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Original Article

Conjugation of Cortistatin Peptide with Gold Nanoparticles Synthesized to Investigate Anti-Inflammatory Effects in Allergic Asthma

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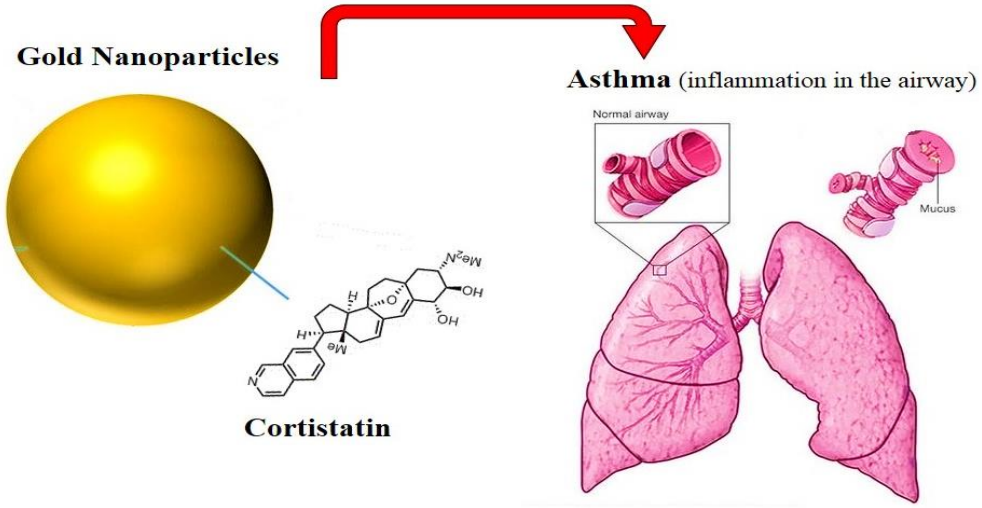
ABSTRACT

In this study, conjugation between Cortistatin peptide and gold nanoparticles was performed for effective drug delivery of peptide to lung tissue to investigate its anti-inflammatory anti-asthmatic effects. To create conjugation between metal nanoparticles and biomolecules, there are different strategies based on chemical or physical interactions, which are selected according to the desired goals. Attaches based on chemical interactions have more specificity and selectivity, and for the intended purpose, drug delivery to the lung tissue is more desirable. This work performed conjugation based on chemical interactions using linker molecules to achieve higher specificity and selectivity. The binding of Cortistatin peptide to gold nanoparticles in this work was 80%, which indicates that the binding through the linker molecules is well done.

GRAPHICAL ABSTRACT



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Introduction

Asthma is an inflammatory disease of the respiratory tract and one of the most common non-communicable diseases that causes many problems for all age groups, from infancy to old age. This disease is the most common chronic disease in childhood [1,2]. In asthma, inflammation and spasm in the airways of the lungs are the main problems. This inflammation causes swelling and closing of the airways and thickening of the pulmonary secretions (mucus) [3,4]. Nowadays, with the development of nanotechnology, attempts have been made to use nano material for therapeutic purposes in order to have the greatest effects on the target tissue with the least amounts of drugs [5-9]. In the case of asthma, a similar procedure is underway and efforts have been made to produce nanoparticles or so-called nano-sprays containing anti-asthma drugs [10,11]. Nano-drugs are obtained by improvement and modification of traditional drugs with nanotechnology or by using new nanocomposites, including biomolecules attached to nanoparticles. At present, the priority of asthma treatment has changed and the use of bronchodilators has shifted to treating inflammation and reducing airway responsiveness [12-16]. One of the biomolecules that have anti-inflammatory properties having been studied in recent years is the Cortistatin peptide. Cortistatin peptide is a cyclic neuropeptide with a structure similar to somatostatin and is known as a potential endogenous anti-inflammatory factor. Cortistatin controls the production of anti-inflammatory mediators and, like somatostatin, is synthesized as a pro-peptide and then processed into the final form and its genes are produced by gene replication. They can bind to all five somatostatin receptors. In addition to its pharmacological and functional properties similar to somatostatin, Cortistatin has distinct functions, such as induction of slow-wave sleep, reduced motor activity, and activation of selective cation current [17].

