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Multifunctional Thio-Stabilized Gold Nanoparticles for Near-Infrared Fluorescence Detection and Imaging of Activated Caspase-3

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Abstract

Background: Gold nanoparticles (AuNPs) are commonly used in nanomedicine because of their unique spectral properties, chemical and biological stability, and ability to quench the fluorescence of organic dyes attached to their surfaces. However, the utility of spherical AuNPs for activatable fluorescence sensing of molecular processes have been confined to resonance-matched fluorophores in the 500 nm to 600 nm spectral range to maximize dye fluorescence quenching efficiency. Expanding the repertoire of fluorophore systems into the NIR fluorescence regimen with emission >800 nm will facilitate the analysis of multiple biological events with high detection sensitivity.

Objective: The primary goal of this study is to determine if spherical AuNP-induced radiative rate suppression of non-resonant near-infrared (NIR) fluorescent probes can serve as a versatile nanoconstruct for highly sensitive detection and imaging of activated caspase-3 in aqueous media and cancer cells. This required the development of activatable NIR fluorescence sensors of caspase-3 designed to overcome the nonspecific degradation and release of the surface coatings in aqueous media.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

SUPPORTIVE/SUPPLEMENTARY MATERIAL

Not applicable.

Method: We harnessed the fluorescence-quenching properties and multivalency of spherical AuNPs to develop AuNP-templated activatable NIR fluorescent probes to detect activated caspase-3, an intracellular reporter of early cell death. Freshly AuNPs were coated with a multifunctional NIR fluorescent dye-labeled peptide (LS422) consisting of an RGD peptide sequence that targets $\alpha_v\beta_3$ -integrin protein ($\alpha_v\beta_3$) on the surface of cancer cells to mediate the uptake and internalization of the sensors in tumor cells; a DEVD peptide sequence for reporting the induction of cell death through caspase-3 mediated NIR fluorescence enhancement; and a multidentate hexacysteine sequence for enhancing self-assembly and stabilizing the multifunctional construct on AuNPs. The integrin binding affinity of LS422 and caspase-3 kinetics were determined by a radioligand competitive binding and fluorogenic peptide assays, respectively. Detection of intracellular caspase-3, cell viability, and the internalization of LS422 in cancer cells were determined by confocal NIR fluorescence spectroscopy and microscopy.

Results: Narrow size AuNPs (13 nm) were prepared and characterized by transmission electron microscopy and dynamic light scattering. When assembled on the AuNPs, the binding constant of LS422 for $\alpha_v\beta_3$ improved 11-fold from 13.2 nM to 1.2 nM. Whereas the catalytic turnover of caspase-3 by LS422-AuNPs was similar to the reference fluorogenic peptide, the binding affinity for the enzyme increased by a factor of 2. Unlike the $\alpha_v\beta_3$ positive, but caspase-3 negative breast cancer MCF-7 cells, treatment of the $\alpha_v\beta_3$ and caspase-3 positive lung cancer A549 cells with Paclitaxel showed significant fluorescence enhancement within 30 minutes, which correlated with caspase-3 specific activation of LS422-AuNPs fluorescence. Incorporation of a 3.5 mW NIR laser source into our spectrofluorometer increased the detection sensitivity by an order of magnitude (limit of detection ~ 0.1 nM of cypate) and significantly decreased the signal noise relative to a xenon lamp. This gain in sensitivity enabled the detection of substrate hydrolysis at a broad range of inhibitor concentrations without photobleaching the cypate dye.

Conclusion: The multifunctional AuNPs demonstrate the use of a non-resonant quenching strategy to design activatable NIR fluorescence molecular probes. The nanoconstruct offers a selective reporting method for detecting activated caspase-3, imaging of cell viability, identifying dying cells, and visualizing the functional status of intracellular enzymes. Performing these tasks with NIR fluorescent probes creates an opportunity to translate the *in vitro* and cellular analysis of enzymes into *in vivo* interrogation of their functional status using deep tissue penetrating NIR fluorescence analytical methods.

Keywords

Gold nanoparticles; nanomedicine; tumor selectivity; near-infrared fluorescence; molecular imaging; caspase-3; apoptosis

1. INTRODUCTION

Cells naturally regulate their population by a process known as programmed cell death or apoptosis [1–3]. This natural control mechanism counters undue cell proliferation and facilitates the destruction of malfunctioning cells. However, a variety of aberrant biological processes can stimulate or inhibit apoptosis, resulting in abnormal cell death or proliferation, as in cancer [4, 5]. The discovery of a link between apoptosis and cancer has led to the development of new anticancer drugs [6–8]. In addition, advances in molecular biology

have unraveled the roles of caspases, a family of cysteine aspartate-specific proteases, in apoptosis. Particularly, the upregulation of executioner caspases, such as caspase-3 [9], indicates the onset of caspase-mediated apoptosis [10–12]. The diagnostic relevance of this enzyme has led to the development of a variety of biological assays and methods to detect and quantify its cellular expression.

Optical methods are particularly attractive for these studies because Förster resonance energy transfer (FRET) or related mechanisms enhance the assessment of the functional status of diagnostic enzymes with high sensitivity and specificity [13, 14]. These FRET-based methods have largely focused on the use of organic dyes and fluorescent proteins [15, 16]. For example, elegant FRET-based methods to image caspase-3 activation have been developed using monomeric red fluorescent protein (mRFP) and enhanced green fluorescent protein (eGFP) [15, 17]. Cleavage of the caspase-3 sensitive site separates the fluorophores, leading to enhanced fluorescence of the mRFP in apoptotic cells. Analogous bioluminescence resonance energy transfer (BRET) mechanisms have been developed [18]. Although highly sensitive, these methods require the transfection of the host cells with constituent reporter plasmids. In addition to the potential to constitutively modify the normal physiology of the cells, the requirement to transfect cells with plasmids poses translations challenges to *in vivo* human application.

