Polyethylene Glycol-Coated Gold Nanoparticles as DNA and Atorvastatin Delivery Systems and Cytotoxicity Evaluation

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1. Introduction

Currently, nanomedicine is a field that has great interest in the scientific community [1], since it has found application in many areas of science such as medical imaging [2], tumor targeting [3], drug delivery [4, 5], and biosensors [6], among others. Particularly, nanoparticles have been widely studied for medical purposes; however, the main problem consists in finding the materials with optimal biocompatibility [7]. It has been found that gold nanoparticles (AuNPs) are one of the most innocuous nanoparticles for living organisms [8], and it is suspected that most of the nanoparticles enter into the cells by endocytosis [9]. Nonetheless, the cytotoxicity of most of AuNPs depends on several parameters such as size, tissue distribution, penetration capacity, tissue absorption, and cell types [10].

It has been reported that AuNPs with sizes between 4 and 5 nm may have toxicity due to penetration into the nuclear compartment where they could bind to DNA; for instance, the 1.4 nm AuNPs have a strong toxic potential on DNA [11]. On the other hand, gold nanoparticles with sizes of 18 and 20 nm show greater cell viability in some kinds of cell cultures [11].

Chithrani et al. [12] showed that the diameter of gold nanoparticles influences the cellular uptake in a HELA cell culture; as the size of the AuNPs increases (from 14 to 50 nm), the concentration of the nanoparticles into the cell is greater, the maximum cellular uptake occurs at the size...
of 50 nm, and subsequently, there is a decrease in the cellular uptake (sizes from 70 to 100 nm). In addition, several researches have reported an improvement in the cellular uptake of gold nanoparticles by using a biopolymer coating [13].

Nanoparticle surface coated with polymers can regulate and improve their stability, solubility, and focalization [14]. In vivo studies suggest that nanoparticles without covering can agglomerate due to different substances present in biological fluids which could cause an obstruction of small capillaries. Some conditions that the polymer must fulfill are as follows: it must be biocompatible, it must not generate an immune response, and it must be biodegradable by the organism [15].

Hereby, gold nanoparticles have potential applications as a gene delivery vehicle with multiple purposes [13]. The transfection efficiency of a plasmid is the rate between the number of cells which express the genetic information inserted by a vehicle and the total number of cells. Currently, there has been a search for nonviral gene vehicles which present the best characteristics, high transfection efficiency, and low cytotoxicity [16]. The nonviral gene vehicles commonly used for transfection studies are organic molecules; for instance, cationic liposomes have acceptable transfection efficiency and high cell viability [17]. However, gold nanoparticles have also been considered for this purpose [18].

Furthermore, gold nanoparticles have been used as drug delivery systems due to their easy functionalization with a drug which uses the nanoparticles as the carrier [19]. In some cases, the bond produced between the nanoparticle and the drug can be easily hydrolyzed by the cell enzymes and release the drug into the cell.

In vitro viability assays are used to measure the proportion of living cells after a potentially traumatic procedure; they are dependent on the alteration of the permeability of the membrane that is determined by the incorporation of agents that are normally impermeable, [20]; for instance, the trypan blue method is based on a blue dye which stains the dead cells due to the damage to the membrane permeability.

In this work, the transfection study of plasmids pSV-β-Gal and pIRE2-EGFP by using gold nanoparticles coated with polyethylene glycol (PEG) as a gene delivery vehicle was performed. The gold nanoparticles were synthesized by chemical reduction at two sizes, 20 and 40 nm. The transfection study was evaluated by means of a cell culture of embryonic kidney cells (HEK 293). In addition, the cell uptake of the PEGylated gold nanoparticles loaded with a fluorescent drug, the atorvastatin which is a drug for cholesterol synthesis inhibition, was evaluated. Furthermore, the viability study of the cells incubated with the PEGylated gold nanoparticles was performed by a trypan blue technique.

2. Methods

The synthesis of gold nanoparticles was performed by a chemical reduction method [21] using sodium borohydride and cetyltrimethylammonium bromide (CTAB) as reducing agents [22, 23]. All glassware was washed with aqua regia (150 mL of HCl and 50 mL of HNO₃) and rinsed with MiliQ water before use. In addition, all AuNPs were synthesized by using a 25 mM stock solution of gold (III) chloride hydrate (Sigma-Aldrich, USA).

