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A549 Alveolar Carcinoma Spheroids as a Cytotoxicity Platform for Carboxyl- and Amine-Polyethylene Glycol Gold Nanoparticles

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ABSTRACT

Gold nanoparticles (AuNPs) present with unique physicochemical features and potential for functionalization as anticancer agents. Three-dimensional spheroid models can be used to afford greater tissue representation due to their heterogeneous phenotype and complex molecular architecture. This study developed an A549 alveolar carcinoma spheroid model for cytotoxicity assessment and mechanistic evaluation of functionalized AuNPs. A549 spheroids were generated using an agarose micro-mold and were characterized (morphology, acid phosphatase activity, protein content) over 21 culturing days. The 72-h cytotoxicity of carboxyl-polyethylene glycol- (PCOOH-) and amine-polyethylene glycol- (PNH₂-) functionalized AuNPs against Day 7 spheroids was assessed by determining spheroid morphology, acid phosphatase activity, protein content, caspase-3/7 activity, and cell cycle kinetics. Spheroids remained stable over the experimental period. Although the A549 spheroids' volume increased while remaining viable over the culturing period, structural integrity decreased from Day 14 onwards. The PCOOH-AuNPs lacked cytotoxicity at a maximum concentration of 1.2×10^{12} nanoparticles/mL with no prominent alteration to the cellular processes investigated, while the PNH₂-AuNPs (at a maximum of 4.5×10^{12} nanoparticles/mL) displayed dose- and time-dependent cytotoxicity with associated loss of spheroid compactness, debris formation, DNA fragmentation, and a 75% reduction in acid phosphatase activity. Differentiation between cytotoxic and non-cytotoxic AuNPs was achieved, with preliminary elucidation of cytotoxicity endpoints. The PNH₂-AuNPs promote cytotoxicity by modulating cellular kinetics while destabilizing the spheroid ultrastructure. The model serves as a proficient platform for more in-depth elucidation of NP cytotoxicity at the preclinical investigation phase.

1 | Introduction

Lung cancer is considered a large contributor to cancer-related mortality worldwide [1], with an estimated 238 340 new cases

diagnosed in the United States in 2023 [2]. According to Statistics South Africa (2008 to 2019 report), lung cancer was the leading cause of cancer-related mortality in South Africa, being the third-most diagnosed cancer in males and the seventh-most

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diagnosed cancer in females [3]. Although there are several treatments available, the diagnosis and chemotherapeutic treatment of lung cancer remain challenging [4].

Chemoresistance is a major obstruction in cancer treatment [5, 6], with acquired resistance being evident in several cancer types [7]. Tumors also possess innate resistance to chemotherapy [7] due to physical barriers preventing drug penetration through the cellular layers [8]. Cancer cells further alter cell cycling and apoptotic processes to prevent chemotherapeutic-induced cytotoxicity [9], which contributes to the preferential targeting of actively proliferating cells [10]. The extracellular matrix also alters cellular signalling [9, 11] to protect solid tumors [5] and modulate cellular proliferation and differentiation in response to chemotherapy [8].

One promising way to address and overcome these obstacles is to improve the delivery of chemotherapy to the tumor by employing nanomedicine [8]. Nanomedicine, which uses nanoparticles (NPs) for a range of theragnostic ends, is an attractive field of study for drug delivery and directed therapy [12]. In oncopharmacotherapy, for example, the non-specific nature of chemotherapeutic drugs, leading to the systemic circulation thereof, often leads to severe adverse effects [13]. However, by employing NPs with specific functional groups, a more targeted approach is achieved [14, 15]. This allows for greater delivery of drugs to targeted organs; reduction of non-specific effects; improved efficacy; and a decrease in side effects [12]. Altering the physicochemical properties allows for more amenable solubility, in vivo stability, and biodistribution of the drug [16]. However, concerns have been raised as to the toxicity of NPs, therefore necessitating cytotoxicity screening [17–19]. Without functionalization, NPs may present with non-specificity and the inability to reach the desired target, resulting in excessive and non-specific cytotoxicity [17]. For example, oxidative stress has been observed for some NPs in keratinocytes [20], macrophages, and monocytes [21]. Additionally, inflammation may occur in exposed cells containing chemically inactive NPs [22].

Gold nanoparticles (AuNPs) are frequently studied in medical applications [23, 24] due to their promise as therapeutic delivery vehicles. The AuNPs have several appealing qualities, including unique photo-optical properties for biomedical optical imaging [24, 25] and sensitization properties in radiation therapy [26]. Although some AuNPs lack cytotoxicity [16, 27, 28], biocompatibility still needs to be considered during drug development, and these compounds cannot be assumed safe until proven so [22]. Cells might be exposed to the AuNPs for extended periods of time due to particle internalization [27, 29], and AuNPs cause cellular damage via the induction of oxidative stress and subsequent up-regulation of inflammatory genes, highlighting the need for cytotoxicity screening [30].

The A549 alveolar carcinoma cell line was selected for experimental assays as continuation of previous research by the group [31], seeing that 3D culturing was known to be successful [32, 33] and that cellular uptake does occur [34]. The study aimed to modify and characterize an A549 alveolar carcinoma spheroid model for AuNP-induced cytotoxicity assessment using 14nm carboxyl-polyethylene glycol (PCOOH)-liganded AuNPs (PCOOH-AuNPs) and 20nm amine-polyethylene glycol (PNH₂)-liganded AuNPs (PNH₂-AuNPs).

