Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *88* (11) 1065–1087 (2023)
JSCS–5681
Review
Published 10 November 2023

REVIEW

Chemically-assisted DNA transfection methods – An overview

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(Received 21 December 2022, revised 21 January, accepted 9 April 2023)

Abstract: Non-viral chemical-based methods for *in vitro* cell transfection are commonly used to incorporate foreign gene of interest into mammalian cells due to numerous benefits – high efficiency, low cost and simple methodology. These powerful transfection methods generally do not possess safety risks as virus-based, and cell toxicity is significantly reduced. To obtain transfectants, host cells are usually treated with biocompatible DNA carriers such as calcium phosphate, cationic lipids, DEAE-dextran, polyethylenimine or dendrimers, classifying these methods based on chemical reagents used. All these different approaches are based on the similar principle, namely formation of encapsulated amphiphilic complexes between DNA and various particles, following cell uptake, most likely mediated by endocytosis. Depending on the aim and design of experiment, the choice of appropriate method is made. This review article outlines strategies of the most widely used chemical transfection techniques, pointing out advantages and limitations of different DNA carriers, also findings of researchers as how to optimize and enhance efficiency of gene delivery procedure. With methodology constantly being improved, transfection methods described here find their main, biomedical application in gene therapy, a promising way to introduce functional copy of exogenous gene to genetically defective target cells.

Keywords: calcium phosphate; cationic polymers; gene delivery; lipofection; non-viral transfection.

CONTENTS

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https://doi.org/10.2298/JSC221222019B

1. INTRODUCTION

A widely used laboratory technique called transfection underlies the introduction of foreign nucleic acids into host cells and the study of gene and protein expression in cellular environment. Chemically-assisted transfection methods catalyze intracellular trafficking of nucleic acids through the use of various compounds and serve as chemical tool that enables advancement in drug discovery research.[1] The basis of this chemical method is interaction between negatively charged nucleic acids and positively charged ions of chemical reagents.[2]

Exogenous DNA must cross different barriers prior to nuclear uptake and gene expression. Unprotected plasmid DNA would be degraded inside the cell by nucleases in a very short period of time, so it has to be encapsulated with appropriate carriers/particles or condensed with high packing density polycationic particles.[3] Avoiding enzymatic degradation depends on the stability of the complex between DNA and transfection agent, as well as the cell type. Upon entering the cell by endocytosis, the endosome matures and vesicle fusion between matured late endosome and lysosome occurs. As a result of increased osmotic pressure and destabilization of lysosomal membrane, endosome escapes in the next step. After bursting, vesiclular content is released into the cytoplasm.[4] Following burst of endolysosome, complexes further enter the nucleus by not fully understood mechanism and plasmid DNA is released into the nucleus, resulting in transcription of gene of interest.[5,6] On the other side, destiny of complexes that did not successfully leave the endolysosome is degradation by lysosomal enzymes.[4]

Expression of the functional eukaryotic protein in bacteria is often a problematic task. Protein expression is significantly improved by using eukaryotic cell cultures due to possibility of post-translational modifications and correct protein folding in this system.[7,8] Introduction of foreign nucleic acids into cultured mammalian cells, enabled by a powerful transfection technique, also known as gene delivery, has revolutionized the study of gene function and expression of specific proteins.[9,10] Furthermore, this technique is promising in the prevention and treatment of genetic disorders and diseases such as cystic fibrosis,[11] hemophilia,[12] dystrophy[13] and cancer,[14] through an innovative approach in biomedicine, namely gene therapy.[15] This up-to-date therapy, where genetically defective target cells are modified by the introduction of an exogenous functional gene, greatly attracts the attention of the scientific community.[11–15] Except repair of genetic damage, with gene therapy treatments it is also possible to treat infectious diseases[16] by inhibiting life cycle stages, as well as malignancies.[14] Although

this type of therapy promises a lot, development of new, effective and safe therapeutics in this field is very slow and demanding.[17]

There are two types of transfection – stable and transient, with the main difference in long-term integration of foreign DNA into the host genome in stable transfection, whereas in transient type transfected gene is not integrated, so expression lasts for only a limited period of time.[18,19] Depending on the purpose and scope of research, choice between creating stable or transient cell lines is made. When required to examine effects of short-term expression of a gene or protein product on a small scale, transient transfection is mainly method of choice as well as in the assays that precede creation of stable cell lines. On the other side, although stable transfection is a complex process, it plays an unavoidable role in revealing mechanism of gene regulation, large scale protein production or pharmacological studies.[20,21] Experimental methods used to create recombinant cell lines can be classified as direct, when the gene of interest is introduced in nucleus directly by microinjection, and indirect, where the transfer of genetic material occurs indirectly through complex formation between DNA and various chemical agents, using viral vectors or by physical force-mediated cell uptake. In addition to this classification, there is another one that classifies transfection methods to biological (use of viral vectors), physical (sonoporation, electroporation, magnetofection, phototransfection) and chemical (use of calcium phosphate, cationic polymers, cationic lipids).[10,19,22]

The main goal of transfection process is to efficiently deliver gene of interest into cells without safety risks. Non-viral carriers are developed as an alternative to viral ones, not only due to their straightforward manipulation, but also due to reduced cell toxicity and absence of immune response or potential mutagenicity.[23] Much effort is put into developing vectors that are not toxic for cells. Motivated by the significance of chemically-assisted transfection methods in biomedicine and importance of availability of summarized information related to their methodology and efficiency improvement, we provide in this paper an overview of currently available and the most commonly used methods for DNA transfection of mammalian cells based on the use of chemical reagents. Short description of the chemically-based transfection methods and their advantages and disadvantages are summarized in the Table I.