2.1. Synthesis of 20 nm Gold Nanoparticles. Synthesis of 20 nm gold nanoparticles was performed by the seed growth method [22]. The seed solution was prepared with 200 μL of the gold stock solution (25 mM) dissolved in 18.7 mL of MiliQ water in a flask with slight stirring, 600 μL of 100 mM sodium borohydride solution and 500 μL of 10 mM sodium citrate solution were added, the stirring continued during one hour at room temperature, and color change from light yellow to light pink was observed. The growth solution was prepared with 625 μL of the gold stock solution and 100 μL of 100 mM CTAB solution (Sigma-Aldrich, USA), and the volume of the mixture was fixed up to 250 mL with MiliQ water. The growth solution was added to the seed slowly in stirring during 2 hours at room temperature. The nanoparticles were purified by centrifugation at 5,000 g for 10 minutes, the supernatant was removed, and the volume was completed with fresh MiliQ water in order to remove the unreacted CTAB. The synthesized gold nanoparticles were stored at 4°C.

2.2. Synthesis of 40 nm Gold Nanoparticles. For the synthesis of 40 nm gold nanoparticles, 500 μL of the gold stock solution (25 mM) was dissolved in 50 mL of water in a flask with slight stirring and a solution of 30 mg of sodium borohydride (Fluka Analytical, USA) in 10 mL of cold water that was added by stirring; the stirring continued during 5 hours at room temperature, until color change from light yellow to light pink was observed. In addition, 1 mL of an aqueous solution of sodium citrate (Sigma-Aldrich, USA) at 1 mM was added to the mixture and kept in stirring for 30 minutes more; it worked as a stabilizing agent. The purification was carried out by centrifugation at 5,000 g for 10 minutes, and the supernatant was replaced with MiliQ water. Finally, the synthesized gold nanoparticles were stored at 4°C.

2.3. Synthesis of the AuNP-PEG Nanoparticles. The synthesized gold nanoparticles were functionalized with polyethylene glycol (SH-PEG-NH₂, MW 2 kDa, Dendritech Inc., USA) following the method reported by Khutale and Casey [24], where the thiol group of the polymer binds to the gold nanoparticles: in a 70 mL of 30 μM of AuNP solution, 1 mL of 1 μM SH-PEG-NH₂ solution was added; this mixture was stirred for 15 min at room temperature. Subsequently, the solution was stored at 4°C overnight to react.

2.4. Nanoparticle Characterization. In metal nanostructures, a phenomenon known as surface plasmon resonance (SPR) can be observed; it is shown in the spectrum as a strong absorption band and corresponds to the wavelengths in which the electrons of nanoparticles vibrate due to the light absorption. This band is characteristic of gold nanoparticles which the electrons of nanoparticles vibrate due to the light absorption. This band is characteristic of gold nanoparticles in regions around 520-540 nm [25]. The optical absorption spectrum of the sample was measured from 400 to 700 nm in a Perkin Elmer spectrometer (Lambda 25) and using MiliQ water as a blank. Furthermore, a stabilizing test of the coated and uncoated gold nanoparticles was performed evaluating the differences between absorption spectra of the particles at different pH. The pH was adjusted by adding 0.2 M citric acid.
acid and 0.1 M sodium hydroxide (Sigma-Aldrich, USA) solutions, respectively. The shape and size distribution of the synthesized nanoparticles were characterized with a JEOL 1011 Electron Transmission Microscope (TEM) by placing 20 \( \mu \)L of the sample on a grid with a formvar membrane and letting it dry at room temperature.

In addition, the characterization of the synthesized gold nanoparticles \( Z \) potential and hydrodynamic diameter was performed by using Zetasizer Malvern Nano Z (Worcestershire, United Kingdom).

