



Impact of Methodological Variations on MTT Assay for Gold Nanoparticle Toxicity Evaluation

Jessica Victoria Martínez-Saráo¹, Samuel Longoria-García²,
Hugo Leonid Gallardo-Blanco³, Celia Nohemí Sánchez-Domínguez²,
and Margarita Sánchez-Domínguez¹ (✉)

¹ Centro de Investigación en Materiales Avanzados S.C. (CIMAV), Parque de Investigación e Innovación Tecnológica, Subsele Monterrey, Alianza Norte 202, Apodaca 66628, México
{jessica.martinez,margarita.sanchez}@cimav.edu.mx

² Departamento de Bioquímica y Medicina Molecular, Facultad de Medicina, Universidad Autónoma de Nuevo León, Monterrey 64460, México
{samuel.longoriagr,celia.sanchezdm}@uanl.edu.mx

³ Servicio de Oncología, Centro Universitario Contra el Cáncer (CUCC), Hospital Universitario “Dr. José Eleuterio González”, Universidad Autónoma de Nuevo León, Monterrey 64451, México
hugo.gallardobl@uanl.edu.mx

Abstract. Gold nanoparticles (AuNPs) coated with PEI-based copolymers have emerged as a promising non-viral vector for gene therapy due to their exceptional biocompatibility and efficient gene delivery capabilities. Green synthesis methods, which utilize environmentally friendly and sustainable approaches, have gained significant attention for producing biocompatible nanoparticles. While AuNPs have been extensively studied, challenges often arise when conducting cell viability assays, particularly using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The inherent properties of nanoparticles, such as their size, shape, and surface chemistry, can interfere with the MTT assay’s mechanism, leading to inaccurate assessments of cell viability. Additionally, the interaction between the MTT reagent and the nanoparticles can further complicate the assay and potentially yield misleading results. To address these challenges, this study aims to optimize AuNP synthesis, establish optimal MTT assay conditions and mitigate interference effects by carefully controlling the synthesis parameters, optimizing the MTT assay conditions, and addressing the potential interference effects. This study aims to provide a reliable and accurate method for evaluating the cytotoxicity and biocompatibility of AuNP-based gene delivery systems. This knowledge will contribute to the development of safe and effective nanomedicine for various biomedical applications.

Keywords: Gold nanoparticles · cell viability · interference · MTT

1 Introduction

Nanoparticles are particles with dimensions ranging from 1 to 100 nm. Their high surface-to-volume ratio facilitates surface functionalization, making them suitable for the delivery of genetic material. Nanoparticles can be classified into various categories

based on their composition, including carbon-based, metallic, ceramic, semiconductor, polymeric, and lipid-based materials. Metallic nanoparticles, especially copper, silver, and gold, are extensively employed due to their well-controlled size and shape, as well as their unique optical properties [1, 2]. Gold nanoparticles are extensively used in biomedical applications owing to their biocompatibility and stability, particularly their efficient delivery of nucleic acids to cells when conjugated with certain polymers. Moreover, the synthesis of these nanoparticles can be accomplished via green chemistry approaches, employing biological resources. This method offers several advantages, including low cost, mild reaction conditions, and the elimination of toxic chemicals, synthetic ligands, and stabilizers [3–5]. Given their biomedical applications, gold nanoparticles must undergo biological testing to demonstrate low cytotoxicity in healthy cells.

Cell viability assays are employed to measure the number of living and healthy cells within a sample by detecting cell proliferation or cytotoxic effects. Numerous assays have been developed to determine and quantify viable cell numbers following a treatment [6]. The colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a prominent method, establishing a linear correlation between cell viability and mitochondrial activity.

Viable cells contain oxidoreductases capable of reducing a yellow tetrazolium salt (MTT) into purple formazan crystals. This reaction is catalyzed by succinate dehydrogenase, a mitochondrial enzyme involved in cellular metabolism [7]. Cell proliferation is quantified based on the measurement of absorbance resulting from the enzymatic reduction of tetrazolium salt. In viable cells, mitochondrial succinate dehydrogenase catalyzes this reaction, producing purple formazan. The amount of formazan generated is directly proportional to the number of metabolically active cells present in the sample [8, 9]. While the MTT assay is a valuable tool for assessing cell viability, it can be susceptible to interference from nanoparticles. These materials can interact with assay components, such as dyes and reagents, leading to inaccurate results [10, 11].