2. CALCIUM PHOSPHATE-MEDIATED TRANSFECTION

Among chemical methods, calcium phosphate (CaP) precipitation is one of the most commonly used due to its numerous advantages.[24] The first method developed for mammalian cell transfection was actually CaP based method initially performed in 1973 by Graham and van der Eb for the introduction of adenoviral DNA into mammalian cells.[25] Adenovirus type 5 DNA was successfully adsorbed by human KB cells in monolayer culture.[25] Widespread use of this transfection method in biomedical research was demonstrated through the gener-

TABLE I. Overview of chemically-based transfection methods

Type of transfection	Description	Advantages	Disadvantages
Calcium phosphate--mediated transfection	Widely used method for introducing foreign DNA into cells that includes mixing DNA with calcium chloride and phosphate ions, formation of coprecipitates and cell uptake.	-Use in biomedical research -Simplicity -Cost-effective -Applicability to large number of cell lines -Biocompatibility -Suitable for stable and transient transfection -Safe use	-low efficiency -Sensitive to changes in pH and salt concentration -Low reproducibility -Phosphate-free medium required -Serum-supplemented medium required -Low efficiency in most primary cell lines
Lipofection	This commonly used transfection method is based on the use of cationic lipids to deliver DNA to eukaryotic cells in the form of vesicles called liposomes.	-High efficiency -The most extensively studied types of DNA carriers -Simplicity -Commercially available reagents -High reproducibility -Suitable for stable and transient transfection -Large scale use -Safe use -Induction of anti-inf-lammatory response	-Cytotoxicity -Inactivation in the presence of serum proteins -Expensive
Cationic polymer--mediated transfection	This method uses cationic polymers such as DEAE--dextran, PEI and dendrimers to form complexes with DNA and introduce nucleic acids into cells of interest via electrostatic interactions.	-Simplicity -Cost-effective -Applicability to large number of cell lines -Structural versatility of cationic polymers -Biocompatibility -Large scale use -Efficient cell recovery after transfection	-Low efficiency (for stable transfection) -Limited to short-term transient transfection -Inactivation in the presence of serum proteins -Expensive synthetic procedures -Cytotoxicity

ation of numerous highly productive recombinant cell lines.[26] Benefits of divalent calcium cations, as DNA carriers in the transfection process, are related to their natural presence in many cells in the organism and physiological acceptability.[27] Principle of this method includes mixing DNA with calcium chloride in phosphate buffer and adherence of resulting DNA-CaP coprecipitates on the surface of the cell membrane.[9]

Endocytosis (Fig. 1) and direct penetration through the membrane are most likely the primary mechanisms of CaP–DNA complex cellular entry.[3,28] One of

the potential mechanisms of endocytotic uptake at the intracellular level is described in the work of Neuhaus *et al.*[4] Using specific inhibitors, Olton *et al.* demonstrated that uptake of NanoCaPs–DNA complexes into the cell and subsequent gene expression were mediated by both endocytosis types, clathrin- and caveolae-dependent, whereas the former one was more highlighted prior to this study.[29]

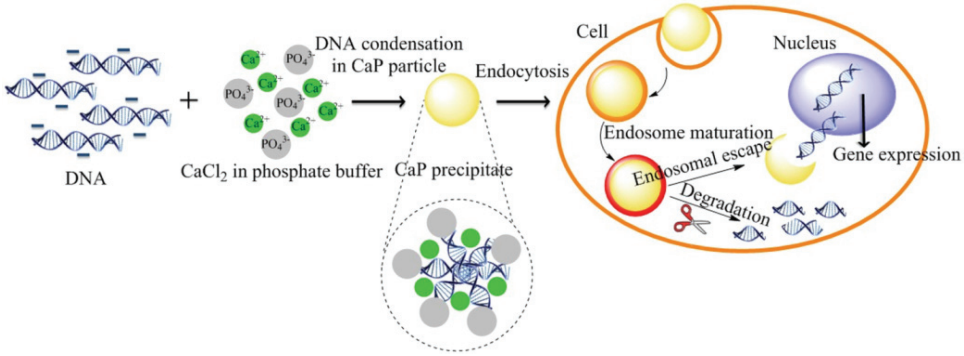

Fig. 1. Proposed endocytotic mechanism of CaP-mediated cell transfection.

This method is characterized by high efficiency, simplicity, low cost, applicability to a large number of cell lines, biocompatibility and it is suitable for both, transient and stable transfection.[27] It is also method of choice when co-transfecting multiple plasmids.[27] Disadvantages include significant changes in transfection efficiency due to small variations in pH and difficulty in reproducing conditions for creating coprecipitates of adequate size and quality.[26] Furthermore, a medium with already high amount of phosphates is undesirable for this type of transfection procedure and this gene delivery method is effective only on highly differentiated cells but not on primary cell lines or animals.[30] Potential difficulties related to transfection of high copy number plasmids by CaP precipitation method are requirements for serum-supplemented medium, often avoided during recombinant protein production in cell lines and low efficiency.[24] The highest efficiency of exogenous DNA uptake was achieved in 80–90 % confluent cells that divide quickly, as well as in those growing in monolayer due to uniform precipitation.[2] Moreover, glycerol and dimethyl sulfoxide (DMSO) are shown to increase efficiency of DNA delivery into some cell lines, however, exposure time to these agents is limited since they may exhibit cytotoxicity.[20,21]

In order to examine optimal conditions and achieve the highest efficiency, the original CaP method has undergone numerous modifications and optimizations since it was published. In the original protocol HEPES buffer (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)) was used.[25] However, many variations in buffer composition were subsequently tested and conditions were individually optimized for specific cell lines. Chen and coworkers significantly

improved effectiveness of this method by replacing HEPES with lower pH buffer, BES (*N*,*N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid).[31] During cell growth in 3 % CO_2 atmosphere at lower pH (6.95), DNA–CaP precipitates are formed gradually and uniformly on the cell surface, resulting in reduced cytotoxicity and increased transfection efficiency, most likely due to uptake of precipitates by a larger number of cells. Furthermore, transfection efficiency was reported by Chen *et al.* to be enhanced with the use of circular plasmids instead of linear ones, that are easily degraded by nucleases from culture medium.[31]