Conversely, the use of exogenous fluorogenic molecular probes could resolve some of the aforementioned problems associated with the cell transfection methods. Given that many complementary biological assays are sensitive in the visible light wavelengths, developing NIR fluorescent sensors for determining the presence and status of enzymes would expand the spectral window that avoids signal interference with standard assays. Moreover, the NIR fluorescent molecular sensors improve the detection of analytes in dense tissues because of the reduced autofluorescence and the low attenuation of light in the NIR region.[19, 20] Previous studies have harnessed the ability of activated caspase-3 to cleave the C-terminal end of aspartic acid in the tetrapeptide sequence (aspartic acid-glutamic acid-valine-aspartic acid; DEVD) [21] to develop activatable NIR fluorescent probes for reporting the activity of caspase-3 [9, 13]. The traditional FRET-based studies use a donor and an acceptor dye to quench the fluorescence before enzyme cleavage. However, the donor-acceptor FRET approach requires a careful selection of the donor-acceptor dye pairs, the stringent positioning of the dyes, or the clustering of the same dye molecules on polymeric materials. Satisfying these requirements is particularly challenging in developing NIR FRET molecular probes for detecting and imaging intracellular enzymes [22]. To overcome these problems, we explored the use of gold nanoparticles (AuNPs) as universal quenchers for NIR imaging of caspase activation by chemotherapeutic drugs.

Plasmonic and metallic properties of AuNPs facilitate a variety of analytical and biomedical applications, including diagnostic assays, biological imaging, and thermal ablation of diseased tissue [23–27]. In addition to the intrinsic plasmonic properties of AuNPs, they can be used to deliver drugs and molecular sensors to specific cells and target tissues. A particularly interesting phenomenon of AuNPs plasmonic effect is the distance-dependent fluorescence-quenching pattern of organic dyes associated with the particles for optical imaging [28, 29]. Complete quenching occurs at proximity to the particle surface, and the

intrinsic fluorescence properties of the dyes are retained beyond the plasmonic region. A fluorescence enhancement zone between these regions boosts the fluorescence quantum efficiency of small organic molecules. Interestingly, the fluorescence-quenching properties of spherical AuNPs are underutilized in NIR ($\lambda_{\text{emission}} > 800$ nm) biological assays and imaging applications. A few reports demonstrated the feasibility of using AuNPs for imaging extracellular [30], intracellular [31], or secreted [32] enzymes. These studies used a FRET-like approach that requires a reasonable overlap of the dye emission with the absorption of spherical AuNPs. In some cases, a secondary fluorescence quencher was added to the AuNPs to minimize background emission from AuNPs or partially quenched fluorescent dye [32]. We demonstrated previously [29] that AuNPs-induced radiative rate suppression [33] extends to fluorophores that do not have spectral overlap with the surface plasmon resonance of spherical AuNPs, such as NIR fluorescent dye, cypate, which emits above 800 nm. This finding suggests that distance-dependent quenching or enhancement of dye fluorescence extends over a wide wavelength range.

In this study, we explored the feasibility of detecting an intracellular enzyme activity based on a combination of dye-proximity fluorescence quenching and targeted delivery to cells, followed by caspase-3 mediated fluorescence enhancement. The AuNPs were coated with a multifunctional NIR fluorescent dye-labeled peptide (LS422) consisting of an RGD peptide sequence for dimeric $\alpha_v\beta_3$ -integrin protein ($\alpha_v\beta_3$) mediated uptake and internalization in tumor cells; a DEVD peptide sequence for detecting activated caspase-3 and reporting the induction of cell death via de-quenching of the NIR fluorescence; and a multidentate hexacysteine sequence for stabilizing and enhancing self-assembly on AuNPs.

2. EXPERIMENTAL

2.1 Synthesis of 13 nm AuNPs

AuNPs were prepared using a previously reported procedure [34]. Briefly, a warm aqueous solution (60°C) of sodium citrate (38.8 mM) was quickly added to a refluxing aqueous solution of HAuCl₄ (1 mM). The resultant solution, which changed color from light yellow to deep red within five minutes, was kept under reflux for 30 min. After cooling to room temperature, the solution was filtered through 0.45 μm Millipore syringe filters to remove any precipitate. Finally, the pH was adjusted to 7 using 0.1 M NaOH, and the filtrate was stored at room temperature.

2.2 Synthesis of Peptide-Cypate Conjugate LS422

The cypate-labeled multifunctional peptide was prepared on solid support by standard Fmoc-peptide synthesis [35]. The side-chain groups of the amino acids were orthogonally protected to allow for a bi-directional synthesis of the peptide. Specifically, Fmoc-Lys(Mtt)-OH was used to introduce the C-terminal lysine. After synthesis of the acetyl-tetradecapeptide, the Mtt group was removed with a mild acid (5% TFA in dichloromethane) to liberate the ϵ -amino group of lysine for the incorporation of DEVD peptide sequence. Cypate dye was coupled to the N-terminus of the branched peptide as described previously [36]. The crude product was purified by HPLC, and the compound was characterized by ESI-MS, HPLC, as well as spectroscopic methods. The purity of LS422 was >95% after

HPLC purification (based on the UV (280 nm) and NIR (780 nm) absorption peak areas). LS422 $C_{113}H_{159}N_{26}O_{31}S_6$: calculated $[M] = 2568$; observed $1285 [M + 2H]^{2+}$, and $857 [M + 3H]^{3+}$.

