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GOLD NANOPARTICLES FUNCTIONALIZED WITH PROTAMINE FOR mRNA DELIVERY

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ABSTRACT

Nucleic acid vaccines introduce foreign genes into the body to produce target proteins using the host cell´s machinery, thereby triggering an immune response. While viral vectors have traditionally been studied for gene delivery, concerns regarding immune response and cytotoxicity have led to the use of non-viral vectors. Gold nanoparticles (GNP) have emerged as a promising candidate due to their low toxicity, high specificity, and therapeutic efficacy. This study presents the synthesis and characterization of GNP functionalized with proteins for mRNA delivery, targeting applications in vaccines and advanced therapies. Gold nanoparticles were synthesized using HEPES buffer as a reducing agent, and complexes between gold nanoparticles, protamine, and mRNA were formed and characterized. The hydrodynamic sizes of the complex varied in the range of 160-280 nm, while their surface charges ranged from -27 to +22 mV. Results showed that the addition of protamine influenced the behavior of the complex, with a tendency towards aggregation at intermediate protamine concentrations. However, the presence of mRNA did not affect the surface charge and size of the system, indicating a lack of interaction between the gold nanoparticle and the nucleic acid. These findings underscore the importance of protamine for facilitating interaction between the components and highlight the potential of GNP as a delivery vehicle for mRNA-based therapeutics.

Keywords: Nucleic acid vaccines. Gold nanoparticles. mRNA delivery. Protamine.

1 INTRODUCTION

Nucleic acid vaccines, such as DNA and mRNA vaccines, work by introducing specific foreign genes into the body, which utilize the host cell's protein synthesis machinery to produce the target proteins, triggering an immune response [1]. Gene delivery strategies utilize both viral and non-viral vectors. Viral vectors such as lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses have high efficiency in DNA transfection and are used to treat diseases like cancer and hemophilia [2,3]. However, they present disadvantages such as immune response and cytotoxicity.

Among non-viral vectors, gold nanoparticles (GNP) have been considered a potentially powerful tool in gene therapy due to their low toxicity, high specificity, and therapeutic efficacy [4]. GNP are defined as stable colloidal nanostructures of gold atom arrangements, with dimensions between 1 and 100 nm [5]. They can come in various sizes and shapes (spheres, rods, cages, stars) and can be functionalized with a variety of biomolecules (proteins, nucleic acids, peptides, antibodies, chemotherapeutic agents), in addition to being biocompatible [6]. GNP can be easily synthesized through physical, chemical, and biological methods.

Due to their versatile surface chemistry, GNP have a wide range of biomedical applications, including detection and imaging, targeted drug delivery and therapeutic agents, inclusion in diagnostic platforms, photothermal therapy, and radiotherapy [7]. Due to their multivalency, GNP can protect unstable drugs and facilitate their delivery to otherwise inaccessible regions of the body. Given their size relative to proteins, GNP can modify cellular processes in ways that small molecules and proteins cannot, allowing them to act as drug delivery agents [8].

Conventional vaccines have been effective in reducing infectious diseases but have limitations such as the risk of reversion in live-attenuated vaccines and the need for multiple doses in inactivated vaccines. These difficulties are exacerbated during outbreaks and in areas with limited healthcare access [9]. In contrast, mRNA vaccines have emerged as a promising alternative since the early studies in 1990, showing efficacy in animal models for diseases like Zika, rabies, Ebola, influenza, cancer, and autoimmune diseases [10]. mRNA vaccines are safe, effective, easy to produce, do not pose a risk of infection or insertional mutagenesis, can be naturally degraded by the body, and their production is faster and cheaper [11,12].

Gold nanoparticles, for therapeutic use, can be functionalized with various molecules to enhance specificity, selectivity, and sensitivity while preventing aggregation and improving biocompatibility [13,14]. Protamines, a group of polycationic peptides rich in L-arginine, are one method of functionalization. Protamine can complex with nucleic acids, protecting from enzymatic degradation in biological systems [15]. Our research group has expertise in conjugating gold nanoparticles with pDNA and protamine.

In this context, the present study aims to synthesize and characterize gold nanoparticles functionalized with proteins for mRNA delivery as a potential vaccine platform. The GNP-Protamine-mRNA complexes will be characterized for their charge, size and shape. The transfection efficacy of these complexes will be investigated through *in vitro* studies by analyzing reporter gene expression.

2 MATERIAL & METHODS

Reagents and biomolecules. Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), HEPES and MEGAscript™ T7 Transcription Kit were purchased from Sigma-Aldrich. The plasmid pVAX1-GFP expressing green fluorescent protein was purifed by using the Promega Wizard® SV Gel and PCR Clean-Up System according to manufacturer's manual.

Gold Nanostar Synthesis. Gold nanostars were synthesized following a protocol from the literature [16]. Briefly, 200 µL of a 20 mM $AuCl_4$ aqueous solution was added to 20 mL of a 40 mM HEPES solution (pH 7.4) and gently agitated for 10 s to ensure homogeneous mixing. The solution was placed in the dark at 25 °C for 1 h to promote nanostar growth. Gold nanostar solutions were centrifuged (30 min, 2400 × g), redispersed in 20 mM and stored at 4 °C until use.