2 | Materials and Methods

2.1 | Reagents

A549 alveolar carcinoma cells (CCL-185) were procured from the American Type Culture Collection (ATCC; Manassas, USA). Gibco Dulbecco's modified Eagle medium (DMEM) and TrypLe were purchased from ThermoFisher (Johannesburg, South Africa). The PCOOH-AuNPs and PNH₂-AuNPs were obtained from Mintek (Randburg, South Africa). Accutase solution, propidium iodide (PI), all salts required for preparation of bicinchoninic acid (BCA) assay reagent A and B, radioimmuno-precipitation assay (RIPA) buffer, bovine serum albumin (BSA) powder, fetal calf serum (FCS), penicillin/streptomycin solution, agarose powder, 9×9 array negative polydimethylsiloxane molds (MicroTissues 3D Petri Dish), sodium citrate dihydrate, citric acid, saponin, Ac-DEVD-AMC powder, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) powder, ethylenediaminetetraacetic acid (EDTA), 3-[(3-cholamidopropyl)-dime thylammonio]-1-propanesulfate (CHAPS), β-mercaptoethanol, and cisplatin were obtained from Merck (Johannesburg, South Africa). The FTA hemagglutination buffer was purchased from BD Biosciences (Sandton, South Africa). The phenylmethylsulphonyl fluoride (PMSF) powder was procured from Sigma-Aldrich (St. Louis, USA).

2.2 | Nanoparticles

2.2.1 | Selection of Nanoparticles

Two AuNPs were selected: 14nm PCOOH-AuNPs and 20nm PNH₂-AuNPs. The PCOOH-AuNPs have previously been shown to lack cytotoxicity and uptake across multiple cell layers of the A549 spheroid model [34], and thus were employed as a non-cytotoxic control.

2.2.2 | Synthesis and Characterization

The 14nm PCOOH-AuNPs were prepared by Mintek (South Africa) by ligand exchange of citrate-stabilized AuNPs with 3 kDa thiolated-PEG-COOH [31] according to published methods [35, 36]. The same process was followed for the synthesis of 20nm amine-polyethylene glycol (NH₂)-liganded AuNPs (PNH₂-AuNPs), where in this case 3 kDa thiolated-PEG (HS-PEG-NH₂) was used for ligand exchange. Interference of AuNPs with the wavelengths and substrates was determined using all experimental settings referred to below in an acellular environment. Little to no interference was noted (Data S1).

2.3 | Cell Culture and Maintenance

2.3.1 | Spheroid Generation

A549 (ATCC CCL-185) spheroids were generated using a modification of the liquid overlay method employed by Fobian et al. [34] using a 9×9 array negative polydimethylsiloxane mold. Molds were autoclaved prior to each microwell generation to ensure sterility. To create the microwell system,

a sterile liquid agarose solution (1.6%, 500 μ L) was pipetted into the mold to create an agarose platform with 81 microwells. Each mold was inserted into a 12-well plate, and 160 μ L of cell suspension (3.8×10^6 cells/mL) was pipetted into the mold. Plates were incubated at 37°C and 5% CO₂ for 24 h while avoiding unnecessary turbulence. After 24 h, DMEM (1 mL) was pipetted alongside the mold to ensure nutrient transfer. Medium was replenished every 4 to 7 days after the spheroids had formed. The spheroids were grown for up to 21 days, with formation, growth, and viability assessed at regular intervals (Days 4, 7, 11, 14, 18, and 21).

2.4 | Characterization of Spheroids

2.4.1 | Morphological and Planimetric Characterization

A Carl Zeiss Axiovert 200M inverted microscope (Carl Zeiss Inc., Oberkochen, Germany) at a 5 $\times$ magnification was used to visualize the A549 spheroids on Days 4, 7, 11, 14, 18, and 21. Micrographs were taken at selected intervals to determine the volume, circularity, and diameter of the spheroids using ImageJ by using the equations:

$$\text{Spheroid volume (V)} = 0.5 \times (\text{length}) \times (\text{width})^2$$

$$\% \text{ change in spheroid volume} = \frac{V \text{ on day } [\#] - V \text{ on Day 4}}{V \text{ on Day 4}} \times 100$$

$$\text{Circularity} = 4\pi \left(\frac{\text{Area}}{\text{Perimeter}^2} \right)$$

2.4.2 | Viability Characterization

Acid phosphatases (APH) are lysosomal enzymes that hydrolyze organic phosphates in acidic environments. A proportional decrease is associated with cytotoxicity. Washed, pooled spheroids ($n = 3$) were re-suspended in 100 μ L PBS and 100 μ L assay buffer (containing para-nitrophenylphosphate [4 mg/mL] and Triton X-100 [0.2% v/v] in citrate buffer [0.1 M]) at 37°C for 90 min. After incubation, 10 μ L sodium hydroxide (1 M) was added to each well to stop the reaction. The absorbance was read at 405 nm (reference: 630 nm) with an ELX800 UV microplate reader (BioTek Instruments Inc., Highland Park, USA) within 10 min of adding sodium hydroxide. All the absorbance values were blank-subtracted, and the APH activity was calculated as a percentage using the following formula:

$$\text{APH activity (\% relative to Day 4)} = \frac{\text{OD sample}}{\text{Average OD of Day 4}} \times 100$$

2.4.3 | Protein Content Determination

The BCA assay was used to quantify the amount of protein present in the spheroids, where the spectrophotometric intensity of the product correlates to protein concentration. Eight spheroids were pooled and washed twice with PBS (100 μ L; 200 g for 5 min). The spheroids were lysed using 100 μ L RIPA buffer (50 mM Tris-hydrochloride [pH 7.4], 150 mM sodium chloride, 1% Triton

X-100, 1% sodium deoxycholate, 0.1% w/v sodium dodecyl sulphate, 1 mM EDTA, and 0.02% w/v Roche complete protease inhibitor cocktail). The spheroids were vortex-mixed, sonicated on ice for 5 min, and centrifuged at 16000 $\times$ g for 10 min. The lysate (as supernatant) was collected and stored at -80°C until use.