In the paper of Jordan *et al.* it was stated that formation of CaP–DNA coprecipitates for successful transfection is possible only in a narrow range of conditions and is mostly dependent on the concentration of calcium and phosphate ions and other physicochemical factors – DNA concentration, temperature and reaction time.[32] Optimized procedures were applied to both, transient and stable transfection, and, using them, greater efficacy than in previous protocols was achieved.[32] Study of Ling *et al.* posed the same question.[30] Key parameters responsible for high transfection efficiency of highly differentiated cells were estimated to be primarily characteristics of the buffer, suggesting HBS buffer with pH 7.10 as an optimal, then presence of fetal bovine serum in the medium, vortexing cells with precipitant particles and glycerol shock, whereas replacement of the consumed medium with fresh before transfecting cells had no effect on transfection potency.[30] Sun *et al.* demonstrated that, with optimization of this method, it is possible to transfect cell types such as primary neurons, a popular target in neural cell biology.[33] Moreover, study of Sariyer provides a high-efficiency protocol for transfection of primary neuronal cultures.[34]

Due to many factors that affect efficiency including pH, salt and DNA content, the period between precipitation and transfection, type of the cell line and the researcher's skills, it is very challenging to standardize the CaP transfection technique.[15] These conditions must be optimized for each cell line and laboratory. Transfection has to be performed shortly after precipitation, otherwise CaP will lose activity when it reaches microcrystal size.[27,28] In order to maintain appropriate size of calcium phopshate particles and inhibit their further growth, functionalization with various organic molecules is preferred.[14] To overcome this problem, several strategies for controlled growth of CaP nanoparticles have been developed, including polymer stabilization or lipid coating.[35] DNA may be adsorbed onto CaP nanoparticles with added additives to maintain their size.[27] Obtaining particle size in submicrometer range facilitates penetration of DNA––CaP complexes through the cell membrane. Additionally, it is of great importance to focus on enhancing stability of nanoparticle-DNA complexes, as well as on biodegradable properties of carrier, especially useful in gene therapy. Working in the field of cancer gene therapy, Liu and coworkers developed modified

formulations of CaP nanoparticles as vectors for efficient DNA delivery into cancer cells.[14,36]

3. LIPOFECTION

Cationic lipid mediated transfection (lipofection) is a bright spot in the field of gene therapy and significantly superior in clinical trials compared to other transfection methods.[37] Therapeutic potential of cationic liposomes was tested many times in clinical trials in patients with cystic fibrosis.[38] In the late 1970s Felgner *et al.* first demonstrated procedure for cell transfection using a positively charged cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), which forms spherical vesicles in aqueous solution with one (unilamellar) or more (multilamerllar) concentric phospholipid bilayers, called liposomes.[39] Cationic lipids are actually the largest and the most extensively studied group of non-viral DNA carriers widely used today. Currently, there is a growing interest in the development of new reagents for successful lipofection and some of them have already found their way to the market. Numerous commercially available cationic lipid transfection reagents from different manufacturers (lipofectamine 3000 or 2000, lipofectin, cellfectin, effectene, FuGENE 6, and DOTAP) vary in price, efficiency and application in different cell types.[40]

Cationic lipids are amphiphilic molecules generally built of three structural domains – positively charged polar heads, hydrophobic domain and linker between them, and even small changes in the structure of any of them can significantly affect transfection efficiency. Cationic head is usually composed of primary, secondary or tertiary amines, but can also contain imidazole and guanidino groups.[40] Byk *et al.* described synthesis of novel cationic lipids as gene delivery agents and conducted structure–activity relationship (SAR) studies to explain differences in transfection potential of geometrically different, asymmetric polyamine groups in cationic heads.[41] Hydrophobic moiety generally represents doubly saturated or unsaturated hydrocarbon chain of varying lengths, which does not have to be symmetrical in structure, whereas linkers are usually esters, ethers, amides or carbamates.[40] A wide range of different linkers, building elements of cationic lipids, is described in detail in review article of Zhi *et al*.[42] Within the liposomes, hydrophobic components of cationic lipids are facing inside of the vesicle and they are protected from aqueous solution by the presence of polar heads on the surface of the molecule. In the central part of the liposome there is a cavity where DNA of interest is packed for delivery and protected from degradation by various enzymes after cell uptake. Review article of Niculescu-Duvaz *et al.* covers SAR studies of cationic lipid domains and helps understanding mechanism of liposome formation and action providing an excellent basis for the rational design of new improved transfection vectors.[40]

Briefly, the mechanism of cationic lipid mediated transfection is divided into several steps: lipoplex formation, membrane binding, entry into the cell, endosomal escape and finally nuclear entry and expression of gene of interest.[40] Electrostatic interactions are formed between liposomes, due to polar heads with overall net positive charge, and negatively charged phosphate groups of transfecting nucleic acids. Penetration of lipid-associated DNA through the hydrophobic cell membrane is facilitated by neutralization of anionic groups and entry into the target cell in the form of lipoplex is probably mediated via endocytosis or fusion (Fig. 2).[38,39] Total charge of lipoplexes is positive enabling them to bind to negatively charged surface of the cell membrane.[39] Following nonspecific electrostatic interaction with the cell membrane lipoplexes are introduced into intracellular compartments dominantly by endocytotic mechanism and this whole process of internalization is mostly influenced by the size of lipoplex. Next phase of lipofection involves endosomal escape which is mediated by flip-flop mechanism due to lipid nature of both, lipoplex and endosomal membrane. In response to the phase behavior of these lipid bilayers, DNA is released after complete neutralization of cationic lipids,[43] which was also investigated and described by Xu and Szoka.[44] Their conclusion was that lipoplexes destabilize endosomal membrane, reducing the intensity of electrostatic interactions between DNA and cationic lipids and releasing DNA to the cytoplasm.[44] There are two theories about the entry of released DNA into the nucleus-by passive transport during cell division, when nuclear membrane is temporarily ruptured, or by active transport through the nuclear pores.[43] Besides endocytosis, dominant pathway for DNA delivery inside the cell – fusion of cationic liposome with the cell membrane is also possible mechanism. Which mechanism of these two will take place depends on the liposomal formulation itself.[38]

