RESEARCH ARTICLE

Evaluation of Synergistic Effect of Cytosine-Phosphodiesterbond-Guanosin-Oligodeoxynucleotide 7909, and Protamine on Transfection Process Mediated by Calcium Phosphate Nanoparticles

Ghassaq T. Al-Ubaidi[1], Ahmed A. Abbas[2], Ali A. Taha[3], Qasim S. Sharhan[1], Israa W. Ahmed[1], Ilham A. Jasim[1]

[1]*Medical Research Unit, College of Medicine, Al-Nahrain University, Alkadhimiya, Baghdad 10001, Iraq.*
[2]*Microbiology Dep., College of Medicine, Al-Nahrain University, Alkadhimiya, Baghdad 10001, Iraq.*
[3]*Biotechnology Dep., University of Technology,10001 Baghdad, Iraq.*

Received: 21th Dec, 19; Revised: 25th Jan, 20, Accepted: 18th Feb, 20; Available Online: 25th March, 2020

ABSTRACT

Bacille Calmette-Guerin (BCG) still the only authenticated vaccine against tuberculosis. Due to its drawbacks, a need for a new formula has emerged. The implication of "Nanovaccinology" is one of the possible alternatives. The non-viral vectors have a low transfection ability. In the context, this work aims to add two adjuvants to a calcium phosphate nanoparticles (CPNPs) functionalized with early secreted antigenic target 6-kilo dalton (ESAT-6) cloned pcDNA3.1(+) plasmid. ESAT-6 gene is specific to mycobacterium tuberculosis complex (MTC) and encodes a T-cell antigen. The adjuvants in practice are Herring protamine and cytosine-phosphodiester bond-guanosine-oligodeoxynucleotide 7909 (CpG-ODN 7909). Each has a different strategy in enhancing immune response; protamine is particulate adjuvant while CpG is an immunopotentiator substance. Nano complex was transfected into THP-1 monocytic cell line after its activation to a macrophage via 100nM PMA. Cellular immune response, interleukin-12 (IL-12), and tumor necrosis factor –alfa (TNF-α) also ESAT-6 protein production were assayed via the Sandwich ELISA technique. Results revealed that CPNPs offer only partial protection to the adsorbed plasmid against enzymatic degradation. Nano complex formula with two adjuvants resulted in significantly higher cellular immune response comparing to formula carrying one adjuvant. In conclusion, the implication of CPNPs in gene delivery accompanied with two adjuvants each possess different strategy, will result in partial protection to the delivered gene with upsurge cellular immune response.

Keywords: CpG-ODN-7909, CPNPs, Monocyte *in-vitro* immune stimulation, Protamine.

International Journal of Drug Delivery Technology (2020); DOI: 10.25258/ijddt.10.1.9

How to cite this article: Al-Ubaidi GT, Abbas AA, Taha AA, Sharhan QS, Ahmed IW, Jasim IA. Evaluation of Synergistic Effect of Cytosine-Phosphodiesterbond-Guanosin-Oligodeoxynucleotide 7909, and Protamine on Transfection Process Mediated by Calcium Phosphate Nanoparticles. International Journal of Drug Delivery Technology. 2020; 10(1):52-59.

Source of support: Nil

Conflict of interest: None

INTRODUCTION

According to the World Health Organization (WHO) reports, tuberculosis still ranks at the top of most serious infectious diseases afflicting mankind. It is a chronic bacterial infection caused by closely related species called MTC, among them *M. tuberculosis* exclusively infects human beings under natural infection while others may infect humans and other hosts, those include (*M. bovis, M. africanum, M. canetti, M. microti, M. caprae,* and *M. pinnipedii*).[1]

Comparative genomic analysis of MTC with the attenuated *M.bovis* BCG, revealed that the attenuation is due to loss of 16 genetic loci from BCG strain, a region of difference -1 (RD-1) through RD-16, RD-1 locus harbors nine genes from Rv3871 to Rv3879, the gene Rv3875 (esxA) encode for major antigenic protein ESAT-6. It is secreted through a member of the type VII secretion system named ESX-1; this secretion system encoded by genes flanking Rv3874 and Rv3875, together with genes lying out the RD-1 region.[2]

Although BCG still a more confident vaccine against tuberculosis, it has several drawbacks. Because it is an attenuated vaccine, so it is not recommended in immunocompromised people. The absence of 16 genetic domains (RD-1 to RD-16) in this vaccine had led to losing relevant T-cell antigens, which are important in eliciting cellular immune response; moreover, its efficacy widely varies in clinical assays.[3]

**Author for Correspondence: ghasaqtariq119@gmail.com*

The DNA vaccine may represent an alternative vaccine to BCG. Naked DNA segment is difficult to enter cells, and they should be complexed with a vehicle. Exploiting of plasmids in gene delivery comparing to viral vectors, are easier, cheaper to be produced, and has a much longer shelf life; moreover, the plasmid integration rate is less than 10^{-5} stable integrants per transfected cell and can be delivered repeatedly.[4] Moreover, transferred free DNA is prone to rapid clearance, enzymatic degradation, so to achieve a high transfection efficiency, the DNA has to be linked to delivery vehicles, among them are the NPs.[5]

Nanoparticles could be engineered, so they target certain cells; use of nanoparticles in vaccination formula guarantees gene entrance through the cell membrane, gene stability, immunogenicity, and targeted delivery with slow and controllable release.[6]

The ward adjuvant comes from the Latin word: "adjuvare", means to help; it is a substance that is able to increase the immunogenicity of a vaccine. Its mechanism of action is thought to be through increasing the antigen uptake by APCs, induce production of immunoregulatory cytokines and chemokines, activation of inflammasomes, induces local inflammation and cellular recruitment to the site of injection. Recently, it is found that some adjuvants have the ability to increase the vaccine formula half-life. According to the mechanism, adjuvants can be classified into four types: delivery system, immune stimulator, combination formula, and mucosal adjuvant.[7]

Protamine is an food and drug administration (FDA)-approved compound can facilitate gene delivery through condensing plasmid when functionalized with different types of nanoparticles. It is recorded to improve viral gene delivery as well.[8]

The CpG-ODN 7909, is a strong stimulator to B-cells, monocyte, macrophage, and dendritic cells,[9] it is one of the Pathogen associated molecular pattern (PAMP), due to its abundance in the microbial genome but its rarity in the mammalian genome.[10]

MATERIALS AND METHODS

Chemicals

For CPNPs synthesis, chemicals including calcium nitrate, $Ca(NO_3)_2.4H_2O$, and sodium citrate $C_6H_5Na_3O_7.2H_2O$ were purchased from Sigma-Aldrich/USA. While Diammonium hydrogen phosphate, $(NH_4)_2HPO_4$, was purchased from Carlo Erba/Italy.

