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Research paper

Synthesis and characterization of a multifunctional gold-doxorubicin nanoparticle system for pH triggered intracellular anticancer drug release

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Abstract

A nanoparticle drug carrier system has been developed to alter the cellular uptake and chemotherapeutic performance of an available chemotherapeutic drug. The system comprises of a multifunctional gold nanoparticle based drug delivery system (Au-PEG-PAMAM-DOX) as a novel platform for intracellular delivery of doxorubicin (DOX). Spherical gold nanoparticles were synthesized by a gold chloride reduction, stabilized with thiolated polyethylene glycol (PEG) and then covalently coupled with a polyamidoamine (PAMAM) G4 dendrimer. Further, conjugation of an anti-cancer drug doxorubicin to the dendrimer via amide bond resulted in Au-PEG-PAMAM-DOX drug delivery system. Acellular drug release studies proved that DOX released from Au-PEG-PAMAM-DOX at physiological pH was negligible but it was significantly increased at a weak acidic milieu. The intracellular drug release was monitored with confocal laser scanning microscopy analysis. In vitro viability studies showed an increase in the associated doxorubicin cytotoxicity not attributed to carrier components indicating the efficiency of the doxorubicin was improved, upon conjugation to the nano system. As such it is postulated that the developed pH triggered multifunctional doxorubicin-gold nanoparticle system, could lead to a promising platform for intracellular delivery of variety of anticancer drugs.

1. Introduction

Cancer is the second most common cause of death worldwide with 14.1 million new cancer cases and 8.2 million deaths in 2012, compared with 12.7 million new cases and 7.6 million deaths in 2008 [1,2]. In spite of all available advanced technology for cancer treatment, chemotherapy is regularly accompanied by an off-target effect on healthy tissues and limits the dose levels of the drug below the therapeutic window to minimize patient discomfort [3]. Generally, nanoparticle based drug delivery systems are developed by the incorporation of active drugs via encapsulation, conjugation or entrapment to a nanoparticle [4–6]. Despite treatment advancements, the development of efficient nanoparticle drug delivery system carrying anticancer drug for chemotherapy has been a top priority in bionanotechnology field due to their potential to selectively accumulate at tumor sites [7]. A targeted drug delivery system may improve the efficacy of chemotherapy via passive targeting due to an enhanced permeability and retention (EPR) effect and could avail of an active targeting system due to receptor-mediated endocytosis mechanism [8–10]. The accumulation of nanoparticles based on their size distribution at tumor sites by extravasation through could also be available targeting vascular defects in cancer cells, this concept known as the enhanced permeability and retention (EPR) effect [11]. In the past years, various pH, reduction and temperature responsive drug delivery systems have been developed to trigger the drug release in tumor cells [12,13].

In this study, we have developed a gold nanoparticle based drug delivery nano-system to alter the intracellular drug release of doxorubicin (DOX) in vitro via an EPR effect. Due to the biocompatibility and unique optical, physical, and electronic properties of gold nanoparticles (AuNPs) they are extensively used for targeted drug delivery in biomedical nanotechnologies [14,15]. It has also been reported that the intracellular drug delivery site can be monitored using surface enhanced Raman scattering (SERS) due to the surface plasmon resonance properties of the AuNPs [16,17]. AuNPs are
often modified with polymers such as poly(ethylene glycol) (PEG) to improve its stability without altering its associated biocompatibility but simultaneously improving its potential in applications in the biomedical field [18–20]. In general, the presence of the PEG polymer is known to enhance the stability of colloidal AuNPs by increasing the electrostatic repulsion between the nanoparticles [21]. The conjugation of a PEG polymer to AuNPs has also been shown to increase the accumulation of AuNPs at tumor sites in vivo by increasing circulation time of nanoparticles in the bloodstream [22]. As such PEG surface conjugation could also help to prevent adverse effects and lower the uptake of spherical AuNPs by the reticuloendothelial system (RES) during circulation in the bloodstream [23,24]. The overall efficacy of drug delivery system depends upon drug loading capacity of nanoparticles, dendrimers are useful for simultaneous conjugation drugs and targeting ligands and their structures offer the potential to increase the drug loading capacity due to its highly branched nature, 3-D spherical morphology, surface multi-functionality and well-defined composition [3,25–29].

