Enhanced cellular uptake and anti-cancer potentials of gold nanoparticles conjugated with cell penetration peptide against lung cancer cells

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Abstract
Gold nanoparticles (GNPs) are often conjugated in the biomedical field with biocompatible peptides, although the effect of biocompatible GNP peptides on cellular responses is still not clearly understood. In the current study, GNPs with / without peptide were used as model probes to investigate the cytotoxicity to the human lung cancer cell line (A457) and human normal breast epithelial cell line (HBL-100). GNPs and GNPs-RGD preparation was confirmed and characterized using UV – VIS spectrophotometer, FE-SEM, FTIR, and TEM. The anticancer effect to A457 cell line was estimated using MTT assay. Our results show that the GNP-RGD had found significant tumor targeted efficacy and decrease in proliferation of A457 cell line compared with HBL-100 which appeared normal growth. Overall, our finding suggests a potential therapeutic effect of GNPs-RGD as a novel anti-cancer drug to be further developed and offer a beneficial targeting therapy.

Keywords: GNPs; GNPs-RGD; Cytotoxicity; A457 cells; HBL-100.
1. Introduction

Lung cancer or lung carcinoma is a malignant tumor of the lung that is characterized by uncontrolled growth of cells in the lung tissues [1]. It a predominant public health problem worldwide is estimated that there were 517,350 men and women living in the US with a history of lung cancer as of January 1, 2019, and an additional 228,150 cases will be diagnosed in 2019. This makes it the most common cause of cancer related death in men and second most common in women after breast cancer [2]. These statistics show that the conventional therapies are not sufficiently effective. There are two main reasons that limit the efficacy of existing treatments: the great complexity and consequent limited understanding of cancer cells, and their resistance to the currently used therapeutics [3]. It is well-known that cancer cells are inherently more vulnerable to chemotherapy than the majority of normal cells, but most anticancer drugs are nonselective and can cause injury to normal tissues. In recent years, efforts have focused on effective and safe cancer therapy by increasing tumor targeting while sparing normal cells. General cures are medicinal drugs that journey via the bloodstream to arriving all portions of the body, and work by distinct mechanisms. For example, chemotherapy drugs typically attack cells that develop quickly. Hormonal remedy works through both blockading or lowering the stage of the body’s natural hormones, which occasionally act to promote most cancers growth. Targeted treatment options work with the aid of attacking particular cancers cells (or close by cells) that commonly assist them grow [4]. There is, therefore, an essential need for new, excessive and better-tolerated agents with chemotherapeutic recreation to targeted tumor cells.

During the earlier years, nanotechnology-based diagnostic and therapeutic structures have provided a possible choice for most cancers’ treatments. Multifunctional nano structures [5]. In conjugation with imaging probes and
concentrated on agents such as antibodies (Abs)/aptamers (Aps), have been currently developed for most cancer treatments [6]. Amongst entirely nanomaterials, gold nanoparticles (GNPs) are ideal candidate for transport of a variety of anticancer agents due to their character homes together with chemical resistivity, low cytotoxicity, biocompatibility, specific optical properties and its highly binding rate to amine and thiol groups, enabling surface modification by biomolecules such as a peptide [7].

Peptides are molecules formed by mixtures of amino acids linked by peptide bonds through a dehydration-condensation reaction. The use of therapeutic peptides over proteins or antibodies depends on many properties, including the size of the peptide and the ability to penetrate the cell membranes. They are characterized by a high specificity, affinity, and activity; they also ensure a reduced drug-drug interaction and are both chemically and biologically diverse. Advantage of their usage in treatment firstly, is that they do not accumulate in the target organs, thereby, reducing the associated drug toxicity. secondly, peptides are that they can be easily synthesized and modified; they are also less immunogenic compared to the proteins or the recombinant antibodies [8]. Several anticancer peptides (e.g. leuprolide, goserelin, and octreotide) have been approved by FDA and many other antitumor peptides are currently being evaluated in various phases of preclinical and clinical trials thus reflecting the high potential of peptides as therapeutics [9].

