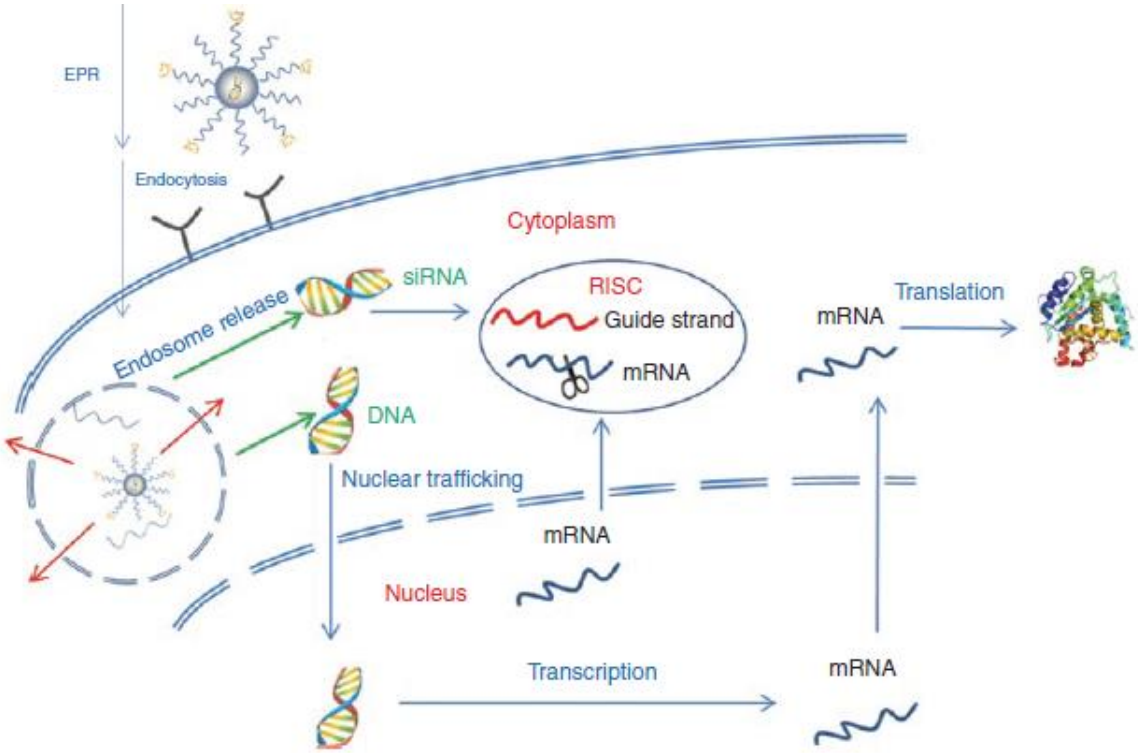

Figure 1: A brief overview of cellular barriers and transfection pathways (Zhang *et al.*, 2012)

2.5. Transfection Methods

Many laboratory systems and methods for transfection have been introduced throughout the years. These systems can be divided into three main groups: physical viral and chemical.

Physical methods for transfection and gene delivery consist of biolistics (aka. gene gun), jet injection, ultrasound, electroporation etc. Physical methods are faster than other non-viral or viral methods. These methods allow a direct penetration and transport of the exogenous nucleic acids and in some cases overcome some hurdles that other methods cannot, although it is problematic to perform a gene transfer in a large scale. Also, physical methods are not effective for transport into the nucleus (Jin *et al.*, 2014).

Virus-mediated transfection, also called transduction is the most commonly used transfection agent in clinical research. The main reason is its high transfection efficiency and target specificity. On the other hand, the drawbacks of virus-mediated transfection are the risk of insertional mutagenesis and steric limitations of the transported nucleic acid. The transduction is achieved by using viral vectors, particles derived from viruses, except the original viral genes. Genes, essential for building the viral particle and infecting the host cell, are retained and genes coding essential proteins for viral life cycle are deleted and replaced with heterologous genes for manipulation of the cells phenotype (Vannucci *et al.*, 2013). The

first ever successful therapeutic transduction was performed in 1990 on two children with severe combined immunodeficiency (adenosine deaminase deficiency). T-cells transduction with retroviral vectors containing a gene for ADA enzyme (Blaese *et al*., 1995).

Chemical transfection methods use mostly calcium phosphate, cationic lipids (CLs) and cationic polymers (CPs). These will be closely discussed in the upcoming chapters. These particles have positive charge and therefore they form complexes with the negatively charged nucleic acids. The complete complex has positive charge which is then moving towards the cell membrane. (Kim and Eberwine, 2010) Main advantages/disadvantages of using chemical and viral methods are mentioned in the table below (tab. 1).

Table 1: Highlighted advantages and disadvantages of transfection methods

GROUP	ADVANTAGES	DISADVANTAGES
Viral		
	low cytotoxicity	insertional mutagenesis
	effective *in vivo*	immunogenicity
	high efficiency	Size limitation of NA cargo
Chemical		
	high efficiency	possible cytotoxicity
	no size limitation	difficult cell targeting
	no insertional mutagenesis	

(Kim and Eberwine, 2010)

3. Transfection Agents

As it was already mentioned, electrostatic interactions are the driving force of forming a thermodynamically quasi-stable formation of the RNA (DNA) and the transfection reagent. Polymers or lipids sterically protect the cargo from nucleases and increases mobility in a cell's cytosol

3.1. Cationic Lipids (CLs)

CLs (Fig. 3) are amphiphilic organic molecules consisting of 3 main domains: hydrophilic head group, which binds the nucleic acid, hydrophobic tail and a linker that connects hydrophobic and the hydrophilic part of the molecule. Liposomes and lipid based vectors appear to exhibit high instability in physiological environment as well as low transfection efficacy.

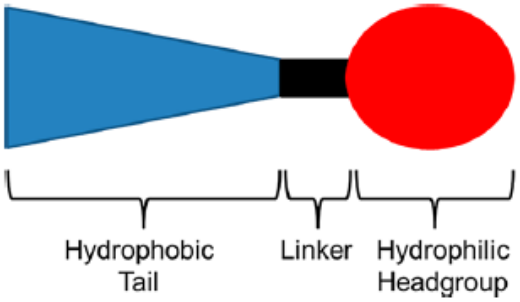

Figure 2: basic structure of common CLs (Jones *et al.*, 2013)

The positive charge, responsible for the electrostatic interaction with RNA (DNA) molecules, is localized either on the hydrophilic head (Fig. 2), on a primary, secondary or tertiary amine group or quaternary salt. A promising substitution for amine groups are phosphonium and arsenium groups. Introduction of these new groups into CLs increased their thermal stability and overall lowered cytotoxicity (Guénin *et al.*, 2000).

