Preparation and cytotoxic activity of resveratrol-gold nanoparticles conjugated to folic acid against MCF-7 cell line

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ABSTRACT: The potential benefits of resveratrol (Trans-3, 5, 4’-trihydroxystilbene) as an anti-cancer are lowered because of its low aqueous solubility, instability and low bioavailability. Recent progresses in nanoparticle technology offer a promising loophole to solve many problems in developing therapeutic agents. The ability of a nanocarrier to selectively bring drug substances to the site of a tumor solely based on passive targeting can be enhanced by coupling a ligand to the surface of the nanocarrier knowingly as active targeting. This research aimed to test the cytotoxic activity of resveratrol conjugated to gold nanoparticles-folate as a carrier. Gold nanoparticles (AuNp) was chosen as a suitable carriers to increase the potency of resveratrol (Rsv) as an anti cancer agent with the help of folic acid (FA). The expression of folate receptors are heightened due to the excessive need of folic acid by certain types of cancer cells. Thus folic acid can act as a targeting agent to help introduce gold nanoparticles to the target cells. Furthermore, the cytotoxic activity of the final conjugate (FA-AuNp-Rsv) will be tested against human breast cancer MCF-7 cell line using MTT chlorimetric test. Gold nanoparticles were fabricated using optimized amount of polyvinyl alcohol (PVA) as a stabilizers. FA were attached to the surface of gold nanoparticles using 4-Aminothiophenol (4-Atp) as a linker. The carboxilic group of FA was first activated using common EDC-NHS reaction and reacted with amine group of 4-Atp to form amide bond. The physical and chemical characterization has been conducted and it was found that the final 86.9 nm nanon conjugate (FA-AuNp-Rsv) were successfully synthesized with good stability and possessed an elevated activity (IC_{50}=21.28 ± 1.04 μM) compared to resveratrol alone (49.94 ± 1.06 μM). Thus this results were hoped to be an adequate addition to the development of nanotechnology in cancer therapy.

KEYWORDS: Cytotoxic activity; gold nanoparticles; resveratrol; MTT method; MCF-7 cells.

1. INTRODUCTION

Breast cancer is the second in terms of the highest cause of deaths which is responsible for about 198,000 deaths a year in a developed country or about 15.4 % deaths around the world. In the United States, 12% or 1 out of 8 women suffers from breast cancer [1]. These facts motivate researches around the world to discover not only pharmacologically active substances but also the right technology to obtain an advanced and better treatment for breast cancer.

Beside the use of synthetic drugs which are mostly happen to possess many side effects especially to the normal tissue, natural substances also have been found out to be beneficial and considered save. Resveratrol or trans-3,5,4’-trihydroxystilbene, a polyphenol derived from stilbene has been proved over decades to have many potentials such as anti-oxidants, anticarcinogenic, antiobesity and also protection against heart and brain diseases [2]. An earlier report proposed that caspase activation and apoptotic induction are resveratrol’s main mechanism that responsible for inhibiting the progression of cancer cells [3]. Despite of its many benefits, many evidence have also risen regarding it’s instability, poor solubility and low bioavailability as a result of rapid metabolism [4]. Nanoparticle based targeting system usually chosen to overcome these problems.

Systemic delivery of anticancer substances as a conventional therapy aimed to increase the amount of therapeutics substance into the site of action which nanocarrier system usually served in this area through its ability to accumulate at tumor site. This term is known widely as enhanced permeability and retention (EPR). Gold nanoparticles (AuNp) is famous as a drug carrier for many cancer drugs through it’s fine nature such as
non-toxic, inert and easily accumulate in the tumor site. Its tunable properties which is usually correspond with the physical attributes can be achieved through different synthesis methods [5]. Gold nanoparticles could be prepared by several different methods. These methods commonly divided in to two categories, the top-down method and bottom’s up method. Upon the fabrication of gold nanoparticles, a stabilizing agent sometimes needed to prevent the particles to aggregate and form a larger particles. One of the most popular chemical method is the one that introduced by Brust et al where gold salts reduced by NaBH₄ to form gold nanoparticles around 2-5 nm [6]. Many drugs and substances can also easily attached to the surface of gold nanoparticles (mostly through amine and thiol groups)[7].