A clear 96-well flat-bottom plate was used to conduct the assay. An aliquot (5 μ L) of the standard (BSA; 0.1 to 2 mg/mL in PBS) or spheroid lysate was added to the wells with 195 μ L BCA working solution. The plate was shaken for 10 min at room temperature and incubated at 60°C for 30 min. The plate was cooled to room temperature, and the absorbance was measured with an ELX800UV microplate reader at 570 nm. Lysate protein content (mg/mL) was interpolated from the BSA standard curve, and dilutions were considered to determine individual spheroid content (μ g/spheroid). The percentage change in the protein content was calculated in relation to the protein content on previous days. For example, the change in the percentage protein content between Day 4 and Day 7 was calculated using the formula:

$$\% \text{ change in protein content} = \frac{\text{Protein (Day 7)} - \text{Protein (Day 4)}}{\text{Protein (Day 4)}} \times 100$$

2.4.4 | Gold Nanoparticle Exposure

The spheroids were deemed appropriate to use at Day 7 as they were compact and maintained their morphological features, which corresponded with previous uptake studies done by the research team [34]. Mature spheroids were carefully removed from the mold and placed into a standard liquid-overlay 96-well plate. The spheroids were exposed to 100 μ L of either PCOOH-AuNPs (6×10^{11} NP/mL, 1.2×10^{12} NP/mL) or PNH₂-AuNPs (1.1×10^{12} NP/mL PNH₂-AuNPs, 2.3×10^{12} NP/mL, 4.5×10^{12} NP/mL) for 24, 48, and 72 h at 37°C and 5% CO₂.

2.4.5 | Effects of Gold Nanoparticles on Spheroids

The effects of AuNPs on morphology, planimetry, viability, and protein content were determined after each exposure time using the aforementioned methods. The negative and vehicle controls included FCS-free DMEM and 1% dimethyl sulfoxide, respectively. Positive controls included saponin (1%, cytotoxicity), cisplatin (100 μ M, cytotoxicity and caspase-3/7), FCS depletion (24 h G₀/G₁-phase block), methotrexate (40 μ M, 24 h S-phase block), and curcumin (24 h, 80 μ M G₂/M-phase block).

2.4.6 | Gold Nanoparticle Effect on Caspase-3/7 Activity as a Surrogate for Apoptosis

As a surrogate for potential induction of apoptosis, caspase-3/7 activity was measured using the acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) conversion assay. Ac-DEVD-AMC is a synthetic tetrapeptide substrate that is cleaved by activated caspases-3/7 to free the bound fluorogenic 7-amido-4-coumarin (AMC). As caspase-3/7 is only activated via pro-apoptotic pathways, fluorescence indicates an induction of programmed cell death. Four pooled spheroids were placed in a 1.5 mL tube and washed with PBS (1 mL) twice via

centrifugation (200g for 5min). Cold lysis buffer (100 μ L) was added to the spheroids and incubated on ice with gentle aspiration every 10min until lysis was observed (approximately 30min). The cell lysate (25 μ L) was pipetted into the well of a black-walled, clear-bottom 96-well plate, and Ac-DEVD-AMC-containing substrate buffer was added (100 μ L). The plate was incubated at 37°C for 4h, and the fluorescence intensity (FI) was measured at an excitation and emission wavelength of 340 and 450nm, respectively. The caspase-3/7 activity was calculated as follows:

$$\text{Caspase - 3/7 activity (fold - change)} = \frac{\text{FI sample}}{\text{FI control average}}$$

2.4.7 | Cell Cycle Distribution Analysis

Flow cytometry-mediated cell cycle analysis enables the quantification of cellular distribution in the different phases of the cell cycle based on differential propidium iodide staining. To yield single-cell samples, the spheroids were dissociated with minor modifications to volumes and incubation periods. Thirty spheroids were pooled in a 1.5mL tube and suspended in PBS (1mL). Pooled spheroids were washed thrice via centrifugation (200 $\times$ g for 5min). The spheroids were dissociated using 900 μ L Accutase and gently mixed by aspiration. The tube was placed in a 37°C heated plate shaker for 10min, after which the mixture was gently aspirated ten times to facilitate dissociation. The dissociated cellular suspension was centrifuged at 200g for 5min, after which the supernatant was decanted, and the pellet re-suspended in FCS-supplemented PBS (1%, 600 μ L). While vortex-mixing, ice-cold absolute ethanol (1.4mL) was added to the tube in a dropwise fashion to fix the cells. The solution was incubated overnight in a refrigerator. Fixed cells were washed and re-suspended in 500 μ L staining solution (80 μ g/mL PI, 0.1% Triton X-100, and 100 μ g/mL DNA-free RNase in PBS) for 40min at 37°C. The samples were analyzed using a Beckman CytoFLEX flow cytometer (Beckman Coulter, South Africa). Deconvolution software (Kaluza) was used to measure DNA distribution, and the CytoExploreR (R-script) was used to analyze data. Cells were classified in different phases of the cell cycle (G_0/G_1 -, S-, and G_2 /M-phase), with the sub- G_1 -phase indicative of DNA fragmentation due to endonuclease activity associated with apoptosis.

2.5 | Statistical Analysis

Each experiment was carried out using a minimum of three biological and technical replicates, allowing for at least nine data points to be generated per sample. Data on the diameter and volume of the spheroids obtained from microscopy results was processed using ImageJ. Results were reported as the mean $\pm$ the standard error of the mean (SEM). Data from the results of all assays was captured using Microsoft Excel and analyzed statistically using GraphPad Prism 5. Statistical analyses for all assays, apart from the cell cycle distribution, were done by performing a Kruskal-Wallis test with post hoc Dunn's test. Cell cycle results obtained from flow cytometry were analyzed using two-way analysis of variance facilitated by R with the use of CytoExploreR, an R script developed by Hammill [37]. Significance was considered as $p < 0.05$.