For cytotoxicity assay, 3-(4,5-dimethylthiazol-*2*-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT), which is a vital stain was taken from Bio-World/USA.

Plasmid pcDNA3.1(+), containing the Cytomegalovirus (CMV) promoter, was chosen to be the cloning vector to the desired gene (ESAT-6). The gene was ordered to be cloned at the polylinker site by Hind III /ECOR-1 with ESAT-6, gene length 306 bp, it was taken from GenScript, USA.

Plasmid was propagated in DH5-alfa *E.coli* taken from MC Lab/USA.

Plasmid purification was done by NucleoBond Xtra MidiEF, Macherey- Nagel, GmbH, Germany.

Adjuvants used are class–B CpG- ODN 2006 (ODN7909), 5'-tcgtcgttttgtcgttttgtcgtt-3', it was taken from Invivogen / USA. The second adjuvant is Protamine Sulphate salt, from Herring, was purchased from Sigma-Aldrich, USA.

Luria-Bertani (LB) broth was prepared, this media consists of Trypton, Yeast extract and NaCl, in 2:1:1 weight ratio. Add 1.5 gm to 100 mL of LB broth of agarose to form the solid structure.Both broth and agar contained 100 µg/ mL Ampicilline as a selective antibiotic.

Transfection step was conducted in THP-1 human monocytic leukemia cell line, taken from the male infant/ European Collection of Authenticated Cell Culture (ECACC). Monocytes were activated to macrophages by Phorbol-12 mystrate-13-acetate (PMA), Santa Cruz, USA. As a positive control the plasmid was transfected to the cell line using Lipofectamin 2000 kit / Invitrogen / Thermo Fischer scientific/ USA.

For the last step, which is cellular immune response inspection, Sandwich ELISA kit for IL-2 and TNF-α were purchased from Elabscience/Biotechnology / China. While for ESAT-6, ELISA kit was taken from Mybiosource/UK.

Equipment

For CPNPs characterization, Scanning probe microscopy SPM-AA3000, (AFM contact mode)/Angstrom Advanced Inc., USA was used.

For EMSA runs, both horizontal gel electrophoresis/ consort N.V./parkland 36/HU13/Belgium used for 1% agarose gel, while Mini-vertical electrophoretic system was used for 10% native polyacrylamide gel.

Methods

Preparation of citrate- stabilized Calcium phosphate nanoparticle

The CPNPs were chemically prepared,[5] characterized by SPM and UV-vis spectroscopy with wavelength ranging 200–1000 nm.

Cytotoxicity

Cytotoxic effect of prepared CPNPs was examined by the vital stain, 3-(4,5-dimethylthiazol-*2*-yl)-2,5-diphenyltetrazolium bromide tetrazolium, MTT.[11] Results were analyzed by an independent T-test- SPSS 20.

Nano complex Synthesis:

- *Conjugation of Plasmid to Protamine*

The cloning vector, pcDNA3.1 (+), was cloned to ESAT-6 gene by the manufacturer. The plasmid was cut in a single site with the restriction enzyme ECOR-1 to give a linear plasmid. Herring protamine sulfate was prepared in 100 ng/µl in sterilized distilled water. Linear plasmid 100 ng/µl was conjugated to the prepared protamine (Pl-Pr) by mixing them in four different weight ratios (Rws') 1: 0.1, 1: 0.5, 1: 0.8, 1: 0.9. Then incubate at 25C°/overnight with continuous shaking.

- *Conjugation of a Plasmid to CPNP*

100 ng/µl of linear plasmid was added to the prepared CPNPs (Pl-CPNPs) in four different volume ratios Rvs', 1:1, 1: 3, 1:5, 1:8. Also, 100ng/µl of circular plasmid was added to prepared CPNPs (Pl-CPNPs) in five different volume ratios Rvs', 1:1, 1:3, 1: 5, 1:7, 1:8. Then, incubated at 25C°/ overnight with continuous shaking.

- *Conjugation of CpG to CPNPs*

A solution of 200 ng/ µL of CpG-ODN-7909 was prepared in sterilized endotoxin-free water. CpG was conjugated to CPNPs in five different Rws' 1:0.1, 1:0.3, 1:0.9, 1.51:, 1:2. The mixtures were then incubated at 25C°/ overnight with continuous shaking.

Verification of Nano complex Formation Using EMSA Assay

It is of significant importance to verify the complex formation success; for this purpose. An electrophoresis mobility shift assay (EMSA) was used. This method is a qualitative assay used to detect DNA-protein interactions, mainly depends on increment in band intensity and change in the molecular weight of conjugated substances. This will be observed by a decrement in band intensity and mobility retardation for the bands of conjugated substances comparing to the free DNA.

Three EMSA runs were conducted; in all of them, the DNA was the target for tagging. So ethidium bromide (EtBr) was a suitable stain for all experiments. Two EMSA runs were conducted in 1% agarose using voltage 70v/15 min. Then 100v/1 hr. One of them to verify plasmid-protamine conjugation while the other is to verify the plasmid-CPNP conjugation. The third EMSA run was designated to investigate the (CpG-ODN 7909)- CPNP conjugation, but because of CpG small size (only 24 nucleotides), this run was conducted in 10 % native polyacrylamide gel.