2.3 Preparation of LS422-AuNPs

A solution of LS422 (0.1 mL; 1.4 mM in H_2O/CH_3CN : 1/1) was rapidly added to 1 mL of AuNPs prepared above. The mixture was vortexed at 600 rpm overnight at room temperature. The crude product was centrifuged at 7,000 rpm for 10 min and filtered (10,000 MWCO, Amicon Ultra-4) to collect the LS422-AuNPs, which was washed with water (6×1 mL). The final product was dispersed in water (1 mL) by sonication.

2.4 Etching of LS422 from AuNPs with KCN

An aqueous suspension of LS422-AuNPs (50 μ L) was added to 10 mM KCN solution (50 μ L), and the mixture was vortexed overnight. The supernatant was used for spectral measurements.

2.5 Characterization of LS422-AuNPs

The size and microstructure of LS422-AuNPs were determined by transmission electron microscopy (TEM), and the presence of chemical groups was determined by Fourier Transform Infrared Spectroscopy (FTIR). The FTIR was performed by dispersing a solution of the AuNPs on a crystal surface, which was allowed to dry. The data were obtained by attenuated total reflectance method. Surface charges of the synthesized materials were measured with a Zetasizer (Nanoseries ZS) by suspending the samples in conductivity grade de-ionized water. The measured pH of the aqueous solution was 4. The absorption and emission spectra were recorded on a spectrophotometer and Fluorolog-3 fluorometer, respectively.

2.6 Determination of Enzyme Kinetics Parameters of LS422-AuNPs

LS422 was digested using commercially available recombinant human caspase 3. All reactions were carried out in an assay buffer consisting of 10 mM PIPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, and 5 mM 1,4-dithiothreitol (DTT). Reactions were performed in a 100 μ L-quartz cuvette using a modified Fluorolog 3 fluorimeter. A 785 nm laser diode was used as the excitation source. The laser diode was temperature regulated during the experiment, and the power output was maintained at 3.50 mW using feedback from an internal photodiode. Stock solutions of LS422-AuNPs determined by cypate absorbance at 780 nm (13.2μ M cypate content using $\epsilon_{780nm}=224,000 M^{-1}cm^{-1}$ in 20% DMSO/80% water) [37] and caspase-3 (26 nM in assay buffer) were used. To determine the total concentration of catalytic sites of caspase-3 ($[E_t]$), the caspase-3 fluorogenic peptide, Ac-DEVD-pNA, was incubated over a range of concentrations (1.25 to 50 μ M) with caspase-3 (254 pM) in the assay buffer (25°C). The resulting increase in absorbance was measured on a microtiter plate reader (Synergy HT, $\lambda = 504$ nm). Initial velocities (v_o) were determined by fitting the initial linear section of the progress curve. The initial velocity (v_o) with respect to substrate concentration was fit to $v_o=k_{cat}[E_t][S]/(K_M+[S])$ (GraphPad Prism 4.0) [38]. To determine the values of k_{cat} and K_M parameters, LS422-AuNPs (660

nM) were incubated with caspase-3 (2.5 nM) in assay buffer at varying concentrations of the competitive inhibitor, Ac-DEVD-CHO (0.5 pM to 0.5 μ M). The fluorescence released by enzyme-mediated peptide cleavage was measured continuously using the modified fluorometer described above. Initial velocities were obtained from plots of fluorescence versus time using only the initial linear portion of the data. The resulting velocities were normalized to the highest and lowest data points and plotted against the log of the inhibitor concentration. They were then fit nonlinearly for one site competition using GraphPad Prism 4.0 software. The experimentally determined IC_{50} , and the mean literature value of the K_i of Ac-DEVD-CHO for caspase 3 (0.23 nM) were then used to calculate the K_M of LS422-AuNPs ($K_i = IC_{50}/(1+[S]/K_M)$, as reported elsewhere [39]. The k_{cat} was calculated using the initial velocity of the uninhibited reaction in the equation $k_{cat} = v_o(K_M + [S])/[E_T][S]$. The raw units of v_o (rfu/s) were converted to M/s by dividing the slope from these plots by the fluorescence corresponding to complete hydrolysis and multiplying it by the substrate concentration [40].

2.7 Solid-Phase RGD Receptor-Binding Assay

The receptor binding assays were carried out using human integrin $\alpha_v\beta_3$ purified protein as described previously [41]. The major difference in our protocol is that we used ^{125}I -c(RGDyK) (0.2 nmol/L) as a tracer instead of ^{125}I -echistatin. Nonspecific binding of ^{125}I -c(RGDyK) was 5-10% of the total binding. The 50% inhibitory concentrations (IC_{50}) calculated by nonlinear regression analysis (sigmoidal dose response with a variable slope) using GraphPad Prism software. The IC_{50} values were corrected with a modified Cheng-Prusoff equation $K_i = IC_{50}^n / (1 + [radioligand]^K / K_D, radioligand)$, where n is the Hill Slope and K is the slope parameter of the displacing ligand [42, 43]. Not more than 10% of the added ^{125}I -RGDyK was bound, and the binding was at equilibrium to justify using the modified Cheng-Prusoff equation for the K_i calculation. We determined the K_D of the radiolabeled peptide was 0.4 nM. Two independent binding assays were performed in triplicates ($n=6$) per sample, except for LS167 for which three independent binding assays were performed. The results are presented as the mean of the K_i values with the standard deviation.