3 | Results

3.1 | Establishing a 3D Model for A549 Spheroids

The A549 spheroids were successfully formed by Day 4 when using the micro-mold liquid overlay method and were maintained up to Day 21, as supported by microscopy images (Figure 1A). There was a significant ($p < 0.0001$) increase in circularity from Day 4 (0.6) to Day 7 (0.9) (Figure 1B), after which the spheroids remained stable till Day 14. From Days 14 to 18, there was a steady decrease in circularity to 0.8, which stabilized until Day 21. Microscopy confirmed the clearly defined spheroid edges and high opacity, indicative of compaction (Figure 1B). The spheroid volume initially decreased from Day 4 ($2.64 \times 10^8 \mu\text{m}^3$) to Day 18 ($1.86 \times 10^8 \mu\text{m}^3$; $p < 0.05$), before marginally increasing to $2.00 \times 10^8 \mu\text{m}^3$ on Day 21 (Figure 1C). Spheroid diameter was relatively unchanged from Day 4 (538.71 μ m) to Day 7, after which a significant increase ($p < 0.001$) was observed until Day 22 (759.61 μ m) (Figure 1D). Day 7 was regarded as the optimal day for AuNP exposure since the spheroids were fully formed and displayed the least variability in diameter, volume, and circularity for the three-day maximum exposure period. Additionally, spheroids maintained structural integrity and viability for long enough to permit exposure to AuNPs for 72h. The acid phosphatase (APH) activity initially increased by 31.9% from Day 4 (0.11) to Day 7 (0.14), after which it plateaued until Day 14 (Figure 1E). A sharp increase of 95.4% in activity was observed at Day 18 (0.21), compared to Day 4, which decreased to an activity similar to what was observed for Day 11 (0.13). Protein content initially increased by 30.0% from Day 4 (2.5 μ g) to Day 7 (3.2 μ g) and continued to increase until Day 21 (0.59 μ g) (Figure 1E).

3.2 | Effects of Gold Nanoparticle Exposure on A549 Spheroids

3.2.1 | PCOOH-Liganded Gold Nanoparticles

The PCOOH-AuNPs non-significantly ($p > 0.05$) decreased circularity (Figure 2A), suggesting minimal alterations at the concentrations tested. The PCOOH-AuNPs at the lowest concentration tested (6×10^{11} NP/mL) non-significantly increased spheroid volume after 48h (11.34%) (Figure 2B), while the higher concentration of PCOOH-AuNP (1.2×10^{12} NP/mL) did not (Figure 2B), though it is within the technical variation of one another.

The lower concentration of PCOOH-AuNPs (6×10^{11} NP/mL) caused a significant ($p < 0.05$) decrease of 34.40% in protein content after 24h, with this effect plateauing till 48h, after which protein content reverted to baseline levels (Figure 2C). The higher concentration (1.2×10^{12} NP/mL) of PCOOH-AuNPs resulted in a significant decrease ($p < 0.01$) in protein content of 35.45%, which diminished over time to become comparable to the negative control after 72h (Figure 2C).

The 6×10^{11} NP/mL PCOOH-AuNPs decreased APH activity non-significantly by 20.57% between 24 and 48h; however, this reverted to baseline by 72h (Figure 2D). The higher concentration of PCOOH-AuNPs (1.2×10^{12} NP/mL) induced the same

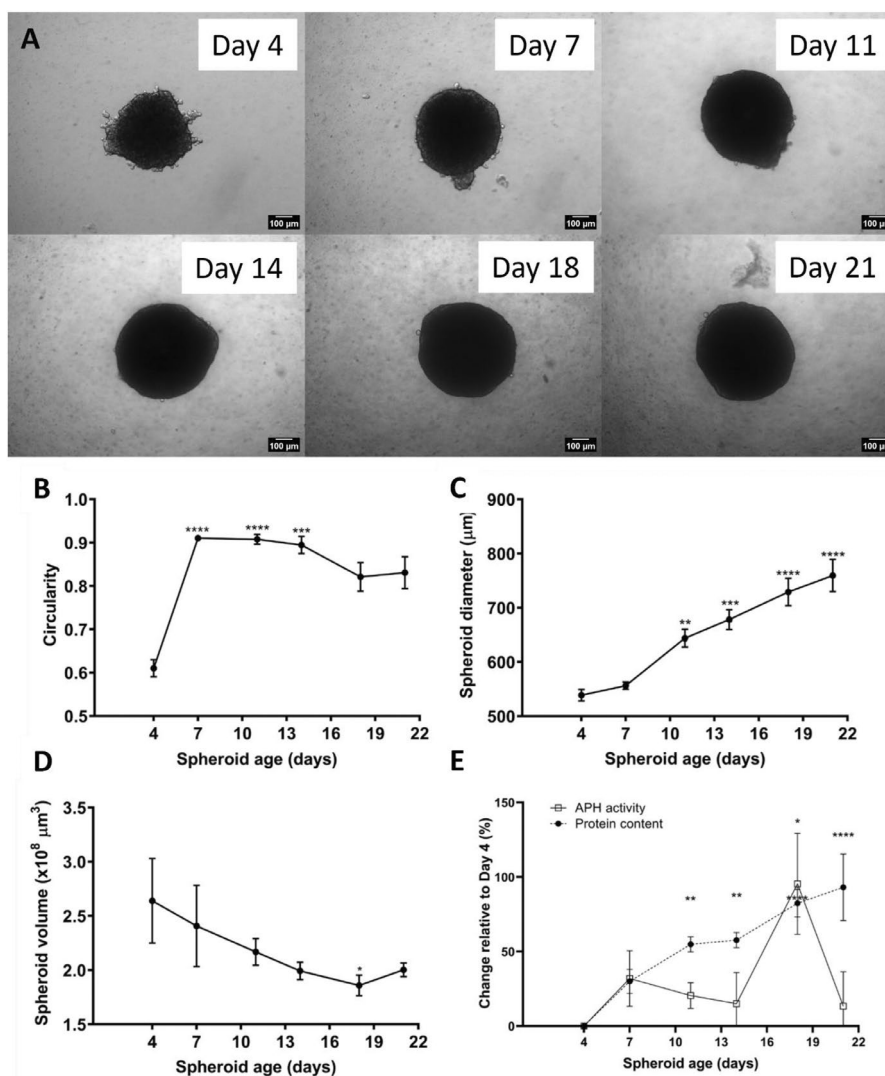


FIGURE 1 | The growth of A549 spheroids over 21 days. Phase-contrast microscopy images where Day 7 is indicative of the exposure time for nanoparticles in later experiments (5× objective; scale bar = 100 μm; A). Changes in spheroid circularity (B), diameter (C), volume (D), and acid phosphatase and protein content (E). Significance indicated as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$ as compared to Day 4.

