The *In Vitro* Therapeutic Activity of Ellagic Acid-Alginate-Silver Nanoparticles on Breast Cancer Cells (MCF-7) and Normal Fibroblast Cell (3T3)

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**ABSTRACT**

The present work involves the development of EA-Alg-AgNPs nanocomposite based on ellagic acid (EA) as active compound. Silver nitrate was taken as the metal precursor (AgNPs) and sodium alginate (Alg) as a reducing agent. The EA-Alg-AgNPs nanocomposite was characterized using transmission electron microscopy (TEM), zeta potential, and *in vitro* release kinetics. The particles thus obtained were spherical in shape and having an average particles size of 10 nm, zeta potentials of $-8.2 \text{ mV}$, and the release kinetics of EA from nanocomposite was following Hixson-Crowell kinetics models with $R^2 = 0.9956$. The cytotoxicity potential of free EA, Alg-AgNPs and the EA-Alg-AgNPs nanocomposite may be determined using a normal mouse fibroblast cells (3T3) and breast cancer cells (MCF-7). EA-Alg-AgNPs nanocomposite demonstrated a decreased cytotoxicity effect when compared to free EA on MCF-7 cells with 15.3% cell viability at $128 \mu\text{g/mL}$; compared to 33.5% cell viability in a direct EA exposure. It is worth mentioning the cytotoxicity of Alg-AgNPs against MCF-7 shows 28% viability at $128 \mu\text{g/mL}$.

**KEYWORDS:** Silver Nanoparticles, Ellagic Acid, Sodium Alginate, Sustained Release, 3T3 Cells, MCF-7 Cells.

**1. INTRODUCTION**

Synthesis metal nanoparticles has become one of the most interesting areas of scientific research in recent years, this is due to their applications in various fields such as bio-labelling,¹ catalysis,² and drug delivery.³ Silver nanoparticles is one of the example on the metal nanoparticles have received considerable attention in the photo-catalysts,⁴ catalysts,⁵ antibacterial,⁶ biosensor,⁷ and bioimaging by enhanced Raman scattering.⁸ The unique properties of silver nanoparticles such as large specific surface area, high fraction of surface atoms and slow-release drug carriers leads to enhanced antibacterial activity⁹ and used in drug delivery.¹⁰

The common method for the preparation of AgNPs was called chemical method, this method occurs in presence of reducing and stabilizing agents. The reducing agent include sodium borohydride,¹¹,¹² hydrazine,¹³ and N,N-dimethylformamide.¹⁴ In addition, stabilizing agents include triphenylphosphine,¹⁵ citrate¹⁶ and polyvinylpyrrolidone.¹⁷ Recently, researchers found that some of the reducing agents harmful on the environment as well as have biological risks,¹⁸ and also the stabilizers are toxic and difficult to dispose.¹⁹ With the increasing of biological risks of reducing and stabilizing agents, different research groups develop green method.
for the preparation of nanoparticles. In this method the natural polymers was used as reducing and stabilizing agent. Currently, many natural polymers like chitosan and there derivatives,20 soluble starch,21–23 sodium and calcium alginate.9,23 polyvinyl alcohol,24 and tween 8025 have been used in the green preparation of nanoparticles.

Silver nanoparticles have been extensively investigated to facilitate the intracellular delivery of therapeutics.10 Cui with his group used silver nanoparticles as the antitumor drug (9-aminoacridine-9AA) release materials. The study shows both silver nanoparticles and 9AA drug can inhibit the growth of Hela cells. In addition silver nanoparticles can slow down the antiproliferation effect on Hela cells at low concentration of antitumor drugs.10

Similar to last study, the antitumor drug ellagic acid is chosen as the drug model in this paper. We have incorporated alginate on the surface of silver nanoparticles and then bind the EA with the polymer. The study was also investigating the cytotoxicity of the nanocomposite and show if binding the EA with nanocarriers will increase the biological activity or not.