In this study, gold nanoparticles were synthesized using a green synthesis approach which employs a copolymer based on mesquite gum and polyethylenimine as a reducing and stabilizing agent. The cytotoxicity of these nanoparticles was evaluated in kidney tissue of African green monkey cells (VERO cells). To determine the most suitable methodology for assessing cytotoxicity in this specific nanoparticle-cell system, various MTT assay conditions were explored.

2 Experimentation

2.1 Gold Nanoparticles Synthesis

To synthesize gold nanoparticles (AuNPs), a copolymer composed of mesquite gum and polyethyleneimine (GM-CBX-PEI) was employed as a reducing and stabilizing agent; the synthesis of this copolymer was carried out based on the method by Pinilla *et al.* [12]. An aqueous solution of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was prepared at a concentration of 1 mM. The copolymer and 5 mL of the gold solution were added to this solution and homogenized using a vortex mixer until complete dissolution of the copolymer. Subsequently, the mixture was incubated in a temperature-controlled water bath with constant stirring. This process resulted in a solution containing purplish-red AuNPs. Finally, the

obtained AuNPs@GM-CBX-PEI dispersion was placed in Sartorius Vivaspin 6 tubes and centrifuged in an Eppendorf 5810 R centrifuge (3000 rpm, 15 min). Four washes with deionized water were performed, followed by a final wash with deionized water at 4000 rpm for 15 min. This procedure was carried out to remove any residual substances that were not part of the nanoparticles, such as unbound copolymer and residual chlorine.

Then, these nanoparticles were characterized by ζ (zeta) potential to measure the stability of nanoparticles in solution and its surface charge, Dynamic Light Scattering (DLS) to determine the size of nanoparticles and UV-Vis Spectroscopy to provide information about the composition and structure of nanoparticles.

2.2 MTT Assay

VERO cells were seeded at a density of 10,000 cells per well in 96-well plates and cultured in 100 μ L of DMEM. The plates were incubated at 37 °C in a 5% CO₂ atmosphere for 24 h. To ensure reproducibility, each sample was tested in triplicate. After 24 h, the AuNPs@GM-CBX-b-PEI treatment was added. The treatment-to-medium ratio in each well is shown in Table 1. After an additional 24 h, a sufficient volume of medium-MTT solution was prepared for all wells. The medium-nanosystem supernatant was removed from the wells, and 100 μ L of the medium-MTT solution was added. The plate was then incubated under standard conditions for 3 h. After the incubation period, a solution was added to dissolve the formazan crystals. Table 1 outlines the specific modifications made to the standard MTT assay protocol in this study. Finally, the absorbance of each well was measured at 570 nm and 651 nm using a microplate reader. The absorbance obtained at 570 nm was subtracted from the absorbance obtained at 651 nm. This resulting absorbance was used in the following formula.

$$\%Cell\ viability = (Abs_{sample} * 100) / (Abs_{control}) \quad (1)$$

Table 1. MTT assay protocol variations.

	Protocol	Variations			Reference
		Medium-nanosystem	Medium-MTT	Solvent to dissolve formazan crystals	
1	Nanomedicine lab protocol (Our work) *	95 μ L medium and 5 μ L nanosystem	10 μ L of MTT per 100 μ L of medium	100 μ L of acidified isopropanol	N/A

(continued)

Table 1. (continued)

	Protocol	Variations			Reference
		Medium-nanosystem	Medium-MTT	Solvent to dissolve formazan crystals	
2	Roche			100 µL solubilization buffer	[13]
3	Babaei, A			50 µL of DMSO at a concentration 1 mg/ml	[14]
4	Alafaleq, N. O			100 µL of acidified isopropanol	[15]
5	Meléndez-Villanueva, M.A			100 µL of DMSO at a concentration of 10 mg/ml	[16]
6	Modified nanomedicine lab protocol 1	80 µL medium and 20 µL nanosystem	10 µL of MTT per 100 µL of medium	100 µL of acidified isopropanol	N/A
7	Maicas, I	Wash well with PBS 1X three times and then add medium-nanosystem solution			[17]
8	Modification of nanosystem preparation **	Nanosystem was diluted in medium instead of water			N/A
9	Modified nanomedicine lab protocol 2	95 µL medium and 5 µL nanosystem	A fresh, unopened stock of MTT reagent was used for the assay	100 µL of acidified ethanol	
10	Modified nanomedicine lab protocol 3		10 µL of MTT per 100 µL of medium		

* Protocol developed by the nanomedicine laboratory at Universidad Autonoma de Nuevo Leon.
** Nanosystem concentrations were initially prepared by diluting deionized water. To further investigate the interferences in MTT, subsequent dilutions were made using culture medium.