trend in APH activity, with an initial increase from 24 to 48 h (16.14%, non-significant), followed by a return to baseline after 72 h (Figure 2D).

After 24 h, the caspase-3/7 activity was reduced, albeit non-significantly ($p > 0.05$) for both concentrations of PCOOH-AuNPs (Figure 2E). From 48 h onwards, the caspase-3/7 activity returned to baseline for both concentrations of PCOOH-AuNPs (Figure 2E). In comparison to the negative control, no statistically significant ($p > 0.05$) differences in cell cycle distribution were observed for either the 6×10^{11} NP/mL or 1.2×10^{12} NP/mL PCOOH-AuNPs. The majority of cells (83.37%) were in the G_0/G_1 phase, with approximately 8% cycling from S to G_2/M phase over the 72-h exposure period (Figure 3).

3.2.2 | Amine-Polyethylene Glycol (NH₂)-Ligated AuNPs

The PNH₂-AuNPs significantly ($p < 0.001$) decreased circularity by 38.84% after 24 h (Figure 4A) and then plateaued, suggesting

a loss of shape and structural integrity. The PNH₂-AuNPs increased in volume by 10.24% after 24 h. The effect was similar to that of the positive control (Figure 4B), except that the effect occurred after a shorter exposure period.

The PNH₂-AuNPs decreased protein content non-significantly ($p > 0.05$) at 72 h by 34.63% (Figure 4C). The APH activity of the 4.5×10^{12} NP/mL PNH₂-AuNPs-exposed spheroids after 24 h was similar to the negative control (Figure 4D). A non-significant ($p > 0.05$) decrease of 28.34% in APH activity was observed after 48 h, which was noted up to 72 h (83.99%; $p < 0.001$) (Figure 4D).

The lowest concentration (1.1×10^{12} NP/mL) of PNH₂-AuNPs caused a non-significant ($p > 0.05$) decrease in caspase-3/7 activity over the first 48 h; however, a significant reduction ($p < 0.05$) of 0.37-fold was observed after 72 h (Figure 4E), compared to the negative control. The higher concentrations (2.3×10^{12} NP/mL and 4.5×10^{12} NP/mL) of PNH₂-AuNPs caused a significant decrease ($p < 0.001$) of 0.82 and 0.74-fold, respectively, in caspase-3/7 activity over 72 h, compared to the negative control (Figure 4E).

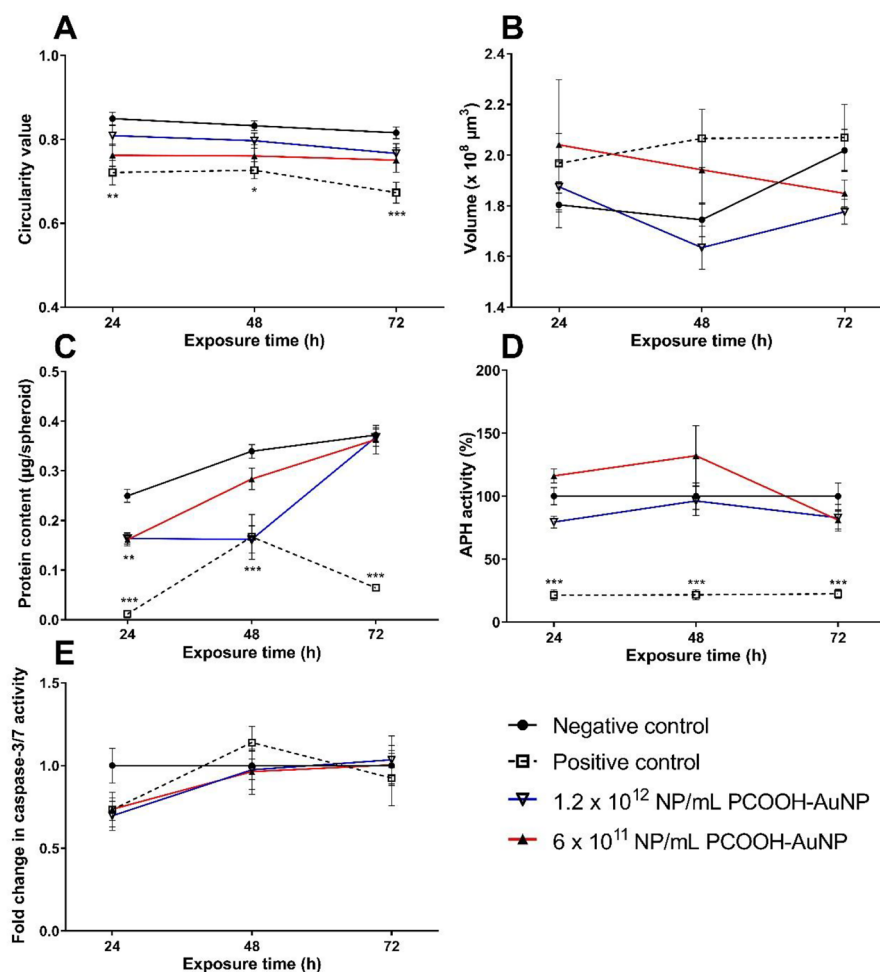


FIGURE 2 | Effect of PCOOH-liganded gold nanoparticles on the circularity index (A), volume (B), protein context (C), acid phosphatase activity (D), and caspase-3/7 activity (E) of spheroids over a 72 h exposure period. Significance indicated as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ as compared to the negative control.