2. MATERIALS AND METHODS

2.1. Materials

Sodium alginate (molecular weight ~500,000) and hydrated ellagic acid (97% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The AgNO₃ was obtained from Guanghua Sci-Tech Co., Ltd. (Guangzhou, People’s Republic of China). Dimethyl sulfoxide (DMSO) was purchased from Ajax Finechem (Sydney, Australia) with 0.1% water content used as solvent. All reagents were of analytical grade and used without further purification. Deionized water was used to prepare all polymer solutions.

2.2. Preparation of the Alginate-Stabilized AgNPs

1.5 wt% sodium alginate of was dissolved completely in 100 ml of deionized water for an hour. Subsequently, 50 ml of an aqueous silver nitrate (AgNO₃) solution (0.05 M) was dropped into the sodium alginate solution with magnetic stirring. These solutions were heated at 60 °C. A 1.5 mL solution of NaOH (1 M) was added, the suspension immediately turned to dark brown color which indicates the formation of AgNPs. The reaction was continued for 30 Minutes and the obtained suspensions were washed and centrifuged using a high Centrifuge Machine at 18,000 RPM and AgNPs powder was kept for further use. The product was donated as Alg-AgNPs (alginate coated silver nanoparticles).

2.3. Preparation of the EA-Alg-AgNPs Nanocomposite

Typically, the appropriate amount of hydrated EA (0.0013 mol) was dissolved in DMSO followed by 10 minutes stirring and heating for 40 °C. The EA-Alg-AgNPs nanocomposite was prepared by mixing a solution of EA (5 mg/ml) with a known weight of each Alg-AgNPs (40 mg/mL). The solution was magnetically stirred at room temperature for 18 hours to facilitate EA uptake. The products were separated by the use of centrifuge and washed three times using deionized water and were denoted as EA-Alg-AgNPs (loaded ellagic acid on the alginate coated silver nanoparticles).

2.4. Ellagic Acid Quantities Loaded and Released from EA-Alg-AgNPs Nanocomposite

Ellagic acid release profiles from the nanocomposite were determined at room temperature using a phosphate buffered saline solution (PBS) at a concentration 0.01 mol/L at pH 7.4. About 85 mg of the nanocomposite was added to 500 mL of the PBS media. The cumulative amount of ellagic acid released into the solution was measured at preset time intervals at λₘ₉₅ = 258.4 nm using a Shimadzu UV-1601 spectrophotometer at Isra University.

To compare the release rate of ellagic acid from the EA-Alg-AgNPs nanocomposite, with the physical mixture which contained ellagic acid with Alg-AgNP, 3.0 mg of the physical mixture was obtained by mixing 0.65 mg of ellagic acid with 2.4 mg of Alg-AgNP to compare the release from EA-Alg-AgNPs nanocomposites. The release of the active, ellagic acid was determined as described above.

2.5. Cell Culturing and MTT Cytotoxicity Assays

Normal mouse fibroblast cells (3T3) and breast cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained and cultured in DMEM medium with 10% fetal bovine serum (FBS), 15 mmol L⁻¹ L-glutamine, 100 units ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin to maintain cells at 37 °C and 5% CO₂ in a humidified incubator. Media were changed every two days and at 90%, confluent cells were seeded into a 96-well plate at 1 x 10⁵ cells ml⁻¹ and kept overnight for cell attachment. The old media was discarded and 100 μL of the new medium containing pure EA, Alg-AgNP, and EA-Alg-AgNPs was used to treat the cells, while wells containing media and cells were only used as controls. For each experiment, freshly prepared stock solutions of EA, Alg-AgNP, and EA-Alg-AgNPs were used for the treatment. A stock of 10 mg ml⁻¹ (in PBS) was made. Using DMEM, the desired concentrations of the media needed for the treatment were made through serial dilution (0–128 μg ml⁻¹). The MTT assay was used 24 hours post-exposure to determine the toxic effect of these agents.