2.7 Cell Lines

The human non-small cell carcinoma cell line A549, the human mammary carcinoma caspase-3 negative cell line MCF-7 [44], and the mouse mammary carcinoma cell line EMT-6 were purchased from American Type Culture Collection. A549 cells were maintained in Ham's F12K medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10% fetal calf serum, 100 units/mL Penicillin, and 100 units/mL Streptomycin. MCF-7 and EMT-6 cells were maintained in minimum essential medium (MEM) with 2 mM L-glutamine and Earle's BSS adjusted to 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, with 10% fetal calf serum, 100 units/mL Penicillin, and 100 units/mL Streptomycin.

2.8 Internalization Studies

Cells were grown on Lab-Tek slides. To induce apoptosis, cells were pre-treated for 24 h at 37°C with 25 μ M paclitaxel in 0.1% DMSO. Nanoparticles (1 μ M) were added to non-paclitaxel and Paclitaxel treated cells, and the cells were incubated for an additional

1, 4, 8, or 24 h. Inhibition of caspase-3 was achieved by pre-treating some cells with 100 μM Z-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)-FMK (MP Biomedicals) for 30 min before addition of the nanoparticles. A 10 mM stock solution of the inhibitor was prepared by dissolving 1 mg of the inhibitor in 15 μL DMSO and 135 μL water. The stock solution was then diluted 1:100 with media, for a final DMSO concentration of 0.1%. At the end of the incubation, the cells were rinsed with PBS containing 1 mM CaCl_2 and imaged live with an Olympus FV1000 microscope using a 780 nm laser and a 20X objective with 3X zoom. After imaging, the cells were mounted with Prolong Gold mounting medium with a coverslip. Mounted slides were imaged with a 60X water immersion objective. Images of each group of slides were acquired with the same microscope settings during a single imaging session, allowing for qualitative comparison of the relative fluorescence.

2.9 Quantification of LS422-AuNPs Cleavage by Caspase-3

To quantify the levels of basal and induced caspase-3 enzymatic activity and LS422-AuNPs cleavage in tumor cell lines, cultured A549 cells were treated with Paclitaxel at three concentrations (1.5, 6, and 25 μM) for 24 h. The cells were then incubated with LS422-AuNPs (1 μM) in 50 μL of the appropriate media in a 96-well black-sided micro plate. Caspase-3 activity and LS422-AuNPs cleavage were monitored at selected time intervals over a 24-h period using the Odyssey Infrared Imaging scanner at 800 nm emission and 780 nm excitation. To normalize cell number per well, cells were stained for 5 min at 37°C with Sapphire-700 and DRAQ5 prior to analysis. Sapphire-700 and DRAQ5 staining was detected in the 700 nm channel of Odyssey imaging scanner and the relative fluorescence intensity was used to determine the normalization factor per well for quantitative analyses of LS422-AuNPs cleavage. Experiments were performed twice in triplicate with the appropriate controls ($n = 6$).

To validate the cleavage specificity of caspase-3 for the LS422-AuNPs, inhibition studies were performed with 10 μM of a reversible caspase-3 inhibitor, Ac-DEVD-CHO. Prior to LS422-AuNPs treatment, cultured MCF-7 and A549 cells were incubated with 10 μM Ac-DEVD-CHO for 20 min. A subset of each cell line was treated with Paclitaxel for 3 h and another group was left untreated. Both drug-and non-drug treated groups were then incubated with nanoparticles for 30 min. Before image analysis, the cells were stained for 5 min at 37°C with Sapphire-700 and DRAQ5 to normalize cell number per well. Fluorescence intensity was measured at designated time intervals over a 24-h period. Experiments were performed in triplicate ($n = 3$).

2.10 Cell Proliferation Assay

Tumor cell growth and LS422-AuNPs uptake after paclitaxel treatment were evaluated using the CyQuant® Cell Proliferation Assay Kit. Briefly, drug-treated cells, which had been previously incubated with LS422-AuNPs and stained with Sapphire-700/DRAQ5, were gently washed (2X) with PBS/1mM Ca solution. The cells were frozen at -70°C , and after two freeze-thaw cycles, 200 μL of diluted (1:20) lysis buffer solution and diluted (1:400) CyQuant® GR stock solution were added to each well and incubated for 10 min at room temperature.

3. RESULTS AND DISCUSSION

3.1. Design, Synthesis and Characterization of Multifunctional AuNPs

Caspase-3 hydrolyzes the tetrapeptide sequence DEVD at the C-terminal aspartic acid [21]. Unlike previous studies that sandwiched donor-acceptor dyes between the DEVD peptide sequence to generate a FRET effect, we used AuNPs as quenchers of the NIR fluorescence of cypate [19]. As caspase-3 is an intracellular enzyme [45], it is imperative to deliver LS422-AuNPs into cells. Cell-penetrating peptides could be used to achieve this goal [9], but the internalization is highly nonspecific. TAT peptides could also deliver their cargo into the nucleus, potentially resulting in immunologic reactions. RGD peptides can internalize imaging agents and drugs to the cytosolic compartment of cells [35, 41] where caspase-3 resides [45]. Moreover, RGD containing peptides target $\alpha_v\beta_3$ that is upregulated on the plasma membrane of highly proliferating cancer cells [46], facilitating their use as motifs for both tumor-specific delivery and endocytosis of the nanoparticles. To be effective, the dye-labeled DEVD and RGD peptides must be strongly anchored to the AuNPs to prevent nonspecific fluorescence activation due to premature detachment from the AuNPs. Thiolates are widely used to coat AuNP surfaces with ligands because they strongly bind to gold surfaces [30, 34]. For this reason, we incorporated a hexacysteine peptide sequence into the structural framework of the multifunctional peptide, LS422 (Fig. 1A).