***In* vitro transcription of mRNA.** *In vitro* transcription was performed following the MEGAscript™ T7 Transcription Kit protocol, using the previously purified PVAX1-GFP template. The reaction was incubated at 37°C for 4 hours, and the product was treated with DNase. The final products were quantified by fluorimetry and analyzed by agarose gel electrophoresis to confirm the synthesis of full-length mRNA.

RNA:Protamine:Gold Conjugates. In Eppendorf tubes, 1 µg of mRNA was incubated with different masses of protamine (1µg/µl) at room temperature. After 10 minutes, 40 µL of gold nanoparticles were added, and the final volume was adjusted to 100 µL with 40 mM HEPES buffer. Right after, the samples were analyzed for size, polydispersity, and surface charge.

Dynamic Light Scattering and Zeta Potential Measurements. The nanoparticles' hydrodynamic diameters were evaluated by dynamic light scattering (DLS) at a temperature of 25 °C in a polystyrene cuvette, using Zetasizer Nano-ZS90 equipment. In the same way, the nanoparticles' surface potential was quantified using their electrophoretic mobility collected at 25 °C using the same equipment. All samples were diluted 1:10 with ultrapure water and the readings were performed in triplicate.

Transmission Electron Microscopy. The transmission electron microscopy (TEM) analyses were done using a JEOL JEM 2100 microscope. The samples were prepared by dripping onto a Formvar/carbon supported copper grid, mesh 300, and then dried in a desiccator containing silica for 24 hours prior to analysis.

3 RESULTS & DISCUSSION

The star-shaped geometry expected for gold nanoparticle synthesis using HEPES buffer as a reducing agent can be observed in Figure 1A. In Figure 1B, the agglomeration state of the complex is observed, caused by the presence of protamine in solution, which are strongly basic proteins containing a high amount of the amino acid arginine. In Figure 1C, there is an observed shadow around the nanoparticles, composed of biological material, indicating the presence of protamine and mRNA.

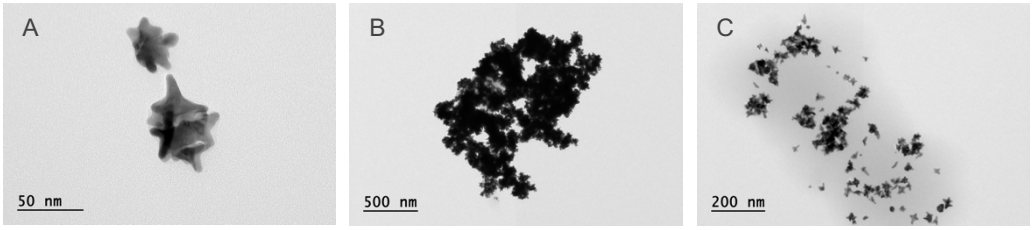

Figure 1 Transmission Electron Microscopy of GNP conjugated with mRNA and protamine. A: GNP + mRNA; B: GNP + Protamine (5 µg) + mRNA; C: GNP + Protamine (40 µg) + mRNA.

The particle size and zeta potential of GNP before and after addition of protamine and mRNA are shown in Table 1. The surface charge was reduced to -25.8 ± 2.0 mV to -13.8 ± 13 mV upon addition of protamine, indicating colloidal instability. The particle size also increased significantly upon conjugation; starting from 20 µg of protamine, the nanoparticles exhibited a particle size of 260–280 nm, indicating a tendency towards the formation of aggregates.

Table 1 Particle Size, Polidispersity and Surface Potential of GNP before and after conjugation of mRNA and protamine.

Sample	Intensity (nm)	Number (nm)	PdI	Zeta (mV)
GNP	184.4 ± 15.7	134.0 ± 31.4	0.364 ± 0.002	-27.1 ± 0.36
GNP + mRNA	162.5 ± 16.9	159.2 ± 18.1	0.568 ± 0.054	-25.8 ± 2.0
GNP + Protamine (0.5 µg) + mRNA	179.3 ± 33.2	178.8 ± 33.0	0.681 ± 0.126	-13.8 ± 13
GNP + Protamine (5 µg) + mRNA	172.1 ± 5.82	172.4 ± 6.12	0.919 ± 0.081	5.50 ± 1.5
GNP + Protamine (20 µg) + mRNA	276.6 ± 76.5	275.7 ± 75.0	0.857 ± 0.126	22.3 ± 7.2
GNP + Protamine (40 µg) + mRNA	257.9 ± 32.1	257.7 ± 31.7	0.919 ± 0.071	20.1 ± 7.3

4 CONCLUSION

Through this study, we can conclude so far that the amount of protamine added to the complex of gold nanoparticles and mRNA influences the nanoparticle's behavior. There is an observed tendency for aggregation at intermediate values of protamine, as indicated by the increase in size and decrease in the surface charge modulus of the complex. As the mass of protamine increases, the system shows a tendency to return to stability.

It is also possible to observe that the presence of mRNA does not affect the surface charge and size of the system, suggesting that there is likely no interaction between the gold nanoparticle and the nucleic acid. This is because both elements have a negative surface charge, which confirms the need for protamine for interaction between them.

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