Nano-medicine and the drug delivery system are branches of nanotechnology having received a lot of attention recently and are advancing rapidly [18,19]. Nano-drugs are often used to improve drug delivery in the body and targeted drug delivery to target tissues. The nano-drug delivery system is the delivery of the drug to the target tissues at a specific time and in a controlled dose, which is dramatically safer and much more effective than distributing the drug throughout the body. Nano-drug delivery reduces side effects and consumes lower doses. Metal nanoparticles have been widely used in drug delivery and detection of biofeedback and biosensors. Different particles are produced and evaluated from various metals, but gold and silver are the most widely used. These particles can be produced in different sizes and shapes with a small particle size distribution [20-22]. One of the unique features of these particles is the change in their optical behavior by changing the particle size. Surface change of these particles is very easy and different ligands such as sugars, peptides, proteins and DNA can bind to these particles [23,24]. Gold nanoparticles have many applications in pharmaceutical and medical purposes. Stability, high dispersibility, non-toxicity, environmental friendliness, and optical adjustment have many advantages over other nanoparticles. Both covalent and non-covalent modes can be used to bind peptides to gold nanoparticles. In dative bonding, a covalent bond is obtained between nanoparticles and peptide-free sulfhydryl groups [25]. In the covalent mode, binding intermediates or adapter molecules, such as avidin and biotin, may be used to form the complex [26,27]. In the present work, the anti-inflammatory peptide Cortistatin was conjugated to gold nanoparticles via covalent bonding by linker molecules. This conjugation was performed by creating an aldehyde active group on Cortistatin and a hydrazine group on gold nanoparticles. Considering that most peptide drugs for the treatment of asthma in the airway environment are proteolysis under the influence of mucous fluid and are destroyed by airway enzymes and that they cannot leave their effects, attempts have been made to increase the density of the desired peptide by conjugating gold nanoparticles to it, due to the increasing use of Nanoparticles in the treatment of asthma and according to studies. The resulting conjugate

compound can pass through the mucous fluid and reach the airway cells, leaving therapeutic effects [28,29].

Our aim in this work is accordingly the conjugation of Cortistatin peptide to gold nanoparticles to investigate its anti-asthmatic effect in animal models of asthma.

Methods

Synthesis of gold nanoparticles

The synthesis of spherical gold nanoparticles was performed according to the previously described method [30]. In short, the aqueous solution of boiling gold chloride is reduced by a steady increase in sodium citrate until a red color appears. By changing the ratio of gold chloride and trisodium citrate, gold nanoparticles of any size can be synthesized. The size of the nanoparticles is very important. Because particles smaller than 50 nanometers are exhaled out of the lungs. Larger particles, like foreign bodies, are trapped in the upper airways and cannot reach the airways or, if they reach the airways, are trapped by the phagocytic system in the lungs. A suitable range of our goals is 80-150 nm.

Bioconjugation of Cortistatin peptide with gold nanoparticles

Covalent bonding via linker molecules was used to bind the peptide to the gold nanoparticles. To do this, aldehyde active groups on Cortistatin and hydrazine active groups on nanoparticles were created to conjugate by connecting the two active groups.

1 g of gold nanoparticles, the average nanoparticles size of 137 nm, and 0.5 mg of peptide each (peptide and nanoparticles) separately to 10 ml of an aqueous solution containing 0.1 M of sodium phosphate and 0.1 M of NaCl in 7.4 pH were increased. Each of the SANH (Succinimidyl 4-hydrazino nicotinate acetone hydrazone) (Bio-world, USA) and SFB (Succinimidyl 4-formyl benzoate) (Bio-world, USA) compounds were also dissolved separately in DMF (Dimethylformamide) (at a concentration of 20 g per SANH and SFB per liter of DMF). In the next step, 10 ml of the prepared SFB solution was added to the solution containing Cortistatin peptide (prepared in the previous step) and similarly, the SANH solution was added to the solution containing gold nanoparticles (prepared in the previous step). These solutions are placed at room temperature for 2-3 hours and can be stored at 4°C or frozen. After these steps, the aldehyde group covered the entire peptide and the hydrazine group covered the entire nanoparticle, and then the two could be joined according to the following method. Each of the peptides (containing aldehyde group) and nanoparticles (containing hydrazine group) were placed in citrate buffer (containing 100 mM Sodium-citrate, 150 mM hydrochloric acid) at pH 6, which is suitable for the formation of hydrogen bonds. Both solutions were then mixed. The reaction was carried out for 2 hours at room temperature. Purification and separation of the nanoparticle-bound peptide from the peptide were performed using only a dialysis membrane with a molecular weight cutoff (MWCO) of 3.5 kDa. Finally, a high-speed centrifuge could be used to ensure the complete purification of the peptide-bound nanoparticles [31]. FTIR spectroscopy and Zeta sizer were used to confirm the formation of the peptide-nanoparticle conjugate.