The trifunctional peptide (LS422) was built on a lysine backbone through orthogonal peptide synthesis. The AuNPs were synthesized in aqueous media and capped with citrates to prevent agglomeration. Analysis of the AuNPs using TEM shows a narrow size distribution of ~13 nm in diameter (Fig. 1B and 1C). Metal-affinity driven self-assembly between the citrate-capped AuNPs and multidentate thiols afforded the desired LS422-AuNPs nanomaterial. Displacement of the stabilizing citrate by LS422 was further evidenced by a color change from the bright red AuNPs to the dark red coloration after capping with the dark green LS422 (Fig. 1D). TEM images and size distribution analysis show that the core size and size distribution of the AuNPs before and after the peptide conjugation were similar. The modular nature of the peptide synthesis and the ease of self-assembly on the AuNP surface favor using this approach for preparing nanomaterials for sensing diverse intracellular and extracellular proteases.

We used zeta (ζ)-potential measurement to determine the net AuNPs surface charge in aqueous solution. The ζ -potential of the citrate-capped AuNPs changed from -25 mV to about +18 mV in LS422-AuNPs (Fig. 2A), indicating a change in the surface coating of the AuNPs at pH 4. Spectroscopic methods were also used to further characterize the chemical and spectral features of LS422-AuNPs. The FTIR was performed by dispersing a solution of the AuNPs on a crystal surface, and the mixture was allowed to dry before using the attenuated total reflectance method for data acquisition [47, 48]. Attachment of LS422 to AuNPs was confirmed by the presence of vibrational bands of peptide amide bonds I and II at 1650 and 1535 cm^{-1} , respectively (Fig. 2B) [49]. Absorption spectroscopy revealed a strong band at 520 nm in water, which is attributable to AuNPs surface plasmon resonance (Fig. 2C). The absorption peak at 788 nm and a characteristic shoulder peak at 722 nm are from the cypate-labeled LS422. The small red shift of the AuNP surface

plasmon absorption and the broadening of the LS422 peaks reflect the interaction between the AuNPs and the proximal cypate molecule on LS422. In phosphate-buffered saline (PBS), the spectral profiles of LS422 and LS422-AuNPs were similar to that of water, but the citrate-stabilized AuNPs were less stable in PBS, as indicated by the broadening and shifting of the absorption peak for the nanoparticles (Fig. 2D). This could be attributed to the high ionic strength of the solution, which minimizes the charge repulsion between the citrate ions on AuNPs, leading to particle aggregation. The steady spectral profiles of LS422-AuNPs in both water and PBS demonstrates the stabilizing effect of LS422 peptide on the AuNPs in the salt-containing buffer. By adjusting the ratio of LS422 and AuNPs used in the exchange reaction and washing off the unbound peptide, the amount of LS422 loaded on the AuNPs can be controlled (Fig. 2E). Using the molar absorption coefficients (ϵ) of AuNPs at 520 nm ($4.21 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$ in water) [50] and cypate at 788 nm ($159,000 \text{ M}^{-1} \text{ cm}^{-1}$ in water) [51], we determined a loading of forty LS422 molecules per AuNP. Despite the significant difference between the absorption of AuNPs (520 nm) and the chromophore in LS422 (780 nm), the fluorescence of LS422 attached to the AuNPs was efficiently quenched (>95%; Fig. 2F). The quenching efficiency was retained with few LS422 molecules on AuNPs. Subsequent etching of LS422 from the AuNPs using KCN regenerated the intrinsic fluorescence of LS422 (Fig. 2F). Unlike the fluorescence quenching approach with a donor-acceptor FRET method, AuNPs are capable of quenching the emission of spectrally different fluorophores, thereby allowing the attachment of multiple emitters to each AuNP. Although a variety of fluorescence quenching mechanisms have been attributed to AuNPs [27], we postulate that the AuNP-mediated quenching of disparate LS422 fluorescence is mostly due to the influence of the plasmon field on the excitation decay rate and change in the quantum yield of cypate [29].

3.2 LS422-AuNPs Transforms Low Affinity to High Avidity Binding of LS422 to $\alpha_v\beta_3$ Integrin

Although previous studies have shown that cyclic RGD peptides have higher $\alpha_v\beta_3$ binding affinity than their linear analogues [41, 52], we incorporated a linear RGD peptide analogue into LS422 to simplify the peptide synthesis. We anticipated that the multivalency of AuNPs would enhance the avidity of an otherwise low $\alpha_v\beta_3$ binding linear RGD peptide. To test this hypothesis, we determined the binding affinity of a standard cyclic RGD peptide, a cypate-labeled cyclic RGD peptide (LS167) [53], the multifunctional peptide LS422, and the nanoparticles construct LS422-AuNPs. Cypate and AuNPs were added in the assay as negative controls. We prepared and used radiolabeled ^{125}I -c(RGDyK) peptide [53] as a radiotracer for the competitive $\alpha_v\beta_3$ binding assay. In contrast to the nonspecific binding of AuNPs and cypate, the RGD containing peptides displayed a log-dose dependent inhibition of ^{125}I -c(RGDyK) (Fig. 3A).

