Research Article

Biological Impact of the Interaction of Functionalized and Bioconjugated Gold Nanoparticles of Different Sizes on HeLa and SH-SY5Y Human Cell Lines

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Nanotechnology opens new perspectives in many science and technology fields through new materials, such as metal nanoparticles. The biomedical field is one of these areas where nanoparticles offer promising application in the diagnosis and therapy of disease, generating biosensors for disease detection, bioimaging, and drug delivery. However, the controversy about whether nanoparticles are inert or exhibit different degrees of toxicity related to their physicochemical properties remains a subject of study and debate. This work shows how gold nanoparticles and nanorods were simultaneously functionalized with 4-aminothiophenol (4-ATP) and methoxypolyethylene glycol thiol (mPEG-SH). Then, folic acid (FA) gold nanoparticle bioconjugates caused diverse biological effects on HeLa and SH-SY5Y cell cultures after 24 h of incubation, when they were evaluated in the range of gold concentrations from 0.17 to 350 μM. We found notable changes in cell metabolic activity, viability, and biomass. 16 nm nanoparticles produced the most enhanced damage for functionalized (AuNPs) and bioconjugated (AuNPs-FA) nanoparticles, which we expected to be more biocompatible when coated with FA. Besides, epifluorescence images showed damage to F-actin microfilaments, adhesion and cell attachment loss, morphology changes (cells became round and detached), presence of blebs, and rupture of cell membrane. These results suggested that both AuNPs and AuNPs-FA have significant biological impact on HeLa and SH-SY5Y cell cultures, whereas gold nanorods showed significant changes only in the metabolic activity of SH-SY5Y cells when they were exposed to gold nanorods of 23 and 27 nm in length with a diameter of 5 nm, and crystal violet did not show evidence of toxicity.

1. Introduction

As a bulk metal, gold has a reddish yellow color. This noble metal is the most inert material and the least reactive, exhibiting high biocompatibility in the interaction with biological organisms, as is the case of the human body [1, 2]; however, as nanoparticles (NPs), it displays fascinating physical properties, such as strong optical absorbance and...
scattering due to NP size, morphology, environment, and surface functionalization [3–5].

NPs are modulated by the localized surface plasmon resonance (LSPR), which refers to the general manipulation of metallic nanostructures to modulate and control light below the diffraction limit. Nowadays, it is possible to produce colloidal NPs with a wide variety of sizes and geometrical forms to tune the LSPR frequency from the ultraviolet (UV) to the near infrared (NIR) region, and their potential applications have shown an impressive development [6–8]. Plasmonic NPs provide a platform for the development of biosensors [9, 10], immunoassays [11, 12], clinical chemistry [13, 14], detection and control of microorganisms [15], cancer cell photothermolysis [16], targeted delivery of drugs [17], and optical imaging and monitoring [16–18]. For all this, metal NPs have attracted the attention of the biomedical field, impacting applications such as disease detection, treatment, and drug delivery [19]. On the other hand, many efforts have been made to elucidate the effects of NPs on human health, but reports show controversial results [20]. Up to now, there is little data on nanomaterials’ safe use and regulatory guidelines concerning their possible toxic effects [21, 22]. While some reports affirm to have observed convincing toxicological results, others show that they are entirely biocompatible. Since it is undeniable that NPs are part of our day-to-day, more in vitro studies can be helpful to evaluate and determine their toxic effects [23–28] before they can be evaluated in animal models or moved on to testing in human beings [29]

In this work, we study the interaction of gold nanoparticles (AuNPs) and nanorods (AuNRs) of three different sizes and their surface chemistry for two cases: when functionalized with 4-aminothiophenol (4-ATP) and methoxypolyethylene glycol thiol (mPEG-SH) and when AuNPs bioconjugated with folic acid (FA). HeLa and SH-SY5Y cell lines were selected to evaluate the cytotoxic effects of these gold NPs.

2. Materials and Methods

2.1. Chemical Reagents. All compounds chloroauric acid (Gold III, ≥99 %), silver nitrate (AgNO₃, ≥99 %), sodium citrate tribasic dehydrate (ACS, reagent, ≥99 %), mPEG-SH (molecular weight Mw=5000), cetyltrimethylammonium bromide (CTAB), hydroquinone (ACS, reagent-Plus, ≥99 %), N-hydroxysuccinimide (NHS, 98 %), 4-ATP (≥97.0 %), FA (≥97 %), and N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide and hydrochloride (EDC, ≥98 %) were supplied by Sigma Aldrich (Toluca, Mexico), and they were used without any further purification.

2.2. Gold Nanoparticles: Synthesis, Functionalization, and Bioconjugation. A slight modification of the method proposed by Bastús et al. was followed to synthesize gold nanoparticles [30]. First, a solution of 150 mL of sodium citrate at 2.2 μM was heated to its boiling point; then, 1 mL of HAuCl₄ (25 mM) was added, and the solution started to change color until the characteristic ruby red color appeared, indicating the presence of seed gold nanoparticles. Afterwards, the colloidal solution temperature was brought down until it stabilized at 90 °C, and 1 mL of HAuCl₄ was injected. We let the solution react for 30 min, and again, 1 mL of HAuCl₄ was injected; after ≤x2009;30 min, we proceeded to dilute the colloid by extracting 55 mL, adding 53 mL of Milli-Q water, and adding 2 mL of sodium citrate solution at 60 mM, thus obtaining the first sample. Figure S3 in the supplementary material shows a schematic representation of the synthesis of gold seed solution and the first growth of gold nanoparticles. The process was repeated two times more to obtain three different sizes of nanoparticles, with each cycle producing NPs of a bigger size. Figures S4 and S5 in the supplementary material show the schematic representation of the synthesis of the second and third growth of gold nanoparticles. Besides, the supplementary material contains information about estimating gold concentrations and the number of NPs/mL of each colloid. The excess of sodium citrate was eliminated by centrifuging the colloid three times, at 4000, 3500, and 3000 rpm, respectively.