Linker is commonly an ether, ester, amide or carbamate. It is generally acknowledged, that ether linkers, for their high stability and therefore low biodegradability, are more delivery efficient and cytotoxic than ester linked lipid vectors. A development of pH sensitive, enzyme sensitive and light sensitive linkers aims for nucleic acid release and biodegradation to both lower the cell toxicity and increase the transfection efficiency. (Martin *et al.*, 2005; Nagasaki *et al.*, 2003)

Cationic lipids are often accompanied with neutral helper lipids (NHLs). CLs are able to create ion pairs with membrane anionic phospholipids, which leads to destabilisation of the membranes via a change in the formation of two phases, lamellar (L_α) and inverted hexagonal (H_{II}). NHL's role in transfection is to help the complex to escape from the endosome and a release into the cytosol by assisting the hexagonal H_{II} formation. (Ewert *et al.*, 2005; Hui *et al.*, 1996)

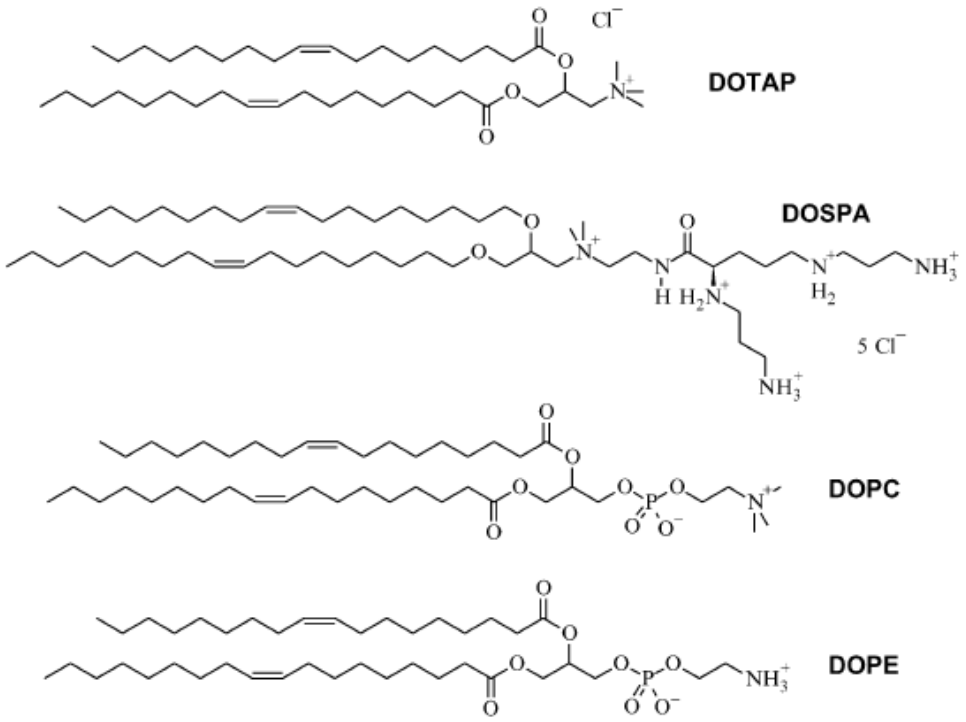

Figure 3: Some of the commonly used CLs for transfection; 2,3-Dioleyloxypropyltrimethylammonium chloride (DOTAP); 2,3-Dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propylammonium chloride (DOSPA); 1,2-Dioleoyl-*sn*-glycerophosphatidylcholine (DOPC); 1,2-Dioleoyl-*sn*-glycerophosphatidylethanolamine (DOPE); (Ewert *et al.*, 2005)

3.2. Cationic Polymers

Polycationic polymers represent a large set of synthetic NA carriers such as polypeptides, polyethyleneimine, dendrimers, chitosan, etc. When using CPs, molecular weight should be considered. An increase of CP's molecular weight positively correlates with efficacy of gene delivery and with cytotoxicity. The cytotoxicity increases with increase of CP's molecular weight despite the same N:P ratio, where N, nitrogen, represents the amount of polymeric nanocarrier and P, phosphorus, represents the amount of nucleic acid present in the complex. It has been observed that certain modifications (*e.g.*, hydroxylation) of CPs lower the carrier's cytotoxic properties and increases their RNA or DNA complexing ability(Ma *et al.*, 2010).

Poly(*L*-lysine) (PLL) is a biocompatible polypeptide with primary amino groups, which can be protonated and therefore interact with the negatively charged nucleic acids. Complex of a PLL and a nucleic acid, despite it is well biodegradable, is toxic to the cell and tends to aggregate, depending on the environmental salt concentration. Additionally, comparison of linear PLL and dendritic PLL showed that dendritic formation exhibited to mediate 100-fold

greater gene expression than the linear PLL (Yamagata *et al*., 2007). Because the dendrimer showed weaker compaction with the nucleic acid compared to the linear PLL, the nucleic acid therefore dissociated from the vector during the endosomal escape.

Polyethyleneimine (PEI) is a polycation molecule widely used in gene delivery including, but not limited to, DNA or siRNA. PEI molecules contain primary, secondary and tertiary amines. This gives PEI possibility to complex both DNA or siRNA with outstanding transfection efficiency, higher than other CPs (Boussif *et al*., 1995). Nevertheless, *in vivo* and *in vitro* toxicity has been registered. Therefore to reduce PEI's toxicity, polyethyleneglycol (PEG) was grafted onto the PEI molecule, which lead to lowering the cytotoxicity (Kunath *et al*., 2002). The length of PEI polymers can be extended to induce gene delivery.However, PEI polymer extension also induces cytotoxicity and *vice versa* (Xia *et al*., 2009a). PEG also serves as a protection of the carrying complex from opsonisation by the RES and stabilizes the complex.

Another cationic polymer is poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) with tertiary amine groups. To overcome drawbacks related with cytotoxicity and also low gene silencing efficiency, when transfecting siRNA, Gary et. al. prepared "micelleplexes", micelle-based siRNA vectors from PEG-PDMAEMA-poly(*n*-butyl acrylate) triblock, which *in vitro* lead to more efficient gene silencing, lower cytotoxicity and enzyme resistance, when compared to PDMAEMA or PEG-PDMAEMA (Gary *et al*., 2011)

Some cationic polymer-nucleic acid complexes may form positively charged complexes. These cationic complexes aggregate with negatively charged proteins leading to higher toxicity. To prevent this setback, neutral, water-soluble complexes like *N*-(hydroxypropyl)methacrylamide (HPMA) are used as copolymers for the gene delivery (Dash *et al*., 1999; Duncan, 2006).