The IC_{50} values were corrected with a modified Cheng-Prusoff equation [42]. Not more than 10% of the added ^{125}I -RGDyK was bound, and the binding was at equilibrium to justify using the modified Cheng-Prusoff equation for the K_i calculation. We determined the K_D of the radiolabeled peptide as 0.4 nM, which was used to calculate the K_i of the RGD peptides. Interestingly, the binding constant of LS422 improved 11-fold from 13.2 nM to 1.2 nM in LS422-AuNPs (Table 1). This enhancement of the linear RGD peptide

binding to $\alpha_v\beta_3$ probably resulted from the availability of multiple RGD peptides for binding to the receptor because of their peripheral location on the AuNPs [54]. Control study using a non-RGD peptide, cypate-octreotate [19, 36], exhibited >100 fold decrease in the binding affinity, demonstrating the specificity of the molecular interactions between the RGD peptides and $\alpha_v\beta_3$. LS167 (cypate-labeled c(RGDfK)) showed a small improvement in the binding affinity relative to c(RGDyK), which could be attributed to the presence of hydrophobic cypate, which can enhance molecular interactions between the ligand and the receptor. Although cypate on its own does not bind $\alpha_v\beta_3$, its peptide conjugates appear to enhance their selective uptake in tumors in small animals [35]. This study demonstrates that incorporating linear RGD peptides in the multifunctional LS422-AuNPs enhanced its binding affinity to $\alpha_v\beta_3$, an indication that the nanoconstruct will facilitate the internalization of the AuNPs in $\alpha_v\beta_3$ -positive cells.

3.3 Conjugation of LS422 peptide to AuNPs enhanced the binding of LS422 to caspase-3 while retaining its catalytic efficiency

Having demonstrated that the binding affinity of the linear RGD peptide was improved significantly after attachment to AuNPs, we investigated the ability of caspase-3 to recognize the DEVD substrate in LS422-AuNPs. Unfortunately, conventional methods of determining enzyme kinetic parameters by varying the substrate concentration over a large range that extends at least ten-fold past the substrate's K_M is not suitable for this study because of a potential inner filter quenching effect of cypate dye at high concentrations. Therefore, the kinetic parameters of caspase-3 were obtained at relatively low substrate concentrations (<1 μM). However, working at these low concentrations presented a different challenge. Traditional fluorescence microtiter plate readers with lamp-based excitation sources do not provide adequate light intensity to excite the sample and the detectors have limited sensitivity in the NIR wavelengths above 700 nm. To circumvent this problem, we incorporated a 3.5 mW NIR laser source into our Horiba Jobin Yvon Fluorolog-3 spectrofluorometer (see Experimental section). The modification resulted in an order of magnitude increase in sensitivity (limit of detection ~0.1 nM of cypate) and significantly decreased the signal noise relative to xenon lamp. This gain in sensitivity enabled the detection of substrate hydrolysis at a large range of inhibitor concentrations (Fig. 3B). At 3.5 mW setting, we did not observe photobleaching of the cypate dye.

Using a published procedure [9], we determined the caspase-3 enzyme parameters k_{cat} , K_M , and k_{cat}/K_M of LS422-AuNPs as $0.9 \pm 0.2 \text{ s}^{-1}$, $4 \pm 1 \text{ }\mu\text{M}$, and $2.3 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$, respectively. The results showed about a threefold decrease in K_M for LS422-AuNPs compared with standard substrates Ac-DEVD-AMC ($K_M = 9.7 \text{ }\mu\text{M}$) and Ac-DEVD-pNA ($K_M = 11 \text{ }\mu\text{M}$) [38, 55], indicating an enhancement in the binding of the LS422-AuNPs to caspase-3. A similar decrease in K_M was previously observed for a caspase-3 substrate possessing two fluorescent dyes and cell-permeable TAT peptide sequence (TcapQ647 $K_M = 1.5 \text{ }\mu\text{M}$) [9]. These findings suggest that modifications such as the addition of relatively large fluorophores may improve K_M values for the caspase-3 enzyme. This is consistent with the observation that cypate also increased the ligand binding affinity to $\alpha_v\beta_3$. In contrast to the K_M , the catalytic turnover of caspase-3 by LS422-AuNPs was similar to those of standard fluorogenic substrates for caspase-3, Ac-DEVD-AMC ($k_{\text{cat}} = 0.75 \text{ s}^{-1}$) and Ac-

DEVD-pNA ($k_{\text{cat}} = 2.4 \text{ s}^{-1}$) [38, 55]. Although the peptide in LS422-AuNPs was efficiently cleaved by caspase-3 ($k_{\text{cat}}/K_{\text{M}} = 2.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$), the gain in K_{M} was offset by the loss in catalytic efficiency, resulting in comparable $k_{\text{cat}}/K_{\text{M}}$ values for LS422-AuNPs, and the aforementioned acylated fluorogenic substrates. These results demonstrate that conjugation of the peptide sequence to the AuNPs did not hinder the catalytic efficiency of caspase-3 and bolsters the use of AuNPs as nanosensors for monitoring the activity of this and other proteases.