After that, we proceeded to carry out the functionalization in the following way. Stock solutions of mPEG-SH at 2 mg/mL and 4-ATP at 0.59 μM were prepared, and after that, 10 mL of the cleaned nanoparticles was poured into a glass vial. Immediately, 50 μL and 10 μL each of the mPEG-SH and 4-ATP solutions were simultaneously added, and the colloid remained under magnetic agitation for 24 h. After that, we dialyzed the colloid (Spectra/Por membranes, 12-14 kD) against water for three days to eliminate unbonded reagents. Bioconjugation was carried out as follows: stock solutions of FA in dimethylsulfoxide (0.1 mM), NHS (0.1 mM) and EDC (0.1 mM) in Milli-Q water were prepared. Then, 450 μL of FA was first activated with 160 μL of EDC under magnetic agitation for four hours, while in another glass vial, 8 mL of AuNPs and 160 μL of NHS were poured under magnetic agitation for four hours. Finally, the activated FA was added to the functionalized NPs for bioconjugation, and the colloidal solution was left for 24 h under agitation; bioconjugated nanoparticles were dialyzed against water (Spectra/Por membranes, 12-14 kD) for three days. Henceforth, when the labels AuNPs and AuNPs-FA appear, we mean functionalized and bioconjugated NPs, respectively, while indices 1, 2, and 3 refer to the first, second, and third samples of colloid NPs; for example, 1-AuNPs-FA means the first sample bioconjugated with FA.

2.3. Gold Nanorods: Synthesis and Functionalization. Five milliliters of gold seed colloid were prepared following the method of Nikoobakht and El-Sayed. Briefly, it consists in preparing 5 mL of HAuCl₄ solution (1 mM), adding 5 mL of CTAB (0.2 M), and finally, under strong mechanical agitation, adding 300 μL of NaOH (0.1 mM) and NaBH₄ (0.1 mM) [31], to be then used after one hour of aging. Afterwards, gold nanorods were obtained following the work of Vigerdman and Zubarev’s and Picciolini et al.’s method, with slight modifications [32, 33]. First, a HAuCl₄ solution (25 mL, 1 mM) was added to a CTAB solution (25 mL, 0.133 M), and subsequently, we added a silver nitrate
concentrated by centrifugation were analyzed using TGA. Methanol at 7.8 M and functionalized gold nanoparticles were also included in the toxicology analysis; then, bioconjugated gold nanorods were prepared following the same protocol carried out for gold nanoparticles, but they display short colloidal stability, which is unsuitable for toxicology analysis; then, bioconjugated gold nanorods were excluded from the toxicology analysis.

3. Characterization

3.1. A UV–Visible Spectroscopy Measurement. UV-Vis extinction spectra of gold nanoparticles and nanorods were monitored by means of a StellarNet XPP2000 portable spectrometer; 1 mL of each colloidal sample was diluted with Milli-Q water at a rate of 11, and the sample was poured into a quartz cuvette with a path length of 1 cm.

3.2. Scanning Electron Microscopy Measurements. The samples for scanning electron microscopy (SEM) analysis were prepared by dropping 3 μL of each sample onto a glass slide coated with a thin layer of aluminum. The samples were dried at room conditions and then analyzed using a JEOI scanning electron microscope (model JSM-7800F). Particle size analysis was carried out using ImageJ free software.

3.3. Zeta Potential. The measurement of Z-potential determined the surface charge of AuNPs, AuNPs-FA, and AuNRs. Measurements were carried out using the Z-potential analyzer (Z-sizer Nano ZS, Malvern Panalytical), samples were diluted with Milli-Q water at a rate of 1:4.

3.4. Raman Spectroscopy. An InVia Renishaw micro-Raman spectrometer (using an excitation laser at 785 nm, with 10 mW of power, exposition time of 10 s, two accumulations, and a 20x objective) was used to analyze the gold nanoparticles, before and after they were bioconjugated, and the functionalized gold nanorods. For the analysis, a sample of 5 μL of each colloid was dropped onto the surface of an electro-polished aluminum slide.

3.5. Thermogravimetric Analysis. A Discovery TGA (TA Instruments) was used in the temperature range of 25-700 °C, heating at the rate of 10 °C per minute in an air atmosphere. Samples of pristine mPEG-SH, 4-ATP prepared in methanol at 7.8 M and functionalized gold nanoparticles concentrated by centrifugation were analyzed using TGA.

4. Cell Culture and Toxicity Studies

4.1. HeLa and SH-SY5Y Human Cell Cultures. For this study, HeLa (Human cervical cancer cell line, ATCC®CCL-2®) and SH-SY5Y (Human neuroblastoma cell line, ATCC®CRL-2266™) cell lines were selected to assess the gold nanoparticle’s toxicological effects, since HeLa cells are one of the most frequently studied lines of cells when evaluating nanoparticle toxicity, and SH-SY5Y cells retain the ability to differentiate into neuronal cell types upon treatment with various agents. Besides, some studies have shown that cell neurons have been affected by the environmental presence of contaminant NPs, as well as having reported that gold nanoparticles in neurological research offer new strategies for disease treatment [34]. However, many of the neurotoxicology effects are still unknown due to the lack of work in this field.

HeLa and SH-SY5Y cells were cultivated in the following fashion. 20,000 cell/mL were grown in a 25 cm² flask (Corn ing) with DMEM (Dulbecco’s Modified Eagle Medium, Gibco, USA) and DME: RPMI (1 : 1 v/v), respectively, to later be supplemented with fetal bovine serum 10% (Gibco, USA) and incubated at 37 °C, in a 95% air and 5% CO₂ atmosphere. Even though SH-SY5Y cell lines can form floating clusters of neuroblasts, mostly these cells grow as adherent cells under the conditions described in this protocol.