4. Nanoparticles as Transfection Agents

Nanoparticles (NPs) are colloidal, submicronic particles. Nanoparticles possess high cellular uptake properties. Research has shown the ability of nanoparticles to penetrate through submucous layers, even the blood-brain barrier. In gene delivery, nanoparticles manifest as excellent vectors, owing to their size and their overall versatility, whether it is cargo protection, bioimaging, biodegradability and biostability. (Panyam and Labhasetwar, 2003). Moreover, nanoparticles *in vivo* can be modified with ligands for specific cellular/tissue active targeting. In passive targeting, nanoparticles can penetrate through fenestrations in the endothelium, *e.g.* in pathophysiological conditions. Their low clearance

allows nanoparticles to be active in the circulation system for long period of time (Moghimi *et al*., 2001)

4.1. Organic Nanoparticles

Virus-like particles (VLPs) are multi-subunit self-assembling protein nanoparticles with a structure highly resembling the native viruses. These particles lack viral nucleic acids, therefore the chance of insertional mutagenesis is eliminated. Disadvantages of using VLPs are adverse immunological features, production difficulties steric limitation of the cargo (Zeltins, 2012). Production of recombinant capsid proteins has been developed in both prokaryotic and eukaryotic systems (Santi *et al*., 2006). Panda *et al*. produced Hepatitis E VLPs in *Escherichia coli*, which expressed pORF2 gene. The expressed pORF2 proteins were self-assembled into column-like VLPs, into which cargo was encapsulated (Kapur *et al*., 2012; Panda *et al*., 2015).

Liposomes, nanoparticles consisting of cationic lipids and anionic nucleic acids, form themselves through self-assembly. Nucleic acid firstly interacts with the lipid monolayer, then the cargo "wraps" itself into lipid monolayer. This process is driven by electrostatic forces between both cationic and anionic molecules. (Oberle *et al*., 2000). It is noteworthy to mention possible liposomes (and other nanocarriers) modifications, which sterically shield the complex from a hostile environment, such as the use of polyethyleneglycol (PEG). On the other hand, PEG modified liposomes fail to protect the carrying nucleic acids inside the cell, where they rapidly degrade in the cytosol and in the endosome (Remaut *et al*., 2007). In order to promote endosomal escape, liposomes can be modified to lower their pK_a. Lowering the pK_a of the complex in slightly acidic environment of the endosome causes it to become positively charged. Protonated lipids interact with the endosomal membrane similarly as the NHLs, changing the lamellar (L_α) and inverted hexagonal (H_{II}) phases, leading to destabilisation of the endosome by its fusion with the liposomes, thus releasing the cargo from the endosome (Bell *et al*., 2003; Koltover *et al*., 1998).

Self-assembling three-dimensional polymers, polymersomes, were first introduced in 1978 (Buhleier *et al*., 1978). Dendrimersomes are stable, rather easy to create carriers with pre-determined size. It is worth mentioning the existence of Janus dendrimers, named after an ancient roman god, prepared by coupling hydrophobic and hydrophilic branched segments, which form themselves through self-assembly into stable bilayer nanoparticles. For their anisotropic nature, Janus nanoparticles are able to encapsulate hydrophilic and

hydrophobic molecules, thus showing a potential of transporting different cargos in drug delivery (Garbuzenko et al., 2014; Yang et al., 2014a).

4.2. Inorganic Nanoparticles

Inorganic nanoparticles, compared to liposomes or dendrimersomes, are resistant to enzymatic degradation and they can withstand overall adverse conditions (Kneuer *et al*., 2000).

Gold nanoparticles (AuNPs) draw research attention, not only for imaging and clinical diagnostics, but also for their chemical stability, electron density and when charged by further modifications, high affinity to nucleic acids. AuNP-oligonucleotide complexes are less prone to nucleases' activity in comparison with bare oligonucleotides, most likely due to the steric limitations, increasing the nucleic acid's lifetime *in vitro*. By controlling the amount of nucleic acid bound to the AuNP, the complex's binding properties can be altered. Rosi *et al*. demonstrated, that particles with larger amount of oligonucleotides attached lead to more efficient gene silencing, better than the commercially available lipid agent Lipofectamine 2000, despite the fact, that most of the nucleic acid molecules remained attached to the AuNPs. (Rosi *et al*., 2006). The AuNPs can be modified with PEG chains. Lee *et al*. prepared siRNA-AuNP complexes with PEG by introducing *N*-succinimidyl 3-(2-pyridyldithio)propionate to the terminal NH_2 group of PEGylated AuNP and finally by adding HS-siRNA linked by disulphide linker. These complexes showed a great salt stability and low aggregation (Lee *et al*., 2009).

Also iron oxide nanoparticles (IONPs) show outstanding abilities in gene delivery mainly for their biocompatibility, magnetic properties and high potential in surface modifications. IONP's magnetic properties are essential for MRI (magnetic resonance imaging). The most commonly used iron oxides are maghemite (Fe_2O_3) or magnetite (Fe_3O_4). To bind the desired nucleic acid, IONP must be modified with a polycation layer. Liu *et al*. used tris(acetylacetonato)iron(III) for IONP synthesis and alkyl-PEI for surface modification for siRNA complexation, achieving well dispersed NPs with high gene delivery efficiency and low toxicity. (Liu *et al*., 2011).

Mesoporous silica nanopartilces (MSiNP) present interesting properties – MSiNPs can load nucleic acids either on their large surface area or on their interior surface. Therefore, a promising dual delivery method emerges. Torney *et al*. prepared MSiNP carrying inducible

marker gene for GFP (green fluorescent protein) with β-oestradiol[1]. Silica materials present a safe and biodegradable approach in gene delivery, showing rapid dissolution in physiological environment (Finnie *et al*., 2008; Torney *et al*., 2007).

Another nanoparticles, nanodiamonds, show promising results in transfection and in bioimaging, owing to their physical and chemical properties and the possibility of surface modification with various functional groups.

5. Nanodiamonds as Transfection Agents

Carbon, "the building block" of nanodiamonds, has six electrons in $1s^2$ $2s^2$ $2p^2$ electronic configuration, meaning that the atom has four valence electrons and two core electrons. When the electronic configuration is sp^2 hybrid, the carbon atoms form a planar, hexagonal structure – graphite – where the carbon creates three σ-bonds and the π-electrons form a delocalized cloud over the graphitic plane. In case of sp^3 hybridization, the carbon atom forms a tetrahedral diamond structure with all four valence electrons participate in a formation of σ-bonds. The absence of free electrons causes the NDs' chemical inertness, except the shell, where the sp^3 carbon atoms are terminated either by graphite, by hydrogen or hydroxyl and other oxidizing agents (Badea and Kaur, 2013; Jiang and Xu, 1995).

There are multiple approaches to ND synthesis, including chemical vapour deposition (CVD) NDs, high-pressure high temperature (HPHT) NDs and detonation NDs (DNDs).

Typically, to prepare HPHT NDs, synthetic micron-sized diamond powder, ~100-200 μm in diameter, is milled with ceramic beads under high pressure (8 bar). The diameter of HPHT NDs varies depending on specific conditions during their preparation (~10 nm) (Boudou *et al*., 2009).

When comparing with HPHT NDs, DNDs are smaller particles (~4 nm). NDs are obtained from explosives containing carbon. The explosions are situated in chambers filled either with water or an inert gas. The product of the explosions, detonation soot, consists of 4nm NDs, graphite and impurities (Dolmatov, 2001). Impurities are removed by oxidizers (peroxides, nitric acid, sulfuric acid). Nanodiamonds tend to aggregate into large-scale particles (100-200 nm). To create a stable suspension of nanodiamonds particles, DNDs are milled with ceramic microbeads and consequently sonicated in H_2O, where they remain well-dispersed (Eidelman *et al*., 2005; Krüger *et al*., 2005).