3 Results and Discussion

3.1 Colorimetric Correlation with Gold Nanoparticle Dimensions

Guided by the literature correlation between solution color and nanoparticle size (Fig. 1), the initial synthesis focused on achieving a reddish hue, targeting nanoparticles within the 20–60 nm range [18]. Figure 2 shows the change in color from the gold solution to the expected purplish-red solution of gold nanoparticles. Subsequent DLS and ζ potential measurements were conducted to verify the size and surface charge of the nanoparticles obtained under these conditions.

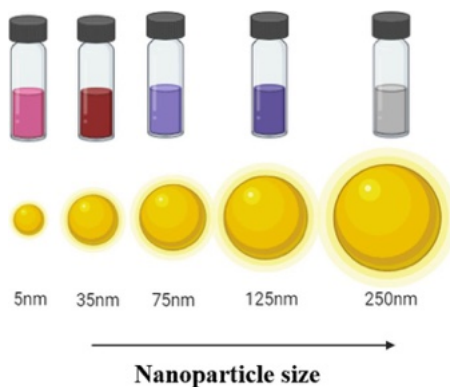


Fig. 1. Correlation between solution color and gold nanoparticle size.

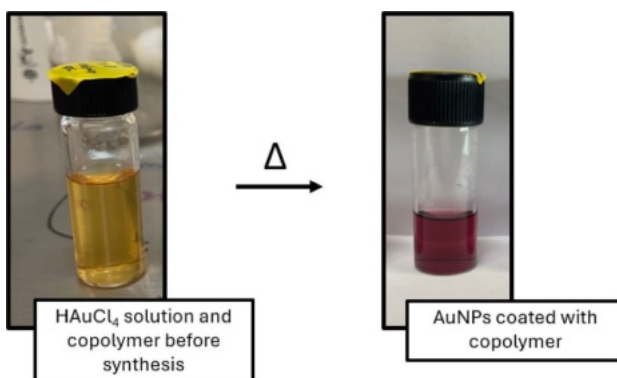


Fig. 2. Synthesis of AuNPs.

3.2 Size and Surface Charge Analysis of Nanoparticles by DLS and Zeta Potential

Dynamic Light Scattering (DLS) was employed to determine the average hydrodynamic diameter of the nanoparticles (Table 2). Nanoparticle solutions were diluted in water to

a pH of 7.5 prior to analysis. The obtained values were approximately 28 nm, falling within the expected range, thus confirming the correlation between color and particle size. Furthermore, the narrow size distribution, with 90% of the particles having a similar size, indicates good colloidal stability. The positive ζ potential, attributed to the protonated amino groups of the PEI component in the copolymer, is crucial for subsequent conjugation with negatively charged RNA molecules for biomedical applications, enabling electrostatic interactions [19].

Table 2. DLS and ζ potential for AuNPs

	Hydrodynamic diameter average (nm) $\pm$ standard deviation (% Volume)			ζ potential (mV)
	Peak 1	Peak 2	Peak 3	
AuNPs GM-CBX-b-PEI	27.56 $\pm$ 4.54 (90.47%)	292.67 $\pm$ 33.43 (9.53%)	-	+6.32 $\pm$ 0.93

3.3 Validation of Gold Nanoparticles Formation by UV-Vis Spectroscopy

UV-Vis spectroscopy confirmed the formation of gold nanoparticles from $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$. The initial peak at 320 nm, attributed to AuCl_4^- , disappeared upon reduction by GM-CBX-b-PEI, while a new peak emerged at 520 nm, characteristic of the surface plasmon resonance of gold nanoparticles. This indicates the reduction of Au^{3+} to Au^0 and subsequent nanoparticle formation. The observed surface plasmon resonance (SPR) at 520 nm is consistent with literature reports for gold nanoparticles of similar size [20–22] (Fig. 3).

3.4 Optimal Methodology Determination for GM-CBX-b-PEI-Coated Gold Nanoparticles by MTT Assay

MTT assay was conducted to identify the optimal methodology for GM-CBX-b-PEI-coated gold nanoparticles. Table 3 outlines the experimental protocols, while Fig. 4 visually represents the coloration of each well, comparing treated samples to their respective controls. It’s important to note that some controls were shared across multiple protocols, as their influence was deemed negligible in certain cases.