The cell lines were exposed to twelve different concentrations of AuNPs and AuNPs-FA (350–0.17 μM), and ten concentrations to AuNRs (175–0.34 μM). Additionally, one positive control (cells exposed to H₂O₂ at a concentration of 50 μM) and one negative control (cells not exposed to NPs) were prepared for each cell type. The tests were carried out in triplicate to evaluate cell viability: XTT analysis, crystal violet assay, and trypan blue exclusion test. Besides, a qualitative analysis of the cytoskeleton damage, Phalloidin-FITC staining, was performed.

4.2. XTT Assay. The metabolic activity of cell lines not exposed and exposed to AuNPs, AuNPs-FA, and AuNRs was quantified using the 2, 3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay. The reduction of XTT by living cells incubated at 37 °C for 90 min yields a water-soluble formazan-colored product that can be spectrophotometrically measured at 490 nm (Epoch BioTek).

4.3. Crystal Violet Assay. Crystal violet staining is a method to detect the attached cells using a crystal violet dye (CV, water-soluble cationic dye), which binds to proteins and DNA. The exposed and nonexposed cell lines with AuNPs, AuNPs-FA, or AuNRs were fixed with 100 % methanol and then removed, after 15 min; later, 0.01% CV (v/v) were added and incubated for 5 min. The wells were then gently washed with sterile ultrapure water, and 33 % acetic acid (v/v) was added to release and dissolve the stain (CV). The absorbance of the obtained solution was measured using a spectrophotometer (Epoch BioTek) at 570 nm.
4.4 Trypan Blue Exclusion Method. Trypan blue is a dye used to distinguish between live and dead cells; the dye is not absorbed by healthy viable cells, but it enters the cells with cell membrane damage. To measure cell viability of line cells exposed to AuNPs or AuNPs-FA using trypan blue (0.4 % prepared in 0.81% sodium chloride and 0.06 % potassium

Figure 1: Absorbance spectra of three different sizes of NPs and NRs: A) AuNPs, B) AuNPs-FA and C) AuNRs.

Figure 2: SEM images of AuNPs (a, b, and c) and AuNPs-FA (d, e, and f) prepared on a glass substrate coated with a thin layer of aluminum. Scalebar: 10 nm.
phosphate, dibasic; Sigma-Aldrich), we chose two concentrations of NPs, 10.94 μM, and 175 μM. The number of blue-stained cells and the number of total cells were counted in a hemacytometer under a microscope inverter (Primo Vert, Carl Zeiss).

4.5. Cytoskeleton Staining. Qualitative analysis of the damage to the cytoskeleton was performed with Phalloidin-FITC (Fluorescein Isothiocyanate Labeled) staining. Phalloidin binds F-actin with high selectivity, while fluorescein provides green fluorescence. Cell lines were plated and grown on coverslips (Nunc™ Thermanox™) after exposure to 10.94 μM and 175 μM AuNPs, or AuNPs-FA for 24 h, and subsequently, the cells were fixed with 4 % p-formaldehyde plus 0.05 % glutaraldehyde solution for 20 min, to later be permeabilized with 0.5 % Triton X-100 in PBS (pH7.0); additionally, Phalloidin-FITC stained the cells for 30 min at darkroom temperature. Samples were prepared on a glass slide with VECTASHIELD Antifade Mounting Medium to prevent photobleaching. Finally, the samples were analyzed using an epifluorescence microscope (Leica, DMLS) with an AxioCam ICc1 camera (Carl Zeiss) using a B filter (Ex/Em 450-490 nm).

4.6. Statistical Analysis. Statistical analysis was performed by means of a one-way ANOVA analysis, and a Tukey Kramer test was used as a post hoc way of determining significant differences between treatments (*p < 0.05), through the use of JMP commercial software.

5. Results and Discussion

5.1. UV-Vis Spectroscopy and SEM. NPs coated with an organic molecule like 4-ATP have different advantages: the thiol group has good affinity to gold atoms, and the amine group can attach more complex molecules for bioconjugation. However, some authors have reported that colloidal nanoparticles functionalized only with 4-ATP and dispersed in water offer limited stability [35, 36]. In order to improve NP stability, molecules such as mPEG-SH, mercaptopundecanoic acid, mercaptopropionic acid, or serum bovine albumin (BSA) are used. However, BSA tends to

![Figure 3: SEM images of AuNRs prepared on a glass substrate coated with a thin layer of aluminum. Scalebar: 10 nm.](c)

<table>
<thead>
<tr>
<th>Sample</th>
<th>LSPR (nm)</th>
<th>Au-concentration (μM)</th>
<th>Concentration (NPs/mL)</th>
<th>Diameter size* (nm)</th>
<th>Length (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D-AuNPs</td>
<td>518</td>
<td>490.1</td>
<td>2.33 × 10^{12}</td>
<td>16</td>
<td>—</td>
</tr>
<tr>
<td>2D-AuNPs</td>
<td>520</td>
<td>632.0</td>
<td>1.33 × 10^{12}</td>
<td>21</td>
<td>—</td>
</tr>
<tr>
<td>3D-AuNPs</td>
<td>522</td>
<td>721.0</td>
<td>7.99 × 10^{11}</td>
<td>26</td>
<td>—</td>
</tr>
<tr>
<td>1D-AuNR1</td>
<td>903</td>
<td>461.6</td>
<td>3.75 × 10^{12}</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>2D-AuNR2</td>
<td>894</td>
<td>459.5</td>
<td>2.50 × 10^{12}</td>
<td>5</td>
<td>27</td>
</tr>
<tr>
<td>3D-AuNR3</td>
<td>859</td>
<td>457.0</td>
<td>1.74 × 10^{12}</td>
<td>6</td>
<td>30</td>
</tr>
</tbody>
</table>

* For AuNRs, the core means the diameter of AuNRs. See Figure S7 for geometric details of AuNRs.
form a protein corona, covering the entire nanoparticle and screening the small molecules that can be found on their surface, thus limiting their potential usefulness to link other molecules.