It can be noticed that EMSA runs were designated to examine the conjugation of each two substances separately, this is because the conjugation of all (four) substances will result in bands disappearance, due to EtBr disability to intercalate itself in between the DNA.

The Complete Nano-complex Synthesis that will be used in Transfection

After making sure of conjugation success of each two substances separately, in a second step, the complete nano complex [plasmid –CPNPs- protamine – (CpG-ODN-7909)] is prepared to depend on the previous strategy and as following, 10 µL of 1µg/ ml ESAT-6-cloned-pcDNA 3.1(+) was mixed with 9 µl of 100ng / µl protamine sulfate. This is (complex-1) incubated at 25C°/ overnight with continuous shaking. Added the complex-1 to 80 µl of prepared CPNPs. Incubated at 25C°/ overnight with continuous shaking, this is (complex-2). Then mix complex-2 with 25 µL of 200 ng/µl of CpG-ODN 7909. Incubated at 25°C/overnight with continuous shaking, this is complex-3. This nano complex, which contains the four components, is ready for transfection (mixture-A).

Two other mixtures were prepared in the same method mentioned above, but in one of them, the CpG is waved this is mixture B, while in the second mixture, protamine is waved this is mixture C. Plasmids were also transfected using Lipofectamine 2000 kit to compare results.

Role of CPNPs in Protecting DNA:

It is intended to examine the role of CPNPs in protecting the plasmid from macrophage DNase**.** For this linear experiment plasmid with two different concentrations 100 ng/µL and 30 ng/µL, also a complete (circular) plasmid with 100 ng/µL concentrations, were tested.

For linear plasmids (100 and 30 ng/µL), they were cut first by ECOR-1, then both linear and circular plasmids were treated with DNase, according to the manufacturer.

Converting non-competent E. Coli DH5-alfa into competent cells

This step is to make the bacteria capable of taking up the plasmid. The noncompetent bacteria were taken by a wood stick and cultured on the LB agar, incubated at 37°C/ overnight. A single colony was taken and cultured it in 5 ml of LB-broth. Incubated at 37°C/overnight with continuous shaking at 300 rpm. This is named the starter culture. After detection of the blurry broth. 1 ml of saturated broth was subcultured into 50 mL of LB-broth. Incubated at 37°C to the early log phase, this took about 4 hours also with shaking at 300 rpm. When the culture reaches an OD 600 of about 0.5 absorbances, at these measurements, the bacterial density is about 10^6 cells/ml (for *E.Coli*), which is ideal for transformation efficiency. Chilled the flask on the ice for 20 minutes and then collected the cells by centrifugation at 3500 rpm at 4°C for 10 minutes. Prepared the transformation and storage buffer (TSB); this is consists of LB broth 6.1 PH with 10% PEG, 5% DMSO, 10mM $MgCL_2$, and 10 mM $MgSO_4$. Resuspended the cells pellet in 10 ml of cold TSB solution and kept it on ice till being used (within hours). At the end of this step, competent cells are ready to be transformed, and any further step was done under cold conditions (-4°C). Otherwise, bacterial transformation efficiency would be strongly affected.

Propagation of pcDNA3.1(+) Incompetent DH-5alfa cells:

The plasmids were reconstituted with sterilized distilled water to make 0.2 µg/µl stock solution. Chilled the plasmid on ice for 20 minutes. For transformation, in a tubes on ice added aliquots of purified plasmid and competent cells by adding 0.5 µL of stock plasmid to 1 mL of competent cells, to get final concentration 100 ng/ml, left on ice for 10 minutes. Incubated overnight at 37°C in shaker/ incubator. Spread 200 µl on Ampicillin containing LB-agar, incubated overnight at 37°C.Put a single colony in 5 ml LB-broth, incubated overnight at 37°C, with a loosely tide cap to allow aeration. Inoculated 2.5 mL to 50 mL of LB-broth, incubated overnight at 37°C, with a loose tide cap to allow aeration.

Centrifuged at 3500 rpm for 10 minutes, discarded the supernatant, and kept the pellet. The plasmid from the cells pellet was extracted using the Nucleobond kit, which is an endotoxin-free plasmid DNA purification kit, the procedure is according to the manufacturer.

Transfection of Nano Complex in THP-1 Cell Line

- *THP-1 Monocytic Cell Line Maintenance*

THP-1 monocytic cells were maintained in complete growth medium, RPMI-1640 medium supplemented with 10% Fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin, checked in advance for the PH 7-7.6. Incubated at 37C° in an atmosphere containing 5% CO2. Cells were passaged and reseeded at 50% confluence twice a week.

- *THP-1 Monocytic Cell Line Activation to Macrophages*

The THP-1 monocyte cell line is usually found in a suspension form. This makes the transfection process to those cells problematic. Therefor it was aimed to activate them to macrophages to achieve adherent cells; this could be accomplished by the addition of 100ng/ml of PMA.

THP-1 monocytes were activated to macrophages as following, THP-1 cells were dispensed in a 12 well plate, 200 µl/well. To each well added 200µl of FCS free- RPMI medium containing PMA to get final concentration of 100 ng/ml of PMA. After 72 hrs of incubation at 37C°, cells were visualized under inverted microscopy to check cells adherence and morphological changes remarkable to macrophages. Changed the media with FCS free- RPMI medium (without PMA). Upon this step, cells are ready for transfection.