The arginine (R)–glycine (G)–aspartic acid (D) (RGD) tripeptide as an adhesion motif that is presented in many proteins of the extracellular matrix (ECM) and its role in cell-cell, integrin interaction, and cell-matrix interactions, driven the development of a broad diversity of RGD peptides and peptidomimetics for potential therapeutic applications. Since RGD binds to the αvβ3 integrin, resulting inhibition of angiogenesis, tumor growth, and metastasis [10]. With the
purpose of improving the anticancer effectiveness by enhance the targeting efficiency, we developed a probe based on GNPs directly surface-functionalized with a tumor homing SH–RGD via Au–S bond then test its affects against human lung cancer cell line (A457) and human normal breast epithelial cell line (HBL-100).

2. Materials and Methods

2.1 Cell lines and reagents
The cell lines, human lung cancer (A457) and human normal breast epithelial cell line (HBL-100) were supplied by Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq. Tetrachloroauric acid trihydrate, trisodium citrate, SH-RGD peptide, trypsin-EDTA, fetal bovine serum, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenylterazolium (MTT), Rosswel Park Memorial Institute (RPMI)-1640 was ordered from Euro Clone (Milan, Italy). Antibiotics such as penicillin and streptomycin were provided from (Biosource International, Nivelles, Belgium). MTT Kit, (Intron Biotech, Korea), The remaining chemicals and reagents were applied with analytical grades.

2.2 Preparation of gold nanoparticles
Gold nanoparticles (GNPs) are synthesized according to Ahmad with some modification [11]. GNPs are produced by reducing chloroauric acid (HAuCl4) in a liquid. After dissolving HAuCl4, a reducing agent (trisodium citrate dihydrate) was added in the solution with constant stirring. After reaction with reducing agent, gold ions Au3+ reduced to neutral gold atoms and the color of the solution altered to wine red, indicate the formation of GNPs. For formation of uniform gold nanoparticles, fast stirring of solution was done.
2.3 Synthesis of GNPs-RGD

GNPs prepared in previous step were modified by the thiol-terminated peptide SH-RGD. In a distinctive reaction, 4 mL of GNPs in a phosphate-buffered saline (pH = 7.5) were degassed for 30 min. Then, 4 mL of a solution of SH-RGD peptides was prepared in deionized water at concentration (220 µg mL⁻¹), before being degassed. The GNPs were added to the peptide solution. This solution was then stirred for 16 hrs. at 25 °C to remove the unreacted SH-RGD peptides from the solution, then centrifuged at 11,000 rpm for 30min, the supernatant was decanted, and the particles were resuspended in distilled water. This process was triplicated. GNPs-RGD collected from this process were suspended in 4 mL of deionized water, sterilized by passing through a 0.22 µm syringe filter (Corning Inc., Corning, NY, USA), and stored in the dark at 4°C.

2.4 Characterization of gold nanoparticles and gold nanoparticles modified with RGD peptide

The synthesis of GNPs and GNPs-RGD was confirmed primarily by using Hitachi U-2910 spectrophotometer (Tokyo, Japan) to measure the UV – Vis spectrum (range of 200–900 nm). The FTIR test was achieved with an attenuated total reflection mode using (8400S, Shimadzu, Japan, spectral range = 4000–400 cm⁻¹, resolution = 4 cm⁻¹). Field Emission Scanning Electron Microscopic (FE-SEM) analysis was shown using MIRA 3 TESCAN (Brno -Czech Republic) to recognize the morphological features of the synthesized. To identify the morphological features and distribution of the synthesized, Transmission Electron Microscopic
(TEM) images were measured using JEOL JEM-1010 (JEOL Ltd., Japan) operated at 400 kV.

2.5 Cell lines culture and maintenance
Cytotoxicity assay (in vitro), used RPMI-1640 medium added with 10% FBS, 20 mM HEPES and 2 mM L-glutamine, was used to culture the MCF-7 and WRL-68 cells via tissue culture flasks (T 25 cm²; Falcon, USA) (5% CO2; 37 °C).

2.6 Cytotoxicity against cell lines
Flat-bottom culture plates (96-well; Falcon, USA) were used to seeding the cancer cell line suspension (200 μL, 1×10⁵ cells mL⁻¹). Cells were stay in the Log. growth phase (48 h) and then exposed for 24 h to treatment with the tested concentrations of GNPs and GNPs-RGD. Then, cells in wells were marked with MTT in PBS (100 μL; 10-15 min; 37 °C). Subsequent discarding and washing of the dye with tap water, air bubbles were mixed in DMSO (50 μL; 10 min). Cell cultures on a microplate reader (ELx 800, Bio-Tek Instruments Inc., USA) were then exposed to absorbance measurement at a wavelength of 492 nm according method presented by Vijayakumar and his co-workers [12].