In the MTT assay, the treatment media were discarded after 24 hours and MTT-containing media were added at 5 mg ml⁻¹ PBS and a volume of 100 μL per well, and the
plates were incubated at 37 °C in a 5% CO₂ humidified incubator. Two hours later, a detergent reagent (DMSO) was then added to the cells to stop the conversion and solubilize the formazan. The amount of formazan correlates directly with the number of viable cells after treatment. Absorbance of the formazan that is formed is taken at a wavelength of 570 nm using a multiwall microplate reader. The experiment was performed in triplicate, and the result was expressed as the mean ± SD.

\[
\text{cell viability (\%) = \frac{\text{Average of treated/average control}}{\text{average control}}} \times 100\%
\]

2.6. Instrumentation

Powder X-ray diffraction patterns were used to determine the crystal structure of the AgNPs in the range of 30–85 degrees on an XRD-6000 diffractometer (Shimadzu, Tokyo, Japan) using CuKα radiation (λ = 1.5406 Å) at 30 kV and 30 mA. Fourier transforms infrared spectroscopy (FTIR) spectra of the ellagic acid, Alg-AgNP and EA-Alg-AgNPs materials were recorded over the range of 400–4000 cm⁻¹ on a Thermo Nicolet Nexus, Smart Orbit spectrometer using the KBr disc method. Thermogravimetric analysis was carried out using a Mettler Toledo 851e instrument (Switzerland) with a heating rate of 10 °C/min, in 150 μL alumina crucibles and in the range of 30 °C–900 °C. Scanning Electron Microscopy was used to observe the surface morphology of the samples using a NOVA™ Nano SEM 230 (FEI, Hillsboro, OR) scanning electron microscope. The zeta potential was measured at room temperature by dynamic light scattering (DLS), using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The mean particle size of the samples was obtained using a transmission electron microscope (Hitachi H-7100, Tokyo, Japan) at an accelerating voltage of 80 and 200 kV. UV-Vis spectra were measured to determine the controlled release study, using an ultraviolet-visible spectrophotometer Shimadzu UV-1601 at Isra University.

3. RESULTS AND DISCUSSION

3.1. X-ray Diffraction (XRD)

Figure 1(A and B) shows the XRD patterns of Alg-AgNPs and EA-Alg-AgNPs nanocomposite, respectively. The XRD peaks for Alg-AgNPs and EA-Alg-AgNPs nanocomposite in the 30–85 degrees angle range ascertained that the peaks in 38.28°, 44.34°, 64.64°, 77.51° and 81.88° can be attributed to the 111, 200, 220, 311 and 222 planes of silver nano-crystalline, respectively. These peaks can be readily indexed to a face-centered cubic structure of silver (XRD for Ag with Ref. No. 00-087-0719). Two peaks was observed at 31.67° and 45.14° in Figure 1, indicates presence of Ag₂O as impurities. However, the diffraction peaks of EA-Alg-AgNPs nanocomposite are broader and lower intensity comparing with Alg-AgNPs.

The theoretical value of lattice constant “a” for face cubic center of silver can be calculate using Eq. (1)

\[
a = \frac{4}{\sqrt{2}} \times r
\]

Where r for silver was 144 pm. By using the above equation, the lattice constant “a” was 4.073 Å. The experimental lattice constant ‘a’ was calculated from the most intense peak (111) of the XRD pattern is 4.078 Å. Both theoretical and experimental lattice constant ‘a’ are in very well agreement.

The average particle size (L) of silver nanoparticles can be calculated using Debye-Scherrer equation:

\[
L = \frac{0.94 \lambda}{\beta \cos \theta}
\]

Where 0.94 value is the Scherrer constant, λ is the X-ray wavelength (1.5418 Å), β is broadening of diffraction line measured at half of its maximum intensity (in radian), and θ is Bragg diffraction angle (in degree). From the Scherrer equation, the average particle size of Alg-AgNPs and EA-Alg-AgNPs was 8.4 and 16.2 nm, respectively.