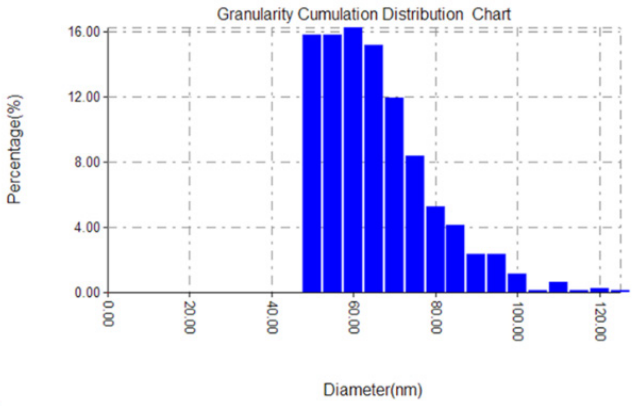


Figure 1: Size distribution of citrate stabilized CPNP solution.

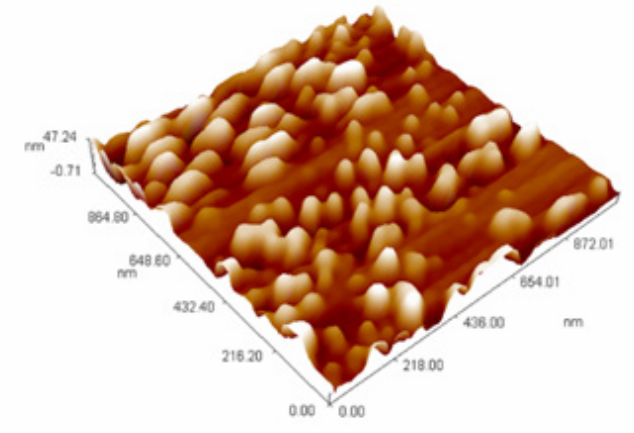


Figure 2: Top view of CPNPs shows distorted spherical shape using SPM.

- *Detection of Cellular Immune Response and ESAT-6 production*

Sandwich ELISA technique was used to verify IL-12 and TNF-α. ESAT-6 protein production was verified in the same technique as an indication for transfection success. For ELISA results, statistical analysis was done by "SPSS-20", ANOVA test.

RESULTS

Synthesis of CPNPs Solution

A solution with stoichiometric concentration ratio of calicium(20mM) and phosphate (12mM), corresponding to hydroxyapatite $Ca_5(PO_4)_3OH$(Ca:PO4, n:n = 1.66). According to this reaction, it was possible to synthesize a colorless calcium phosphate colloidal solution at $3.3* 10^{-3}$ M. Particle size and shape were analyzed by scanning probe microscopy (SPM). The synthesized particles lie in the nanoscale, ranging in size from 50 nm to 125 nm in diameter, about 31.6% of particles sizing 50 nm–55 nm in diameter, about 31.3% of particles sizing 60 nm–65 nm in diameter, about 20.2% of particles ranging 70–75 nm in diameter. While the remaining percentage of NPs (16.9 %) ranging in their size from 80–125 nm in diameter (Figure 1). It was found that the colloidal solution particles posses a distored spherical shape with an average size of 63 nm (Figure 2).

The prepared colloidal solution was analyzed using scanning UV-vis spectroscopy with wavelength values ranging from 200 nm to 1000 nm. Results showed that the maximum light absorbance was 0.376 at wavelength 295 nm. They were indicating that maximum λ is observed within the UV-region (Figure 3).

Cytotoxicity Assay

Cytotoxic effect of CPNPs was measured against the THP-1 cell line using the MTT stain. Results showed that the highest concentration of a colloidal solution ($3.3* 10^{-3}$ M) showed no cytotoxicity, cell viability was 100%. For MTT assay, statistical analysis was conducted by the "SPSS-20" statistical program in the dependent T-test (Figure 4).

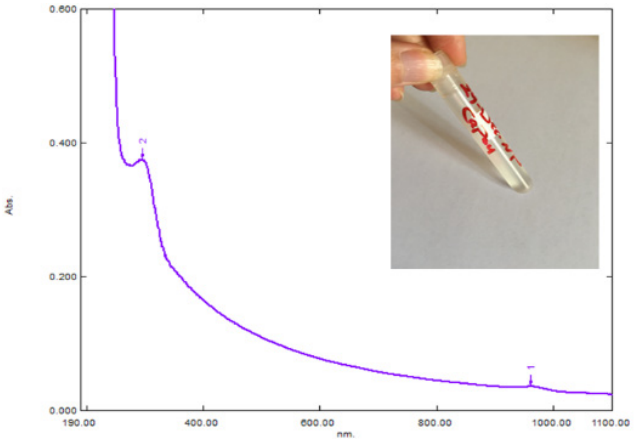
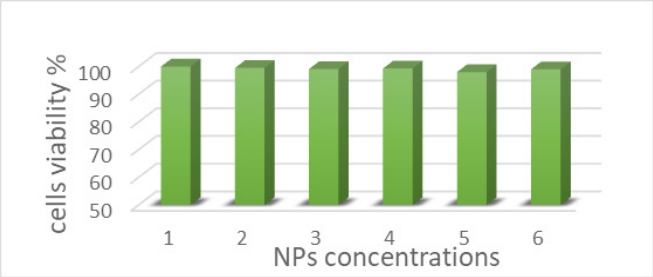


Figure 3: UV-VIS spectrum for colorless CPNPs colloid showing maximum absorbance at 295 nm.



1- PNP con. Zero, 2- CPNP con. 0.2 mM, 3- CPNP con. 0.4 mM, 4- CPNP con. 0.8 mM, 5- CPNP con.1.6 mM, 6- CPNP con. 3.3 mM

Figure 4: MTT assay shows THP-1 cells viability when challenged against CPNPs.

Detection of Plasmids-protamine Conjugation using EMSA

It is of significant importance to ensuring the success of complex formation for those four substances. For this purpose, the EMSA was applied. The conjugation of each two substances was tested separately.

DNA marker used ranging in size from 25 kb- 250 bp and unconjugated linear plasmid were also run. The ESAT-6 cloned plasmid full size is 5744 bp (Figure 5, Lane-1, and Lane-2). Linear plasmid was conjugated to protamine (Pl-Pr) in four different weight ratios (Rws') 1: 0.1, 1: 0.5, 1: 0.8, 1: 0.9.