2.7 Statistical analysis
A one-way ANOVA (Tukey Test) variance analysis was performed to examine whether or not that variance in the group was significant, statistical significance was clarified as * p 0.05 or * * p 0.01. Data were expressed as mean ± standard deviation, and Graph Pad Prism version 8 (Graph Pad Software Inc., La Jolla, CA) analyzed statistical significances.
3. Results and discussion

3.1 Synthesis gold nanoparticles and RGD-modified gold nanoparticles (GNPs–RGD)
Gold nanoparticles (GNPs) were prepared in the present study by reducing gold salts by citrate. As illustrated in Fig. 1, pale yellow to red gradually changed colour. This change has been utilized as an indicator for GNP formation. The diffuse of GNPs on the surface of gold nanoparticles in a polar solvent such as water caused by the negative surface charge of citrate ions. These citrate-stabilized GNPs are able to undergo irreversible aggregation with thiolate ligands during the functionalization process. Tween 20 preceding the modification to avoid aggregation to conquer this problem. Finally, we prepared a probe (GNPs – RGD) using a functionalized one-step method. SH – RGD was conjugated via Au – S bonds on the surface of the GNPs. This method, compared with conventional targeted peptide modified methods, simplifies the synthetic steps and reduces the reaction time along with cost. This shift in color was probably caused by the appearance of the absorption band Localized Surface Plasmon Resonance (LSPR); a characteristic feature for metal nanoparticles such as gold. The GNPs are so small that electrons can't move around freely as its gold in bulk. Because this motion is restricted, the particles react with light differently [13].

3.2 Characterization of gold nanoparticles (GNPs) and the probe (GNPs–RGD)

3.2.1 UV-VIS spectrophotometry and Fourier transform infrared spectroscopy (FTIR) analysis
Using a UV–visible spectroscopy, the successful conjugation was confirmed. As illustrated in Fig. 2, red line GNPs and green line GNPs–RGD at 527 and 536 nm wavelengths, respectively. Depending on gold-citrate ratio, the citrate reduction method can produce GNPs with a peak of 520-530 nm. The red-shift from 527 nm to 536 nm, which might attribute to RGD peptide that changed the refractive index around GNPs, which indicated a successful surface modification of GNPs. These results consist with Yang and his colleagues [14], who mentioned that the protein or peptide conjugation on GNPs result in a shift to a longer wavelength due to increased particle size. In contrast, the maximum absorbance increases with increasing particle sizes [15]. To further verify the attachment of SH–RGD to GNPs were characterized using Fourier transform infrared spectroscopy (FTIR). As shown in Fig. 3A and 3B, the GNPs and GNPs–RGD have same characteristics peaks (peaks are common in both), except amide I bond (1631 cm⁻¹, carbonyl stretch vibration) and amide III bond (1384 cm⁻¹, C–N stretch vibration) only appeared in GNPs–RGD (Fig. 3B), which illustrated the successful conjunction of SH–RGD on the surface of GNPs.

### 3.2.2 Field emission scanning electron microscopy (FE-SEM) and Transmission electron microscopy (TEM)

The morphology of the particles, the size and shape of GNPs and GNPs–RGD were evaluated using FE-SEM. GNPs showed well-separated, smooth spherical-shaped particles with an average size of ~20 nm. All particles were distributed almost as single particles, and were clearly homogeneous. No agglomerated particles or aggregates were detected (Fig. 4A). This may be attributed to citrate reduction of GNPs in a polar solvent. The negative surface charge from the citrate ions on the surface of gold nanoparticles cause easily disperse of GNPs [16]. The GNPs–RGD of spherical particles monodispersed was seen with an average size of
~30 nm, (Fig. 4B), this confirm the presence of SH-RGD peptide resulted in fluctuating the size from 20 to 30 nm, but not affect the morphology and distribution of the with GNPs. On the other hand, the TEM images showed the GNPs and GNPs–RGD (Fig. 5A and 5B, respectively) had a uniform spherical morphology. The conforming histograms of the hydration kinetic diameter size distribution (insets) of nanoparticles exposed that the GNPs and GNPs–RGD possessed a spherical shape with a mean diameter of 20.2 and 30.3 nm, respectively, with a relatively narrow size distribution. TEM confirmed that no aggregation occurred after the conjugation of SH–RGD with GNPs.