[1] β-oestradiol – a steroid inducing the expression of GFP in plants

When using CVD method, nanodiamonds are grown in a form of nanofilms or ultracrystalline films. These films present tools with high mechanical resistance and chemical inertness in biocompatible coating (Espinosa *et al*., 2003; Gracio *et al*., 2010; Qin *et al*., 1998).

HPHT NDs and CVD NDs, DNDs have a great tendency to aggregate. To create stable colloids of, DNDs, CVD and HPHT NDs in the suspension, repulsive forces must take place between the individual particles (Krüger *et al*., 2005). *Ergo* aggregate size reduction and nanodiamonds surface modification to increase these forces are required. The non-diamond carbon (graphite) removal from the ND surface also increases colloidal stability. The oxidation can be performed *via* mineral acid treatment or by gaseous oxygen or the air. Besides the non-diamond carbon, these procedures remove also organic aggregation-promoting impurities. (Shenderova *et al*., 2006).

Zeta potential is a key quantitative indicator of the colloidal stability. It is a potential difference between the disperse medium and the stational layer of fluid attached to the dispersed particle. Particles in a suspension with ζ-potential lower than –30 mV and higher than 30 mV are generally considered as stable colloids (Gibson *et al*., 2009).

NDs are biocompatible and show a great potential in optical bioimaging, owing to their specific optical and magnetic properties in association with defects in nanodiamond lattice. *Nitrogen-vacancy centres* (NV centres) were created under high temperature annealing in vacuum for migration of vacancies, which were created during electron irradiation by cleaving carbon-carbon bonds and removal of carbon atoms from the atoms from the lattice. The vacancies move towards nitrogen centre, thus they form NV centres. The NV centres are responsible for ND red or near-infrared fluorescence of wavelength (~700nm). These centres, made of neighbouring nitrogen and a lattice defect called a "vacancy". Since they show high resistance to photobleaching and photoblinking, NDs prove to be an excellent tool for bioimaging (Davies and Collins, 1993; Gruber *et al*., 1997).

The vacancies are formed via by irradiation of the ND, usually with electrons (~2 MeV), protons (~3MeV) and helium ions(~40KeV) (Boudou *et al*., 2009; Gruber *et al*., 1997), which then travel through the lattice until they get trapped by the nitrogen atoms present in the lattice during the thermal annealing, thus creating thermally stable NV centres (Slepetz *et al*., 2010).

A major drawback of fluorescent NDs is their low brightness. New methods enhancing the fluorescence ND properties have been reported (Chi *et al*., 2011; Havlik *et al*., 2013; Stursa *et al*., 2016).

Fluorescent NDs photostability properties are unchanged even after long-time exposure to a light source. In this field fluorescent NDs surpass other bioimaging particles. The high energy irradiation creates NV centres not only on the ND surface, but also under it. That is why, when ND surfaces are modified, NDs retain their fluorescencent properties.

Except the red/infrared fluorescence, a green fluorescence (~600 nm) can be emitted from DNDs, which contain N-V-N centres. The green fluorescence is stable, resistant to photobleaching (Mkandawire *et al*., 2009; Opitz *et al*., 2010).

5.1. Modifications of nanodiamonds surfaces

Surface modifications are a necessity for proper complexation of anionic nucleic acids and ultimately for successfully mediated gene delivery. Colloidal stability in electrolytes is a crucial property when the nanoparticle is *in vitro* or *in vivo*. Unmodified NDs in electrolytes (buffers, cytosol, etc.) tend to aggregate due to their lability, arising from the imbalance between van der Waals forces and the Coulomb forces. Destabilization can be prevented by modifying the ND surface with charged molecules, which increase the electrostatic forces in the environment, or by steric hindrance introduced upon coating the surface with polymers(Liang *et al*., 2011; Slegerova *et al*., 2014).

The individual surface properties of ND vary between the DNDs, CVD NDs and HPHT. The surfaces of NDs are either oxidized or hydrogenated after their synthesis (Arnault *et al*., 2011). Nanodiamonds mostly have oxygenated surfaces, due to an application of water during cooling after the detonation (DNDs) and additionally by oxidizing purification (metallic impurities, sp^2 carbon) of the product (Butenko *et al*., 2006; Pichot *et al*., 2008). The consequence of this process is the formation of functional groups on the nanodiamond surface, *e.g*., carboxyl-, carbonyl-, hydroxyl- groups (Mochalin *et al*., 2009; Pichot *et al*., 2008), although some sp^2 carbon atoms are present on the ND surface in different forms (double bonds or graphite-like structures) (Osswald *et al*., 2006). To completely eliminate carbonyl and carboxyl group from the surface, subsequent hydrogenation at high temperatures is applied. Even though CVD NDs have initially hydrogen atoms attached to their surfaces, however when separating the nanoparticles from the film, hydrogen is partially expelled from the ND surface, thus ND becomes oxidized as well (Landstrass and Ravi, 1989a, 1989b; Neu *et al*., 2011). Carboxyl-, keto- and hydroxyl- group are applicable for adding a variety of functional groups to the ND surface. The –OH modification is probably the most investigated for further functionalizations (Krüger *et al*., 2005; Zheng *et al*., 2009).

The amination of the nanodiamond surface is being highly researched for binding of some functional bioactive molecules. Although the direct amination of the ND surface has proven to be highly difficult with low effectivity and the reaction mechanism is not yet clear (Sotowa *et al*., 2004; Zhang *et al*., 2006). Halogenated surfaces of NDs show improved solubility and seem to represent a good input material for grafting other moieties via nucleophilic substitution, *e.g*., moieties with terminal amino- and carboxyl- group. Especially then fluorine-halogenated NDs, as fluorine proves itself to be an outstanding leaving group, although the reaction mechanism is yet mostly unknown (Liu *et al*., 2004). The substitution of fluorine atoms by amines requires extreme conditions (up to 470 °C), therefore other methods are typically used to achieve similarly modified NDs. The substitution can also be carried out with chlorine atoms instead of fluorine, although harsh environment (300 °C) was necessary (Sotowa *et al*., 2004).

By attaching amino- or OH- groups to the carboxylated ND, amides and esters respectively, are formed on the ND surface. The reaction between the carboxyl- and amino-groups requires auxiliary agents. Consequently, long hydrocarbon chains may be attached to the nanodiamond surface through the amide groups, thereafter the NDs are soluble in hydrophobic solvents. A newly emerged property of a ND modified with octadecylamine (ODA) is its ability to emit blue light (450 nm) after UV light excitation. (Mochalin and Gogotsi, 2009).

Hydroxyl groups on ND surface are utilized for a large variety of reaction, often for formation of ethers, for example by reaction of ND-OH with alkyl-chlorides (Liu *et al*., 2010).

