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Synthesis and Characterization of L-Lysine Conjugated Silver Nanoparticles Smaller Than 10 nm

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Abstract

A rapid and convenient batch method for synthesizing lysine-conjugated silver nanoparticles of approximately 5 nm of size was developed. Nanoparticles of size less than 100 nm exhibit significant medical potential. L-Lysine demonstrates potential for therapeutic applications and silver nanoparticles are an optimal choice for drug delivery because of its intrinsic anti-platelet, anti-bacterial and anti-inflammatory capabilities. Current synthesis protocols for Lysine-capped particles under 10 nm are time consuming and tedious and allow only for the synthesis of small quantities of particles. The synthesis of Lysine-capped silver nanoparticles was based on the reaction in which AgNO₃ was reduced by excess NaBH₄. L-Lysine, a known essential amino acid, served as the capping agent to minimize initial aggregation. The particles were then separated by size chromatography. Capping occurred through the amide bond on L-Lysine as determined by FT-IR. The conjugation of the particle to the amide bond is important, since this leaves the amino group of Lysine open to further modifications. The particles were further characterized in regards to their shape, size and stability. Finally we demonstrated that the synthesized particles exhibit limited to no toxicity in cells, using HEK 293 cell line as a model system. Our synthesis protocol can be successfully used for scale-up and synthesis of high quantities of nanoparticles.

Keywords

Silver; L-Lysine; Nanoparticles; Toxicity; Synthesis

1. INTRODUCTION

The aim of this study was to synthesize and characterize silver nanoparticles of less than 10 nm conjugated to L-Lysine (AgNP) in a rapid and convenient way that can be scaled up easily.

Nanotechnology has found significance with many current fields of industry including engineering, physics, optics, and the emerging subfield of nanomedicine.^{1,2} One of the major

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areas of emphasis in the current field of nanomedicine is the development of improved drug delivery systems.³ Nanoparticles have gained much attention due to their properties, which can be maximally optimized in drug delivery systems. Nanoparticles, foremost, are advantageous for their small particle size allowing entry across multiple biological barriers in the body.⁴

Particle size, is of utmost importance, due to its influence on uptake and drug mobility. In addition, they exhibit large surface to volume ratio allowing maximum binding and adsorption of drug conjugates. In accordance with Mie's theory, there is a strong correlation between increase of surface atoms with decreasing particle size.⁵ Nanoparticles could also be useful in flexible drug administration routes and increased drug retention times⁶ advocates that nanoparticles are optimal in their abilities of carrier capacity and interaction with hydrophobic and hydrophilic substances in treatment for diseases such as tuberculosis (which is known as the disease of the poor). Advantages such as decreased dosage frequency and increased bioavailability that nanomedicine offers could improve treatment in epidemics. Silver nanoparticles are currently employed in a variety of uses from cosmetics to domestic products because of silver's inherent therapeutic properties previously mentioned. These advantageous characteristics make silver nanoparticles a worthy contender for a drug delivery vehicle. Silver and L-Lysine are already implemented in alternative forms of medicine for treating conditions such as canker sores.^{7, 8} Not only does L-Lysine serve as a capping agent for silver nanoparticles but it is also considered advantageous for its biocompatibility for entry and cellular uptake into the nucleus which is well documented in current literature⁹ demonstrates that silver nanoparticles have potential in the area of suppressing metastasis. In addition, a recent study reveals lysine conjugates using phototherapy methods possessed the ability to selectively kill human kidney cancerous cells. L-Lysine is known to have disruptive action in the nitric oxide system found in the brain and may be instrumental in treating schizophrenia.¹⁰ L-Lysine also plays a role in decreasing Ca excretion and Ca absorption thereby making it a possible candidate in the treatment of osteoporosis. L-Lysine has antiviral properties that have been shown to suppress outbreaks of Herpes Simplex Virus (HSV).¹¹ The AgNPs synthesized in this study are approximately 5 nm in size and are conjugated at the amide bond to the silver leaving the amino bond of lysine free for further conjugation. Moreover, the particles showed stability for 2 days at 37 °C and exhibited limited toxicity to liver cells. This makes the particles very valuable for the drug targeting.

2. PARTICLE SYNTHESIS

2.1. Materials

Sephadex G-50 beads (Pharmacia), Silver Nitrate (AgNO_3) was purchased from Fischer Scientific, monohydrate L-Lysine and Sodium Borohydride (NaBH_4) was purchased from Acros Organics.

2.2. Methods

Glassware was cleaned and scrubbed with hot water and dish soap and rinsed thoroughly. Glassware was then rinsed with 75% ethanol and allowed to air dry. 15 ml of 0.002 M of ice

cold NaBH_4 was poured into a 50 ml flask surrounded by ice on a stir plate. 5 ml of 0.001 M AgNO_3 was then slowly dripped into stirring solution. Stirring was immediately stopped after AgNO_3 addition. Stabilization was performed immediately via the addition 250 μl 0.2 M-Lysine was immediately added to the solution initially producing red color and then to a dark yellow brown solution after mixing.