2.5. Synthesis of the AuNP-PEG-pDNA. The pSV-\( \beta \)-Gal was isolated from Escherichia coli DH5 bacteria strain; the purification of the plasmid was performed by means of the alkaline lysis method using the kit Ultra Clean MoBio® (California, USA). Different quantities of pDNA (100, 200, 300, and 400 ng) were added to the synthesized nanocomposites for 30 minutes, and this interaction was evaluated with electrophoresis in agarose gel 0.8% at 90 V by 40 minutes.

Gold nanocomplex were formed due to the pDNA bound to the PEGylated gold nanoparticles by means of charge interaction between the amine group of the SH-PEG-NH\(_2\) and the negative charges of the phosphate on the DNA helix. The scheme of the synthesis of AuNP-PEG-pDNA can be observed in Figure 1.

2.6. Synthesis of the AuNP-PEG-At. In order to activate the carboxyl group of atorvastatin (Sigma-Aldrich, USA), 1 mg of NHS (N-hydroxysuccinimide, \( C_4H_5NO_3 \)) and 1.78 mg of EDC-HCl (N-(3-dimethylaminopropyl)-N\(^{\prime}\)-ethylcarbodiimide hydrochloride, \( C_8H_{17}N_3 \cdot HCl \)) were added to 2 mL of a solution of atorvastatin (1 mg/mL); this mixture was stirred for 30 minutes. Subsequently, the activated atorvastatin was added to 50 mL of the AuNP-PEG 14 \( \mu \)M. The synthesis scheme of the AuNP-PEG-atorvastatin is shown in Figure 2.

2.7. Cellular Transfection Study. The gold nanoparticles coated with PEG and the pDNA (Au-PEG-pDNA) were incubated with human embryonic kidney cells (HEK 293 cell culture) in order to evaluate the transfection efficiency. The HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, USA) in an atmosphere of 5% \( CO_2 \) at 37°C; the cells were incubated in 96 well plates.
where the transfection was performed with 90% confluence (12,000 cells per well). 20 μL of gold nanocomplex and 30 μL of medium were added to each well for 2 hours; subsequently, the volume was fixed to 150 μL with DMEM and it was incubated for 48 hours. After, the medium was removed from the wells, the cells were washed twice with phosphate-buffered saline (PBS) 1x, and 50 μL of a mixture of 0.4 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, Sigma-Aldrich, USA) in a solution of 5 mM potassium ferricyanide (Meyer, Mexico), 5 mM potassium ferrocyanide (Meyer, Mexico), and 2 mM magnesium chloride (Meyer, Mexico) dissolved in PBS 1x was added; the cells were incubated for 24 hours.

For the transfection study, the pSV-β-Gal bounded to the vehicle (the AuNPs) allows the cells to produce the β-galactosidase enzyme [26]. The activity of this enzyme in histology with X-gal yields insoluble blue compounds similar to indigo as a result of enzyme-catalyzed hydrolysis [27]. The transfected cells were stained for the enzymatic activity of β-galactosidase expression of pSV-β-Gal, and the cells were counted in three different fields by an image processing software developed in Matlab®.

On the other hand, the pIRES2-EGFP after the expression produces into the cell a green-fluorescent protein which stains the transfected cells [28]; the transfection efficiency was evaluated by using the fluorescence microscope Carl Zeiss Axio Vert.A1.

In addition, 30 μL of a lipofectamine reagent (Sigma, USA) bounded to the plasmid was used as a positive control.

2.8. Cell Uptake Study of the Atorvastatin. The cells were incubated with the same conditions, and they interacted with 50 μL of the Au-PEG-At for 24 hours; then, they were washed with PBS and observed with the fluorescence microscope.

Gold nanoparticles loaded with atorvastatin, which enter into the cells, stain the cytoplasm due to the atorvastatin molecule which presents fluorescence at 310 nm (excitation and 385 nm emission). The cell uptake efficiency was evaluated with the micrographs from 3 different fields by using the image processing software developed in Matlab®. In addition, 50 μL of 1 mg/mL free atorvastatin solution was used as a control.