3.2. Infrared Spectroscopy (FTIR)

Figure 2(A–C) shows the FTIR spectra of Alg-AgNPs, EA-Alg-AgNPs nanocomposite, respectively. From the literature, sodium alginate given bands at 1609 and 1326 cm⁻¹, this is due to asymmetry and symmetry stretching COO⁻. The absorption band at 1083 cm⁻¹ is corresponded to C–O–C stretching mode from the glucosidic units. In the case of Alg-AgNPs (Fig. 2(A)), the bands of asymmetry and symmetry COO⁻ was shifted to
1598 and 1326 cm$^{-1}$, respectively. The peak of 1083 cm$^{-1}$ shift to 1081 cm$^{-1}$ indicating that carboxyl groups are involved in the synthesis and stabilization of AgNPs. The appearance of carbonyl functionality after formation of AgNPs from reaction of sodium alginate with silver nitrate can be clearly visualized by the appearance of a new band at 1789 cm$^{-1}$ in the FTIR spectrum of Alg-AgNPs (Fig. 2(A)). This indicates the presence of a six-membered cyclic ketone.
The FTIR spectrum of EA at Figure 2(B) shows an absorption band at 3557 cm$^{-1}$, attributed to the stretching mode of OH groups in the phenol. A broad absorption bands between 3500–2750 cm$^{-1}$ due to stretching of C–H aromatic ring and hydrogen bond between EA molecules. A band at 1700 cm$^{-1}$ can be attributed to stretching of C=O. Bands at 1620–1511 cm$^{-1}$ are due to aromatic rings. A band at 1196 and 1058 cm$^{-1}$ is due to ester linkage of C–O stretching and another band at 758 cm$^{-1}$ is due to C–H aromatic stretching.

FTIR spectrum of EA-Alg-AgNPs nanocomposite (Fig. 2(C)) shows characteristic bands of pure EA together with other band of Alg-AgNPs. This indicates that the EA has been loaded on the surface of Alg-AgNPs. A band for C=O slightly shifted in position to 1695 whereas the C–O ester linkage shifted to 1193 and 1050 cm$^{-1}$ and the C–H aromatic rings bands was shifted to 750 cm$^{-1}$.

In the producer of AgNPs, sodium alginate molecules played the roles of both reducing and stabilizing agents. When silver nitrate was mixed with sodium alginate solution, silver ions could be bound to alginate molecules throw ion–dipole interactions. The dipole was due to the electron-rich oxygen atoms of polar hydroxyl groups of alginate. The development of carbonyl functional group after reaction of sodium alginate with silver nitrate indicates the presence of a six-membered cyclic ketone.

This formation process takes by oxidation of secondary OH groups present in alginate skeleton. At same time, the silver ions undergo reduction to form AgNPs. Therefore, the electron-rich oxygen atoms in the hydroxyl and ether groups of alginate bind tightly with metal clusters and nanoparticles via electrostatic interactions as shown in Figure 3. The EA molecules as shown in Figure 3 was bind with alginate molecules throw hydrogen bonds.

### 3.3. Determination of the Zeta Potential and Size Distribution Properties

Figures 4(A–C) shows the zeta potential measurement of the Alg-AgNPs, EA and EA-Alg-AgNPs nanocomposite dissolved in deionized water, respectively. The zeta potential for Alg-AgNPs, EA and EA-Alg-AgNPs were $-24.0$, $-15.7$ and $-8.2$ mV, respectively. The negative zeta potential of Alg-AgNPs suggest that the surface charge of the nanoparticles was dominated by the adsorption layer of alginate, which preventing the Ag nanoparticles from aggregation through electrostatic repulsion. This result was very clear at Figure 5(A). Loaded EA on the surface of Alg-AgNPs decreased the negative zeta potential from $-24.0$ mV to $-8.2$ mV, therefore the EA-Alg-AgNPs nanocomposite have more aggregation comparing with Alg-AgNPs.

![Fig. 5. TEM images of Alg-AgNPs (A), and EA-Alg-AgNPs (B).](image-url)
Figures 5(A and B) shows the TEM morphologies of Alg-AgNPs, and EA-Alg-AgNPs nanocomposite, respectively. The average size of the particles is 10 nm for both samples.