3.3 Cytotoxicity of gold nanoparticles (GNPs) and the probe (GNPs–RGD) against human lung cancer (A457) human normal breast epithelial cell line (HBL-100).

Safety of nanoparticles was an important parameter to detect their potential for further translational development. To assess the cytotoxicity of GNPs and GNPs-RGD, we chose the human lung cancer (A457) cell lines compared with human normal breast epithelial cell line (HBL-100). Fig. 6A showed that the treated A457 cells GNPs and GNPs -RGD at concentrations ranged from100 to 400µg mL$^{-1}$ for 24 hours exhibited a reduction in cell viability in a dose-dependent manner of these compounds. The lowest A457 cell viability (%) noted at concentration 400 µg mL$^{-1}$ were 63.9% and 57.2% by GNPs and GNPs -RGD respectively. The GNPs -RGD showed significantly the most potent cytotoxic activity with IC$_{50}$ value of 216.4 µg mL$^{-1}$, whereas IC$_{50}$ values of GNPs was 163 µg mL$^{-1}$. In contrast, GNPs-RGD at 400 µg mL$^{-1}$ showed significant (P ≤ 0.05) weak cytotoxicity (less than 12%) on human normal breast epithelial cell line (HBL-100), compared with GNPs at same concentration that show cytotoxicity about 24.1% (Fig. 6B).
GNPs are considered to be a particularly valuable source of effective anti-proliferative and cytotoxic for different cell lines. Due to the small size of GNPs, it is able to enhance permeability and retention (EPR) effect and ability to easily enter into cells by transport mechanism like or by accumulated in the extracellular space between the basal plasma membrane then, inhibited cells growth by inducing apoptosis and necrotic phenotype [17]. On the other hand, a new study showed the increasing cytotoxicity of GNPs-RGD, compared to GNPs. This may be attributed to RGD peptide, it has a highly receptor affinity and selectivity with improved biostability to integrins which play a vital role in the interaction of extracellular matrix protein with the cell surface and in cell–cell adhesion in vertebrates [18]. Integrins are composed of an α- and a β-subunit; many of which recognize binding partners through an RGD sequence, the RGD peptide in GNPs-RGD can bind to MCF-7 cell integrins and cause cellular growth inhibition. The less cytotoxic effect of GNPs-RGD on the human normal breast epithelial cell line (HBL-100) may be attributed to the normal expression of RGD peptide receptors on its surface compared with cancer cell [19]. These results indicated that potent cytotoxicity and antitumor activity of GNPs-RGD that can be used as a good potential as an anticancer agent.

4. Conclusion

This study demonstrated the synthesized GNPs have good stability and excellent biocompatibility, the RGD peptide can be conjugated to GNPs by direct cross-linking using a simple method. The GNPs-RGD had high cytotoxicity toward lung cancer (A457). In contrast, it had low cytotoxicity toward human normal breast epithelial cell line (HBL-100). Those results highlight the capacity to fabricate cytocompatible conjugated GNPs with cell penetration peptide, which can be
beneficial for the development of new treatments such as anticancer treatments. Additional studies to well understand the effect of such conjugated GNPs should confirm their efficiency as a cancer therapeutic and their capacity to specifically target cancer cells. Finally, we suppose that our study will provide an innovative idea for the single step synthesis of other nanomaterials and multifunctional used for tumor imaging or therapy.

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Figure 1. Gold nanoparticles preparation. (Suspensions of gold salt solution. (B) gold nanoparticles formation. (C) Stabilize Gold nanoparticles by tween 20. (D) The formation of Gold nanoparticles conjugated RGD (GNPs-RGD). The lower scheme showed the mechanisms of GNPs preparation and conjugation.
Figure 2. UV-vis absorption spectra of GNPs and GNPs–RGD.
Figure 3. FTIR analysis GNPs and GNPs–RGD.

Figure 4. FE-SEM image of GNPs (A) and GNPs–RGD (B).
Figure 5. TEM image of GNPs (A) and GNPs–RGD (B).

Figure 6. Cytotoxicity effect of GNPs and GNPs–RGD on lung cancer cell line (A547) (A) and on human normal breast epithelial cell line (HBL-100) (B). After 24 hours incubation at 37 °C. *, P 0.05; **, P 0.01.