On the other hand, FA has been used to bioconjugate nanoparticles and nanorods for their use as biomarkers for cancer cell detection and hyperthermia therapy; however, gold nanorods exhibited short colloidal stability after their bioconjugation, and so we kept them out of subsequent tests. The folate receptor (FR) is a biomarker that is overexpressed in many tumors (i.e., lung, kidney and ovarian cancers) [37, 38].

Our AuNPs, AuNPs-FA, and AuNRs were analyzed using UV-Vis absorbance spectroscopy. Figure 1 shows the absorbance spectra of the different AuNPs, AuNPs-FA, and AuNRs, with the numbers 1, 2, and 3 preceding the abbreviations AuNPs, AuNPs-FA, and AuNRs to point out the different sizes; besides, if label FA precedes AuNPs, it means bioconjugate or if it is absent means only functionalization.

It is well known that the LSPR shifts to red wavelengths as a function of NP size, as well as when the environment changes due to a change in the index of refraction. Here, we can observe, in Figures 1(a) and 1(b), that our colloids exhibit a significant shift in the order of 3 nm of the plasmon band after bioconjugation, due to the anchoring of FA.

Figures 2(a)–2(c) show the SEM images of AuNPs, whereas Figures 2(d), 2(e), and 2(f) show those of AuNPs-FA, while Figures 3(a)–3(c) show the functionalized AuNRs. The estimated average size for each particle, before and after bioconjugation, has the same value, for the organic material on the particle’s surface does not change the average size of the core; after the corresponding analysis of the micrographs, we found that the mean sizes were (a) 16 ± 2 nm, (b) 21 ± 3 nm, (c) 26 ± 2 nm, (d) 16 ± 2 nm, (e) 21 ± 3 nm, and (f) 26 ± 2 nm, whereas, for AuNRs, their size in length (l) and diameter (d) were (a) \( l = 23 \pm 3 \) nm, \( d = 5 \pm 1 \) nm, (B) \( l = 27 \pm 4 \) nm, \( d = 5 \pm 1 \) nm, and (c) \( l = 30 \pm 4 \) nm, \( d = 6 \pm 1 \) nm, respectively. Scalebar: 10 nm.

5.2. Zeta Potential and Raman Spectroscopy. Zeta potential is related to the net surface electric charge that nanoparticles have. It offers information to determine the colloidal stability of NPs and understand the colloid system’s performance under a variety of conditions; besides, zeta potential provides indirect information about the surface chemistry of NPs. Zeta potential measurement was carried out for colloidal NPs before and after bioconjugation. Fresh NPs stabilized with sodium citrate exhibited a zeta potential value of -41.0 mV, while the same NPs showed a mean value of -30 mV after functionalization (AuNPs); finally, bioconjugated AuNPs-FA yielded a mean value of -15 mV. The potential zeta values clearly show a change in the nanoparticle’s surface chemistry, giving us indirect information about bioconjugation with FA. In turn, mean value of potential zeta for AuNRs after the synthesis was +56 mV, but after functionalization, values were 25, 39, and 30 mV for 1D-AuNRs, 2D-AuNRs, and 3D-AuNRs, respectively.

Furthermore, Raman spectroscopy was used to analyze the NPs before and after bioconjugation. For their corresponding analysis, each colloid was dialyzed for two days using spectra/pore membranes (with a molecular weight cut of 12-14 kD) to eliminate the unbonded molecules. Then, one drop of each colloid was dropped onto the surface of an aluminum substrate, and twenty spectra were acquired. Figure 4 shows the mean SERS spectra of 3D-AuNPs, 3D-AuNPs-FA, and 3D-AuNRs. The spectra look like very
similar, with some intense peaks at around 1078 cm\(^{-1}\), assigned to \(\nu(C-S)\); at 1175 cm\(^{-1}\), assigned to \(\delta(C-H)\); at 1378 cm\(^{-1}\), assigned to \(\nu(C-C)\) and \(\delta(C-H)\); and at 1586 cm\(^{-1}\), assigned to \(\nu(C-C)\) that appear in the three cases; however, we found significant differences between functionalized and bioconjugated NPs (shown by the black arrows). In the supplementary material, Figures S1 and S2 show the essential differences between the spectra of functionalized and bioconjugated nanoparticles. These spectral regions were analyzed by means of Gaussian deconvolution, with the first one being centered at 1078 cm\(^{-1}\) and the second at 1586 cm\(^{-1}\). For the case of stretching vibration band \(\nu(C-S)\), deconvolution analysis shows that two Gaussian peaks describe the Raman signal behavior in both cases; the Gaussian component intensity ratio is \(I_{1078}/I_{1067} = 6\) for 3D-AuNPs, and \(I_{1078}/I_{1067} = 7\) for 3D-AuNPs-FA; the band at 1078 is always very intense, but the band ratio is higher in the case of 3D-AuNPs-FA. In turn, for the second zone, deconvolution is carried out with two Gaussians, which describe the band assigned to the stretching vibration of \(\nu(C-C)\), but this time, significant variations can be observed in the relative intensities; the intensity ratio between these two bands is \(I_{1585}/I_{1573} = 1.92\) for 3D-AuNPs and \(I_{1587}/I_{1577} = 0.77\) for 3D-AuNPs-FA. This behavior agrees with that reported by Fasolato et al. [39], and it happens because the phenyl ring \((C-C)\) stretching from the benzene ring band is very susceptible to being influenced by the type of chemical substituent, and by its electronic properties, such as in the case of FA. In our case, the change in intensity of these bands is not very intense because mPEG-SH also is covalently attached to the NP surface, and 4-ATP is not the only molecule coating the surface of the NPs, but this band offers enough information about how FA is bonded to 4-ATP. Besides nanorods show the bands at 1078 and at 1586 cm\(^{-1}\), we also observed that some bands assigned to 4-ATP were enhanced at 1139 cm\(^{-1}\) assigned to \(\delta(CH)\), and at 1431 cm\(^{-1}\) assigned to \(\delta(CH)\) and \(\delta(CC)\), which means that 4-ATP is bonded to AuNRs (shown by the gray arrows).