Strumina et al. described the use of a nanoparticle-cored dendrimer to design platforms for drug delivery nanosystems [30], we report a novel pH-responsive PEGylated dendrimer modified drug conjugated AuNPs as a smart drug delivery system for chemotherapeutic purpose. Currently reports on the combined use of a PEG polymer and PAMAM dendrimers with doxorubicin for cancer therapy [31,32] are lacking in literature and little detail into their interactions in vitro are discussed. Huang et al. have studied the intracellular behavior of nanoparticles using confocal laser scanning microscopy (CLSM) to develop the nanoparticle based drug delivery systems [33]. Li et al. described photothermal-chemotherapy using PEGylated dendrimer-doxorubicin conjugated gold nanorod drug delivery system [34]. This system required a linker to conjugate doxorubicin with five steps, in the present study the nanosystem was synthesized in four steps without the aid of a linker but using a modified PEG as a stabilizing agent. We describe the synthesis of Au-PEG-PAMAM-DOX conjugate as a drug delivery vehicle and tracking of intracellular drug release using confocal laser scanning microscopy (CLSM) images.

2. Experimental section

2.1. Materials and chemicals

Gold(III) chloride trihydrate (HAuCl₄·3H₂O), trisodium citrate dehydrate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) HCl, N-hydroxysulfosuccinimide (NHS), O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyleuronium hexafluorophosphate (HBTU), diisopropyl ethylamine (DIPEA) and doxorubicin hydrochloride (DOX) were purchased from Sigma Aldrich (Dublin, Ireland). PAMAM dendrimer succinic acid 10% in water solution (G4, MW 20615) was purchased from Dendritech Inc. (USA), thiolated PEG (SH-PEG-NH₂) MW 5 kDa was purchased from Laysan Bio (USA). Cell culture media, all supplements, Fetal Bovine Serum, diisopropyl ethylamine (DIPEA) and doxorubicin hydrochloride (DOX) were purchased from Sigma Aldrich (Dublin, Ireland). A series of pH solutions were prepared by diluting one phosphate buffered saline tablet in 100 mL of ultrapure water. A series of NaCl solutions based on concentration were prepared by dilution of 1 M stock solution of electrolyte. NaCl (1 M) stock solution was prepared by dissolving 0.5844 g sodium chloride in 10 ml of ultrapure water.

2.2. Synthesis of gold nanoparticles (AuNPs)

Briefly, 100 ml of 1 mM HAuCl₄·3H₂O solution was brought to the boil with vigorous stirring and 8 ml of 38.8 mM sodium citrate was added, there was a resultant color change of the solution from pale yellow to deep red. Boiling was continued for 10 min, after which heating was removed and the solution stirred for a further 15 min [35]. The produced nanoparticles were stored at 4 °C until required.

2.3. Functionalization of AuNPs with PEG thiol compound

To functionalize the AuNPs, in a 30 μM of 70 ml AuNPs solution, 1 μM of 1 ml SH-PEG-NH₂ (MW 5 kDa) was added and the solution was stirred for a further 15 min. Then the mixture was kept at 4 °C overnight to react [21]. The solution was then dialyzed using a dialysis membrane (MWCO 30 kDa) to remove the unreacted PEG, the sample was washed three times with ultrapure water and the purified product stored at 4 °C.

2.4. Colloidal stability of thiolated Au nanoparticle (Au-PEG) based on pH and salt test

UV-absorption spectroscopy was used to study the effect of change in pH and NaCl concentration on colloidal stability of Au-PEG in aqueous environment [21]. A series of pH solutions were prepared by adding NaOH or HCl to the buffer solution (pH 7.4). The stock solution, 1 x phosphate buffer solution (pH 7.4) was prepared by dissolving one phosphate buffered saline tablet in 100 mL of ultrapure water. A series of NaCl solutions based on concentration were prepared by dilution of 1 M stock solution of electrolyte. NaCl (1 M) stock solution was prepared by dissolving 0.5844 g sodium chloride in 10 ml of ultrapure water.