3.4. SEM Analysis
Figures 6(A and B) show the SEM images of the Alg-AgNPs and EA-Alg-AgNPs nanocomposite at 10,000× magnification, respectively. Figure 6(A) shows sheets morphology, where the morphology of the nanocomposite was very different than the Alg-AgNPs. The SEM images for EA-Alg-AgNPs given rod shaped (Fig. 6(B)).

3.5. Thermogravimetric Analysis (TGA)
The thermogravimetric analysis thermograms (TGA) of free EA, Alg-AgNPs and EA-Alg-AgNPs nanocomposite were shown at Figure 7. From the literature, the TGA of sodium alginate shows two steps of weight loss. The first weight loss occurred in the temperature range of 106–190 °C due to loss of volatile products like dehydration. The second-stage of weight loss occurs in the range of 219–261 °C, which attributed to the decrosslinking of polymer networks, formation of a carbonaceous residue, and finally yields Na₂CO₃ as char. TGA curve of Alg-AgNPs was given in Figure 7. For the peak below 100 °C, this is concomitant by 10% weight loss due to desorption of moisture between Ag clusters and alginate bilayer. The dominant weight loss of the sample occurred in temperature region between 190 and 500 °C. It is generally attributed to the evaporation of water and alginate outer layer component. The third step of 16.6% weight-loss in above 500 °C attributed to alginate inner monolayer decomposition.

TGA thermogravimetric analysis obtained for EA shows three weight losses. The first step weight loss can be attributed to the removal of bonded water with hydrogen bond at temperature maxima of 112 °C (8.2%). The second and third of weight losses of EA occurs at 463 °C (39%) and 596 °C (17.5%).

As shown by the TGA curve for the EA-Alg-AgNP nanocomposite (Fig. 7), the mass loss below 100 °C
was low (2.1%) due to the removal of the absorbed physical and chemical water. The nanocomposite began to degrade at approximately 100 °C, and final decomposition occurred at approximately 900 °C, 79% total mass loss. The mass loss for the Alg-AgNP was lower than for the EA-Alg-AgNP nanocomposite; this result confirms that the EA was loaded onto the Alg-AgNPs surface.

3.6. In Vitro Study of Ellagic Acid Release from the EA-Alg-AgNP Nanocomposite

The loading percentage of EA-Alg-AgNP nanocomposite was analyzed using a UV-Vis spectrophotometer and the data were calculated using calibration curve. The data show that the EA-Alg-AgNP nanocomposite is composed of 21%. The sustained release of EA from the nanocomposite into the release media was performed in 0.01 mol/L

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**Fig. 9.** Data fitting for ellagic acid release from EA-Alg-AgNP nanocomposite using five different kinetic models at pH 7.4.
Table I. The correlation coefficients ($R^2$) obtained by fitting the ellagic acid anion release data from the EA-Alg-AgNP nanocomposite in PBS solutions at pH 7.4.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Saturation release (%)</th>
<th>Pseudo-first order</th>
<th>Pseudo-second order</th>
<th>Higuchi model</th>
<th>Hixson-Crowell model</th>
<th>Korsmeyer-Peppas model</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA-Alg-AgNP</td>
<td>100</td>
<td>0.9600</td>
<td>0.9847</td>
<td>0.9296</td>
<td>0.9956</td>
<td>0.9767</td>
</tr>
</tbody>
</table>

concentration pH 7.4 of sodium saline solution. The affinity of anions in the aqueous saline solution is higher than the EA towards the nanocomposite; therefore, EA anions can be ion-exchanged with the anions in saline solution. Generally, the release rate is initially rapid, and then slows until it reaches equilibrium. Maximum release was achieved at about 300 min. The physical mixture of EA and Alg-AgNPs exposed to pH 7.4 environments showed the release of EA very quickly, within 14 minutes (inset for Fig. 8). The release rate of EA from the nanocomposite was slower than from the physical mixture, indicating that the nanocomposite has the potential to be used for the controlled release of antimicrobial agents.