Besides, thermal behaviors of the mPEG-SH, 4-ATP and functionalized AuNP samples upon heating in an air atmosphere were investigated using TGA to estimate the approximate number of mPEG-SH molecules per nanoparticle. Figure 5(a) shows the characteristic TGA curves of mPEG-SH and 4ATP, as can be seen, thermal decomposition of mPEG-SH starts at 200°C, and weight loss increases rapidly from this temperature up to about 350°C. The TGA curve of 4-ATP in ethanol shows three regions; in the first one, from 90 to 133°C, ethanol still is evaporating, while in the second region, thermal decomposition starts, increasing rapidly.

**Figure 5**: TGA analysis of: (a) pristine mPEG-SH and 4-ATP in ethanol and (b) 1, 2, 3D-AuNPs.

**Table 2**: TGA analysis of functionalized AuNPs, and the estimated number of 4-ATP molecules per NP.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial weight ((\mu)g)</th>
<th>Residual weight ((\mu)g)</th>
<th>Weight loss ((\mu)g)</th>
<th># (mPEG-SH)/NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D-AuNP</td>
<td>82.85</td>
<td>74.09</td>
<td>8.0</td>
<td>538.9</td>
</tr>
<tr>
<td>2D-AuNP</td>
<td>274.20</td>
<td>258.90</td>
<td>15.3</td>
<td>655.3</td>
</tr>
<tr>
<td>3D-AuNP</td>
<td>290.70</td>
<td>279.00</td>
<td>11.7</td>
<td>897.0</td>
</tr>
</tbody>
</table>
from 350 to 600°C, which also includes the third region. On the other hand, the TGA curves of functionalized gold nanoparticles show that their thermal decomposition happens in the temperature range from 200 to 280 °C; after that, it seems that no weight losses take place. The sensitivity of the TGA system is in the order of 0.1 μg, and the mass of 4-ATP is in the order of 5 to 10 ng, due to the sensitivity of the TGA system, the curves show mainly the thermal decomposition of mPEG-SH. Therefore, the number of mPEG-SH per NP can be calculated using the TGA curves following the method of Bajaj et al. [40], see supplementary file. From the results obtained, 538.9, 655.3, and 897.0 molecules per NP were estimated for samples 1D-AuNPs, 2D-AuNPs, and 3D-AuNPs, respectively. As we mentioned before, due to the sensitivity of TGA and of fluorescence analysis through fluorescein [41], it is not possible to find out the number of 4-ATP molecules bonded to our nanoparticles. Table 2 shows a summary of the estimated molecules of mPEG-SH per NP.

5.3. Biological Response of HeLa and SH-SY5Y Cells to AuNPs and AuNPs-FA. Cell metabolism is the sum of biochemical processes that either produce or consume energy at the cellular level; then, a change in metabolism can impact cellular functions. In this work, HeLa and SH-SY5Y cells were incubated with AuNPs and AuNPs-FA, at Au concentrations from 0.17 to 175 μM, and after that, mitochondrial metabolic activity changes due to interaction with NPs were measured utilizing XTT. Figures 6(a) and 6(d) show the dose-response curves of HeLa cells for 1D-AuNPs and 1D-AuNPs-FA, respectively; in them, the maximum concentration at which no statistical differences against the negative control were observed was found to be 2.73 μM for 1D-AuNPs and 1.36 μM for 1D-AuNPs-FA. Figures 6(b) and 6(e) show the dose-response curves for 2D-AuNPs and 2D-AuNPs-FA, displaying reduced metabolic activity throughout the range of concentrations.

On the other hand, when HeLa cells were incubated with 3D-AuNPs (Figure 6(c)), we found significant differences in metabolic activity against the negative control at lower concentration values; only the last four highest concentrations did not show evidence of changes in metabolic activity. Nevertheless, in 3D-AuNPs-FA (Figure 6(f)), we did not find differences against the negative control.

The XTT assay on HeLa cells showed that 16 nm NPs (1D-AuNPs and 1D-AuNPs-FA) exhibited significant metabolic activity changes at high concentrations (Figures 6(a) and 6(b)), while 21 nm NPs 2D-AuNPs and 2D-AuNPs-FA showed statistical differences through all the range of concentrations (Figures 6(c) and 6(d)). We hypothesize that the cell uptake of 21 nm NPs is more efficient than the other sizes we evaluated, and it might account for the significant

Figure 6: Effects of functionalized and bioconjugated NPs on the metabolic activity of HeLa were measured using an XTT assay. HeLa control: cells not exposed to NPs (-), cells exposed to H2O2 50 μM (+). Bars indicate the standard deviation (SD) from 3 replicates. The data are represented as mean ± SD, significantly different from control (-) at *p < 0.05. We found that only 2D-AuNPs and 2D-AuNPs-FA had experienced significant change through all the concentrations.
changes in mitochondrial metabolic activity, which may indicate cell toxicity. For instance, Chithrani et al. evaluated AuNPs of different sizes in HeLa cell cultures, and they found that 50 nm NPs showed the maximum uptake [42]; however, Rieznichenko et al. showed that 20 and 30 nm gold NPs exhibit higher uptake than 40 nm NPs when evaluated in U937 tumor cells [43]. Finally, our sample of 3D-AuNPs-FA did not show remarkable changes in metabolic activity throughout the concentration range. In point of fact, Chen et al. exposed HeLa cells to different sizes of citrated-coated gold NPs (3, 5, 12, 17, 37, 50, and 100 nm), but MTT assay did not show any changes in cell viability [44]. Similar results were found by Qu and Lü in dermal fibroblast cells [45] and Uboldi et al. using A549 and NCI-H441 cells [46].