2.5. Modification of Au-PEG with carboxylated PAMAM G4 dendrimer

To modify the Au-PEG NPs, a water solution of PAMAM-COOH (2 ml, 2 mg) was mixed with EDC-HCl (1.78 mg) and NHS (1 mg), and the solution was stirred for 30 min to activate the carboxylic group of PAMAM at room temperature. This solution was then added into the Au-PEG NPs solution (50 ml, 14 μM AuNPs conc.) and was stirred for a further 48 h [36,37]. The solution was then again dialyzed using dialysis membrane (MWCO 30 kDa) to remove the unreacted PAMAM and stored at 4 °C.

2.6. Conjugation of doxorubicin hydrochloride (DOX) with Au-PEG-PAMAM NPs

The prepared Au-PEG-PAMAM NPs (30 ml) were stirred at 0 °C for 5 min, then HBTU (3.5 mg) and DIPEA (4.31 μl) were added and stirring was continued for 10 min at 0 °C. A solution containing doxorubicin (1 ml, 2 mg) was added to the mixture and the reaction was stirred for 48 h at room temperature protected from the surrounding light [38]. The final solution was again then dialyzed using a dialysis membrane (MWCO 30 kDa) to remove the free DOX and stored at 4 °C.

2.7. Drug conjugation efficiency

The efficiency of drug conjugation was calculated by a direct method using the absorption of the DOX at 480 nm. Briefly, the unknown drug concentration of the nano-carrier system was determined using a calibration curve based on series of known DOX concentrations. The drug conjugation efficiency was then calculated using following equation,

\[ \text{Conjugation efficiency} \% = \frac{\text{amount of drug in micelle}}{\text{total amount of drug in feed}} \times 100\% \] (Eq. 1)
2.8. Characterization of drug delivery system and its intermediate

The absorption measurements at 480 nm and fluorescence measurements at 471 nm were carried out using a Spectramax M3 multi-mode microplate reader (Molecular Devices, USA). The hydrodynamic size, polydispersity index (PDI) and zeta potentials of the nanoparticles were measured using Zetasizer Nano analyser (Malvern Instruments, Worcestershire, UK). Scanning electron microscopy analysis was performed using Hitachi SU 6600 FESEM instrument. The TEM samples were prepared by spin coating of nanoparticle solution onto prewashed silicon substrates at 1000 rpm for 20 sec and dried in air in a dust free environment. TEM analysis was carried out using FEI Tecnai F30, with an accelerating voltage of 300 kV. The TEM images were obtained by placing a drop of sample on a coated 400 mesh Copper grid and evaporated in air at room temperature. The point resolution is 0.19 nm in TEM mode. Confocal images were taken using Zeiss 510 (Oberkochen, Germany) laser scanning microscope.

2.9. pH dependent in vitro drug release studies

The pH dependent drug release of DOX loaded Au-PEG-PAMAM-DOX nanosystem was verified using a pH 4.0 citric acid and a pH 7.4 phosphate buffer solutions and monitoring the DOX release as a function of time for 96 h. Briefly, the Au-PEG-PAMAM-DOX nano-system (200 μl) was loaded into a mini dialysis kit (MWCO 25 kDa); the mini dialysis kit was then placed in 4 ml of corresponding buffer solution and incubated at 37 °C. 250 μl of solution from each buffer was harvested overtime with subsequent replacement of equal volume of fresh buffer at the different time interval. The release of the DOX was then quantified by measuring its absorbance using absorption spectroscopy at 480 nm and the concentration of the released DOX estimated with the aid of a standard curve.

2.10. Cell culture

The A549 cell line (human lung adenocarcinoma) was purchased from ATCC (ATTC, No.: CCL-185) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal bovine serum (FBS), 45 IU ml⁻¹ penicillin and 45 IU ml⁻¹ streptomycin in a humidified 5% CO₂ incubator at 37 °C.