Electrophoretic run for those mixtures on 1% agarose showed that as the protamine to plasmid weight ratio increases as the band intensity decreases in reciprocal proportion, possibly because the protamine prevents the ethidium bromide from intercalating itself to the DNA, resulting in vaguer bands.

As the protamine to plasmid weight ratios increase as the band's position retards comparing to that of the free plasmid band. It is also found that part of the protamine-plasmid mixture still found just beyond the wells. This could be due to the fact that resulted conjugate have a higher molecular weight comparing to that of the free plasmid. Moreover, since protamine is a positively charged protein, its mobility by electrophoresis toward the anode is weak. Both those causes resulted in mobility retardation of protamine-plasmid conjugate comparing to the free plasmid.

Detection of CPNP- plasmid Conjugation using EMSA

DNA marker used ranging in size from 25kb- 250 bp and unconjugated linear plasmid were also run. The ESAT-6 cloned plasmid full size is 5744 bp (Figure -5, Lane-1, and Lane-2). A linear, 100 ng/µl plasmid is linked to the CPNPs in four different (Pl:CPNP) volume ratios Rvs' 1:1, 1:3,1:5, 1:8.

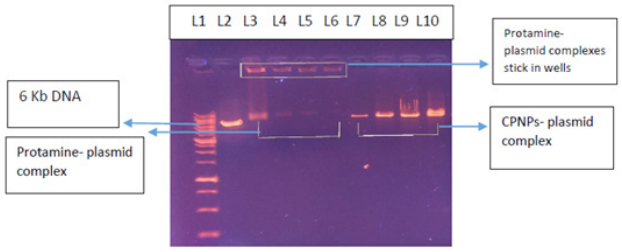
When those mixtures were run on 1% agarose gel, results showed that bands intensity are affected in a reciprocal proportion to the CPNPs volume, that it decreases gradually as the CPNPs: Pl volume ratios increase. Moreover, the bands composed from Pl:CPNP conjugates locate in a position slightly higher from band contains free plasmid (Figure -5, lane- 7, lane-8, lane-9 and lane 10, respectively).

The same experiment of CPNPs linkage to plasmids was applied on complete (circular) plasmids also. The same results were recorded, both band's intensity and mobility are affected in a reciprocal way by an increment of conjugated CPNPs volume but with the presence of several bands in the same line. This because what is called the sticked DNA that is supposed to due to damage and /or supercoiling of DNA strands (Figure 6). In the presented work, such bands were detected, but it is suggested that their presence indicates plasmid coiling rather than damage. Because when circular plasmids were cut in a single- site, only one band appeared as previously shown (Figure 5).

Those findings indicate the success of complex formation between plasmid and protamine in different weight ratios.

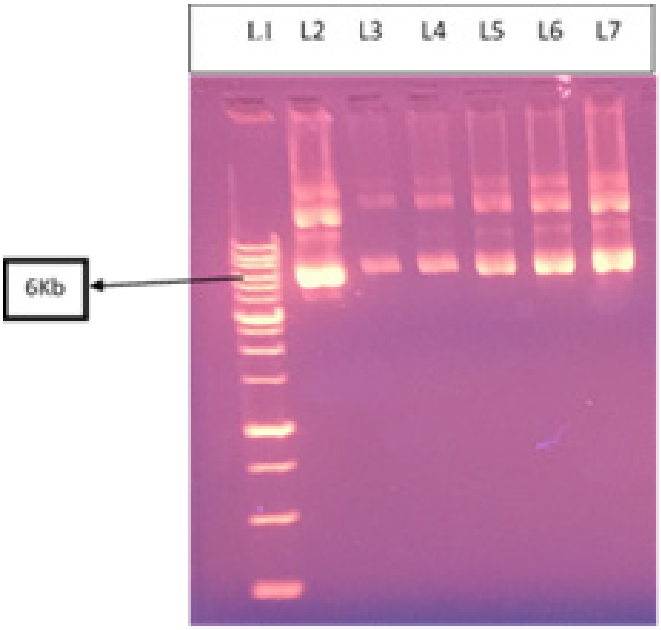
Detection of CpG- CPNPs conjugation using EMSA

Due to the significantly small size of CpG-ODN 7909 (24 nucleotides), verification of success of CPNPs conjugation to



Lane-1: Ladder, **Lane-2:** free plasmid (control), **Lane-3:** Pl: Pr. 1:0.1 Rw, **Lane-4:** Pl: Pr. 1:0.3 Rw, **Lane-5:** Pl: Pr. 1:0.8 Rw**, Lane-6:** Pl: Pr. 1:0.9 Rw. **Lane-7:** Pl: CPNP 1:8 Rv, **Lane-8:** Pl: CPNP 1:5 Rv, **Lane-9:** Pl: CPNP 1:3 Rv, **Lane-10:** Pl: CPNP 1-1 Rv.
(Note: Plasmid used in this experiment is linear).

Figure 5: EMSA assay shows the mobility shift of Pl: Pr and Pl: CPNP conjugate comparing to the free plasmid.



Lane -1: Ladder, **Lane-2:** Free circular-plasmid (control), **Lane-3:** Pl: CPNP 1:8 Rv, **Lane-4:** Pl: CPNP 1:7 Rv, **Lane -5:** Pl: CPNP 1:5 Rv., **Lane-6:** Pl: CPNP 1:3 Rv, **Lane-7:** Pl: CPNP 1-1 Rv.
(Note: Plasmid used in this experiment is circular).

Figure 6: EMSA assay shows mobility shift of Pl:CPNP conjugates comparing to free plasmids.

CpG should be conducted in 10 % native polyacrylamide gel rather than 1% agarose.