Figure 7 shows the dose-response curves of SH-SY5Y cells exposed to NPs within the same range of concentrations. Unlike HeLa cells, SH-SY5Y cells exposed to 1D-AuNPs and 2D-AuNPs (Figures 7(a) and 7(b)) and to 1D-AuNPs-FA and 2D-AuNPs-FA (Figures 7(d) and 7(e)) showed reduced metabolic activity throughout the range of concentrations, but when cells were exposed to 3D-AuNPs and 3D-AuNPs-FA, the highest concentrations at which we did not find statistical differences against the negative control were at 1.36 μM and 0.34 μM, respectively (Figures 7(c) and 7(f)). These results suggest that SH-SY5Y cells are more sensitive than HeLa cells when exposed to functionalized and bioconjugated NPs. Analogous results have been reported, showing that not all cell cultures have the same answer to interaction with NPs; for example, Vecchia et al. observed that after exposing B16-F10 and HeLa cells to citrated gold NPs, those cells were more resistant to exposition to NPs [47].

It has been reported that FA is a biomolecule needed for DNA nucleotide synthesis and cell division, and that folate receptors are overexpressed in some cancer cell lines and thus can be used as a cancer biomarker [34–35], and some works have also evidenced the high biocompatibility of NPs coated with FA; however, in our case, the bioconjugated NPs (AuNPs-FA) show significant toxicological damage to both cell lines. PEG derivatives have also been used to functionalize gold NPs for their biocompatibility, but contrary to this affirmation, some works showed that they are not bio-compatible at all.

Due to the low stability of gold NPs coated with 4-ATP, very few works have reported the toxicological effects of gold NPs coated with 4-ATP. One example is Mansoori et al., who showed that the survival rate of HeLa and MCF7 cell lines incubated with gold NPs functionalized with 4-ATP and bioconjugated with FA did not show significant changes in cell survival even after four hours [48]. Furthermore, Cardoso et al. showed good stability and biocompatibility with
gold NPs coated with 4-ATP and BSA [27]. Our results obtained from XTT assays are not conclusive at all, but they still offer important information on the toxicity of these NPs, suggesting that more tests need to be carried out to get more information related to the possible damage effects of gold NPs and the coatings we used here.

5.3.1. Biomass and Cellular Viability: Crystal Violet and Trypan Blue Exclusion. As it is well known, crystal violet staining is only to be used on adherent cells. It does not differentiate between live and dead cells on the plate; dead cells (detachment of adherent cells) are simply eliminated by washing off, and the remaining stained cells are presumed to be alive. Therefore, crystal violet’s biomass can be used to infer cell viability levels [49, 50]. Figure 8 shows the dose-response curves for HeLa exposed to 1D-AuNPs and 2D-AuNPs (Figures 8(a) and 8(b)) and to 1D-AuNPs-FA and 2D-AuNPs-FA (Figures 8(d) and 8(e)). In this case, we did not find significant biomass changes for any of the concentrations compared to the negative control; however, when cells were exposed to 3D-AuNPs and 3D-AuNPs-FA (Figures 8(c) and 8(f)), they showed reduced biomass throughout the range of concentrations. It seems that for HeLa cells, size is the most crucial factor to cause cell damage, contrary to the results obtained from XTT, where we were unable to conclude whether size is the main factor for toxicity, and we suppose that uptake can be the most critical factor. Some authors have reported that smaller NPs are more genotoxic than bigger ones. For example, Pan et al. and Lebedová et al. showed that toxicity is size-dependent; they found that NPs of smaller sizes are more toxic than bigger ones [51, 52]. However, Daniel and Astruc found that larger NPs can indirectly induce DNA damage, even without entering the nucleus [53].

On the other hand, the dose response of SH-SY5Y cells exposed to 1D-, 2D-, and 3D-AuNPs and 1D-, 2D-, and 3D-AuNPs-FA, at the same concentrations as above, did not show significant differences for any of the sizes (Figure 9).

Trypan blue is a stain used to quantify live cells by labeling dead cells exclusively. Because live cells have an intact cell membrane, trypan blue cannot penetrate their membrane and enter the cytoplasm, whereas in dead cells, trypan blue passes through the porous cell membrane and enters the cytoplasm; under light microscopy analysis, only dead cells are colored in blue. To measure cell viability by means of trypan blue staining, we chose two concentrations of AuNPs and AuNPs-FA for each size.
Figure 9: The average crystal violet optical density dose-response curve for SH-SYSY cells exposed to 1D-, 2D-, and 3D-AuNPs (a, b, and c, respectively) and 1D-, 2D-, and 3D-AuNPs-FA (d, e, and f, respectively). SH-SYSY control: cells not exposed to NPs (-), cells exposed to H₂O₂ 50 μM (+). Bars indicate the standard deviation (SD) from 3 replicates. The data are represented as mean ± SD, significantly different from control (-) at *p < 0.05.

Figure 10: Relative viabilities of HeLa cells after being incubated with (a) 1D-, 2D-, and 3D-AuNPs and (b) 1D-, 2D-, and 3D-AuNPs-FA at 10.94 and 175 μM. HeLa control: cells not exposed to NPs (-), cells exposed to H₂O₂ 50 μM (+). Bars indicate the standard deviation (SD) from 3 replicates. The data are represented as mean ± SD.
**Figure 11:** Relative viabilities of SH-SY5Y cells after being incubated with (a) 1D-, 2D-, and 3D-AuNPs and (b) 1D-, 2D-, and 3D-AuNPs-FA at 10 and 175 μM. SH-SY5Y control: (−) cells not exposed to NPs, (+) cells exposed to H₂O₂ 50 μM. Bars indicate the standard deviation (SD) from 3 replicates. The data are represented as mean ± SD.