2.11. Time dependent cellular studies using confocal laser scanning microscopy

A549 cells (0.5 ml) were seeded at a cell concentration of 1 × 10⁵ cells/ml in to 35 mm glass bottom culture dishes and allowed to attach the glass substrate for 3 h after which 1.5 ml fully supplemented medium was added and the cells were incubated for 24 h. Cells were then exposed to both free DOX and Au-PEG-PAMAM-DOX in a time dependent manner (4 h, 12 h and 24 h). For this, the culture medium was removed and the cells washed with 2 ml of PBS three times, cells were then exposed to the compounds under test and incubated for the desired time point. After the exposure was completed, the exposure solutions were removed and washed again three times with PBS prior to staining. The exposed and control cells were then stained with NucRed® Live 647 ReadyProbes, the cells were then incubated for 20 min and finally washed three times with PBS. After staining, the cells were fixed by addition of Formalin solution onto the cells for 10 min. After fixation, formalin solution was removed and the cells were washed twice with PBS and imaged on a Cells were then imaged in 2 ml of PBS by using CLSM. The nuclear region of the cells was stained by NucRed ready probe with excitation at 633 nm, emission observed between 649 and 799 nm and DOX was excited by 488 nm, its emission recorded between 560 and 615 nm.

2.12. Cell viability assay

The cytotoxic evaluation of free doxorubicin, Au-PEG-PAMAM-DOX, Au-PEG-PAMAM-DOX was monitored with the aid of the MTT assay. For cytotoxicity measurements, A549 cells were seeded in 96 well plates (Nunc, Denmark) at a density of 1 × 10⁵ cells/ml for 24 h exposure, 4 × 10⁴ cells/ml for 72 h exposures respectively, in 100 μl of medium containing 10% FBS. Three independent experiments were conducted and eight replicate wells were employed per concentration per plate. Following 24 h of cell attachment, plates were washed with 100 μl/well phosphate buffered saline (PBS) and further treated with 100 μl/well of free doxorubicin (1.25–10 μg/ml), Au-PEG-PAMAM-DOX (1.25–10 μg DOX/ml, 0.0875–0.7 nM AuNPs), Au-PEG-PAMAM (0.0875–0.7 nM AuNPs), prepared in media for 24 h and 72 h respectively. After 24 h and 72 h exposure, the medium for the controls or test exposures were removed, the cells were washed with PBS and 100 μl of freshly prepared MTT in media (10 mg/ml of MTT in media without FBS) were added to each well. After 3 h incubation, the medium was discarded and the cells were rinsed with PBS and 100 μl of MTT fixative solution (DMSO) were added to each well and the plates were shaken at 240 rpm for 10 min. The absorbance was then measured at 595 nm in a microplate reader.

2.13. Statistical analysis

At least three independent experiments were conducted in triplicate for all experiments. Test results for each assay were expressed as percentage of the unexposed control ± standard deviation (SD). Control values were set at 100%. Difference between the control and samples were evaluated using the statistical analysis package GraphPad Prism 7.0 (GraphPad Software, Inc., USA). Statistical significant difference was set at p < 0.05. Normality of data was confirmed by one way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. Cytotoxicity data for MTT assays were fitted to a sigmoidal curve and four parameter logistic model to calculate the IC₅₀ values and they were reported as ±95% confidence interval. IC₅₀ were estimated using GraphPad Prism 7.0.

3. Results and discussion

3.1. Synthesis and characterizations of drug delivery system Au-PEG-PAMAM-DOX

As stated, we have developed a pH-sensitive Au-PEG-PAMAM-DOX nanosystem with the goal of modifying the cellular uptake mechanism and enhancing the chemotherapeutic efficacy of DOX at cancer sites in the acidic compartments (Fig. 1). The PEGylated gold nanosphere PAMAM dendrimer-doxorubicin conjugate (Au-PEG-PAMAM-DOX) was prepared through a multistep synthesis process (Fig. 2). The colloidal gold nanoparticles (AuNPs) were prepared using a citrate reduction method of gold chloride, AuNPs have a strong affinity towards polymer functional groups such as CN, NH₂ and SH and can be stabilized with these polymers through covalent bonding to the AuNPs. The produced citrate capped AuNPs were then coated with a thioltated PEG polymer in order to enhance its stability in water. Biocompatibility of the nanoparticles is improved by modifying the surface of nanoparticle with dendrimer molecule. After the PEG stabilization, the surface of AuNPs modified with a carboxylated PAMAM G₄ dendrimer through amide linkage with the amine group of PEG polymer via an EDC coupling reaction. Finally, the DOX was conjugated with...
the carboxylic acid group of the PAMAM through amide bonding by using DIPEA as a base and HBTU as a coupling agent to obtain the final Au-PEG-PAMAM-DOX carrier system (Fig. 2).