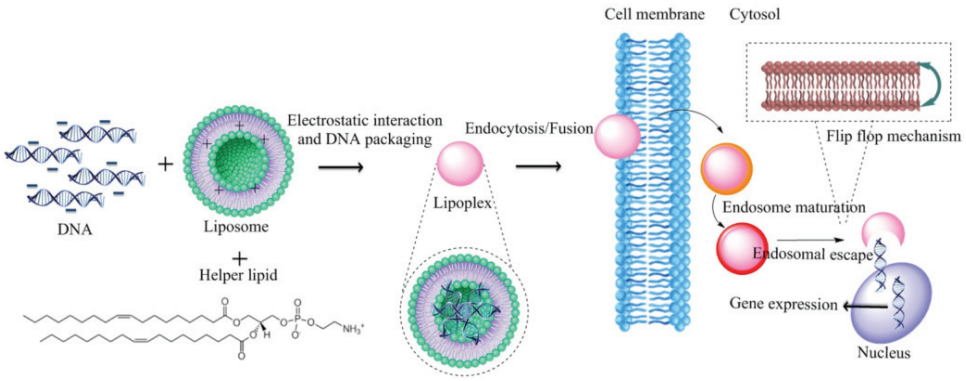

Fig. 2. Proposed mechanism of cationic lipid-mediated transfection.

Key features of this cationic lipid-mediated transfection process are simplicity, high efficiency (especially in adherent cell lines), reproducibility and

applicability to both, transient and stable transfection types, as well as to *in vivo* models, where it has shown lower efficacy indeed.[39,45] Genetic material owes protection from degradation to stability and structural properties of liposomes, but in order to avoid possible cell toxicity, it is necessary to find balance between optimal conditions, crucial for transfection efficiency and potential toxic effect on cells.[46] As expected, when considering the principle of this method, one of the essential factors for successful lipofection is optimal ratio between liposomal and DNA component. However, if this ratio moves in favor of the first component, cytotoxicity may occur.[45] Liposomes are suitable for large-scale production, their use is safe [47] and, significantly, there are no limitations to the size of the DNA molecules that can be transfected.[48]

Depending on the cell type, this technique has proven to be up to 100 times more effective than transfection using CaP or cationic polymers.[39] Routinely for performing this procedure, cells are grown in serum-free media. It is likely that negatively charged serum proteins interact with cationic liposomes and destabilize them, dramatically reducing efficiency of transfection process. Yang and Huang separated serum proteins by chromatography and tested efficacy of lipofection in the presence of both fractions.[49] Except introducing serum-free media, that is impossible in *in vivo* conditions, they suggested overcoming this problem by increasing the charge ratio between cationic lipid reagent and DNA molecule or by adding positively charged polylysine to neutralize multiply negatively charged serum proteins.[49] Ross *et al.* attempted to overcome reduced transfection potential in the presence of serum by controlled growth of lipoplexes and concluded that key parameter for successful transfection is appropriate size of lipoplex, crucial for association with the cell membrane.[50] Since it was shown that inhibition of lipoplex formation by serum proteins is actually the cause of lower efficiency of lipofection-mediated gene transfer, they developed a protocol for fine regulation of lipoplex growth in polyanion-containing medium that stopped at a crucial moment by serum supplementation.[50]

Higher transfection efficiency achieved by combining cationic liposome with “helper” neutral lipid was discussed in many published studies.[51–53] The most commonly used helper lipids for enhancement of cationic liposomes transfection potency are unsaturated phosphatidylethanolamines (PE), such as dioleoyl-PE (DOPE). This molecule is believed to facilitate fusion of cationic liposomes from lipoplexes with endosomal membrane, followed by release of DNA into the cytoplasm. The efficiency of fusogenic lipid is believed to be result of its ability to form structural forms similar to membrane fusion intermediates and destabilize it.[53] Hui *et al.* attempted to explain complex role of helper lipids comparing efficiency of PE and phosphatidylcholine (PC) in transfection of CHO cells.[53] According to their results, former lipid led to rapid and premature aggregation of complexes in the medium resulting in formation of too large granules

to enter the cell, while transfection efficiency was higher in the presence of PC as helper lipid. Gradual formation of PC agregates was directed at the cell surface and after reaching appropriate size, granules were introduced into the cell. They also pointed out that endocytosis together with all other factors affecting this process is crucial for successful transfection, while fusion of cationic liposomes with the cell membrane is secondary and insufficient for entry of DNA complexes into the cell in the absence of endocytosis.[53]