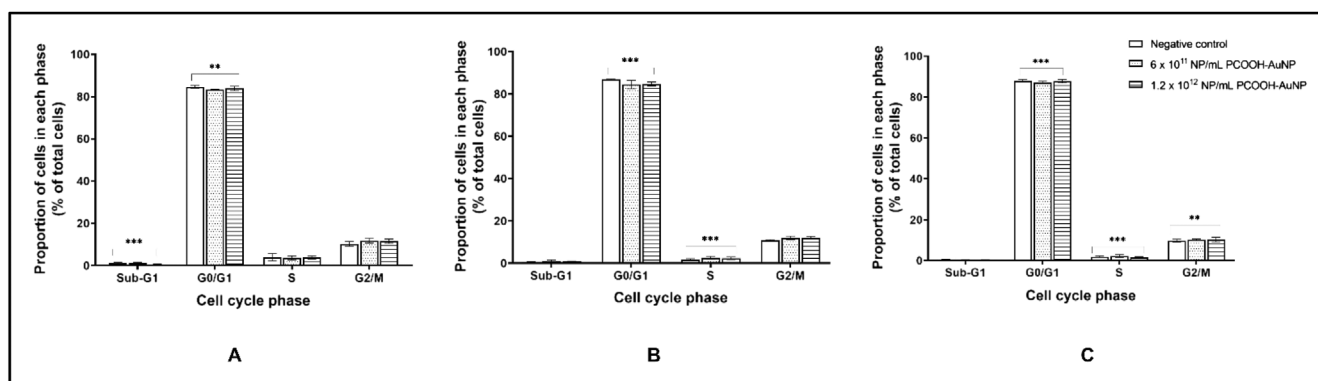


FIGURE 3 | Effect of PCOOH-liganded gold nanoparticle treatment on the distribution of A549 cells within the cell cycle of Day 7 A549 spheroids after 24 h (A), 48 h (B) and 72 h (C). Significance indicated as ** $p \leq 0.01$; *** $p \leq 0.001$ as compared to the negative control.

In comparison to the negative control, no statistically significant ($p > 0.05$) differences in cell cycle kinetics were observed after incubation with 2.3×10^{12} NP/mL or 1.2×10^{12} NP/mL PNH_2 -AuNPs. The majority of cells (84.68%) were in the G_0/G_1 phase, with a low percentage cycling from G_2/M to S-phase over the 72-h exposure period (Figure 5). However, the PNH_2 -AuNPs at 4.5×10^{12} NP/mL resulted in a time-dependent and significant ($p < 0.001$) increase in cells in the sub- G_1 phase, with a steady reduction in cells in

the G_0/G_1 (significant; $p < 0.01$), S (non-significant; $p > 0.05$), and G_2/M phases (only significant at the 72 h time point; $p < 0.01$) from 24 h onwards (Figure 5). The proportion of cells in both the sub- G_1 and G_0/G_1 phases was significantly affected by the 4.5×10^{12} NP/mL PNH_2 -AuNPs. After 24 h, a significant ($p < 0.001$) increase in the sub- G_1 phase and decrease ($p < 0.01$) in the G_0/G_1 phase was noted. A similar trend was noted in the phases after 48 h. Ratiometrically, the percentage of cells relative to the G_0/G_1