The MTT assay provided valuable insights into the optimal methodology for GM-CBX-b-PEI-coated gold nanoparticles. Using DMSO and solubilization buffer to dissolve formazan crystals resulted in bubble formation, incomplete crystal dissolution, and interference with absorbance readings. Wells treated with PBS-MTT, instead of Medium-MTT, failed to exhibit the expected purple color, turning yellow and potentially leading to inaccurate low cell viability assessments.

As expected, increasing the nanoparticle concentration in Protocol 6 led to decreased cell viability compared to lower concentrations. Interestingly, Protocols 1 and 7–10

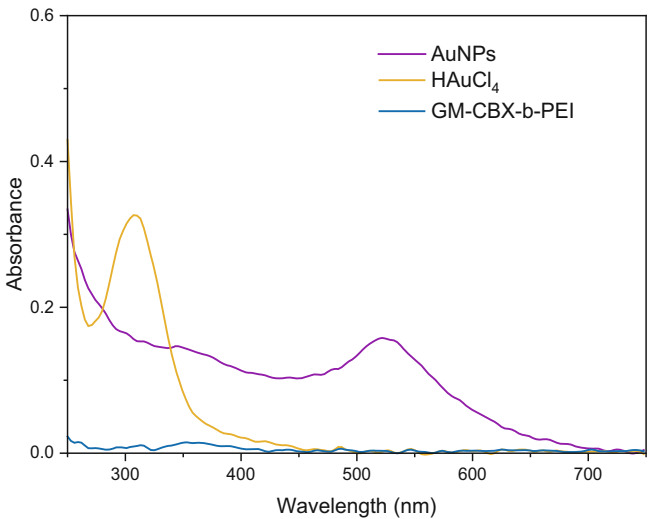


Fig. 3. UV-Vis spectra of GM-CBX-b-PEI-coated gold nanoparticles and its precursors.

Table 3. Overview of experimental methodologies for MTT assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS											
B	PBS	DMEM Medium+Cells (without treatment), Nanomedicina lab protocol	DMEM Medium+Cells (without treatment), Babaei, A.	DMEM Medium+Cells (without treatment), Melendez-Villanueva, M.A.	DMEM Medium+Cells (without treatment), Modified nanomedicine lab protocol 3	Roche protocol	Alafaleq, N. O. protocol	Modified nanomedicine lab protocol 1	Modification of nanosystem preparation protocol	Modified nanomedicine lab protocol 3	DMEM Medium +MTT (without cells)	PBS
C												
D												
E												
F												
G		DMEM Medium+Cells (without treatment), Roche	DMEM Medium+Cells (without treatment), Alafaleq, N. O.	DMEM Medium+Cells (without treatment), Modified nanomedicine lab protocol 2	Nanomedicin e lab protocol	Babaei, A. protocol	Melendez-Villanueva, M.A. protocol	Maicas, I. protocol	Modified nanomedicine lab protocol 2		DMEM Medium (without cells)	
H		PBS										

showed no significant differences, indicating that nanoparticle preparation in water or medium does not significantly impact the results. Additionally, variations in MTT stock solutions did not affect the outcome, highlighting the importance of proper storage conditions. An extra PBS wash before adding Medium-MTT had a negligible effect on the results. Finally, while both isopropanol and ethanol were effective in dissolving formazan crystals, microscopic examination revealed residual crystals with ethanol, suggesting that isopropanol may be a more efficient solvent (Fig. 5).