Investigation of the percentage of peptide binding to nanoparticles

The basis of this work was the assay of the unbound free peptide by the Bradford method [32], based on the specific binding of the dye to the protein. The specific dye in this method was Comassi Brilliant Blue G 250 (250 G Comassi Brilliant Blue), which normally does not absorb light at 595 nm, but when attached to the protein, shows strong absorption in the 595 nm region. The binding of the dye to the protein was completed in a short time of about 2 minutes and the resulting complex remains stable in the solution for up to 1 hour.

For the assay, 0.1 ml of the solution containing the unbound peptide (the high-speed centrifuge supernatant of the final nanoparticle-peptide solution described in section 2-1-2) was used. The peptide-nanoparticle solution was centrifuged at 20,000 rpm. The supernatant was then used to determine the amount of unbound peptide. It was poured into a separate tube and mixed with 5 ml

of Kumasi Blue dye solution and its absorbance was immediately read at 595 nm by spectrophotometer. The best time to read the results is 5-30 minutes, and it is better to use a glass cuvette. Before performing the above step, the Standard absorption curve was plotted using different concentrations of peptide and then the concentration of the solution was obtained using the standard curve. If the absorbance read by the device is outside the standard range of the device, the sample is diluted with distilled water.

Determination of peptide release from nanoparticles at different pHs

The inside of the bronchi has a weakly acidic pH (pH of about 6.5) but may change due to other factors involving the respiratory tract [33]. For this purpose, peptide release was investigated at physiological pH 7.4, neutral pH 7, and acidic pH 6.6.

Phosphate buffer was used to adjust the desired pHs, and the pH was adjusted with a pH meter. Three different pH modes were prepared in three separate microtubes. Twelve series of these three microtubes were prepared to examine each (three series of different pH) at intervals of 2, 4, 6 to 24 hours later. 10 ml of the peptide-nanoparticle solution was removed and 200 µl of it was poured separately into each of the 36 microtubes. All microtubes were slowly dilated at 37 °C. At the specified times, 3 microtubes related to different pHs were removed and centrifuged at 20,000 rpm for 15 minutes, and the adsorption of the supernatant at 260 nm was read by a spectrophotometer. Finally, the released peptide concentration was determined according to the standard curve. The release percentage was calculated according to the following formula [34,35]:

(Total peptide/ peptide released) ×100 = peptide release percentage

Results

Covalent bonding created conjugation between gold nanoparticles and Cortistatin peptide by linker molecules. This binding is specific, the conjugate compound is not easily affected by other available biomolecules, and the biological reaction is easier to control. The reaction between the active hydrazine group and the active aldehyde group leads to the formation of a hydrazone bond. The formation of the hydrazone bond was examined by FTIR spectroscopy. The peak located in 1264/11 is related to the formation of hydrazone bonds (C=N) [36-38].

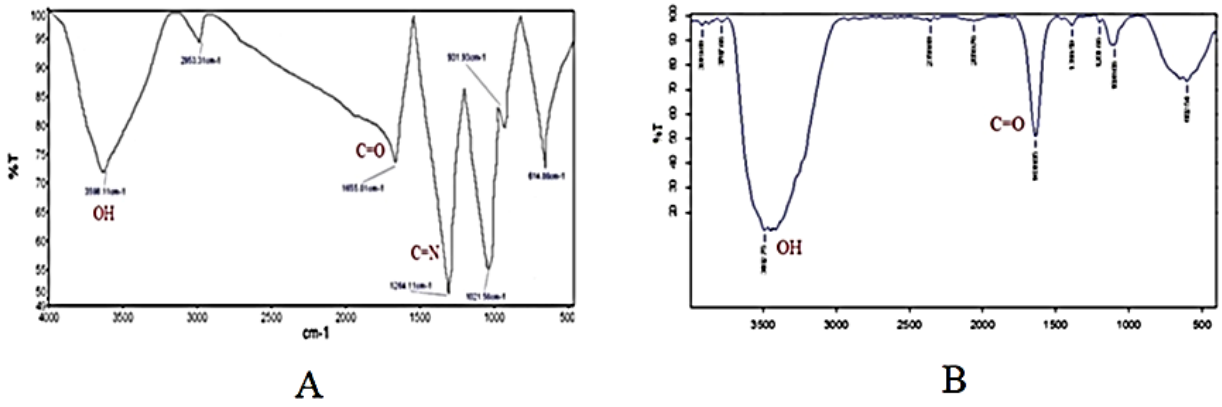
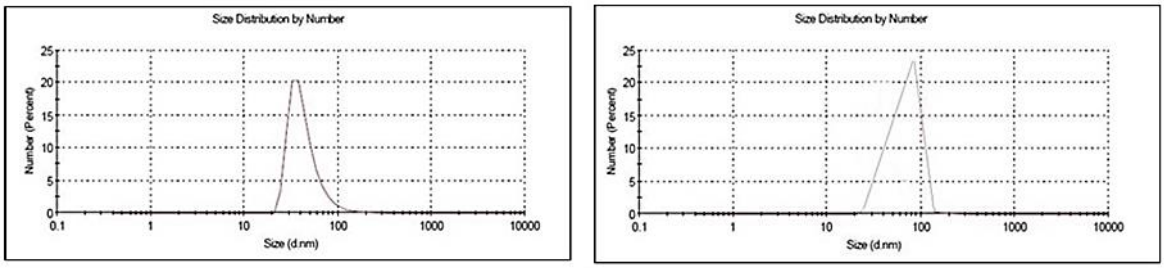