3.4 LS422-AuNPs internalizes in cells and detects activated Caspase-3

The high $\alpha_v\beta_3$ binding affinity and the excellent caspase-3 kinetic parameters of LS422-AuNPs demonstrate that the RGD and the DEVD components of the multifunctional peptide were effectively recognized by their respective targets. Cellular evaluation of the LS422-AuNPs requires the use of cell lines that are $\alpha_v\beta_3$ and caspase-3 positive as well as the negative control cell lines. To address this need, we used three cell lines with differential expression of the biological targets. The human non-small cell carcinoma cell line A549 is both $\alpha_v\beta_3$ and caspase-3 positive [13, 56, 57]. This cell line served as a positive control for RGD-mediated internalization and caspase-3 fluorescence enhancement. In particular, treatment of A549 cells with Paclitaxel upregulates the expression of activated caspase-3, allowing this cell line to be used for monitoring treatment response [56, 57]. We also used the human mammary cell line MCF-7, which expresses $\alpha_v\beta_3$ [41, 58] but not caspase-3 (including pro-caspase-3) owing to a 47-base pair deletion within exon 3 of the CASP-3 gene [59]. Therefore, the internalization of LS422-AuNPs in MCF-7 cells is not expected to enhance fluorescence emission before or after paclitaxel treatment. To assess the effect of nonspecific internalization of the nanoparticles in caspase-3 positive cells, we used the murine mammary carcinoma cell line EMT-6, which is $\alpha_v\beta_3$ -negative [60] but caspase-3 positive [61]. In this case, nonspecific internalization should result in fluorescence enhancement in the presence of activated caspase-3. Cells were first treated with Paclitaxel (25 μM) for 24 h to induce apoptosis before adding LS422-AuNPs.

Live cell microscopy of the different cell lines after incubation with LS422-AuNPs is shown in Fig. 4. Relative to the untreated control (Fig. 4A), we found that LS422-AuNPs exhibited fluorescence enhancement in A549 cells within 1 h post paclitaxel treatment and continued to increase in both the number of cells and intensity over time (Fig. 4B,C). The fluorescence in these cells was diffuse, suggesting that LS422 was not confined in intracellular vesicles, which would be indicated by punctate intracellular signals. The exact mechanism of escape from the endosomal pathway is not clear at this time. To determine the specificity of the observed enhancement, we treated the cells with a caspase-3 inhibitor (Z-Asp(Ome)-Glu(Ome)-Val-Asp(OMe)). As expected, inhibition of the caspase-3 activity was evidenced by its inability to activate the LS422 fluorescence (Fig. 4D,E). At similar time points, fluorescence enhancement was not observed in MCF-7 and EMT-6 cells in the presence or absence of paclitaxel treatment (Fig. 4F-I). This finding is supported by other studies demonstrating that MCF-7 can undergo cell death through a non-caspase-3 mediated apoptosis pathway [59]. After 24 h, nonspecific uptake was predominant in the Paclitaxel treated cells, including EMT-6. Thus, specific uptake of LS422-AuNPs should be determined within 4 h post-incubation. Residual fluorescence, representing less than 5%

fluorescence intensity above background, was observed in the caspase-3 positive cells in the absence of paclitaxel treatment after incubating for 4 hours. This residual fluorescence could be attributed to the increased basal caspase-3 in the caspase-3 positive EMT-6 and A549 cells [62].

3.5 LS422-AuNPs fluorescence activation inversely correlates with cell proliferation

To quantify the response of LS422-AuNPs to drug-induced activation of caspase-3, cultured MCF-7 cells were treated with Paclitaxel for 24 h to activate caspase-3, followed by incubation with the nanoparticles. The fluorescence was monitored at selected time intervals over a 24-h period using the Odyssey Infrared Imaging scanner at 800 nm. Free cypate was used as a positive control, and negative control nanoparticles containing EEVE peptide, which is not a caspase-3 substrate. In the absence of paclitaxel treatment, A549 cells treated with 1.5 μM of LS422-AuNPs showed a 2-fold fluorescence increase relative to MCF-7 cells (Fig. 5A). At 25 μM of Paclitaxel, A549 cells treated with LS422-AuNPs (1 μM) showed a >4-fold fluorescence enhancement relative to MCF-7 cells. The stark contrast between induced peptide cleavages in MCF-7 versus A549 cells demonstrates the enhanced caspase-3 activity in A549 cells [56]. Time-dependent inhibition of paclitaxel-induced activation of LS422-AuNPs cleavage revealed a 4.5-fold decrease in fluorescence signal in A549-cells after 2 h (Fig. 5B).

Assessment of the correlation of tumor cell proliferation and LS422-AuNPs fluorescence activation after paclitaxel treatment revealed that drug-induced caspase-3 cleavage of LS422-AuNPs delineates the differential effects of chemotherapy on tumor cell proliferation (Fig. 5C). Drug-treated cells, previously incubated with 1 μM LS422-AuNPs and stained with Sapphire-700/DRAQ5, were scanned on the Odyssey Imaging scanner at 800 nm to visualize free cypate in solution. Fluorescence intensity was recorded at 528 nm using 485 nm excitation light. Cell proliferation was determined using CyQuant® Cell Proliferation Assay Kit. Increasing the concentration of Paclitaxel correlated with a global decline in A549 tumor cell proliferation. The fluorescence from cleaved LS422 correlated inversely with the number of A549 proliferating cells. A decrease in cell proliferation could be attributed to the induction of cell apoptosis, as evidenced by NIR fluorescence enhancement because of a progressive caspase-3 cleavage of DEVD. The half-maximal effective concentration ($\log \text{EC}_{50}$) in A549 cells was determined to be $19.7 \pm 0.25 \mu\text{M}$. The inverse relationship between NIR fluorescence enhancement and cell proliferation highlights the potential of using LS422-AuNPs as molecular probes for measuring and delineating differential tumor response to chemotherapy.

CONCLUSION

Our study reveals that caspase-3 efficiently recognized and cleaved the DEVD peptide-sequence in LS422-AuNPs. The preferential affinity for caspase-3, photostability of the NIR fluorophore when attached to AuNPs, and tumor targeting specificity with the RGD-peptide sequence combine to support the use of LS422-AuNPs as a reliable nanosensor for detecting effector caspase-activity in biological systems. Application of NIR NIR fluorescence sensing

eliminates high background signal and overcomes a drawback of using strategies involving UV/vis-excitable fluorophores [63].