**Figure 12:** Cytoskeleton HeLa response to different sizes of AuNPs and AuNPs-FA at the two different concentrations 10.94 and 175.0 μM. Besides, we write in each case the corresponding concentration in NPs/mL. HeLa cells were stained with Phalloidin-FITC (Fluorescein Isothiocyanate Labeled), which binds F-actin with high selectivity. Epifluorescence images of negative control (−) correspond to nonexposed cells, and positive controls (+) were cells exposed to 50 μM H₂O₂ for 30 min to induce a cytotoxicity response. HeLa cells exposed to AuNPs (a–f) and AuNPs-FA (a’–g’) for 24 h showed NP aggregation (red arrow), alterations in morphology (blue arrows), debris cells (yellow arrows), and membrane blebbing (white arrows).
Figure 10 shows the percentage of viable cells from the interaction of HeLa cells with AuNPs and AuNPs-FA, measured using trypan blue, where the negative control is taken as 100%. We found that the lower percentage of viable cells happens for the interaction with the smallest size of NPs, giving 60.3% for 1D-AuNPs and 72.3% for 1D-AuNPs-FA; meanwhile, the highest cell viability was 83.1% for 2D-AuNPs and 80.7% for 2D-AuNPs-FA.

On the other hand, Figure 11 shows the cell viability results for SH-SY5Y cells exposed to 1D-, 2D-, and 3D-AuNPs and to 1D-, 2D-, and 3D-AuNPs-FA. We found again that NPs with the smallest size yielded lower percentage values of cell viability, <50% for AuNPs and AuNPs-FA, and for both tested concentrations, 10.94 μM and 175 μM, while the 1D- and 2D-AuNPs and 1D- and 2D-AuNPs-FA samples showed cell viability values <80%, but higher than 60%, which may be indicative of the toxicity behavior of our NPs.

5.4. Cytoskeleton Response to AuNPs and AuNPs-FA. Besides the metabolic activity and cell viability assays, we conducted epifluorescence microscopy analysis to investigate any significant changes produced in the cytoskeleton by interacting with gold NPs. As it is well known, actin filaments take part in the regulation of internal cell architectures, as well as of some cellular functions, such as proliferation, adhesion, and differentiation, and some of these changes can be detected by analyzing epifluorescence images. The analysis was carried out using FITC-phalloidin to label the cytoskeleton (F-actin microfilaments) for both cell lines before and after exposure to 1D-, 2D-, and 3D-AuNPs and to 1D-, 2D-, and 3D-AuNPs-FA at 10.94 μM and 175 μM.

Figures 12 and 13 show the epifluorescence micrographs of negative (-) and positive (+) controls of HeLa and SH-SY5Y cells, respectively. In the negative control, we observed that the F-actin microfilaments of the HeLa and SH-SY5Y monolayer cells were well-formed and presented good
adhesion, exhibiting their natural shape and size, which means that morphology was normal; actin filaments also showed good integrity. On the other hand, positive controls showed significant damage on the F-actin microfilaments, as well as morphology changes (blue arrow), and proliferation seemed to be diminishing, because we observed a reduced number of cells in the images, which significantly reduced fluorescence emission through the cytoskeleton disruption observed in positive controls of HeLa and SH-SY5Y cells.

The epifluorescence micrographs of HeLa cells exposed to 1D-AuNPs, 2D-AuNPs, and 3D-AuNPs at 10.94 μM are shown in Figures 12(a)–12(c), while those exposed to a concentration at 175 μM are shown in Figures 12(a)–12(f), respectively. We found a loss of adhesion and cell attachment, and changes in morphology are evident; for example, cells became round (blue arrows). Moreover, we found black points in the cells (red arrows), which evidences efficient internalization of NPs, and NPs aggregation also took place (see the case of 1D-AuNPs), along with additional damage to some cell membranes, as were blebs on the cell membrane (white arrows) and loss of cell adhesion.

Figures 12(a)–12(f) show the epifluorescence micrographs of HeLa cells exposed to 1D-AuNPs-FA, 2D-AuNPs-FA, and 3D-AuNPs-FA at the same concentrations as above, showing significant toxic effects. For example, at the lower concentration, all sizes of AuNPs-FA (Figures 12(a)–12(c)) seem to penetrate the cell, because black points of NPs aggregation are observed (red arrows); besides, slight changes in morphology and cell adhesion are also perceived. However, the behavior changes for NPs at the high concentration (Figures 12(d)–12(f)) since substantial loss of adhesion and cell attachment is observed for all the three sizes of AuNPs-FA. Changes in morphology and disruption of the cytoskeleton are evident, and now, in the cases of 1D-AuNPs-FA and 2D-AuNPs-FA, debris cells (yellow arrows) can be found, just as in the case of 2D-
Figure 15: The average crystal violet optical density dose-response curve for HeLa cells exposed to 1D-, 2D-, and 3D-AuNRs (a, b, and c, respectively), and dose-response curve for SH-SY5Y cells exposed to 1D-, 2D-, and 3D-AuNRs (d, e, and f, respectively). Controls: cells not exposed to NPs (-), cells exposed to H₂O₂ 50 μM (+). Bars indicate the standard deviation (SD) from 3 replicates. The data are represented as mean ± SD, significantly different from control (-) at *p < 0.05.
AuNPs-FA (white arrows) there is presence of blebs; moreover, there is also loss of cell junctions. In all cases, we observe the presence of aggregates of nanoparticles.

SH-SY5Y cells were exposed to functionalized and bioconjugated NPs at 10.94 and 175 μM (Figure 13). The images show that 1D-AuNPs and 3D-AuNPs at 10.94 μM (Figures 13(a) and 13(c)) caused significant toxic effects, and we can see changes in morphology, as evidenced by rounded or enlarged cells (blue arrows) and blebs (white arrows); on the other hand, the sample with 2D-AuNPs (Figure 11(b)) seemed not to cause significant changes to the cytoskeleton of SH-SY5Y cells. However, when the concentration increases, see Figures 13(d), 13(e), and 13(f), it seems that toxic effects appear for all three sizes of AuNPs, and although, we were unable to get a good view of the F-actin fiber, cell adhesion and loss of cell union were observed; besides, the cell showed a significant change in morphology (blue arrows), membrane blebs (white arrows), and black points of NP aggregation (red arrows).