Figure 1: FTIR spectrum of the A) Gold nanoparticles attached to the Cortistatin peptide, B) Gold nanoparticles. The appearance of peak 1264.11 cm-1, which belongs to the C=N group, confirms the formation of hydrazone bonds

In addition, to confirm the binding of gold nanoparticles to the peptide, the average size of the nanoparticles attached to the peptide was measured with a Zeta sizer. After binding of the peptide to the gold nanoparticles, the average size of the nanoparticles increased.



A B

Figure 2: The average size of gold nanoparticles (A). The average size of gold nanoparticles attached to the Cortistatin peptide (B). The increase in mean size in Figure B, confirms the bond formed between the gold nanoparticles and the Cortistatin peptide

The concentration of unbound peptide was measured by the Bradford method and it was found that 80% of the primary peptide was bound to gold nanoparticles.

The release rate of peptide Cortistatin from gold nanoparticles was measured in vitro for 24 hours and every two hours for three different pHs, 6.6, 7 and 7.4. In the early hours, in 6.6 pH release it occurred more rapidly, but over time, within 24 hours, the release rate was the same in all three pHs (Fig. 3)

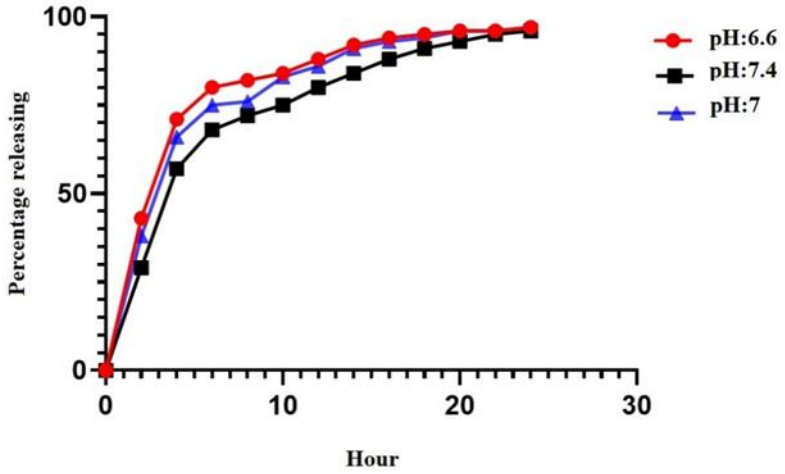


Figure 3: Percentage of Cortistatin released from gold nanoparticles in vitro for 24 hours and every two hours for three different pHs

Discussion

In our study, conjugation of peptides to nanoparticles was performed via linker molecules to create covalent bonds. Such bonds are strong enough and are not easily affected by environmental conditions or the presence of other biomolecules. In addition, the binding efficiency of the peptide to the nanoparticle was acceptable by about 80%. Several methods are used to conjugate nanoparticles into biomolecules. In a 2003 study by Sokolov et al., a molecular biosensor was used to detect cervical cancer cells using conjugation antibodies and gold nanoparticles for Real-time vital imaging. In this work, the desired antibody and gold nanoparticles were incubated in a buffer with 7.4 pH to create conjugation. The formation of a complex between antibodies and the binding between gold nanoparticles and antibodies was done through non-covalent modes. To study the formation of conjugate composition, specific optical changes of gold nanoparticles in the UV-Vis spectrum were followed. A red shift in surface plasmon resonance also indicates conjugation. These optical changes are associated with changes in the local refractive index around the particle after binding to the monoclonal antibody. Another symptom of conjugation is the stability of the conjugated nanoparticles in the saline solution in

a dispersible manner, because the bare gold suspension can be detected by a rapid change in color from red to blue as soon as saline results from the accumulation of nanoparticles [39].