5.1.1. Non-covalent Surface Modifications

Non-covalent modifications present an interesting way to functionalize the nanodiamond. Non-covalent modifications are very flexible and when compared with covalent modifications, they easily establish specific functional group to the ND surface. The NDs present hydrophilic properties with a high amount of oxygen incorporated in the surface-linked functional groups. These structures are prone to create noncovalent hydrogen bonds with other polar molecules. Except the interaction via hydrogen bonds, NDs can adsorb molecules by electrostatic forces based on their individual charge. The formation of hydrogen bonds between the ND and large biomolecules leads to their immobilization. The non-covalent modifications can be easily achieved by incubation of the NDs in a serum containing albumin (Perevedentseva *et al*., 2011), insulin (Shimkunas *et al*., 2009), cytochrome c (Huang and Chang, 2004) or DNA molecule (Purtov *et al*., 2008) with satisfying colloidal stability.

Exploiting the above-mentioned immobilization can be used to coat the ND surface with a homogenous polymer. For example, PLL can be used to enwrap the ND with primary amines, which can be then functionalized or when protonated, can interact with negatively charged molecules (Vaijayanthimala *et al*., 2009). Electrostatic forces can be used to create complex of differently charged molecules ND: PEI: siRNA (Chen *et al*., 2010a). To prepare PEI functionalized NDs, NDs must be oxidated (air or acid-treatment) and sonicated in ion-free water. The solution of NDs is then mixed with cationic PEI, which forms complex with anionic NDs (Zhang *et al*., 2009).

5.1.2. Covalent Surface Modifications

Formation of covalent bonds between the surface of the ND and to-be-immobilized molecules proves to be important, mostly for the site-specific binding of the immobilized molecule in a favourable position, preventing or decreasing the chance of hampering its function. To eliminate non-specific interactions between the ND and the immobilized molecule, linker or spacer is used (Krueger and Lang, 2012). Conformation of the polymers covering the ND surface can be divided into *brushes*, when polymers are situated densely and *mushrooms*, when polymers are situated sparsely around the ND (Netz and Andelman, 2003).

An interesting utilization of ND-OH is achievable by introduction of glycidol to the nanodiamond, creating a polyglycerol (PG)-functionalized NDs. Glycidol is a compound containing both epoxide and alcohol functional group. ND-OH initiates epoxide opening and chain polymerization at high temperature (140 °C) (Zhao *et al*., 2011). Moreover, ND-OH are often modified with *tetraethylorthosilicate* (TEOS) or trialkoxysilanes, functioning as linkers between the ND and the attached moieties (Krüger *et al*., 2006). These linkers, however, are not stable in slightly acidic pH and are prone to hydrolysis, *e.g*. in endosomes and lysosomes.

The most common reactions for covalent interactions during the ND coating are *amidic coupling* (Boudou *et al*., 2013) and *bioorthogonal reactions* (Rehor *et al*., 2014), like click reaction.

To ensure further specific functionalizations of NDs, a large amount of various subsequent reactions was developed. The first discussed method for further grafting is *click-chemistry*. Click-chemistry is a term introduced by K. B. Sharpless *et al.* to describe modular, high-yielding, „wide in scope “, stereospecific reactions that create by-products that are easy to remove by non-chromatographic methods. Moreover these reactions are favourable because of their tolerance to other functional groups and can undergo in water (Kolb *et al*.,

2001). Barras *et al.* used this method to covalently connect molecules with terminal acetylene group to azide-terminated NDs with copper catalyst (Barras *et al*., 2010).

There are two main pathways for polymer coating of NDs. First is grafting from, where the polymer synthesis takes place directly on the ND surface (HPMA, polyglycerol). Second pathway is, grafting to, where the polymer is synthetized separately from the ND and then attached to the ND surface (PEG) (Lee *et al*., 2013; Slegerova *et al*., 2015).

Zhang *et al.* used grafting from method for radical polymerization for synthesis of 2-(dimethylamino)ethyl methacrylate (DMAEMA) polycation modified ND with different chain lengths for further use in transfection of exogenous material (Zhang *et al*., 2011a). Herein highlighted polymers are shown below (Fig. 4).

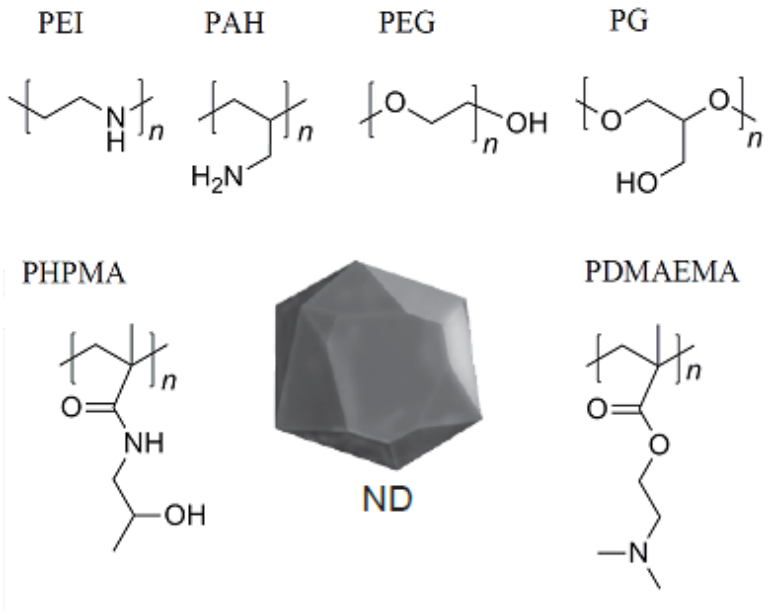

Figure 4: Several strategies for covalent HPHT ND and DND modifications with various polymers.

5.2. Nanodiamonds in transfection

In quick summarization, nanodiamonds are favourable for transfection for their biocompability, surface functionality and fluorescence properties as a fluorescence label. Studies show that the size, shape, surface charge and mostly the surface functionality of nanoparticles determines its intracellular uptake and controls its later fate (Saha *et al*., 2013). NDs were assessed for their cell toxicity in various sizes. These assays proved that NDs are non-toxic to a large variety of cells in comparison with other carbon particles (Mohan *et al*., 2010; Schrand *et al*., 2007). Even though it is generally accepted that NDs exhibit low

cytotoxicity, research shows that NDs with oxidized surfaces may be toxic to the cell. (Wehling *et al*., 2014).

There have been several studies conducted on cellular entry pathway. It has been determined that receptor-mediated endocytosis is the major pathway for cellular entry of NDs by reducing accessibility of ATP. (Schmid and Carter, 1990). When using caveolae-mediated endocytosis, clathrin-mediated endocytosis and macropinocytosis inhibitors, the results show that inhibiting clathrin- and caveolae-mediated endocytosis exhibit almost zero effect on cellular uptake of the ND. On the other hand, when inhibiting the macropinocytosis, ~50% of NDs remain outside the cell, suggesting that macropinocytosis is the major endocytic route for NDs (Chu *et al*., 2015).

After cellular entry, NDs are localized in endosomes for a short period of time and afterwards translocated to cytoplasm and rarely to lysosomes, suggesting early escape from the endosomes. Since NDs do not escape from the lysosomes, causing their damage, rupture and spillage of their content to the cytoplasm (Chu *et al*., 2015). In the following text, several research methods will be thoroughly examined.