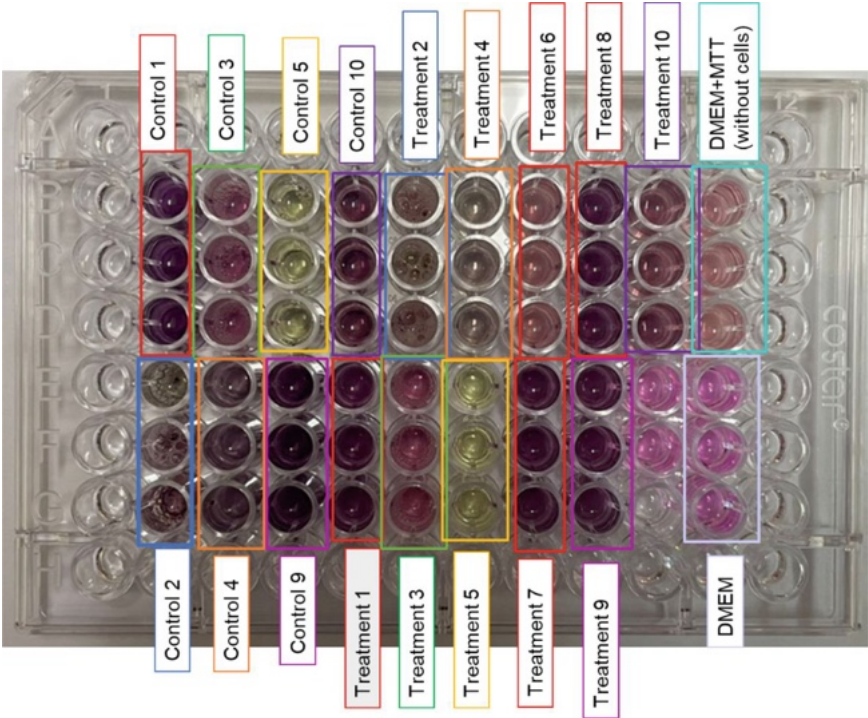


Fig. 4. 96-well plate for experimental methodologies performed in MTT assay.

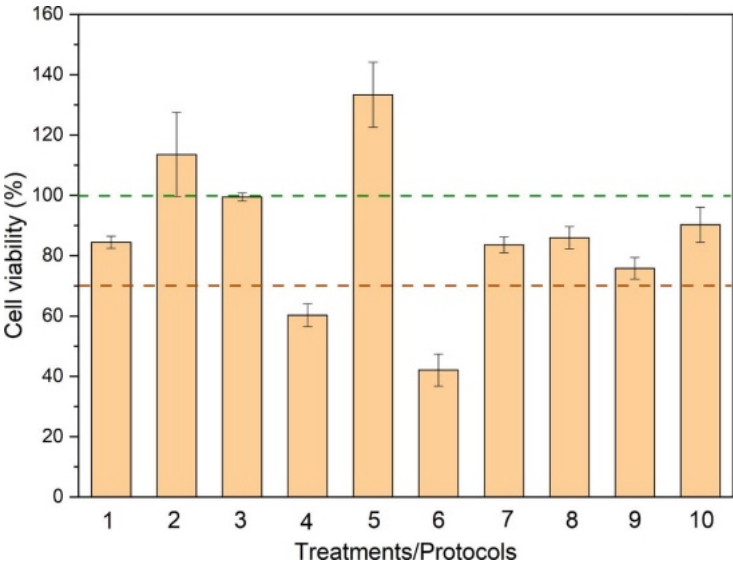


Fig. 5. Cell viability of VERO cell line with MTT assay.

4 Conclusions

In conclusion, this study successfully demonstrated the green synthesis of GM-CBX-b-PEI-coated gold nanoparticles with the desired size (28 nm) and ζ potential, making them suitable for biomedical applications. The optimal conditions for MTT assays were determined for these specific nanoparticles, highlighting the importance of tailoring experimental protocols to individual materials. Protocols 1, 8, and 9 were identified as the most suitable, as they yielded the expected results without interference and demonstrated non-cytotoxic effects, consistent with previous reports. These findings underscore the significance of optimizing experimental methodologies to ensure accurate and reliable results in future research involving gold nanoparticles.