The nanosystem Au-PEG-PAMAM-DOX was fully characterized by using UV–Vis absorption spectra to verify the successful conjugation of PEG-SH, G4-PAMAM dendrimer to AuNPs and loading of doxorubicin drug on nanosystem. Fig. 3a shows the UV–Vis absorption spectra of AuNPs, Au-PEG, Au-PEG-PAMAM and Au-PEG-PAMAM-DOX respectively. The red shift in the absorbance peak of gold nanoparticles observed has been widely attributed to a slight change in the refractive index of the local environment of AuNPs manifesting itself as a red shift in the absorbance spectrum [39]. As shown in Fig. 3a, after conjugation of the PEG, PAMAM and DOX onto the surface of AuNPs there was a resultant in red shift of longitudinal surface plasmon resonance band by 3 nm, 2 nm and 2 nm respectively. The shifts observed in the absorption spectrum of AuNPs were deemed an indication of the successful conjugation PEG, PAMAM and DOX onto the surface of AuNPs. The conjugation efficiency was then verified by absorption spectroscopy monitoring the 480 nm absorption peak of the DOX in Au-PEG-PAMAM-DOX and comparing it to a standard curve and the average loading efficiency was then found to be 48.75% (an average of \( n = 3 \) batches) DOX present in total in the nano-carrier solution.

The DOX loaded nano-carriers and DOX solution were also characterized using fluorescence spectroscopy and compared to an equivalent DOX concentration (10 \( \mu \)g/ml) as shown in Fig. 3b. When compared with the fluorescence spectra of a free DOX solution, the fluorescence of the Au-PEG-PAMAM-DOX nano-system
The particle size of Au-PEG-PAMAM-DOX nanosystem was further characterized by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) as shown in Fig. 51b and Fig. 51c. TEM and SEM micrographs of nanocarrier indicated the size of the nanoparticle around 20–25 nm. The hydrodynamic diameter of all nanoparticles measured using dynamic light scattering. As shown in the Table 51, the mean diameter of all nanoparticles was smaller than 100 nm, with PDI of 0.4–0.6. Nanoparticles with an average particle size of below 100 nm have been shown to be more effective for the passive targeting of tumor sites [37].

At each stage of the synthesis, no agglomeration had occurred but rather a systematic increase in the particle size due to the conjugation of PEG and the dendrimer to the system. As shown in Fig. 3, there was no broadening of the absorption band of nanoparticles observed. The PEG conjugation prevented the agglomeration of gold nanoparticles by increasing the hydrophilicity of these nanoparticles due to the formation of a hydrogen bond between water molecule and conjugated PEG molecule [40]. The addition of the dendrimers further helped to stabilize the nanoparticles and prevent agglomeration [41]. As can be seen in the TEM and SEM micrographs (Fig. 51b, Fig. 51c), the nanosystem Au-PEG-PAMAM-DOX was completely dispersed in water and there was no sign of agglomeration, this was further supported by the consistent dispersibility of the system indicating no aggregation (Fig. 52).

3.2. Colloidal stability of Au-PEG nanoparticle in aqueous solution

The stability of a nanoparticle is a vital attribute for the successful implementation of any potential nanosystem in drug delivery application so extensive efforts were made to improve and verify the stability of the produced materials in this study. As compared with amine compounds, thiol compounds have a strong affinity for AuNPs, which as a result forms stable Au-S covalent bonds [42]. Surface modification of AuNPs with thiolated PEG enhances the biocompatibility of AuNPs, which can as was the case in this study facilitates the further functionalization of nanoparticles with multiple other components such as dendrimers, antibodies and enzymes.