Alhaddad *et al.* used 50nm NDs for transfection of siRNA into cells of Ewing sarcoma, a disease causing bone cancer in children. siRNA molecules are homologous to EWS-Fli1 mRNA coding gene, which is expressed as a chimeric protein. The NDs were oxidatively treated and non-covalently modified with PEI and poly(allylamine hydrochloride) (PAH). Naked NDs exhibited -28mV zeta potential, ND-PAH +31mV and ND-PEI +27mV and the size increased to 130nm and 120nm respectively. The increase in their size is supposedly caused by aggregation of NDs. The quantity of amino-groups was determined to be 274 μmol (ND-PEI) and 173 μmol (ND-PAH) per gram of NDs. The maximal concentration of siRNA adsorbed to the ND-PAH particles was carried out when mass ratio was 1:70. On the other hand mass ratio of ND-PEI:siRNA was 1:120. In conclusion, because of the adsorption capacities and zeta potentials, ND-PEI shows inferior siRNA adsorbing ability, when compared to ND-PAH. The cytotoxicity of both NDs and naked NDs was evaluated on fibroblast cells. ND-PAH exhibited some cytotoxicity without adsorbed siRNA. This effect can be elucidated by the positive charges, which are neutralized by positively charged siRNA adsorption, thus the cytotoxicity decreases. Again, ND-PAH is more toxic, when not neutralized, than the ND-PEI, because of their positive zeta potentials. The cytotoxicity of all ND-siRNA complexes was evaluated (<30%) and compared with lipofectamin:siRNA (45%) under the same conditions. For evaluation of siRNA (labelled with fluorescein isothiocyanate, FITC) cellular uptake, complexes with fluorescent NDs (fNDs) were observed through a confocal microscope. ND-

PAH and ND-PEI did not internalize into the cell, therefore the optimal mass ratio has been set to 1:25 and 1:75, respectively. Intensity of the green plot (FITC) and red plot (fNDs) has been evaluated, indicating some co-localization. Results show that siRNA is being released in a form of small particles, not detectable due to the fluorescence intensity. Later, FITC signal co-localized with fND signal strongly decreased to a stable minimum in the case of ND-PEI. In the case of ND-PAH, the signal decreased very slowly, probably for its desorption ability, which is lower, when compared with ND-PEG complexes. Alhaddad *et al*. also found out in another study, that gene silencing is observed only when ND-PEI:siRNA enter the cell *via* macropinocytosis, thus proving previously mentioned ND internalisation pathway. On other hand, ND-PAH was not affected by macropinocytosis inhibition. The experiment showed that ND-PAH and partially ND-PEI are involved in clathrin-mediated endocytosis (Alhaddad *et al*., 2012). The ability of siRNA to interfere with the EWS-Fli1 mRNA was evaluated by quantitative PCR of the mRNA. The free siRNA did not exhibit any inhibition of the expression. When vectorised with ND-PAH or ND-PEI, expression was decreased to 50% and 45% respectively. When lipofectamine was used as a vector, expression decreased to 35%. Although when in medium with serum, lipofectamine:siRNA shows only 20% inhibition of expression. ND-PEI:siRNA promotes 50% inhibition under the same conditions (Alhaddad *et al*., 2011), thus proving that ND-PEI is the best of these three examined vectors for transfection under physiological conditions for its lower cytotoxicity, better desorption and the expression inhibition mediated by siRNA

Zhang *et al.* modified NDs non-covalently with crosslinked non-toxic PEI (800Da) by simple adsorption mediated by hydrogen bonds and electrostatic interactions with oxidized surfaces of the NDs. Another set of NDs was functionalized covalently with (3-aminopropyl)-trimethoxysilane. Firstly, the carbonyl functional groups of NDs were reduced to hydroxymethyl groups succeeded by silanization using the (3-aminopropyl)-trimethoxysilane, creating ND-NH_2 particles. Size of naked fNDs was 50 nm, because of cluster formation. Functionalized ND-PEI800 increased 2-fold in their size when compared to naked fNDs. Plasmid DNA was electrostatically immobilized on modified nanodiamonds. The zeta-potential of both modified ND:pDNA complexes was measured. When N:P ratio corresponded to 5:1, neutral ζ potential was observed. When ratio increased above 5:1, the ζ potential of both modified complexes with pDNA increased to a stable level between 35-45 mV. HeLa cells were transfected with the pDNA carrying gene for luciferase. When both PEI and R-NH_2 modified NDs are compared with the naked NDs, their ability to enter the cell is increased by ~50% after 5 h of incubation. Cytotoxicity of all ND particles was evaluated and compared with 800Da PEI polymer and

25kDa PEI polymer. When incubated with ND, ND-PEI800 and PEI800, the HeLa cell viability exceeded 80%. When PEI25k was used, the cell showed <40% viability with N:P ratio 3-fold lower than with other vectors. When all vectors mentioned in above were complexed with pDNA their cytotoxicity was lowered. Zhang proposes that the cytotoxicity may be caused by aggregated micrometre-large particles with disruptive effect on the cellular membrane and nucleus. The gene expression efficiency induced by the transfected pDNA decreased in the following order: ND-PEI800 > PEI800 > ND-NH_2 > ND > naked DNA. ND-PEI800 showed up to 400-fold more efficient than ND-NH_2 and 800-fold more efficient than naked NDs. Although PEI25k expressed ~3-times higher transfection ability, PEI25k is also more cytotoxic when compared to ND-PEI800 or PEI800 (Zhang *et al*., 2009). When compared to experiment by Alhaddad *et al*., the higher cellular uptake and lower cytotoxicity may be caused by the physiology of the cancerous cells.

Chen *et al.* prepared (initially carboxylated) NDs modified with crosslinked PEI800 non-covalently by mixing an excess PEI800 with NDs in ratio of 20:1. After functionalization of NDs with PEI, siRNA was added. ND-PEI complexes showed positive ζ potential. siRNA was electrostatically immobilized on the ND-PEI particles. ζ potential of complexes with siRNA changed to neutral charge and subsequently to positive charge with the increase of ND-PEI present. *Via* gel electrophoresis, the complete loading of siRNA to ND-PEI was determined to be at 1:5 w/w ratio siRNA:ND-PEI. Although at this ratio, complexes aggregated into 2611nm sized particles. For cellular internalization 1:3 w/w ratio was used in breast cancer cell culture. At this ratio, 97% of ND-PEI:siRNA complexes was internalized, although GFP knockdown reached 23%, probably due to slow release of siRNA. For comparison, lipofectamine-mediated cellular entry and GFP knockdown reached 99% and 45%, respectively. When in environment with serum, ND-PEI showed better transfection properties in comparison with lipofectamine, confirming the above-mentioned result. Lipofectamine-mediated transfection resulted in 26% cytotoxicity, whereas ND-PEI800 caused only ~3% cytotoxicity(Chen *et al*., 2010b).