Finally, when SH-SY5Y cells were exposed to AuNPs-FA (Figures 13(a)–13(f)), they showed significant toxic effects. Even though many cells are observed in the image, we can see morphology changes (blue arrows), in the shape of rounded or enlarged cells. The 1D-AuNPs-FA sample (Figures 13(a) and 13(d)) shows that the smallest NP size causes significant damage to F-actin microfilaments, loss of cell union, and bleb formation (white arrows), along with morphology changes with both concentrations, while for 2D-AuNPs-FA and 3D-AuNPs-FA sizes (Figures 13(b) and 13(c)), we were unable to observe a toxic effect at 10.94 μM, respectively. Meanwhile, when SH-SY5Y cells were exposed at the concentration of 175 μM (Figures 13(e) and 13(f)), once again we observed significant changes in cell adhesion and cell attachment, significant changes to the F-actin microfilaments, changes in morphology, and the presence of membrane blebbing after interaction with the three NP sizes.

Finally, functionalized gold nanorods of three different sizes were also evaluated by XTT and crystal violet assays to evaluate their toxicity on HeLa and SH-SY5Y cells. Figure 14 shows the XTT dose-response curves of HeLa and SH-SY5Y cells for 1D-, 2D-, and 3D-AuNRs. When AuNRs were incubated in HeLa cells, we observed that HeLa cells exposed to gold nanorods showed significant differences with respect to negative control in their metabolic activity (Figures 14(a)–14(c)). Nevertheless, when SH-SY5Y were incubated with AuNRs, we found that SH-SY5Y cells were more sensitive to AuNR toxic effects, mainly for samples 1D-AuNRs (Figure 14(d)) and 2D-AuNRs (Figure 14(e)), producing significant changes in cell viability. On the other hand, sample 3D-AuNRs did not show significant changes after being exposed to SH-SY5Y cells, see Figure 14(f).

Figure 15 shows the CV assay dose-response curves for HeLa and SH-SY5Y cell cultures exposed to samples 1D-, 2D-, 3D-AuNRs. We did not find significant changes for both cell cultures exposed to the three AuNRs, except when HeLa and SH-SY5Y cells were exposed to higher concentrations of gold nanorods (Figures 15(a), 15(b), 15(d), and 15(e)).

6. Conclusions

For this work, we prepared gold nanoparticles and nanorods of three different sizes. Their surface chemistry was modulated by functionalizing them with 4-ATP and mPEG-SH molecules and bioconjugated with FA by attaching it to 4-ATP (for the case of AuNPs), leaving a free methyl group methoxy-PEG-SH. The findings of this work showed that our functionalized and bioconjugated AuNPs cause toxicological effects in both cell cultures, HeLa and SH-SY5Y cells, after 24 h of exposition with gold nanoparticles, since mitochondrial metabolic activity, cell viability (cellular membrane permeability), and biomass (cell adhesion) showed significant changes in comparison to the negative control. Epifluorescence micrographs revealed loss of cell adhesion, damage to the F-actin microfilaments, and morphology changes; for example, some cells became round and detached, there was formation of blebs, and cell membrane rupture appeared mainly for high NP concentrations. These toxicological effects were observed almost for all concentrations, but mainly at high values, with 1D-AuNPs and 1D-AuNPs-FA being the most toxic. Amrolia et al. [54] and Liu et al. [55] found that free 4-aminophenol and PEG oligomers and PEG-based monomers have different chemical and physical properties, leading to potential toxicity due to the production of reactive oxygen species (ROS). While gold nanorods cause significant changes in HeLa cells’ metabolic activity for the three AuNRs, AuNRs showed significant toxicological effects only for SH-SY5Y cells when exposed to samples 1D-AuNRs and 2D-AuNRs for all concentrations.

We did not observe significant changes or toxicological effects of gold nanorods in other cases. Finally, the CV assay did not show significant changes in biomass except for the highest concentration of AuNRs in both cell cultures.

Data Availability

The data supporting this work is available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors’ Contributions

JL P-M, J C M-E, A P A-A, and J T-R contributed to the conception and design and data acquisition, analysis, and interpretation; drafted the manuscript; and critically revised the manuscript. J C V-C, J P C-D, and LL V-F contributed to the data analysis and interpretation and critically revised the manuscript. I S-T contributed to TGA analysis and interpretation. All authors have read and agreed to the published version of the article.
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Supplementary Materials

Figure S1: deconvolution of the Raman spectra of the (C-S) stretching band from 1050 to 1110 cm⁻¹, obtained with the 785 nm laser. Two Gaussian bands describe the stretching peak, and the bands are centered at 1079 cm⁻¹ and 1067 cm⁻¹ (A), 1078 cm⁻¹, and 1063 cm⁻¹ (B). Figure S2: deconvolution of the Raman spectra of the (C-S) stretching band from 1500 to 1620 cm⁻¹, obtained with the 785 nm laser. Two Gaussian bands describe the stretching peak, and the bands are centered at 1585 cm⁻¹ and 1573 cm⁻¹ (A), 1587 cm⁻¹ and 1577 cm⁻¹ (B). Figure S3: synthesis of gold seed solution and first growth of gold nanoparticles, concentration of Au (0.49 mM) and nanoparticle’s concentration, C₁₈ = 2.332 × 10¹² NPs/mL. Figure S4: synthesis of gold nanoparticles’ second growth, concentration of Au, C₂₆ = 0.632 mM, and nanoparticle’s concentration, 1.33x 10¹³ NPs/mL. Figure S5: synthesis of gold nanoparticles’ third growth, concentration of Au, C₆₃ = 0.721 mM, and nanoparticle’s concentration 7.99 × 10¹¹ NPs/mL. Figure S6: figure shows the synthesis of (a) gold seeds, (b) gold nanorods 1D-AURs, (b) gold nanorods 2D-AuNRs, and (c) gold nanorods 3D-AuNRs. Figure S7: geometry of gold nanorods. (Supplementary Materials)

References