The stability of the produced particles the effects of different environment on the particles were monitored, initially the particles were placed into a NaCl solution with concentration range from 0.02 to 0.1 M and the stability of AuNPs was monitored with absorption spectroscopy. As the size of the nanoparticle increases, the UV–vis absorption spectrum showed a red shift due to aggregation caused by shortening of the bond between the AuNPs [21]. As shown in Fig. 4a, the absorption spectra of citrate capped gold nanoparticle was noted to broaden at higher concentrations (0.08 M and 0.1 M) such broadening of the absorption spectrum is due to aggregation of nanoparticles in the salt solution, which indicates its instability in a NaCl solution. When the absorption spectra of Au-PEG were monitored, it did not display any peak broadening or red shift in wavelength, as shown in Fig. 4b, indicating an increased stability when coated with the PEG, aggregation of nanoparticles as was observed in the absence of the PEG. In this study, as the concentration of NaCl solution was increased, the surface charge of nanoparticle decreased and aggregation of nanoparticle was observed, which is due to a reduction in the electrostatic repulsion of the citrate capped AuNPs in the salt solution. The stability of AuNPs can be enhanced with an increase in the electrostatic repulsion and steric hindrance, when the citrate capped AuNPs and thiolated AuNPs were compared, the presence of the PEG-SH increases the steric hindrance around the AuNPs and improves its stability in a salt solution [21].
The stability of AuNPs as a function of pH was also monitored by varying the pH over a range of 5.4–9.4. It was noted that the AuNPs capped with PEG-SH did not exhibit any change in their absorption spectrum over the tested range pH 5.4–9.4, as shown in Fig. 4d. In contrast, the absorption spectrum of the citrate capped AuNPs changed with pH, as shown in Fig. 4c indicating a higher stability of the thiolated AuNPs in acidic as well as alkaline environments when compared to the citrate capped AuNPs.

3.3. Acellular DOX release study

The ultimate desire of any nano-carrier is to release the payload (drug) in a controlled fashion as such the kinetics of Au-PEG-PAMAM-DOX drug delivery system was monitored by a dialysis method. The nano-system was incubated in a physiological pH (pH 7.4) buffer solution and an acidic (pH 4.0) buffer solution to mimic cellular lysosomal compartments in vitro. Fig. 5 shows the DOX release profiles from Au-PEG-PAMAM-DOX at pH 7.4 and at pH 4.0 as a function of time. There was negligible release of the DOX from Au-PEG-PAMAM-DOX at pH 7.4 conditions. In contrast, we observed an approximately 50% DOX release from the nanosystem after 96 h at pH 4.0 which we postulate is due to cleavage of the amide bond between the DOX and dendrimer. The release profile data demonstrated that there was a higher drug release from nanoparticles and more importantly the drug was released in a controlled manner over 96 h at pH 4.0. When compared to that of a physiological pH 7.4, as such the produced system shows great potential for further development in targeted applications.

To understand the drug release kinetics, the release data were fitted with zero order kinetics model at pH 4. For slow drug release kinetics, zero order model is more useful [27]. Release kinetics were analyzed by plotting the release data vs. time by using following equation,

\[ Q_t = Q_0 + K_0 t \]  

(\text{Eq. 2})

where \( Q_t \) is the amount of drug dissolve in time \( t \), \( Q_0 \) is the initial amount of drug in the solution, \( K_0 \) is the release constant. The value of \( K_0 \) was determined to be 0.2083 (\( R^2 = 0.9618 \)). Conclusively, drug was slowly released through nanocarrier due to cleavage of amide bond in the acidic condition.

3.4. Cell viability study

The in vitro cytotoxicity was evaluated by the MTT assay, for free doxorubicin, Au-PEG-PAMAM and Au-PEG-PAMAM-DOX were all calculated and the component parts of the Au-PEG-PAMAM-DOX system used as reference for the produced nano-system in order to evaluate if the overall system was more effective than the free DOX. All compounds were tested over a concentration
range of 1.25–10 μg/ml (or DOX equivalent AuNPs concentration) and were exposed to the A549 cells for periods of 24 h and 72 h.  
As can be seen in Fig. 6, Au-PEG-PAMAM-DOX, free DOX and Au-PEG-PAMAM exhibited both a dose and time dependent cytotoxicity profiles. The Au-PEG-PAMAM was noted to have little effect on cells and caused a statistically significant reduction in viability, it was deemed of low magnitude and to be due to conjugated PAMAM dendrimer [43,44] on the surface of the material. The cell viability was noted to decrease as a function increased exposure time and was verified by the decreasing IC50 values as a function of exposure time as shown in the Table S2. Interestingly the Au-PEG-PAMAM-DOX exhibited a higher cytotoxicity than free DOX and Au-PEG-PAMAM nanoparticles indicating that the final system improves the efficacy of the DOX. The Au-PEG-PAMAM-DOX yielded an IC50 value of 1.79 μg/ml significantly lower than that of the free DOX, which yielded a value of 4.0 μg/ml and the Au-PEG-PAMAM yielding an IC50 of 24.5 after 24 h exposure. In terms of cell viability levels the Au-PEG-PAMAM-DOX induced 69.76%...
cell death when compared to 58.40% cell death induced by free DOX equating to an approximately 12% increase in the efficacy of the DOX (Fig. 6A). After 72 h exposure, the Au-PEG-PAMAM-DOX gave an IC50 of 0.31 μg/ml compared to a value of 0.40 μg/ml for the free DOX and 14.9 μg/ml for the Au-PEG-PAMAM. While the difference in the viability levels were not so apparent after longer exposures the results suggest that the creation of the nano-carrier system could significantly alter the speed at which the DOX kills the cell as evident by the differing IC50 values in the 24 h exposures.