Bertrand *et al.* prepared hydrogenated NDs (ND-H) by mixing DNDs with hydrogen plasma. Originally 7nm ND-H particles aggregated to 30 nm in diameter particles with net zeta potential +55 mV. siRNA binding properties were assessed and showed the minimal weight ratio for binding 80% of siRNA – 50:1. Internalization into Ewing sarcoma cells efficacy of ND-H was evaluated by using fluorescent siRNA labelled with FITC. Free siRNA was not observed inside the cell. When w/w ratio was >10:1, ND-H was detectable in the cytosol. The cellular entry *via* clathrin-mediated endocytosis, endosomes and macropinocytosis pathway

was confirmed by TEM analysis. Some of the ND-H:siRNA complexes were localized outside the vesicles, suggesting vesicular escape. For evaluation of expression interference, siRNA with homologous sequence for EWS/FLI-1 mRNA was transfected. When cells were treated with ND-H:siRNA, 70% inhibition of gene expression was observed. At mass ratio 50, cationic ND-H lowered cell viability by more than 50%, whereas ND-COOH showed lower cytotoxicity. This may be due to aggregation of cations onto the cell membrane (Bertrand *et al*., 2015).

Zhang *et al.* covalently coated NDs with polymeric DMAEMA brushes (Fig. 5). To achieve carboxy-group on ND surface, NDs were oxidatively treated with H_2SO_4 and HNO_3.

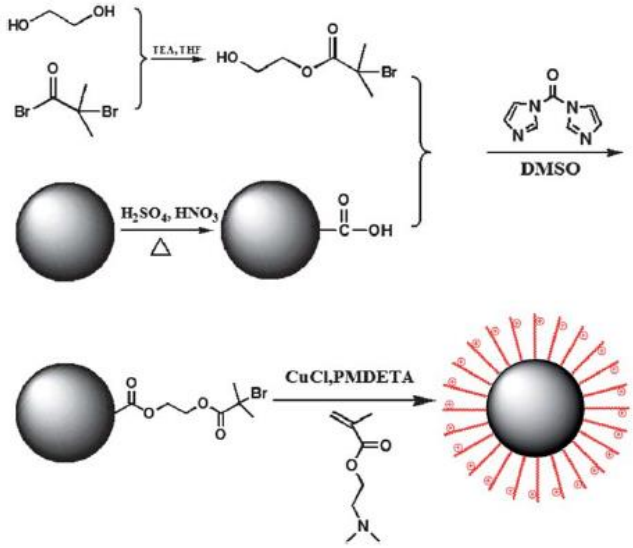

Figure 5: Scheme of ND-pDMAEMA brushes synthesis route; tetrahydrofuran (THF); tetraethylamine (TEA) (Zhang *et al*., 2011b)

Hydroxyethyl-2-bromoisobutyrate was added into solution of ND-COOH particles dispersed in dimethyl sulfoxide (DMSO), thus creating *nanodiamond initiator* ND-Br. Finally, two different amounts of DMAEMA (DMAEMA-1 with higher concetration and DMAEMA-2 with lower concetration of the monomer in a solution) was introduced to ND-Br at w/w ratio 100:1. *Via* zeta potential analysis, raw NDs exhibited -4mV charge, ND-COOH -17 mV, ND-PDMAEMA-1 +42.7 mV and PDMAEMA-2 +51.3 mV. The pDNA complexation ability was evaluated by gel electrophoresis. ND-PDMAEMA-1 and -2 retarded pDNA at weight ratio 0.8 and 0.3, respectively, thus proving that ND-PDMAEMA are able to condense nucleic acids at very low weight ratio. In comparison with PDMAEMA homopolymer immobilized pDNA at the same weight ratio as ND-PDMAEMA-2. The size of vector:DNA complexes was investigated by TEM imaging. ND-PDMAEMA-1, -2 and PDMAEMA with weight ratios 10, 6, 10 were 143 nm, 52 nm and 205 nm in diameter. Zeta potential of vectors with complexated DNA decreased in following order: ND-PDMAEMA 2 > ND-PDMAEMA-1 > PDMAEMA. The unexpected reason of low ζ pontential of PDMAEMA:pDNA comlexes can be caused by its topological structure,

where polymer chains bury into the core of the condensate, thus lowering the surface positive charge. To examine stability of the complexes, heparin was used (charged polyanion) to competitively replace DNA in the complex. Gel electrophoresis output showed ND-PDMAEMA to surpass PDMAEMA in complex stability. To test the vectors protection property of the DNA, DNase I was used. ND-PDMAEMA:DNA complexes showed almost no degradation. PDMAEMA:DNA complexes, on the other hand, degraded almost entirely. Luciferase reporter gene was used for *in vitro* gene transfection efficacy of the vectors. Indisputably, ND-PDMAEMA-2:DNA surpassed other vectors, including PEI25k, most efficiently at 6:1 weight ratio. After endocytosis, a small portion of ND-polymers was detected by laser-scanning confocal microscopy inside the nucleus, which is favourable for gene delivery. Cytotoxicity of vectors was assessed. When w/w ratio was up to 2:1, all ND-PDMAEMA complexes showed insignificant cytotoxicity. When the ratio reached 10:1, ND-PDMAEMA-2 caused 74% decrease in cell viability, whereas ND-PDMAEMA-1 showed approx. 10% decrease. This difference can arise from the different net positive charge. Still, the ND-PDMAEMA show to be less cytotoxic and more transfection-effective than PEI25k (Zhang *et al*., 2011b).

6. Other transfections reagents and nanodiamonds

Nanodiamonds were already thoroughly described in previous chapters according to their physical, chemical and biological properties. Herein the transfection abilities of several different nanoparticles will be described in comparison with nanodiamonds.

6.1. Properties of nanoparticles

Like nanodiamonds, MSiNPs are inorganic nanoparticles, which are applicable in transfection. MSiNPs' main advantage in transfection, when compared with NDs, is their large surface area. MSiNPs have diameter of 100 nm in average with surface planes equal to ~900 m^2/g with pores sizing from 2 to 6 nm. These parameters can be altered during the MSiNP synthesis by tuning the reaction conditions and the relative amount of reagents (Kosuge and Singh, 2001). The overall structure of the MSiNP is honeycomb-like with hexagonal porous channels. Another advantage of MSiNPs over NDs is a possibility to immobilize large amount of cargo molecules both on the surface and in the pores of the MSiNPs, thus allowing co-delivery of different moieties. Unlike NDs, MSiNPs do not contain any fluorescence centres. Therefore the MSiNPs require modifications with fluorescent molecule for bioimaging and tracking the nanoparticles during the transfection (Slowing et al., 2008a).

Another inorganic nanoparticle, which is often used in transfection is an iron oxide nanoparticle. Iron oxides are commonly ferromagnetic, which means that they can be permanently magnetized. When size of IONPs is ~15 nm, the ferromagnetic phenomenon is no longer observed. Although the particles still show their superparamagnetic character. An advantage of the IONP is their superparamagnetism, which enables their stability in solutions after the removal of the magnetic field (Gupta and Gupta, 2005). NDs, on the other hand, require their surfaces to be specifically treated to achieve colloidal stability (Krüger et al., 2006). Additionally, the superparamagnetic properties of IONPs are exploited in MRI, where IONPs are utilized as contrast reagents (Chen et al., 2009). Like MSiNPs in comparison with fNDs, IONPs must be modified with fluorescent moieties for imaging through confocal microscopy methods.