2.2.1. Size Exclusion Chromatography—The gel chromatography column is made with a solution of 10 g of Sephadex G50 powder with 200 ml of deionized water. It is optimal to use the gel solution after a few days in order to allow the gel beads to absorb the water. Gelatinous solution is poured down the column and packed to provide an efficient barrier between the stationary and mobile phases of the column. The silver colloidal solution is mixed briefly and then immediately added to the gel column. AgNP is filtered through the gel column by adding deionized water. 5 ml of deionized water is added to the column after 5 ml of sample is eluted from the column. Elution is clear in color indicating that the filtered silver particles are separated and dispersed in water. After addition of D.I. water, elution is collected up to the 8 ml checkpoint. The following fractions of the 9–12 milliliters that are eluted from the chromatography column are collected in separate 1 ml eppendorf tubes. The fraction samples should be protected from light using aluminum foil protection.

2.2.2. Size Distribution—Samples are measured for average particle size using a DTS nano zetasizer (Malvern Instruments, Worchester, UK) according to the manufacturers protocol.

2.2.3. Fourier Transform Infrared Spectroscopy—FT-IR analysis was performed using a Bruker Optics Vertex 70 FT-IR spectrometer with a Hyperion 2000 microscope attachment used in reflection mode and a 15° Schwarzschild objective. 100 scans were taken and averaged to yield each spectrum at a 4 cm^{-1} resolution. Mid-infrared spectra were collected in specular reflectance mode by drop-casting a 2 μl aliquot of each sample onto 100 nm gold-coated coverslips. OPUS v6.0 was used for spectral acquisition. Essential FTIR software (Opperant, LLC) was used to view each spectrum.

2.2.4. AFM—Atomic force microscopy (AFM) (Bruker Inc., Bioscope II AFM, August-Schanz-Straße nominal spring constant of 0.06–0.08 N/m) was used to measure the height of Lysine capped nanoparticles at 4 °C, room tempture and 37 °C over three days. The height indicated the diameter of the spherical nanoparticle. Measurements were taken once a day for three days after synthesizing Lysine capped nanoparticles. 25 nanoparticles from each sample were randomly selected and measured for size in diameter. Representative pictures of AFM were selected to show the size of the Lysine capped nanoparticles over the three day period.

2.2.5. Cell Culture—*Human Embryonic Kidney* (HEK 293) were grown in Dulbecco's Modified Eagle's Medium (Hy-Clone, Pittsburgh, PA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), 0.5% (v/v) L-Glutamine (Cellgro, Manassas, VA) and 1% (v/v) penicillin/streptomycin (Hy-Clone, Pittsburgh, PA).

2.2.6. MTT [3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide]

Assay—A 12 mM MTT stock solution was prepared by mixing 1 mL of sterile PBS to one 5 mg vial of MTT. HEK 293 were seeded in 96 well plate at density of 1×10^5 . 10 μ L of the 12 mM MTT stock solution were added to each well. As negative control 10 μ L of the MTT stock solution was added to 100 μ L of medium alone. Mixtures were incubated at 37 °C for 4 hours. 100 μ L of DMSO were added to each well and mix thoroughly with the pipette. The plate was incubated at 37 °C for 10 minutes absorbance was read at 570 nm.

2.2.7. Statistical Analysis—Values of the standard deviations (SD) and standard error of the mean (SEM) were calculated from the raw data at the 95% confidence level. SEM was used as error bars. Test for significance between data sets was performed via., 1-way ANOVA.

3. RESULTS

3.1. Synthesis of Lysine Capped Nanoparticles

Lysine capped silver particles were synthesized by reduction of AgNO_3 by NaBH_4 followed by capping with L-Lysine. Particle sizes were separated by size chromatography. Eluted fractions from the column were collected up to fraction 12 and were then analysed by the zeta sizer to find the particle size in each fraction. Fractions 10, 11 and 12 yielded the most narrow distribution of particles less than 10 nm in size. The DTS nano zetasizer shows the hydrophobic radius of the synthesized particles. Figure 1 shows a representative image of the particle distribution in fractions 10, 11 and 12.

3.2. Characterisation of the Particles by FT-IR

One of the three fractions was selected for further characterization by FT-IR spectroscopy. FT-IR analysis of silver nanoparticles with conjugated with L-Lysine is shown in Figure 2(C) or unconjugated L-Lysine is shown in Figure 2(A). As control the AgNO_3 was measured (Fig. 2(B)).