Opposite results of efficiency of neutral helper lipids, DOPE and DOPC, in the lipofection approach were identified by Du *et al.* in the study about design of novel lipopolyplex formulations using combination of plasmid DNA, cationic liposomes and peptide component.[51] Increased transfection efficiency was reported in the presence of neutral lipid DOPE instead of DOPC, as claimed above. Peptides are thought to participate in DNA packaging and directing complex to membrane receptors, whereas liposomal component, stabilized by electrostatic interactions between cationic lipid and DNA, presumably causes fusion with endosomal membrane, endosomal escape and release of genetic content into the cytoplasmic region.[51] After cellular uptake of lipo(poly)plexes by clathrin-dependent endocytosis, release of nucleic acids from endosome to cytosol is required in order to avoid endosomolysis, degradation of genetic material by lysosomal enzymes, suggesting the need to promote destabilization of the endosomal membrane and increase lipoplex stability after entering the cell.[54] It has also been noticed that, due to its nature, DOPE promotes formation of inverted hexagonal lipid structures that fuse with lipid bilayer of endosome leading to endosomal escape, DNA release into the cytoplasm and accumulation in the nucleus, whereas non-fusogenic DOPC promotes more stable laminar forms of lipid bilayer leaving lipoplexes trapped within the late endolysosomes.[51,54]

Besides DOPE, effect of cholesterol, as helper lipid molecule, has also been recognized in enhancing transfection efficiency of cationic liposomes.[55] Biological importance of cholesterol is widely known. This molecule is essential building block of membranes, participant in many metabolic and biochemical processes, as well as highly involved in endocytosis.[56] Group led by Safinya explained impact of cholesterol on enhancing transfection efficiency by inducing structural changes in lipoplexes.[48] Increase in cholesterol concentration will lead to a decrease in hydration layer of the lipoplex cationic part that, due to repulsions, acts as a barrier for endosomal fusion. In this manner not only fusion between lipoplex and endosomal membrane is facilitated, but also endosomal release of the complex.[48] Importance of cholesterol in enhancing efficiency of cationic polymer transfection has also been reported. Replacement of poly(allylamine) primary amino group with cholesterol significantly reduced cytotoxicity of this agent and enabled hydrophobic interaction with the cell membrane.[52]

Interestingly, cationic lipids have not only been shown to function as carriers of hydrophilic molecules (DNA), but also interacting with the cell, they can modify different signaling pathways, stimulating immune and anti-inflammatory response.[57] However, there is a lack of information on this topic. Combination of immunostimulating and carrier properties of these molecules is highly recommended in the vaccine field.[58] During their interaction with the main target, cell membrane, physiology of the cell changes, especially at the level of membrane proteins involved in numerous signaling cascades, such as MAPK kinases.[59] Considering large number of synthesized cationic lipids of different structures and various targets of their action, it is obvious that only a small part of their possible activities has been identified to date.

4. CATIONIC POLYMER-MEDIATED CELL TRANSFECTION

Cationic polymer-mediated transfection is a technique used in biochemistry and molecular biology to introduce nucleic acids into cells with many applications in gene therapy,[60] drug delivery and recombinant protein production.[61] Its methodology is relatively straightforward, cost-effective, and can be applicable to a wide range of cell types. Cationic polymers are classified based on their chemical structure, molecular weight, morphologies and charge density to linear, branched, hybrid and amphipathic.[62] Unlike cationic lipids, cationic polymers are water-soluble molecules that create polyplexes by complexation with DNA. Principle of cationic polymer based transfection for the most commonly used reagents is shown in Fig. 3. Resulting complexes are smaller in the size and DNA is more compactly packed than by previously described methods, implying that these advantages may be the key for efficiency.[63,64]Cationic polymers are very flexible molecules that can be prepared as linear or branched form and due to structural versatility it is easy to manipulate with their molecular weight and geometry.[64] Molecular weight of cationic polymers was found to be inversely proportional to their cytotoxicity along with the distance between charged atoms in polymer.[65]

Naturally occurring carbohydrates, such as dextrans, are characterized by biocompatibility and ability to directly bind nanoparticles from polyplex formulations to receptors on the cell membrane.[66] Except biocompatibility, sugar-based nanoparticles, as an alternative to non-viral vectors, exhibit low toxicity, low cost and structural modifications to improve their biological potential also are easily obtained.[67] In addition to dextran, chitosan and gelatin are natural polymers commonly used in biomedicine, whereas structures of synthetic gene delivery carriers are based on polyethyleneamine (PEI) [68], poly-L-lysine (PLL), polyprene and dendrimers.[67] Use of cationic polysaccharides in non-viral transfection procedures, with a focus on chitosan and its derivatives, has been recognized and extensively studied by Liu *et al.*[69]

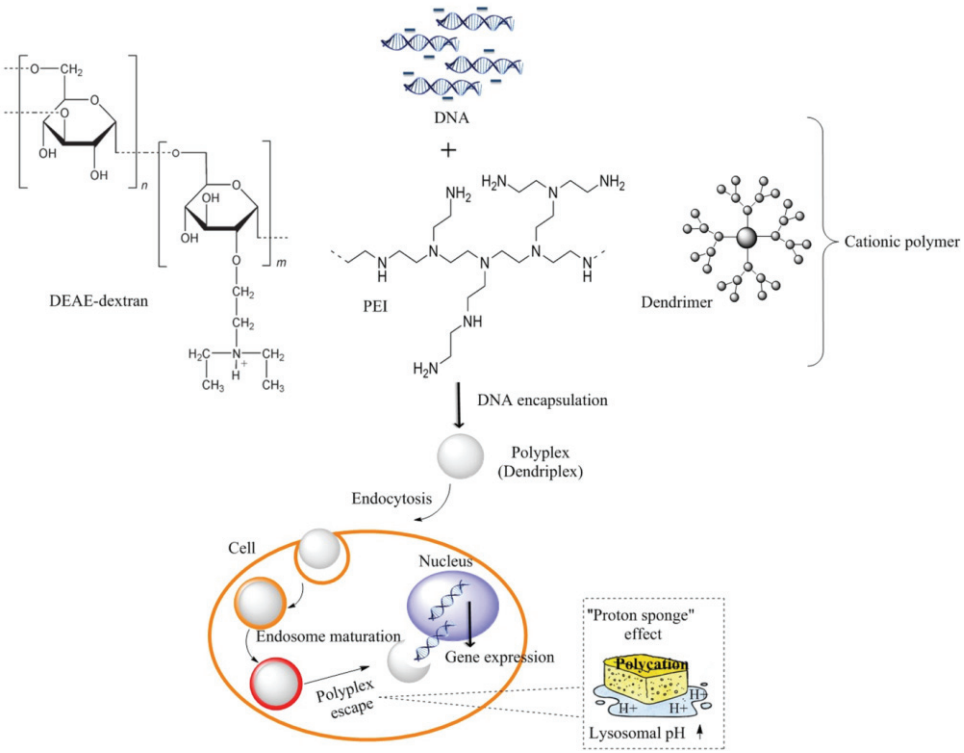

Fig. 3. Cationic polymer based transfection with the most commonly used reagents.