The control sample in this experiment is represented by 200 ng/µL unconjugated CpG- ODN 7909 (Figure 7, lanes-2). While the ladder used is ranging in size from 1000 to 50 bp (Figure -7, Lane-1)

The CpG 200ng/µl was conjugated to CPNPs in 5 different Rws' ranging from 1: 0.1, 1:0.3, 1:0.9, 1:1.5 and 1:2 Rws'. (Figure 7, Lanes-3, -4, -5, -6 and -7, respectively).

The electrophoretic run was conducted in 10% native polyacrylamide gel in a mini-electric vertical system. Results showed that as the CPNPs to CpG weight ratios decrease as the bands' intensity increases, and the band position appears to be closer to the control band (free CpG).

The highest CpG: CPNP Rw (1:0.1) possesses the highest intensity and closest band position comparing to the control band (Figure 7, Lane-3).While the lowest CpG: CPNP Rw (1:2) possesses the lowest intensity and most far position comparing to the control band (Figure -7, Lane-7).

Role of CPNPs in protecting plasmid from DNase

In order to verify the role of CPNPs in protecting the plasmid against macrophage DNase, it was attempted to use a non-CPNP conjugated linear plasmid in two concentrations 100 ng/µL and 30 ng/µL (this in the duplicate form) and CPNP-conjugated circular plasmids (plasmid concentration 100 ng/µl), they were treated first with DNase and then to run them on 1% agarose gel electrophoresis.

Results showed that after DNase treatment to 100 ng/ µL non-CPNP-conjugated linear plasmid for 5 minutes, the plasmid would be cut into small pieces that appear as a smear on the gel, while the non-DNase treated plasmid appears in first lane(Figure 8, Lane-2)

When plasmid concentration was decreased into 30 ng/ µL, the smear was disappeared (Figure 8, lane-4, and lane-6) comparing to their controls (Figure 8, Lane-3, and Lane-5, respectively).

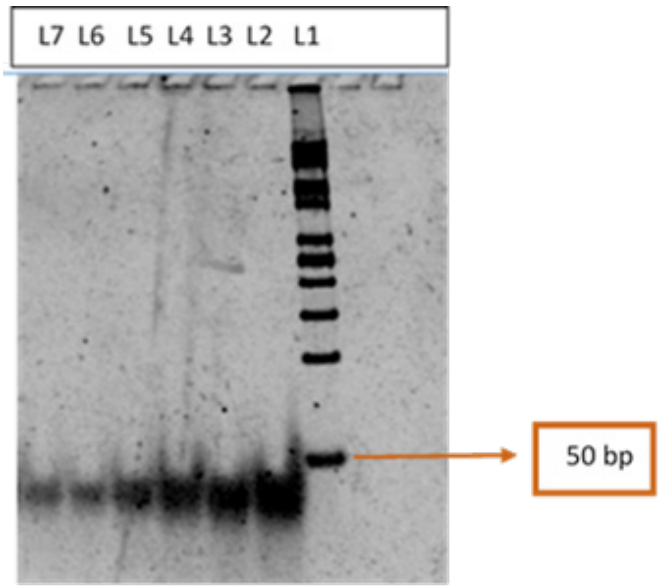
As the plasmid used in transfection should be in complete (circular) form and because both linear and circular plasmids gave the same results, as previous experiments showed, the CPNP conjugation was applied to the complete plasmid in 100ng/µl concentration rather than to the linear plasmid.

It was found that in the CPNP- conjugated circular plasmid, which is treated with DNase, only part of the band is disappeared (Figure 8, lane-8), comparing to non CPNP-conjugated circular plasmid that is treated with DNase which appeared as a smear (Figure 8, lane-2). It can be said that CPNP conjugation may results in partial protection to the DNA when conjugated to it at 8:1 Rv.

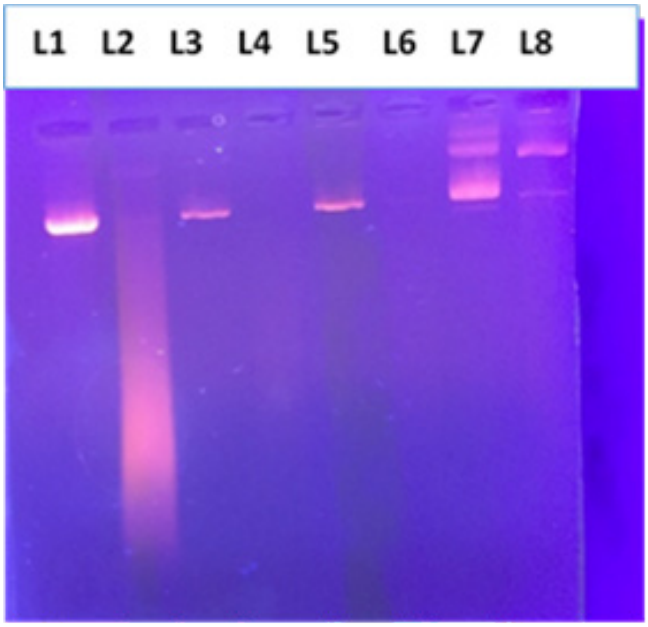
Differentiation of THP-1 Monocyte to Macrophage and Measurement of Cellular Immune Response

After cells incubation with PMA at 37°C for 72 hours, results revealed that treated cells appeared in a bigger size, irregular shape, some of them had a star-like shape and vast majority of cultured cells possessed an adherence performance, comparing to normal cells that possess a suspended circular homogenous shape (Figure 9, A and B).

When THP-1 cell line transfected with the complete nanomixture (CPNP- (CpG-ODN 7909)- Protamine- Plasmid),



Lane -1: ladder, **Lane-2:** Free CpG (control), **Lane-3:** CpG: CPNP 1:0.1 Rw, **Lane-4:** CpG: CPNP 1:0.3 Rw, **Lane-5:** CpG: CPNP 1:0.9 Rw, **Lane-6:** CpG: CPNP 1:1.5 Rw, **Lane-7:** CpG: CPNPs 1:2 Rw.
Figure 7: EMSA assay shows the mobility shift of CPNPs: CpG conjugates comparing to free CpG.