Unlike inorganic NDs, which are not biodegradable, organic nanoparticles, like polymersomes and liposomes are commonly degradable organic nanoparticles. Although studies show that long-term accumulation of NDs in the cell show no cytotoxic effect (Fang et al., 2011). Polymersomes are advantageous for their controlled nucleic acid release, through systematic biodegradation (Corsi *et al*., 2003).

6.2. Cellular uptake

Pathways of internalization of silica nanoparticles are resembling the same endocytic pathways used by NDs during the cellular entry (clathrin-mediated endocytosis, macropinocytosis), although the inhibition of clathrin-mediated endocytosis has low effect on ND internalization. (Chu et al., 2015; Slowing et al., 2006, 2008b). Chu *et al*. proved that unmodified silica nanoparticles remained in endosomes after the cellular uptake and were degraded afterwards. When NDs were used in the same experiment, nanoparticles were found in the cytoplasm, showing their early endosomal escape. The same effect was observed, when both NDs and MSiNPs were internalized at the same time. This might be explained by rupture of the endosomal compartment, which was caused by NDs (Chu *et al*., 2015). When modified, both NDs and MSiNPs achieved >80% cellular entry efficiency (Alhaddad et al., 2011; Slowing et al., 2006; Xia et al., 2009a).

Because of the high variability in the structure and the composition of organic polymersomes, cellular entry of these organic nanoparticles varies greatly. Through inhibition of several pathways, two main uptake mechanisms were determined: caveolae-mediated and clathrin-mediated mechanisms. Therefore, these main endocytosis pathways are different, when compared with NDs. When macropinocytosis, (which Chu *et. al*., proposed

as the main endocytosis pathway of NDs) was inhibited, cellular uptake of polymeric nanoparticles decreased by 30%. The cellular uptake of polymersomes is cell-dependant as well as, cargo-dependant and polymer-dependent (Chu et al., 2015; Nam et al., 2009).

Lipofection, liposome-mediated transfection, is frequently used for siRNA delivery. As well as NDs, liposomes enter the cell *via* macropinocytosis, but also *via* clathrin-mediated endocytosis(Lin et al., 2013). Additionally, in comparison with NDs, liposomes interact with the endosomal membrane and escape the endosome *via* different mechanism. As it was already mentioned, protonated lipids of the liposome interact with the endosomal membrane, by changing between the lamellar and inverted hexagonal (H_{II}) phases. This process leads towards destabilization of the endosome by its fusion with the liposomes and consequently releasing the nucleic acid into the cytosol (Bell *et al*., 2003; Koltover *et al*., 1998). Gene silencing efficiency and toxicity

For evaluation of MSiNPs' siRNA transfection efficiency and subsequent comparison with NDs a study conducted by Xia *et al.* was singled out for its similar conditions with previously reviewed research by Alhaddad *et al.* The assessment of siRNA transfection efficacy was conducted in HEPA-1 cells by knocking down the GFP protein. Gene silencing efficiency of MSiNPs modified with PEI was evaluated by confocal microscopy and it reached 55-60% with very high cell viability (Xia *et al*., 2009b). These results show slightly better gene silencing efficiency than the methods utilizing NDs with similar functional moieties, where Alhaddad *et al* and Chen *et al.* achieved 45% and 23% efficiency, respectively.

IONPs can be also modified with PEI for siRNA transfection. Liu *et al.* transfected siRNA complexed with alkyl-PEI-IONPs into breast cancer cells with stable luciferase expression. The knockdown efficacy of mRNA homologous with siRNA was observed *via* confocal microscope and by MRI. Similarly to PEI modified NDs, the efficiency increased with the increase of N:P ratio, ranging from 20% to 50% with very low cytotoxicity (Liu *et al*., 2011). When IONPs are modified with poly(dimethylaminoethyl acrylate) (PDMAEA) the transfection efficiency of siRNA was approximately 25%. Interestingly, when the IONP-PDMAEA-siRNA complexes were transfected in the presence of magnetic field, the gene expression decreased to ~50%. Owing to a protective shell on top of the PDMAEA layer, complexes demonstrated almost zero cytotoxicity (Boyer et al., 2009). The range of transfection efficiency and cytotoxicity values are again comparable with values of NDs.

Polymersomes are nanoparticles, which are commonly used for delivery of genetic material cells. Recently conducted studies show high transfection efficiency and consequently high polymer-mediated siRNA gene silencing is polymer-dependant. When compared with

the ND-mediated efficiency of gene silencing (up to ~50%), polymersome-mediated efficiency is ranging approximately between 40% – 90%, although this efficiency is often accompanied with high cytotoxicity, whereas NDs exhibit low levels of cytotoxicity (Ge et al., 2013; Kim et al., 2009; Yang et al., 2014b).

Liposomes present several setbacks in gene delivery applications such as leaking of the transfected nucleic acid associated with their long-term instability, in contrast to NDs, which are stable and there was not observed any leakage of nucleic acids from the ND surface (Bradley et al., 1998).

7. Conclusion

This bachelor thesis focuses on transfection of small interfering RNA, a gene delivery method, where siRNA is transported into a cytosol of a eukaryotic cell, where it silences gene expression by RNA interference. Naked siRNA is unable to efficiently pass through the cellular membrane, therefore, it can be delivered by transfection agents. In past few years, NDs were used for siRNA delivery. NDs modified with cationic polymers can adsorb siRNA electrostatically to form complexes able to deliver siRNA into a cell.

Herein, methods of preparation and utilization of modified NDs in transfection were reviewed. There are three types of NDs, HPHT NDs, DNDs and CVD NDs. These NDs are modified either non-covalently or covalently with cationic polymers, for example with PEI, DMAEMA or PAH. When modified covalently, two methods are being distinguished: grafting from, where polymers synthesis takes places on the ND surface, or grafting to, where polymer is synthetized separately. Additionally, other nanoparticles utilized in transfection were characterized and compared with NDs regarding their properties, mechanism of cellular uptake and gene transfection efficacy. IONPs, in comparison to NDs, do not require surface modifications to be colloidally stable. The pathways of cellular uptake of NDs are comparable with other inorganic nanoparticles, in contrast to organic nanoparticles, which use different pathways of cellular uptake. Studies show that the shape of nanoparticles plays a role in endosomal escape. Spiky NDs exhibit more efficient endosomal escape than for example rounded silica nanoparticles. The siRNA transfection efficiency of nanoparticles varies, depending on experiment conditions. NDs mediate gene transfection ability leading to efficient silencing comparable to other inorganic nanoparticles. However, in comparison to other nanoparticles, NDs are advantageous because of their intristic fluorescence properties emitted from NV centres, which exhibit stable fluorescence with no photoblinking or photobleaching and can be therefore observed in cell for unlimited period of time.

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