In this review more details are discussed on three the most prominent types of cationic polymers with superior efficiency compared to others, commonly used as transfection vectors due to their wide range of applications, high charge density, low cytotoxicity and immunogenicity.

5. DEAE-DEXTRAN-MEDIATED CELL TRANSFECTION

Diethylaminoethyl (DEAE)-dextran was among the first reagents used to deliver exogenous nucleic acids (poliovirus RNA) to mammalian cells (primary rhesus monkey kidney cells) over 50 years ago.[70] DEAE-dextran is cationic polysaccharide polymer of bacterial origin. Positively charged DEAE moiety electrostatically binds to DNA of total negative charge, resulting in formation of compact particles, polyplexes.

Due to the presence of three basic groups of different pKa values in the structure, specific association between DEAE-dextran and DNA is dependent on pH and solution ionic strength.[71] As a result of excess positive charge, this soluble complex associates with negatively charged cell membrane and is probably introduced into the cell by a process of nonspecific endocytosis, osmotic shock and change of membrane permeability either assisted by glycerol or DMSO.

Method for transfection of lymphocyte cell lines, described by Smale, is based on an initial incubation with a mixture of DEAE-dextran and DNA, followed by a secondary one in the presence of chloroquine.[63] Chloroquine participates in the neutralization of lysosomal enzymes, prevents acidification in endosomes and thereby inhibits intracellular degradation of plasmid DNA.[63] The increase in transfection efficiency with chloroquine treatment was investigated by Luthman and Magnusson.[72] They assumed that, except increasing pH in lysosomes, this molecule can strongly bind to DNA and protect it from degradation.[72]

Osmotic shock probably facilitates DNA uptake into the cell and prevents its breakdown by action of nucleases from lysozyme, causing pinocytotic vesicles burst due to osmotic imbalance.[73] Takai *et al.* applied combination of DEAE--dextran and osmotic shock, by treating cells with high osmolality buffer, for transient and stable transfection of lymphocyte cell line, with less efficiency and lower gene expression observed in the latter one.[73] DEAE-dextran-mediated DNA uptake is generally limited to a short-term transient transfection, whereas in the case of stable transfection, except low number of successfully transfected cells, toxicity may represent another drawback.[63] In addition to its biocompatibility, beneficial effects of dextran on the lipid status of the organism make it an attractive candidate in drug delivery and gene therapy.[71,74] Examining effect of polysaccharide dextran polymers of wide molecular weights range on transfection efficiency, it was concluded that efficiency positively correlates with increase in molecular weight, without impact on cell viability.[74] Furthermore, DEAE-dextran found its use in lipofection in the design of appropriate delivery carrier systems by stabilizing liposomal vesicles.[75]

Modified cell transfection protocol with DEAE-dextran, published by Shovel and coworkers in the early 1980s, introduced a step of "shocking" cells by exposure to DMSO or glycerol, which significantly increased expression of transfected gene, up to 50 fold.[76] Traditional protocol was also improved by Sussman *et al.* by treating mouse Ltk cells with DMSO at higher pH during initial incubation, resulting in 80 % of cells successfully transfected with thymidine kinase gene from simplex virus.[77] Mack *et al.* published reproducible method for transfecting sensitive adherent human primary macrophages using DEAE-dextran.[78] In this study three critical parameters for successful DEAE-dextran mediated transfection were identified and described: amount of added DNA per transfection, concentration of cationic polymer DEAE-dextran and time of incubating cells in transfection medium. [78]

Due to its great biomedical potential, Onishi *et al.* chose DEAE-dextran as a basis for the development of non-viral carriers for *in vivo* gene delivery.[79] This polymer can be autoclaved, it exhibits low cytotoxic and high transfection activity, and plays protective role against DNases. They performed graft polymer-

ization of methyl methacrylate on DEAE-dextran generating stable and efficient vector in the form of copolymer with significant therapeutic potential.[79]

6. PEI-MEDIATED CELL TRANSFECTION

Among leading and highly diverse group of non-viral carriers in the gene delivery field, PEI attracts attention as cationic polymer with easily modifiable structure that allows fine regulation of its physicochemical potential.[80] PEI is polymer of aziridine, with amino nitrogen atoms (at every third position) available for protonation, contributing to significant cationic potential of this organic molecule and buffering properties in wide pH range.[81] PEI does not have defined center of symmetry.[82] This molecule is easy to handle, low-cost and effective even in the treatment of cells growing in suspension.[5] It can be prepared in two forms, linear and branched, with the latter proven to be more effective in DNA condensation and transfection of mammalian cells and, unless otherwise stated in the literature, it refers to a branched type.[6] Due to branched structure and high density of positive charge, DNA is certainly more easily trapped within this polymer than with linear structures such as PLL and, thus, protected for safe gene transfer to target eukaryotic cell.[81] PLL alone does not show significant transfection efficiency *in vitro* and it mostly requires binding to molecules that will facilitate either cell entry or endolysosomal escape.[82]