In the tumor cell population-based studies, LS422-AuNPs showed an appreciable capacity to detect drug-induced decrease in tumor cell proliferation. The ability to accurately determine the half-maximal effective drug concentration in the examined tumor cell lines demonstrates the effectiveness of using this approach as a potential nanosensor of tumor response to chemotherapy. Our findings in tumor cell lines provide further support for extending this approach to other biological processes such as monitoring the functional status of calpains, cathepsins, and metalloproteases [14, 16]. Although we did not observe LS422-AuNP-induced cytotoxicity at the 1 μ M used in this study, we noticed caspase-3 activation at a higher concentration (10 μ M), indicating the potential use of these nanoparticles as imaging agents at low concentrations and therapeutic agents at higher levels. This observation will be the focus of a future study.

In summary, we developed NIR dye-labeled multifunctional AuNPs that can be internalized in cells by a receptor-mediated pathway and subsequently reported the cellular induction of apoptosis through NIR fluorescence activation. The two-prong approach of combining high $\alpha_v\beta_3$ binding peptides and caspase-3 substrate facilitates the delivery of the nanoparticle to target cells and selectively enhance fluorescence in cells responding to treatment. These results may provide a new strategy for using AuNPs to monitor and detect molecular processes in the NIR region above 750 nm, at which autofluorescence is minimal. The method illustrates the feasibility of enhancing the receptor binding affinity of otherwise low binders by anchoring them on the surface of spherical nanoparticles. To obtain a multivalent effect, all the targeting peptide moieties must be bioavailable for interaction with the target protein.

CONFLICT OF INTEREST

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List of Abbreviations:

AuNPs	Gold nanoparticles
NIR	Near-infrared
FRET	Förster resonance energy transfer
mRFP	Red fluorescent protein
eGFP	Enhanced green fluorescent protein (eGFP)
BRET	Bioluminescence resonance energy transfer
TEM	Transmission electron microscopy

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Fig. (1).

Design and size distribution of AuNPs. Schematic diagram of the multifunctional peptide (LS422) design (A). Structural features of AuNPs consisting of Arg-Gly-Asp (RGD), caspase-3 cleavable Asp-Glu-Val-Asp (DEVD), and hexacysteine (Cys)₃Lys(Cys)₃ peptide sequences. A NIR fluorophore, cypate, was coupled at the *N*-terminus of the branched DEVD peptide. (B) TEM of LS422-AuNPs. Scale bar is 30 nm. (C) Distribution of particle size of LS422-AuNPs determined from TEM analysis. (D) Ruby red and dark red colors of AuNPs and LS422-AuNPs, respectively.

Fig. (2).

Characterizations of AuNPs and LS422-AuNPs. **(A)** ζ -potential measurements of the aqueous solution of AuNPs and LS422-AuNPs at pH 4. **(B)** FTIR spectra of the AuNPs and the LS422-AuNPs. **(C)** Absorption spectra of AuNPs, LS422 and LS422-AuNPs in water. **(D)** Absorption of LS422, AuNPs, and LS422-AuNPs in PBS. **(E)** Absorption of LS422-AuNPs with increasing amounts of LS422 on AuNP surface. **(F)** Fluorescence quenching of LS422-AuNPs and its regeneration after etching of LS422 from the AuNPs.

Fig. (3). Functional binding assays. **(A)** *In vitro* evaluation of peptides and nanoparticles by $\alpha_v\beta_3$ binding affinity assay. **(B)** Inhibition of caspase-3 mediated cleavage of LS422-AuNPs

Fig. (4).

NIR fluorescence microscopy of LS422-AuNPs in A549, MCF-7, and EMT-6 cells. The images show LS422-AuNPs in (A) A549 cells (1 h); (B) A549 cells treated with Paclitaxel (1 h); (C) A549 cells treated with Paclitaxel (4 h); (D) A549 cells treated with Paclitaxel (4 h) with inhibitor; (E) the corresponding bright field image of (D); (F) MCF-7 cells treated with Paclitaxel (1 h), (G) the corresponding bright field image of (F); (H) EMT6 cells treated with Paclitaxel (1 h), (I) the corresponding bright field image of (H). Cells were pre-treated for 24 h at 37°C with 25 μ M paclitaxel in 0.1% DMSO.

Fig. (5).

Effect of chemotherapy on tumor cell proliferation and fluorescence enhancement. **(A)** Cultured MCF-7 and A549 cell lines were treated with the indicated concentrations of paclitaxel for 24 h to induce apoptosis. **(B)** Time-dependent inhibition of induced caspase-3 activation of LS422-AuNPs. Inhibition of caspase-3 activity in A549 cells yields a 4.5-fold reduction in LS422-AuNPs fluorescence signal to a similar value as paclitaxel-treated MCF-7 cells. **(C)** Relationship between tumor cell proliferation and LS422-AuNPs fluorescence enhancement after paclitaxel treatment.

Table 1.

Receptor binding affinity

Compound	K_i (nM $\pm$ s.d.) ^a
c(RGDyK) ^b	2.63 ± 0.967
LS167 ^c	1.62 ± 0.491
LS422	13.2 ± 0.023
LS-Au-422	1.16 ± 0.015
AuNPs	NB ^d
cypate	NB

^a Corrected K_i values were determined using the modified Cheng-Prusoff equation (see experimental methods);

^b ^{125}I -c(RGDyK) was used as a tracer;

^c structure of LS167 is Cypate-c(RGDfK);

^d NB, No specific binding was observed