2.9. Cytotoxicity Test. The cell viability assay was performed in order to know the toxicity of the gold nanoparticles to the cells. The cells were incubated with 20 μL of gold nanocomposites and 30 μL of culture medium during 2 hours; subsequently, they were washed two times with PBS; then, 20 μL of 0.2% trypan blue (Sigma, USA) was added, and finally, after 30 minutes, two more PBS washes were made. The cells stained by the dye (dead cells) were counted by using the image processing software in three different fields. In this case, 30 μL of lipofectamine was used also as a control since it is known that this reagent is cytotoxic.

3. Results

3.1. Gold Nanoparticle Characterization. The absorption spectra of the AuNPs and AuNP-PEG showed a change in the absorption band at longer wavelengths in the spectrum of the PEGylated gold nanoparticles; the optical changes indicate modifications in the nanoparticle surface, and it is attributed to the functionalization of the AuNPs with the polymer. Figure 3(a) shows the UV-visible spectra of the 40 nm gold nanoparticles uncoated and PEGylated.

In addition, the stability of AuNPs as a function of pH was evaluated by varying the pH from 3 to 10. It was found that there are not significant differences between the surface plasmon resonance (SPR) bands of the PEGylated gold nanoparticles coated at different pH. However, the uncoated gold nanoparticles showed changes in the absorption spectra at a pH of 3 and less significance at 10 (Figures 3(b) and 3(c)); this red shift is due to the nanoparticle aggregation. These results confirm that polymer coating improves the stability of the nanoparticles, avoiding the aggregation at different pH conditions; this was also reported by Khutale and Casey [24] who used PEGylated gold nanoparticles as a doxorubicin release system. Stability tests could be used to show the behavior of nanoparticles in colloidal suspension at different pH conditions and salt environment, among other similar physiological conditions. For this reason, it is necessary to find coatings which improve the stability of the colloids at different environments; in this case, it was showed that SH-PEG-NH₂ (2 KDa) coating on gold nanoparticles could be useful for this purpose.

Transmission electron micrographs showed the spherical shape of the synthesized gold nanoparticles and the size distribution of the gold nanoparticles synthesized with CTAB (diameter: 20 ± 5 nm) in Figure 4(a) and sodium borohydride (diameter: 40 ± 5 nm) in Figure 4(b). In addition, Figure 4(c) shows the 20 nm PEGylated gold nanoparticles, a layer which coats the AuNPs can be observed, and this is attributed to the PEG coating. Furthermore, it was obtained that there is a change in the Z potential between the nanoparticles coated and uncoated with PEG (Figure 5(a)); this is because the surface amine group of the SH-PEG-NH₂ provides positive charges to the gold nanoparticles which increases the Z potential in both cases. The 40 nm uncoated gold nanoparticles presented negative potential due to the carboxyl groups of the citrate which coated them; in contrast, the 20 nm gold nanoparticles showed positive Z potential due to the CTAB, which is a cationic surfactant which promoted the growth of the seed solution, and capped the gold with positive charges. In addition, the hydrodynamic diameter of the nanoparticles increases in 37% and 28% due to the PEG which coats the synthesized gold nanoparticles (Figure 5(b)). On the other hand, the obtained averaged polydispersity indexes (PDI) were 0.634 for AuNPs (20 nm) and 0.253 for AuNP-PEG (20 nm) and 0.389 for AuNPs (40 nm) and 0.172 for AuNP-PEG (40 nm). These PDI values validate the measurements of the hydrodynamic diameter; furthermore, they show that the PEGylation decreases the PDI.

3.2. Cellular Transfection and Cytotoxicity. The electropherogram showed that free pDNA does not present retardation in the gel (Figure 6, lane 1 with 400 ng of free pDNA and 2 with 200 ng); however, lanes 4 to 6, where there is interaction
between pDNA and AuNPs, present a delay. In contrast, the 40 nm uncoated gold nanoparticles in lane 3 did not show interaction with the DNA; therefore, the plasmid do not show delay in the gel; this is explained due to the fact that the surface potential is negative which results in a repulsion with the negative charge from DNA. On the other hand, lane 7 shows the interaction of the AuNPs with 400 ng of pDNA; the apparition of bands of the DNA was observed which did not bound to the gold nanoparticles due to the fact that they are saturated and cannot hold all of this DNA amount; i.e., it is the limit quantity of pDNA that can be bounded to the AuNPs.