3.5 In vitro confocal imaging

Drug release and accumulation in vitro was monitored with the aid of confocal laser scanning microscopy coupled with counter staining the nucleus of the A549 cells and monitoring the site of DOX accumulation by tracking the DOX emission as shown in Fig. 7. It has been reported that PEGylated dendrimer nanoparticles are internalized into the cells by an EPR effect and reside within the cell outside the nucleus most likely in the lysosomal compartments of the cell [45]. In the study here free doxorubicin and DOX conjugated nanoparticles showed different cellular uptake mechanisms of the drug by the cells [46]. As can be seen in Fig. 7, after 4 h incubation with the Au-PEG-PAMAM-DOX nanosystem, the drug had accumulated in the lysosomal compartment of cells, indicated by red fluorescence from DOX. These findings verify the results of the acellular DOX release study, which confirmed that the drug was released from the nano system at pH 4.0 (Fig. 5) and support the hypothesis that we have synthesized a pH sensitive release system for DOX. As incubation time increases to 24 h, the red fluorescence from DOX could be observed in the cell nucleus as well as in the cytoplasm as shown in Fig. 7, indicating that the lower pH value triggered the release of the DOX within the lysosomal compartment of the cell which subsequently left the lysosome and accumulated in the nucleus. In contrast, internalization of DOX was observed in the cells within 4 h incubation of free doxorubicin with nuclear accumulation clearly visible in all the exposures up to 24 h as shown in Fig. 7. These findings clearly demonstrate that the Au-PEG-PAMAM-DOX nanosystem released the DOX in controlled manner; it entered initially in to the cytoplasm, accumulated in the lysosomes and after 24 h lysosomal drug release occurred and the drug entered into the cell nucleus. As such the produced pH sensitive Au-PEG-PAMAM-DOX drug delivery system could significantly enhance the efficacy of DOX by a controlled intracellular drug release couple with effective targeting may also reduce undesired side effects, and may lead to a more efficient chemotherapy treatment.

4. Conclusion

In conclusion, we have developed a promising platform for the pH-responsive intracellular drug release in the acidic organelles of cancer cells based on PEGylated PAMAM doxorubicin-gold (Au-PEG-PAMAM-DOX) nanosystem. When compared to citrate coated AuNPs, the PEG polymer coated AuNPs showed improved colloidal stability and biocompatibility with low toxicity. The active agent DOX was conjugated onto the final system with the aid of a dendrimer via an amide bond. The final drug delivery system Au-PEG-PAMAM-DOX showed a pH sensitive drug release, this was subsequently shown to be true for in vitro studies as the nano-carrier was shown to enter the cell through a different mechanism and accumulate in the lysosomal compartments (pH 4–5) of the cells. As such the DOX loaded Au-PEG-PAMAM-DOX nanoparticle altered the cellular uptake mechanism of DOX which had an effect on its associated cytotoxicity making the DOX more effective in short term exposures. This novel multifunctional dendrimer based PEGylated doxorubicin-gold nanoparticle (Au-PEG-PAMAM-DOX) nano-system could provide a new platform for the intracellular release of anticancer drug at the tumor sites.

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Appendix A. Supplementary material

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References