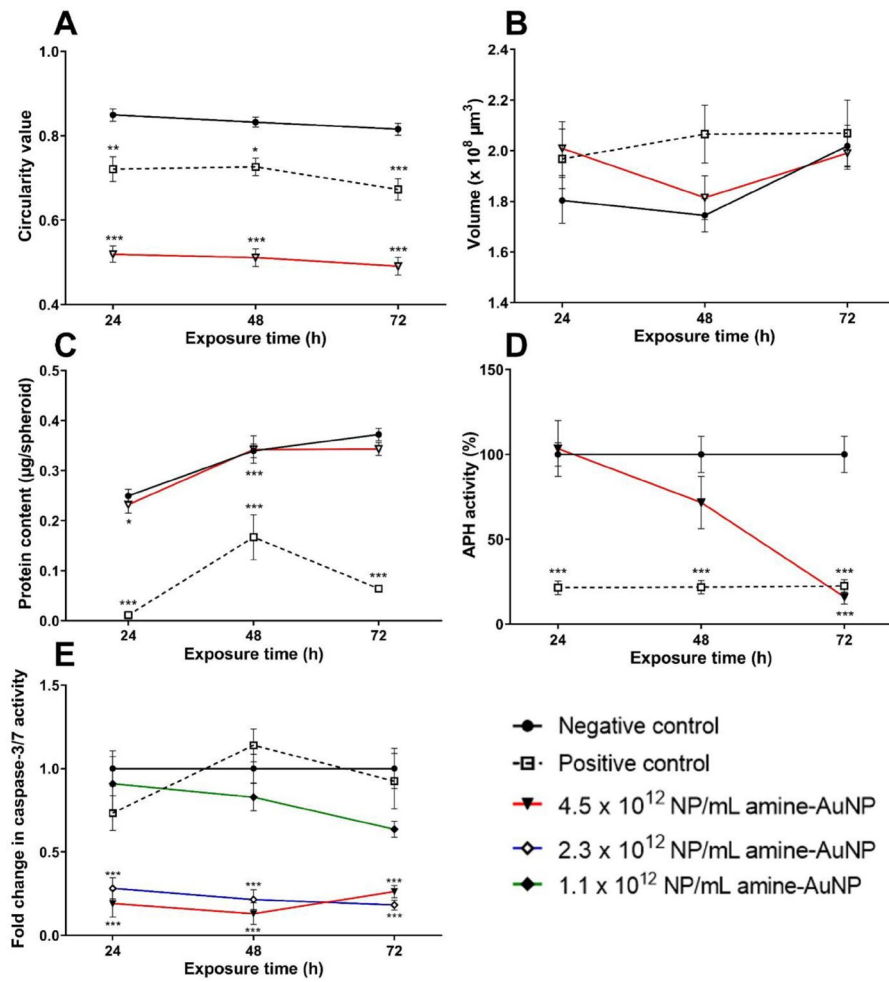


FIGURE 4 | Effect of PNH₂-gold nanoparticles on the circularity index (A), volume (B), protein context (C), acid phosphatase activity (D), and caspase-3/7 activity (E) of spheroids over a 72 h exposure period. Significance indicated as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ as compared to the negative control.

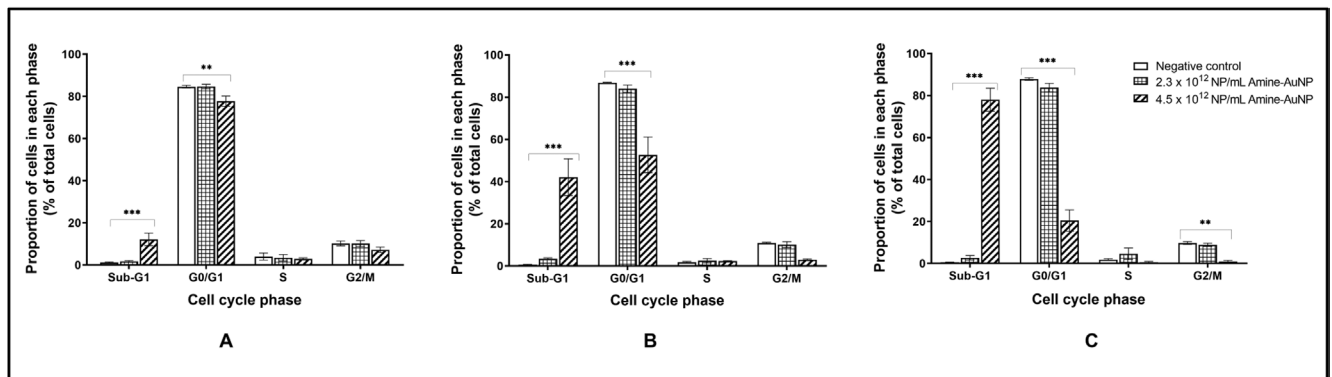


FIGURE 5 | Effect of PNH₂-AuNPs treatment on the distribution of A549 cells within the cell cycle of Day 7 A549 spheroids after 24 h (A), 48 h (B), and 72 h (C). Significance indicated as ** $p \leq 0.01$; *** $p \leq 0.001$, as compared to the negative control.

phase of each respective time point suggests that cells are not cycling into the S and G₂/M-phase, and thus the ratio of cells has increased in the G₀/G₁-phase, except at the 72h time point where the 4.5 x 10¹² NP/mL PNH₂-AuNPs produced the same effect with a significant increase in cells in the sub-G₁ phase ($p < 0.001$) and decrease in G₀/G₁-phase ($p < 0.001$), while at the same time causing a significant decrease in cells in the G₂/M-phase ($p < 0.01$).

4 | Discussion

4.1 | Establishing a 3D Model for A549 Spheroids

To facilitate reproducible high-throughput assessment, homogeneous spheroids are required for biological assays [38]. Although an adapted liquid overlay method [34] was used, the spheroids

formed were often heterogeneous in shape and size. To circumvent this, a modified bulk generation liquid overlay method, using polydimethylsiloxane molds, was employed to generate spheroids, whereby self-assembly occurs due to gravity in a low-attachment environment [39–41]. This method proved more time-efficient and resulted in the formation of up to 81 homogeneous spheroids per single agarose micro-mold.

The A549 spheroids displayed a diameter $> 500\mu\text{m}$, a size at which zonal differentiation and nutrient/waste gradients occur, similar to in vivo tumors [42–44]. The zonal differentiation better reflects the diffusion kinetics of tumors, leading to greater representation of in vivo tumors, albeit influenced by spheroid circularity [45]. Spheroid circularity increased between Day 4 and Day 7, plateaued until Day 14, and systematically reduced thereafter. The latter is indicative of a gradual loss of integrity due to the inherent nature of scaffold-free formation [43]. Previous characterization by the authors reported that A549 spheroids grown using the classic liquid overlay method had a larger average diameter of $702\mu\text{m}$ on Day 7 [34] in comparison to the micro-mold method ($556.32\mu\text{m}$). Although the size of spheroids would be expected to increase during growth [46], the volume of Day 7 spheroids was decreased. Necrotic cores, which are generally seen in spheroids $> 500\mu\text{m}$, form due to differential gradients of oxygen, nutrients, and waste leading to the accumulation of detrimental metabolic byproducts and reduced availability of cellular resources [46]. Such zonal differentiation provides more representation of 3D models in cytotoxicological studies [32, 33]. Given the increased APH activity and protein content, in parallel to the decreased volume, spheroid compaction is more likely due to increased cell–cell interactions [40, 43, 47].