Gel retardation assays allow to know the amount of DNA which could be retained by the nanoparticles, and this is due to the interaction between positive surface charges on the particle and negative charges from DNA. The most common synthesis of gold nanoparticles is by using sodium citrate or borohydride as reducing agents; however, it produces negatively charged gold nanoparticles. Hence, polymer coating is important for binding DNA to AuNPs. For instance, Lazarus and Singh [29] used polyethylenimine (PEI) which provides positive charges to the nanoparticles, due to the presence of many amine groups in the chain, and they showed by means of electrophoresis the interaction between pCMV-luc DNA and 76 nm AuNPs at different mass ratios. In addition, Ahn et al. [26] found the optimal charge ratio of copolymers (PEI and PEG) for DNA delivery by using gel retardation assay as well.

Figure 3: Optical characterization of the gold nanoparticles and stability test. (a) UV-visible absorption spectra of 40 nm AuNPs and AuNP-PEG. (b) UV-vis absorption spectra of AuNPs and (c) AuNP-PEG at different pH.

Figure 7 shows a micrograph of the pSV-β-Gal transfection study of HEK 293 cells using lipofectamine and the AuNP-PEG-pDNA nanoparticles of 20 and 40 nm in diameter as vectors. The transfection study (Figure 7(a)) demonstrated
that the lipofectamine and 20 nm AuNP-PEG (Figure 7(b)) enter into the cells with the pDNA and it allows the expression of the plasmid in which blue stains the cell due to the blue molecule produced by the metabolism of the X-gal substrate. In addition, Figure 7(c) shows the cell transfection of the AuNPs of 40 nm in diameter.

Figure 8(a) shows a micrograph of the cells incubated with the AuNP-PEG-pIRES2-EGFP nanoparticles. The transfection study demonstrated that the AuNP-PEG enter into the cells with the pDNA and it allows the expression of the plasmid which stains the cell due to the transcription of the green fluorescent protein [28].

On the other hand, Figure 8(b) shows the cellular uptake of the atorvastatin using AuNP-PEG particles as carriers. It can be observed that the atorvastatin which enters into the cells with the vehicle helps stain the cytoplasm; however, it was demonstrated that the free atorvastatin do not enter into the cells and then was removed by the PBS; hence, the micrograph did not show fluorescence with the UV lamp.

Furthermore, the cytotoxicity test with trypan blue showed that the synthesized gold nanoparticles have a low toxicity effect to this cell culture, and with the conditions described, the micrograph obtained by the cytotoxicity test (Figure 9) shows a higher proportion of living cells than dead cells (blue-stained cells).

Finally, Figure 10 summarizes the results obtained of the cellular transfection efficiency, drug cell uptake, and cytotoxicity. Lipofectamine showed the highest amount of cellular transfection of both plasmids, but it is cytotoxic (46.64% of dead cells). However, the PEG-coated gold nanoparticles synthetized by both methods showed <10% of dead cells (6.2% and 4.1%, respectively) and the transfection efficiency >45%. It can be also observed that the results of transfection efficiency of the two plasmids are similar; however, in the

**Figure 4:** Transmission electron micrographs of AuNPs. (a) 20 nm gold nanoparticles, (b) 40 nm gold nanoparticles, and (c) gold nanoparticles coated with PEG contrasted by phosphotungstic acid.

**Figure 5:** DLS and Z potential analysis of AuNPs and AuNP-PEG. (a) Z potential and (b) hydrodynamic diameter.
three cases, the transfection of pIRES2-EGFP is greater than the pSV-β-Gal. In addition, smaller gold nanoparticles (20 nm) provide greater transfection efficiency in both kinds of plasmids, as expected, but they have a greater cytotoxicity effect than the 40 nm, and this was also expected due to some publications that agree that the smaller nanoparticles have a greater cytotoxic effect [11].

4. Discussion

Transfection efficiencies of pSV-β-Gal obtained in this work are comparable to those reported by González et al. [30] who used gold nanoparticles capped with different types of chitosan as pSV-β-Gal delivery systems. Chitosan provides more positive charges to the nanoparticles than the SH-PEG-NH₂, due to the fact that the chitosan has an amine group in each monomer of the chain; therefore, the chitosan could retain more DNA and the obtained transfection efficiencies for some types of chitosan are higher than the those reported in this work; however, in some cases, the AuNP-chitosan were cytotoxic.