PEI/DNA complexes enter most of the cells, but only a small number will express protein of interest.[83] Investigating destiny of these polyplexes inside the cell may explain why this occurs and answer a number of other important questions related to the mechanism of action. After endosome formation, polyplexes are released into the cytoplasm, destabilizing the endosomal membrane by "proton sponge" effect.[81] This phenomenon is associated with buffering capacity of PEI molecule and its ability to bind free protons within endosomes.[63] By binding protons PEI leads to an increase in endolysosomal pH that may affect folding of enzymes involved in DNA degradation, whose inactivation leads to release of functional, undegraded nucleic acids. One of the speculations is that surface of these complexes, due to cationic character, adheres to negatively charged phospholipids from nuclear membrane or from membrane fragments of bursted endolysosomes and being introduced into the nucleus by fusion.[5,6]

Optimal PEI/DNA ratio is essential for successful transfection. It has been documented that treating cells by complexes with low ratio showed no significant transfection efficiency, whereas too high ratio resulted in altered cellular morphology and reduced growth.[81] Accordingly, to optimize this method, it is crucial to find optimal ratio between these two components. On the other hand, entering large number of polyplexes by endocytosis may lead to cytotoxicity due to increased concentration of components from damaged endosomal membrane parts, upon endosomal escape.[81] Cytotoxicity of PEI polyplexes may be related to their

charge,[84] especially formation of non-specific interactions in the cell, so modification of their structure is popular strategy to avoid undesirable binding. Circulating serum proteins can bind to PEI/DNA complexes and inactivate them. For these reasons, their surface is modified by non-ionic molecules such as PEG,[85] as well as by various polysaccharides, which also play a role in targeting cells via receptors on the membrane. Patnaik *et al.* performed complexation of PEI with alginic acid and optimized appropriate ratio of these two components to achieve high mammalian cell transfection efficiency and viability.[80] Unmodified PEI molecule showed approximately 2–16 fold lower transfection efficiency compared to PEI-alginate complex against all tested cell lines. Beside reducing polyplex cytotoxicity, this inserted polysaccharide molecule increases transfection efficiency by participating in endosome damage.[80]

Godbey *et al.* provide an overview of physicochemical characteristics of PEI, its complexes and role in transfection of cell lines.[6] The protonability of this nucleic acid carrier was demonstrated to correlate with transfection efficiency. Also, it has been shown that manipulation of balance of components in PEI/DNA complex is essential to overcome attenuation of transfection efficiency caused by off-target interactions with interfering proteins. Although this undesirable binding can be bypassed by modifying complex surface with PEG, polystyrene, ethoxylated glycerol, poloxamer and polylactic acid,[86] significant improvement of transfection efficiency was made by attaching PEI to different transport proteins, such as transferrin.[6] In addition to concentration of H^+, formation of DNA/PEI polyplexes depends on the time of incubation, temperature, medium characteristics and amount of ions and various salts in solution.[83] PEI/DNA polyplexes occur in different forms, including rods and toroids. These two structures determine the uptake efficiency and therefore overall transfection efficiency.[6] Taking into account the fact that all these factors affect transfection efficiency, methods that provide insight into physicochemical characteristics of these complexes are of great importance. Scanning force microscopy was used to determine morphological properties and dimensions of PEI/DNA condensates, as well as to visualize way of DNA packaging and compactness under physiological conditions.[87] On the other hand, using particle tracking techniques (nanoparticle tracking analysis (NTA), dynamic light scattering (DLS)) and electron microscopy and varying incubation time of polyplex formation Dominguez *et al.* found a strong correlation between rate of polyplex aggregation and success of transfection.[5] Together with previously mentioned factors, transfection efficiency is affected by concentration of DNA, as well as by the type of cell being transfected and its environment.[5]

In comparison to CaP method, where presence of serum in the medium is mandatory and complex formation time-consuming, PEI transfection is more suitable for large scale use since it can be performed in serum-free medium and

does not require formation of pre-complexes between PEI and DNA.[88] Comparing transfection efficiency of CHO cells with PEI or CaP, Chenuet *et al.* emphasized higher efficiency of the first method in terms of cell recovery and the latter one related to cell-specific productivity and copy number of integrated plasmids in stable transfection system.[24] Cell recovery after PEI-mediated transfection is more efficient than in the case of CaP, although it was believed that interaction of this polymer with genomic DNA in the nucleus can lead to destabilization of chromosomes and reduced recovery.[24] On the other side, when considering costs of performing on a large scale, this method requires lower budget than lipofection.[88]

7. DENDRIMERS IN CELL TRANSFECTION

Dendrimers represent another gene delivery system of unique structure and will be briefly described here. These molecules are highly branched synthetic polymers of spherical geometry composed of monomeric subunits arranged around central core.[89] Owing to different synthetic strategies, functional groups, monomer units, as well as structure of central dendrimer core and number of concentric layers around it differs.[90] Layer number around central nucleus increases molecular weight of dendritic molecules, reaching values of protein molecular weights,[91] which is important for transfection process because structurally more complex vectors are able to carry and deliver more DNA.