We used FTIR spectroscopy and Zeta sizer to confirm the formation of peptide-nanoparticle conjugate. The addition of peptide to the nanoparticle increased the size of the nanoparticle, which was well visible in the spectroscopy spectrum. The status of the active groups of gold nanoparticles after peptide binding is evident from the comparison of the FTIR spectrum of gold nanoparticles and the FTIR spectrum of nanoparticles attached to the peptide. Peptide to gold nanoparticles occurs through bonding between aldehyde and hydrazine binding groups.

Zhao-Peng Chen *et al*. (2007) used the link between gold nanoparticles and antibodies to build a sensitive immunosensor. The conjugation process in this study occurred through physical interactions including ionic and hydrophobic interactions, resulting from the absorption of negative gold surface charges to positive charges of antibody groups. The UV-Vis absorption curve was used to study the conjugation of gold and antibodies [40]. In 2012, Andre E. James et al., used conjugated gold nanoparticles to monitor nanoparticle-bound biomolecules using Tracking Analysis (NTA) and Dynamic Light Scattering (DLS). For conjugation, the method of incubating nanoparticles with the protein under PI (isoelectric point pH of protein) was used. These nanoparticles-conjugated proteins have been biomarkers used in several emerging diagnostic techniques. Conjugation in this study was performed by self-assembly of proteins on gold nanoparticles through non-specific adsorption due to electrostatic and hydrophobic interactions. Adsorption was affected by many parameters, such as pH, ionic strength, protein PI. The conjugate compound was used as a biomarker in diagnostic processes and NTA and DLS are used to monitor and track it [41].

Vibha Singh *et al***.** (2013) compared the effect of conjugate type, covalent or non-covalent, on the conjugate function of the Gαi1 subunit, from heterotrimeric G-proteins to gold nanoparticles. Covalent conjugation was done through site specific. A peptide bond was formed between the primary N-terminal amine of the protein and the carboxylic acid groups of the gold nanoparticles. Delay of gold nanoparticles electrophoretic mobility in agar gel confirms conjugation. In non-covalent conjugation, gold nanoparticles are coated with DHLA ligand, which creates a negative charge all over the surface. The protein may interact with gold nanoparticles in several directions, or a number of gold nanoparticles may bind to specific protein molecules. Non-covalent binding of the gold nanoparticle -protein complex also delays electrophoretic mobility. Studies show that in non-covalent conjugation, GTPase activity shows up to 0.8-fold decrease in activity, while in covalent conjugation activity increases up to 12-fold. In other words, non-covalent conjugation has an inhibitory effect on GTPase function, while covalent conjugation significantly increases it. It should be noted that conjugation, does not alter the overall structure of the protein. These findings suggest that nanoparticles can actively interact with biomolecules and affect how they work [42].

Sometimes a linker was used for conjugation of metal nanoparticles and biomolecules. One of the advantages of these structures is their being more efficient and stronger bonding of nanoparticles with biomolecules. In a study by Honwei Liao et al., a heterobifunctional cross-linker (LC-SPDP) was used to bind to nanoparticles and then conjugate to antibodies. In this work, gold nano tubes were used to bind to anti-rabbit IgG. Bio conjugates were characterized by independent measurements of the nanorod and antibody concentrations. The LC-SPDP consists of a Pyridildithio group that binds to the surface of the nano tubes and an NHS ester that binds to the primary amine in the antibody. Connections are selective in this type of conjugation [43].

Conclusion

In this study, spherical gold nanoparticles synthesized by citrate reduction were used to conjugate to the Corticating peptide. The binding rate of the peptide to the nanoparticles was 80%, indicating that the binding through the linker molecules was well done. The size of the peptide-nanoparticles composition was adjusted by

controlling the size of the synthesized nanoparticles so that they did not exceed a certain limit because large particles were trapped either in the upper airways and could not enter the airways and bronchi or enter the lungs by the system. Phagocytes were eliminated. Laboratory experiments showed that the peptide-nanoparticle composition was released properly under pH conditions close to lung pH

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Authors' contributions
All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest
There are no conflicts of interest in this study.

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