FT-IR analysis of L-Lysine, shows a peak 1570 cm^{-1} originating from the carboxyl double bond, the 1512 cm^{-1} peak is indicative of an NH bending of the NH_2 . 1340 cm^{-1} is the NH_2 vibration. The last peak in this set is indicative of free lysine at 1409 cm^{-1} .¹² Figure 2(B) displays the spectrum of AgNO_3 and shows a very intense absorption band at 1297 cm^{-1} , which is characteristic of the ion pair, Ag^+NO_3^- .¹³ Binding of Lysine to the silver particle leads to a shift of the vibration at 1570 and 1512 to 1646 and 1524 cm^{-1} . While these vibration at 1409 and 1340 cm^{-1} is unchanged. These data suggest that L-Lysine is bound by the amide group to the silver nanoparticle and that the amino group in the Lysine is available for further conjugation. As recently shown by us BH_4 or uncapped Silver particles do not show any significant FT-IR spectra.¹⁴

3.3. Analysis of the Ag-Np Core Size by AFM

The zetasizer measurements showed a narrow particle distribution less than 10 nm. However, this measurement includes the hydrophobic radius of the particles. In order to analyse the size of the AgNP core we next used AFM. Figure 3 shows a representative AFM

image of the particles. From these images particle sizes were measured and the histogram of particle distribution calculated (Fig. 4). As these data reveal the core of the particles is about 4.7 nm. In order to determine the stability of the synthesized particles, the size was measured after incubating the particles for 1, 2 or 3 days at RT, 37 °C or 4 °C. The experiment showed that the particles aggregate only after 3 days at 37 °C.

3.4. Cell Viability

MTT assay was performed using HEK 293 cells human embryonic kidney cell line for day 0, 1, 2. Figure 5 shows images of control untreated HEK cells as well as treated with two concentrations of AgNP. Concentrations were 3.5 μ M and 35 μ M diluted. Cyclohexamide (10 mg/mL) was added to inhibit the proliferation of cells and served as negative control. Images shown in Figure 5 shows healthy cells with little dead cell debris. Next we performed a MTT assay (Fig. 6) to determine if the particles influence cell metabolism. As shown there is little effect of the particles on metabolic activity. Only at the high concentration of 35 μ M for 3 days a slight decrease in metabolic activity at day 3 is observed. This shows the toxicity of the silver nanoparticles is minimal on the kidney to allow for removal from the body.

4. DISCUSSION

AgNPs with a radius of less than 10 nm size were synthesized via a reduction reaction between sodium borohydride and silver nitrate. These particles may possess the characteristics of a potential drug delivery candidate. In addition, the method of silver nanoparticle synthesis is novel since silver nanoparticles were separated by size through the process of column chromatography. This method is not only unusual but it provides consistent size results in which the eleventh fraction sample provides the optimal product. The reliability of this preparation method allowed for increased validity in size stability. We further showed that the particles exhibit limited toxicity, which is of utmost importance as particle size is influential to many aspects of drug delivery including dissolution rate, efficacy, suspension stability, and viscosity. According to the FT-IR analysis, AgNPs were conjugated to the NH₂ amide group of L-Lysine. AFM imaging revealed the experimental sample as spherical particles and also confirmed the sample's identity as silver nanoparticles. This is essential in order to define the nanoparticle's core size characteristic in terms of diameter. Additionally, the particles only showed a slight effect on cell proliferation at 35 μ M and are non-toxic at low concentrations. Our study has demonstrated success in the innovative method of creating L-Lysine silver nanoparticles in an exact size range. *In vivo* experimentation would prove useful in the further analysis of L-Lysine silver nanoparticles and determination of its biocompatibility as a drug delivery candidate.

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Figure 1. Synthesis of L-Lysine silver nanoparticles measured for size on nano ZS Zetasizer. Results show that at room temperature, particle size consistently remains stable and therefore minimal aggregation occurs. Size stability is significant in drug delivery systems for drug retention.

Figure 2.
FTIR of (A) L-Lysine, (B) AgNO₃ and (C) L-Lysine capped AgNPs.

Figure 3. Representative AFM image obtained from the nanoparticles. From the AFM images a histogram of the particle distribution was calculated.

Figure 4.

(A) Size distribution of silver nanoparticles immediately after synthesis for reductions of AgNO_3 and differing concentrations NaBH_4 and L-Cysteine at room temperature. (B) Silver nanoparticles were measured in three different environments (4 degrees, 37 degrees, and 25 degrees room temperature) and synthesis was performed in triplicate. AgNPs were measured for size frequency after 24, 48, and 72 hours. Results show that there is a consistency in the size frequency of AgNPs.

Figure 5.

Morphology of HEK cells at 48 hours for (A) control (B) 3.5 μM (C) 0.35 μM silver nanoparticle treatment. Morphology for silver nanoparticle treated cells are comparable to that of control cells. (D) Addition of cycloheximide as showed reduced the protein levels and slowed cell growth.

Figure 6. AgNp are non toxic in HEK cells. HEK cells were treated with 0.35 and 3.5 μ M AgNPs. As control cells were untreated or treated with Cyclohexamide. At 1 and 3 days MTT assays were preformed to assess metabolic activity.