Lane-1: Non-DNase treated linear plasmid (100ng/µl). **Lane-2:** DNase-treated linear plasmid (100ng/µl).**Lane-3:** Non-DNase treated linear plasmid (30ng/µL), **Lane-4:** DNase-treated linear plasmid (30ng/ µL) **Lane-5:** duplicate for Lane-3. **Lane-6:** duplicate for Lane-4**. Lane-7:** Non-DNase treated circular plasmid (100ng/µl) conjugated to CPNP in 1:8 Rv). **Lane-8:** DNase treated circular plasmid (100ng/µL) conjugated to CPNP in 1:8 Rv).
Figure 8: Effect of DNase on CPNPs conjugated - plasmid.

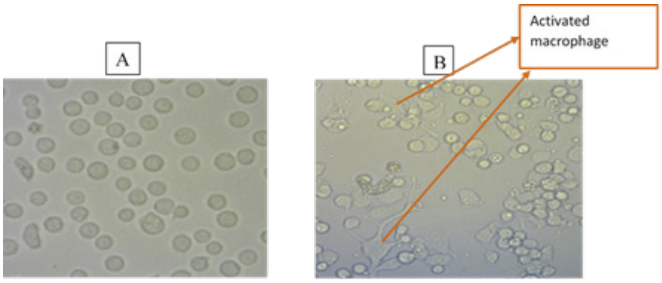


Figure 9: THP-1 monocyte before (A) and after (B) activation with PMA.

Table-1: Contents of the nano mixtures used in transfection.

	Mixtures
1-	CPNP- (CpG-ODN 7909)- Protamine- Plasmid.
2-	CPNP- Protamine-Plasmid.
3-	CPNP- (CpG-ODN 7909)- Plasmid.
4-	Positive control, plasmid transfected by Lipofectamine.

and by measuring the TNF-α level (mean value) in culture media using sandwich ELISA technique and after analyzing data in ANOVA test, it was found that cells produced amounts of TNF-α (88.3 pg/mL) higher comparing to cells treated with mixtures in which CpG-ODN-7909 and protamine were waived, (15.6 and 31 pg/mL), respectively ($p < 0.05$). While not significant difference was recorded compared to the positive control sample (plasmid transfected by Lipofectamine) (132 pg/ml) ($p > 0.05$).

Regarding the IL-12 level produced by cells treated with complete nano mixture, results revealed that those cells produced amounts of IL-12 (208 pg/mL) significantly higher than that produced by cells treated with mixtures lacking CpG-ODN-7909 or protamine (31 and 36.3 pg/ml), respectively, (p-value < 0.05). While no difference was recorded comparing to a positive control sample (172 pg/mL), (p-value > 0.05).

The last parameter that was studied in this experiment is the protein expression, ESAT-6 protein production. It was found that complete nanmixture treated THP-1 cells produced this protein in amount (12.3 ng/mL) significantly higher than the group with waved (CpG-ODN 7909 (1.6 ng/mL), p-value < 0.01 and the group with waved protamine (3.6 ng/mL), p-value < 0.05. But no significant difference was recorded compared to the positive control (9.9 ng/mL),(P-value > 0.05) (Table 1).

DISCUSSION

Due to calcium phosphate nanoparticles (CPNPs) biocompatibility, biodegradability, and their ability for co-precipitation with DNA on the cellular membrane, they are mostly the NPs of choice for gene delivery. However, the only limit for their employment as a conventional transfection method is their low transfection ability.[5]

For that, we focused on improving CPNPs transfection ability by functionalizing them with other components, which are Herring protamine and CpG- ODN 7909 as an adjuvant; each one possesses a different strategy in enhancing immune response while gene was chosen to the putative DNA vaccine ESAT-6. Because the ESAT-6 is T-cell antigens are encoded in RD-1 region present in all virulent strains of *M.tuberculosis* and *M.bovis* genome, but not in BCG (Ahmed, 2012).

In all conjugation steps, EMSA is a qualitative assay used to inspect conjugation success, and results revealed that when protamine or CPNPs weights increase comparing to DNA weight, this causes vaguer band intensity. Also, bands position appears to retard comparing to that of the free DNA band. Mobility retardation is explained by an increment in nano complex molecular weight. Moreover, protamine is a positively charged protein. This is supposed to hamper the nano complex flow toward the anode pole. Both decrement in band intensity and bands mobility retardation considered as signs for complexes formation.

It is believed that vaguer band intensity for the conjugated DNA comes from the truth that CPNPs and protamine binding to DNA molecules will reduce the EtBr ability to intercalate itself. Especially that EtBr intercalate itself in between the hydrophobic interior major grove of the DNA molecule between the two strands for the dsDNA or in the intra-nucleotides of the ss-DNA.[12]

The role of CPNPs in protecting the plasmid adsorbed to the CPNPs from DNase degradation was assessed by EMSA assay also. It was found that this type of conjugation offered only partial protection against DNase degradation.

This finding is in accordance with results documented in work conducted in Delhi University, in which the researchers found that only encapsulation of DNA in the rigid matrix of CPNPs may give complete protection against DNase while DNA absorbance offers a very low level of DNA protection.[13]

Regarding cellular immune response, macrophages treated with nano complex containing both CpG-ODN 7909 and protamine showed production of IL-12 and TNF-α and also ESAT-6 protein in significantly higher levels than macrophages treated with nano complex with one waived component. Those components seem to have a synergistic effect in eliciting a cellular immune response. Macrophages treated with a complete nano complex, produced IL-12, TNF-α, and ESAT-6 in levels comparable to those produced from cells transfected with conventional transfection kit, Lipofectamine. Both CpG-ODN-7909 and protamine possess several features making them ideal to be applied as adjuvants.