More than 100 types of dendrimers have been synthesized and classified into several families – peptide, polyamidoamine, PAMAM, polypropyleneimine, PPI, phosphorus, carbosilane and polylysine or polyornithine dendrimers.[92] Characteristics of listed dendrimer groups have been extensively reviewed in the article of Pedziwiatr-Werbicka *et al*.[92] Binding affinity and stability of peptide dendrimer–DNA complex, and thus transfection efficiency, can be influenced by the type of interactions (covalent or non-covalent) between peptide and non-peptide components in this dendritic molecule.[93,94] Structure of one of the most commonly used peptide dendrimers is based on polylysine.[93] PAMAM is among the most common commercially available cores for dendrimer formation.[93] Core of PAMAM dendrimers contains ammonia or ethylenediamine, where nitrogen is tri- or tetravalent.[91] Controlled polymerization around this central molecule in PAMAM dendrimers leads to gradual formation of structure with spherical molecular architecture and large number of repeating amidoamine units. High density positive charge of primary amino groups on the surface, protonated at neutral pH, enables interaction with biological molecules of polyanionic character, such as DNA.[91]

These molecules, of usually radial symmetry, generally exhibit: low cytotoxicity, good biocompatibility and water solubility making them vectors of choice in transfection process.[95] However, due to high density charge, dendrimers may

become cytotoxic, and to overcome this barrier, they undergo functionalization and coating. In terms of costs, synthetic procedures with these compounds can be expensive.[93] Nature of dendrimer surface affects stability of encapsulatied complexes with DNA, cellular uptake and gene delivery. Dendrimers showed great biomedical potential as drug delivery carriers and transfection agents in both, nucleic acid and protein transfection, as well as in gene therapy.[92] Functional role of these molecules in biomedicine has been described comprehensively in recent work of Mirakabad *et al.*[93]

In the article of Tang and Szoka interaction of dendrimers and other cationic polymer structures with DNA was examined, as well as morphology of resulting complexes.[82] It was observed by electron microscopy that DNA condensation occurs in compact toroidal form. Complexes with degraded polyamidoamine dendrimers were observed as single units, while intact ones were clustered with almost 100 times larger diameter, suggesting that degree of aggregation is dependent on properties of individual polymers.[82,96] Unexpectedly, compared to intact, degraded dendrimers were more successful in transfection, most likely due to increased flexibility.[82] Wang and coworkers modified surface of dendrimer by fluorination introducing fluorine atom into aromatic ring of benzoic acid conjugated with dendrimer.[95] Transfection efficiency was estimated to increase with the degree of dendrimer fluorination.[95] Kwok *et al.* developed new hybrid transfection concept by studying synergistic action of peptide dendrimers and lipids as transfection reagents.[97] Combination of different transfection agents affects increase in their individual efficacy probably due to multiple interactions with DNA and membrane on their way to the cell nucleus. This field has not yet been extensively studied because dendrimer structure modifications are very challenging.[97]

8. CONCLUSION

Transfer of exogenous genes into cultured mammalian cells is a very powerful tool for manipulating DNA and represents an essential step in understanding function and regulation of genes and their products, proteins. Testing using this model system precedes *in vivo* studies and clinical trials converging towards the same milestone – therapeutic use in humans. Gene therapy, designed to introduce exogenous functional copies of genes into cells with damaged protein function represents a real revolution in medicine. Mammalian cell transfection methods are developing exponentially. New generations of transfection technologies are sophisticated and easily amenable to further improvement, such as automation with minimal operator input. In order to become commercially available products, gene delivery carriers have to meet certain criteria and overcome a lot of barriers. Undesirable characteristics of these vectors are induction of immunogenicity and cytotoxicity, whereas it is preferable to be biocompatible, stable,

available for structural modifications and to exhibit significant potential for cargo delivery of molecules of various types and sizes in the cell.

Due to simple handling procedure and reduced costs there is a great interest for developing non-viral methods, especially those based on the use of various chemical agents-inorganic amphiphilic aggregates, cationic polymers or cationic lipids. Their chemical design is constantly undergoing modifications in order to increase transfection efficiency and reduce side effects. Generally, transfection by chemical methods is based on electrostatic interactions between polycationic core of chemical agent and oppositely charged DNA followed by entry of resulting complexes into the cell, most likely via endocytosis. Chemically-assisted approaches described here have their own advantages and disadvantages and choice of the optimal one depends on the purpose of planned experiment. Furthermore, remarkable transfection success is achieved by combination of individual methods and transfection agents, with promising potential in biomedicine. Synergistic action leads to overcoming disadvantages of individual methods and improves their activity. Transfection efficiency may also be increased by removing nonspecifically binding serum proteins from the medium, optimizing size of complexes formed between chemical reagent and DNA, then modifying charge on their surface, inhibiting degradation within the lysozymes, increasing cell permeability, etc. To sum up, *in vitro* non-viral gene delivery to cells by chemical methods represents promising area in biomedical research with methodology constantly being improved.

Acknowledgement. The authors gratefully acknowledge the financial support of the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Grant No. 451-03-47/2023-01/200125).

И З В О Д

ПРЕГЛЕД ХЕМИЈСКИХ МЕТОДА ЗА ДНК ТРАНСФЕКЦИЈУ

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За *in vitro* уношење жељеног гена у ћелије сисара обично се користе невирусне хемијске методе за трансфекцију с обзиром на то да су веома ефикасне, јефтине и једноставне. Углавном немају безбедносне ризике као оне засноване на употреби вирусних вектора, а и токсичност према ћелијама је значајно смањена. Ове методе се класификују на основу хемијских реагенса који се користе за трансфекцију ћелија домаћина. Углавном су то биокомпатибилни носачи ДНК, као што су калцијум фосфат, катјонски липиди, DEAE-декстран, полиетиленимин или дендримери. Иако различити приступи, сви су засновани на формирању инкапсулираних амфифилних комплекса између ДНК и различитих честица, након чега следи улазак у ћелију, највероватније посредован ендоцитозом. У зависности од циља и дизајна експеримента, врши се избор одговарајуће методе. У овом прегледном раду описане су стратегије најчешће коришћених техника хемијске трансфекције. Поред тога, указано је на предности и ограничења различитих

носача ДНК, а наведени су и резултати истраживача добијени током оптимизације протокола у циљу повећања ефикасности испоруке гена. Главна биомедицинска примена овде описаних метода трансфекције је генска терапија, где се дефектни гени замењују функционалним.

(Примљено 22. децембра 2022, ревидирано 21. јануара, прихваћено 9. априла 2023)

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