4.2 | The Effect of PCOOH-Liganded Gold Nanoparticles on A549 Spheroids

The PCOOH-AuNPs lacked cytotoxicity in all assessed parameters, which is supported by Fobian et al. using the lactate dehydrogenase assay [34]. Vetten and Gulumian also noted a lack of cytotoxicity after treatment with PCOOH-AuNPs in monolayer cultures of the BEAS-2B bronchial epithelial cell line [31]. PEGylated NPs have been reported to induce less cytotoxicity compared to their non-PEGylated counterparts [17]. Given the low penetration of PCOOH-AuNPs in A549 spheroids (regardless of the potential for clathrin-mediated endocytosis leading to lysosomal trafficking) [34], it appears that the AuNPs may lack the means to alter intracellular processes or affect the inner structure of the spheroid. The lack of cytotoxicity would increase the attractiveness of the AuNPs as chemotherapeutic drug delivery agents; however, the minimal penetration via transcytosis perturbs its use [34].

The lack of alteration to cell viability is supported by Fobian et al. [34] and Vetten and Gulumian [31]. The lower concentration of PCOOH-AuNPs did affect protein content within the first 48 h, which may be indicative of protein degradation or loss of cells [48, 49], however, this did not translate to cytotoxicity. Covering the NP surface in PEG has been found to reduce protein deposition [50, 51]. The return of protein content to baseline implies that the effect could not be sustained after 48 h of exposure.

4.3 | The Effect of PNH₂-Liganded Gold Nanoparticle on A549 Spheroids

The PNH₂-AuNPs were more cytotoxic than PCOOH-liganded AuNPs, though these AuNPs did not affect protein content. The circularity index of spheroids was reduced significantly by PNH₂-AuNPs, indicating cytotoxicity and a loss of integrity. Although cellular proliferation in the periphery may also reduce rigid circularity due to newly formed cells lacking dense compaction [52], from the morphology a loss of structural integrity was noted. The uptake mechanism of the PNH₂-AuNPs is unknown, which warrants further investigation. Surface charge influences opsonization, which in turn affects plasma protein adsorption [53]. The PNH₂-AuNPs carry a slightly positive charge, with cationic liposomes known to have higher activation than neutral counterparts [54]. Harush-Frenkel et al. found positively charged NPs to internalize more rapidly [55]. The slightly cationic nature of the PNH₂-AuNPs might explain the increased cytotoxicity observed, due to possible increased uptake compared to the PCOOH-AuNPs.

The effect of the PNH₂-AuNPs on spheroid morphology increased after 48 h, suggesting further cell death, such as apoptosis, which is a time-dependent mechanism [56]. When apoptosis is initiated, cell shrinkage occurs, and thus the overall volume of the spheroid decreases [56]. Brünigk et al. found that reduced spheroid volume coincided with reduced viability in HCT116 cells [57]. The observed time-dependence of the PNH₂-AuNPs's effects may be a factor of the uptake mechanism employed, NP design and penetration, and activation of cytotoxic mechanisms [58–60]. Since caspase-3/7 activity was reduced, pro-apoptotic caspase-dependent activity was not present, and thus cytotoxicity may be driven by alternative pathways not assessed in the study. Cell death may be induced via caspase-independent pathways, which do not require the activation of caspase enzymes [61, 62]. Lui et al. found that AuNPs (13 nm) induced cell death by various mechanisms, including caspase-independent mechanisms [63]. Since spheroids are cultured to have contact with other cells and form an ECM, the spheroid may benefit from protection from anoikis (apoptosis incurred by loss of attachment to the extracellular matrix) [64, 65]. Loss of cellular or extracellular matrix contact (such as suggested by microscopy in the degradation of the spheroid structure) may have induced anoikis and subsequently apoptosis [66].

The PNH₂-AuNPs displayed dose-dependent cell cycle disturbances, where only the highest concentration resulted in perturbations. This effect was also time-dependent, which may be due to the gradual inhibition of cell cycle checkpoint factors [67]. Interestingly, the proportion of cells in the sub-G₁ phase increased after exposure to the PNH₂-AuNPs at the highest concentration tested, suggesting DNA fragmentation, which supports the potential for apoptosis. An increased proportion of cells in the sub-G₁ phase has been linked to DNA damage, protein degradation, and cytotoxicity caused by AuNPs [68]. Various AuNPs have been described to inflict DNA fragmentation [69, 70]. Ratiometrically, it appears that cells were not cycling past the G₀/G₁ phase for the first 48 h; however, at 72 h, the 4.5×10^{12} NP/mL PNH₂-AuNPs incurred DNA fragmentation and a block in the G₂/M phase.

5 | Conclusion

The spheroid model for A549 cells was successfully established and optimized via a high-throughput micro-mold system. The spheroids formed and started compacting by Day 4 and were sustained until Day 21. The spheroids remained stable over the three-day exposure period, allowing for cytotoxicity determination. The PCOOH-AuNPs did not have any significant cytotoxic effect on A549 spheroids. Although this finding appears to make them attractive targets for drug delivery vehicles, their uptake and cellular penetration are too low, as previously shown. PNH₂-AuNPs at high concentrations did significantly affect viability and growth measurements, indicating possible cytotoxicity. There is potential for future studies to establish whether the A549 spheroid would employ a different uptake mechanism compared to the findings on the PCOOH-AuNPs. To further elucidate the extent and mechanism of DNA damage observed because of exposure to PNH₂-AuNPs, studies relating to anoikis and alternative mechanisms of cell death should be performed. The study has shown that the A549 spheroids established could reliably differentiate between non-cytotoxic and cytotoxic AuNPs and thus serve as a platform for further development for drug discovery purposes.

Author Contributions

M.P. performed all experimentation, contributed to the conceptualization of the project, analyzed all data, and drafted the manuscript. S.-F.F. aided throughout with the development of the spheroid model and edited the manuscript. V.S. provided scientific insight and edited the manuscript. M.G. provided scientific insight on nanoparticles, funding, and nanoparticle supply and edited the manuscript. W.C. conceptualized the project, provided scientific insight and assistance with data analysis, and edited the manuscript.

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Ethics Statement

The project received ethical clearance from the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria (690/2019).

Consent

It is hereby confirmed that all authors are aware of the contents of this manuscript and provide consent for its publication.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are available upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.