Protamine has a remarkable ability for plasmid condensation,[13] also, it promotes the DNA transportation to the nucleus by means of nuclear localization signal (NLS) sequence.[15] It is believed that protamine is a good cell-penetrating polypeptide due to its richness in arginine residues containing guanidinium functional groups, the positive charge of protamine will pave the way to electrostatic interaction of the complex.[16] Moreover, Role in transfection is mediated by acting as transcription activator protein; it induces gene transfection through targeting certain response elements.[6]

Regarding the TLR-9 ligand role, present work results indicated their absence from the nano complex resulted in a reduction in cellular immune response comparing to the complete nano complex. It looks that their role is similar to that of protamine.

It was documented that signaling via TLR-3, TLR-4, TLR-7, TLR-8, and TLR-9 promotes Th1 immune response, which aids in defense against intracellular pathogens. That is conducted through the release of IL-12 p70 and INF-α and INF-gamma from APCs.[17] Moreover, activation of TLR-7, TLR-8 and TLR-9 decreases T-reg function.[18]

Those TLR-9 and protamine properties underlie their development as putative vaccine adjuvants against infectious disease, and the involvement of both those combinations together is supposed to culminate in a rapid upsurge in cellular immunity.

CONCLUSION

It was also found that DNA absorbance on the CPNPs offers only partial protection against DNase degradation.

Adding products like Herring protamine and TLR-9 ligand CpG-ODN 7909 will enhance the transfection efficiency achieved by CPNP, and the presence of both of those compounds would act in a synergistic mode, resulting in a better transfection level comparing to that obtained by nano complex lacking one of them. Although, absence of significant immune response increment comparing to the positive control, this study represents a platform to consider the multimeric-DNA vaccine as a future antituberculosis vaccine after further improvement steps.

ACKNOWLEDGMENT

On behalf of colleagues participated in this work, I would like to thank Dr. Najmeh Ketabchi and Dr. Mojdeh Safari at Tehran University of medical science/ School of Advanced Technologies of Medicine/Iran, and to Dr Haralambus stamatis, Dr. Angeliki Polydera and Miss Alexandra at Department of Biological Application and Technologies, Iwannina University, Greece, for their scientific advice

REFERENCES

1. Homolka S, Projahn M, Feuerriegel S, Ubben T, Diel R, Nubel U, *et al*. High resolution discrimination of clinical Mycobacterium tuberculosis complex strains based on single nucleotide polymorphisms. PLoS One. 2012;7(7):e39855.
2. Sakamoto K. The pathologyof mycobacterium tuberculosis. Vet Pathol. 2012;49(4):423–39.
3. Ahmad S. Pathogenesis, immunology, and diagnosis of latent Mycobacterium tuberculosis infection. Clinical and Developmental Immunology. 2011 Oct;2011.
4. Hardee CL, Arévalo-Soliz LM, Hornstein BD, Zechiedrich L. Advances in non-viral DNA vectors for gene therapy. Genes. 2017 Feb;8(2):65.
5. Banik M and Basu T.(2014). Calcium phosphate nanoparticles: a study of their synthesis, characterization and mode of interaction with salmon testis DNA. Dalt Trans ,43(8):3244–59.
6. Zhao L, Seth A, Wibowo N, Zhao C, Mitter N, Yu C, *et al*.Nanoparticl vaccines. Vaccine. 2013;32:327–337. doi: 10.1039/c3dt52522h.
7. Apostólico JD, Lunardelli VA, Coirada FC, Boscardin SB, Rosa DS. Adjuvants: classification, modus operandi, and licensing. Journal of immunology research. 2016 Jan 1;2016.
8. Yuri Tsuchiya, Tsuyoshi Ishii, Yoshio Okahata. Characterization of protamine as a transfection accelerator for gene delivery. Journal of bioactive and compatible polymers. 2006;21(6): 519-537.
9. Yuan S, Qiao T, Li X, Zhuang X, Chen W, Chen X, Zhang Q. Toll-like receptor 9 activation by CpG oligodeoxynucleotide 7909 enhances the radiosensitivity of A549 lung cancer cells via the p53 signaling pathway. Oncology letters. 2018 Apr 1;15(4):5271-5279.
10. Jung H, Kim D, Kang YY, Kim H, Lee JB, Mok H. CpG incorporated DNA microparticles for elevated immune stimulation for antigen presenting cells. RSC Adv. 2018;8(12):6608–6615.
11. Neuhaus B, Tosun B, Rotan O, Frede A, Westendorf AM and Epple M. Nanoparticles as transfection agents: a comprehensive study with ten different cell lines. RSC Adv. 2016;6(22):18102–18112.
12. Vardevanyan PO, Antonyan AP, Parsadanyan MA, Davtyan HG, Karapetyan AT. The binding of ethidium bromide with DNA: interaction with single-and double-stranded structures. Experimental & molecular medicine. 2003 Dec;35(6):527-533.
13. Bisht S, Bhakta G, Mitra S, Maitra A. pDNA loaded calcium phosphate nanoparticles: highly efficient non-viral vector for gene delivery. Int J Pharm. 2005;288(1):157–168.
14. Sorgi F, Bhattacharya S, Huang L.Protamine sulfate enhances lipid-mediated gene transfer. Gene Ther. 1997;4:961–968.
15. Chen J, Yu Z, Chen H, Gao J, Liang W. Transfection efficiency and intracellular fate of polycation liposomes combined with protamine. Biomaterials. 2011 Feb 1;32(5):1412-1418.
16. Motta S, Brocca P, Del Favero E, Rondelli V, Cantù L, Amici A, Pozzi D, Caracciolo G. Nanoscale structure of protamine/DNA complexes for gene delivery. Applied Physics Letters. 2013 Feb 4;102(5):053703.
17. Dowling J and Dellacasagrande J. Toll-like receptors: Ligands, cell-based models, and readouts for receptor action. Methods Mol Biol. 2016;1390:3–27.
18. Dowling J, Mansell A. Toll like receptor:the swiss army knife of immunity and vaccine development. clinc transl Immunol. 2016;5(5):e85.