References

1. Ditto, A.J., Shah, P.N., Yun, Y.H.: Non-viral gene delivery using nano-particles (2009). <https://doi.org/10.1517/17425240903241796>
2. Khan, I., Saeed, K., Khan, I.: Nanoparticles: Properties, applications and toxicities. Elsevier B.V. (2019). <https://doi.org/10.1016/j.arabjc.2017.05.011>
3. Bishop, C.J., Tzeng, S.Y., Green, J.J.: Degradable polymer-coated gold nanoparticles for co-delivery of DNA and siRNA. *Acta Biomater.* **11**, 393–403 (2015). <https://doi.org/10.1016/j.actbio.2014.09.020>
4. Usman, A.I., Aziz, A.A., Noqta, O.A.: Application of green synthesis of gold nanoparticles: A review. *Jurnal Teknologi* **81**(1), 171–182 (2019). <https://doi.org/10.11113/jt.v81.11409>
5. Santhosh, P.B., Genova, J., Chamati, H.: Review Green Synthesis of Gold Nanoparticles: An Eco-Friendly Approach. MDPI (2022). <https://doi.org/10.3390/chemistry4020026>
6. Walker, J.M.: Mammalian Cell Viability. Humana Press, Hatfield (2011). www.springer.com/series/7651
7. van Meerloo, J., Kaspers, G.J.L., Cloos, J.: Cell Sensitivity Assays: The MTT Assay, pp. 237–245 (2011). https://doi.org/10.1007/978-1-61779-080-5_20
8. Kamiloglu, S., Sari, G., Ozdal, T., Capanoglu, E.: Guidelines for cell viability assays. *Food Frontiers* **1**(3), 332–349 (2020). <https://doi.org/10.1002/fft2.44>
9. Ghasemi, M., Turnbull, T., Sebastian, S., Kempson, I.: The mtt assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. *Int. J. Molecular Sci.* **22**(23) (2021). <https://doi.org/10.3390/ijms222312827>
10. Almutary, A., Sanderson, B.J.S.: The MTT and Crystal Violet Assays: Potential Confounders in Nanoparticle Toxicity Testing. in *International Journal of Toxicology*, SAGE Publications Inc., pp. 454–462 (2016). <https://doi.org/10.1177/1091581816648906>
11. Kroll, A., Pillukat, M.H., Hahn, D., Schnekenburger, J.: Interference of engineered nanoparticles with in vitro toxicity assays. *Archives of Toxicology* **86**(7), 1123–1136 (2012). <https://doi.org/10.1007/s00204-012-0837-z>
12. Pinilla-Torres, A.M., et al.: Green Synthesis of Mesquite-Gum-Stabilized Gold Nanoparticles for Biomedical Applications: Physicochemical Properties and Biocompatibility Assessment. *Polymers (Basel)* **15**(17) (2023). <https://doi.org/10.3390/polym15173533>
13. Roche: Cell Proliferation Kit I (MTT). Mannheim (2020)
14. Babaei, A., et al.: Gold nanoparticles show potential in vitro antiviral and anti-cancer activity. *Life Sciences* **284** (2021). <https://doi.org/10.1016/j.lfs.2021.119652>
15. Alafaleq, N.O., et al.: Anticancer potential of gold nanoparticles (AuNPs) using a battery of in vitro tests. *Nanotechnol. Rev.* **11**(1), 3292–3304 (2022). <https://doi.org/10.1515/ntrev-2022-0502>

16. Meléndez-Villanueva, M.A., et al.: Virucidal activity of gold nanoparticles synthesized by green chemistry using garlic extract. *Viruses* **11**(12) (2019). <https://doi.org/10.3390/v11121111>
17. Gabas, I.M., Stepien, G., Moros, M., Mitchell, S.G., De La Fuente, J.M.: In vitro cell cytotoxicity profile and morphological response to polyoxo-metalate-stabilised gold nanoparticles. *New J. Chem.* **40**(2), 1039–1047 (2016). <https://doi.org/10.1039/c5nj02775f>
18. Abrica-González, P., et al.: Measurement of the Optical Properties of Gold Colloids by Photoacoustic Spectroscopy. *Int. J. Thermophys.* **39**(8) (2018). <https://doi.org/10.1007/s10765-018-2412-1>
19. Shahidi-Hamedani, N., Shier, W.T., Moghadam Ariaee, F., Abnous, K., Ramezani, M.: Targeted gene delivery with noncovalent electrostatic conjugates of sgc-8c aptamer and polyethylenimine. *J. Gene Medicine* **15**(6–7), 261–269 (2013). <https://doi.org/10.1002/jgm.2718>
20. Gomes, D.S.B., et al.: UV-Accelerated Synthesis of Gold Nanoparticle–Pluronic Nanocomposites for X-ray Computed Tomography Contrast Enhancement. *Polymers (Basel)* **15**(9) (2023). <https://doi.org/10.3390/polym15092163>
21. Shang, Y., Min, C., Hu, J., Wang, T., Liu, H., Hu, Y.: Synthesis of gold nanoparticles by reduction of H₂AuCl₄ under UV irradiation. *Solid State Sci.* **15**, 17–23 (2013). <https://doi.org/10.1016/j.solidstatesciences.2012.09.002>
22. Chan, J.Z., et al.: Green Synthesis of Gold Nanoparticles using Aqueous Extract of Clitoria Ternatea Flower. In: *IOP Conference Series: Materials Science and Engineering*. Institute of Physics Publishing (2020). <https://doi.org/10.1088/1757-899X/808/1/012036>