3.7. Release Kinetics of Ellagic Acid Anions from the EA-Alg-AgNP Nanocomposite

The data of the cumulative release of the EA from nanocomposite was fitted to five kinetic models which generally are described as follows:

1. The pseudo-first-order kinetic model represented in its linear form as Eq. (3)

$$\ln(q_e - q_t) = \ln q_e - k_1 t$$

in which $q_e$ and $q_t$ are the quantity released at equilibrium and the quantity released at any time ($t$), respectively, and $k_1$ is the rate constant of the pseudo-first-order release kinetics.

2. The pseudo-second-order kinetic equation may be represented in its linear form as Eq. (4)

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2 + \frac{q_t}{q_e}}$$

in which $k_2$ is the rate constant of the pseudo-second-order release kinetics.

3. The Higuchi model describes the increased release of the drug from the nanocomposites with the increasing the square root of time

$$q_t = k_H \sqrt{t}$$

in which $k_H$ is the Higuchi rate constant.

4. The Hixson-Crowell model gives the relationship between the cube root of the percentage of drug remaining in the nanocomposites as a function of time

$$\sqrt[3]{M_0 - \sqrt[3]{q_t}} = Kt$$

in which $M_0$ is the initial quantity of drug in the nanocomposite and $q_t$ is the quantity released at time $t$.

5. The Korsmeyer-Peppas model gives the relationship between the log of percentage of drug released and the log of time

$$\frac{q_t}{q_\infty} = Kt^n$$

in which $q_\infty$ is the release at infinite time.

Among the models used in this work, the correlation coefficient ($R^2$) for the Hixson-Crowell kinetic model gave the best fit with value 0.9956, as shown in Table I.

Fig. 10. The cytotoxicity profiles for the EA, Alg-AgNP and EA-Alg-AgNPs after incubation with 3T3 (A), and MCF-7 (B) cells.
3.8. Cytotoxicity Studies

The cytotoxicity of free EA, Alg-AgNP and EA-Alg-AgNPs nanocomposites on 3T3 and MCF-7 cells was shown at Figure 10. In this study, coated AgNPs with alginate (Alg-AgNPs) did not show enough cytotoxicity against 3T3, this result was reported from the literature [41]. The highest concentration of Alg-AgNPs (128 μg mL⁻¹) showed 81.5% viability. Whereas free EA and nanocomposite given 49.5 and 45.3% viability, respectively (Fig. 10(A)).⁴²

The MCF-7 cells were highly sensitive to free EA and EA-Alg-AgNPs nanocomposite compared to 3T3. A deeper look at Figure 10(b) makes it clear that the EA-Alg-AgNPs nanocomposite have a higher tumor suppression efficiency compared to EA itself (IC₅₀ values for EA and nanocomposite have a higher tumor suppression efficiency compared to EA itself (IC₅₀ values for EA and EA-Alg-AgNPs were 21.4 and 10.5 μg mL⁻¹, respectively). The cell viability of the free EA at 128 μg mL⁻¹ shows 33.5%. However, the EA-Alg-AgNPs nanocomposite shows a rapid decrease in cell viability as the concentration increases. The maximum suppression effect of EA-Alg-AgNPs nanocomposite was observed at the concentration of 128 μg mL⁻¹ with 15.3% viability.

4. CONCLUSION

The EA-Alg-AgNPs nanocomposite was prepared by loading Ellagic acid on the surface of Alg-AgNPs. The release of the drug Ellagic acid from nanocomposite was followed Hixon-Crowell kinetic model. The EA and EA-Alg-AgNPs nanocomposite exhibit MCF-7 cells in a dose-dependent manner with IC₅₀ value 21.4 and 10.5 μg mL⁻¹, respectively.

Declaration of Conflicting Interests

The authors report no conflicts of interest in this work.

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References and Notes