Results of pIRES2-EGFP transfection efficiency obtained in this work are comparable to those reported by Liu et al. [31] who used a modification of the vector pIRES2-EGFP by using liposomes as vehicles to evaluate transfection by means of fluorescence microscopy. However, transfection efficiencies of AuNP-PEG evaluated in the present work are slightly higher than those the liposomes.

Furthermore, the obtained transfection efficiency of these plasmids by using AuNP-PEG is higher than the efficiency obtained from many methods used for gene therapy; this shows that the gold nanoparticles coated with PEG could be considered promising DNA vehicles for many medical proposes, for instance, cancer therapy.

On the other hand, the drug cellular uptake by using the gold nanoparticles as carriers is greater in the larger nanoparticles, in contrast with the transfection results obtained; however, it was demonstrated that the free atorvastatin cannot enter into the cells (0% of cell uptake) which besides confirms that both types of AuNPs are effectively performing the role of drug carriers (69.39% and 75.12% of drug cell uptake, respectively). Cellular uptake studies of fluorescent drugs
are semiquantitative methods which allow to know if the carrier molecule is transporting a drug into the cell [32]. Currently, nanocarriers have been used due to the physical and chemical advantages that they have. For instance, Khutale and Casey [24] proposed PEGylated gold nanoparticles as doxorubicin carriers and they showed that these produce higher cellular uptake at short time exposures than the free doxorubicin. In addition, the low cytotoxicity and drug release efficiency obtained promises that these nanoparticles could be used in several applications in medical treatments as drug delivery systems. These results complement those obtained by Patra et al. [33] who showed that gold nanoparticles synthesized with CTAB and coated with PEG are not toxic to Hep2G and BHK21 cell lines but it produces cytotoxicity to A549 cells. In general, gold nanoparticle toxicity depends on the concentration and cell type, but there are many studies which conclude that AuNPs do not have toxicity effects (see Reference [34] and the references within).

5. Conclusions
Gold nanoparticles of two different sizes (20 and 40 nm) were synthesized, the gold nanoparticles were coated with PEG, and it was shown that this coat improves the stability of the gold nanoparticles at different pH; besides, two different plasmid DNAs (pSV-β-Gal and pRES2-EGFP) were bounded to the nanoparticles; the interaction between the nanoparticles and the plasmid (up to 300 ng) was demonstrated by agarose gel electrophoresis; this confirms that the synthetized PEGylated gold nanoparticles could be used as vehicles of these plasmids.
The results of the transfection study between both plasmids were similar, and it was showed that 20 and 40 nm PEGylated gold nanoparticles presented a transfection efficiency comparable with the lipofectamine but the main difference is that the cytotoxicity effect is lower if the PEG-coated gold nanoparticles are employed as vehicles compared with the lipofectamine.

In addition, it was shown that the synthetized AuNP-PEG worked as carriers of the atorvastatin and the drug uptake obtained was ≈70% which demonstrates that these nanoparticles are suitable to be used as drug nanocarriers.

These results show that gold nanoparticles coated with PEG have a great potential use in several medical applications, particularly in gene therapy and drug delivery systems.

**Abbreviations**

- PEG: Polyethylene glycol
- AuNPs: Gold nanoparticles
- pDNA: Plasmid DNA
- AuNP-PEG: PEGylated gold nanoparticles
- AuNP-PEG-pDNA: PEGylated gold nanoparticles loaded with plasmid DNA
- AuNP-PEG-At: PEGylated gold nanoparticles loaded with atorvastatin
- CTAB: Cetyltrimethylammonium bromide
- SPR: Surface plasmon resonance
- TEM: Transmission electron microscopy
- DMEM: Dulbecco’s modified Eagle’s medium
- PEI: Polyethyleneimine

**Data Availability**

The micrographs and gold nanoparticle characterization data used to support the findings of this study are available from the corresponding author upon request.

**Conflicts of Interest**

The authors declare that they have no competing interests.
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