A delivery and targeting system solely based on EPR effect simply can not guarantee that maximum amount of drugs can reach its pharmacological target. Without the help of a specific affinity, nanocarriers would risk to be redistributed in to vascular tissue [8]. While unspecific distribution of drugs can lead to increase of toxicity to the normal cells, ligands such as antibody or fragment, peptide and vitamins could work as targeting agents to help introduce drug substance into specific target location [9]. Folic acid receptors expression are high in over up to 40% of cancer cells type including breast cancer while that of normal cells are considered low [10]. This situation is due to the excessive need for folic acid by these type of cancer. This research aimed to elevate the potency of resveratrol as an anti cancer agent. In this research folic acid (FA) were attached to the surface of gold nanoparticles (AuNp) bearing resveratrol (Rsv) as the active agent. To examine the cytotoxic activity, we have tested the conjugate result (FA-AuNp-Rsv) against breast cancer MCF-7 cells and compared the result to free reveratrol (Rsv). This research was only comparing the potency of resveratrol in conjugated form and the free form in a certain range of concentration. In this case, the cytotoxic activity of bare vehicle (AuNp) whether exist or not was not our strong discussion. FA was attached via amide bond link with 4 Aminothiophenol (4Atp) which act as linker because of it’s good thiols (S-H) affinity with AuNp surface. To our knowledge, this was the first attempt to conjugate resveratrol with gold nanoparticles bearing folate residues and see whether there was an elevated potency. As a complement in this research, in the beginning of the step, effect of different concentration of stabilizers were also be studied to optimize the stabilization of AuNp formation.

2. RESULTS AND DISCUSSION

2.1. Characterization

2.1.1. Visual observation and UV-vis spectroscopic study

Gold nanoparticles (AuNp) formation was fabricated by reducing HauCl₄ solution with NaBH₄ followed by continues stirring for 30 minutes. In the beginning of process, a three varied AuNp samples prepared by the same recipe but different amount of PVA to study how the amount of stabilizers affect particle size and distribution of the synthesized gold nanoparticles. The amount of PVA added in appropriate quantities so that the final concentration of gold was about 50%, 10% and 5% of PVA or less.

PVA is well known as a good stabilizer for metal nanoparticles based on research [11,12]. The reduction of Au⁺ ion was visually confirmed as the reaction mixture turned red ruby within a few minutes. Particle size usually correspond with maximum wavelength (λ_max) observed in UV-Vis spectroscopy while full width half maxima (FWHM) related to size distribution. From the Table 1, it can be seen that λ_max decreased with the increasing amount of PVA while maximum wavelength (λ_max) usually correspond with particle size. This suggested that AuNp will be stabilized properly at concentration 5% or less of PVA concentration. It is also clear that a lower PVA amount will result in broader SPR peak indicating a formation of nanoparticles with broader size distribution.

The amount of optimized PVA before will then be chosen to fabricate AuNp for further steps. As it was mentioned before that conjugating folic acid with AuNp’s surface required two steps. The first step is to conjugate AuNp’s surface with 4Atp as linker. In this conjugation, 4Atp was attached to AuNp’s surface through it’s sulfur atom. Several studies have successfully fabricated gold nanoparticles capped with thiolate molecules and indirectly confirmed the strong affinity of thiol groups against gold surface[13-15]. This conjugation was confirmed visually by the change in colour of the mixed solution from ruby red to deep purple blue as it was seen in Figure 1. SPR characteristic band of a gold nanoparticles linked with various molecules usually exhibit peak around 520-600 nm which is depend on particle size. Since interlinked molecules will also increase the size of a particle, a significance red shift on the SPR band will also be spotted if the attachment is success. Furthermore, an observation of SPR characteristic band change also be observed through the whole conjugation process. It was noticed that there was a significance different between AuNp
before and after conjugation with FA and Rsv where the SPR band shifted from 522 nm for AuNp to 547 nm for FA-AuNp-Rsv conjugate. This result indicating an increase in particle size and also will be confirmed in the next investigation. Subsequently after the final conjugate was obtained, standard stability tests were performed using different medium and condition (pH 7.4; 0.9% NaCl; bovine serum albumin; 1% cysteine). The final conjugate was stable in a long period of time (data not shown) and stored for next investigation.

2.1.2. Fourier transform infra red analysis

The surface modification of AuNp has also been confirmed using FT-IR instruments. Pure PVA showed a characteristic bandwidth of O-H stretching region at 3387 cm\(^{-1}\) while the absorption band was shifted at 3371 cm\(^{-1}\) for AuNp stabilized with PVA. This observed hydroxyl vibrational band in AuNp and the other shifted band confirmed the presence of PVA coating on AuNp’s surface. 4Atp molecule posses a specific absorption band at 2548 cm\(^{-1}\) due to the presence of thiol (S-H) group which was disappeared after conjugation with AuNp’s surface while the other absorption band were either shifted or disappear as well. This result confirmed the conjugation of 4Atp to AuNp has taken place via thiol group. Furthermore, as it was mentioned before that FA and AuNp conjugation take place by forming amida bond between amine group from 4Atp with carboxylic group from FA. Therefore it can be confirmed from the peak with several absorption bands at 1647 and 1678 cm\(^{-1}\) (C=O amide bond) and also broad bands on ranged from 3700 to 3000 cm\(^{-1}\) (N-H vibration of amide bond). This result confirms the formation of amide bond was occurred along with the conjugation of folic acid in AuNp’s surface. On the final step as it can be observed in Figure 2, a characteristic band of pure Rsv at 3306 cm\(^{-1}\) (phenolic O-H groups stretch), 1384 cm\(^{-1}\) (C-O stretching), 1585 cm\(^{-1}\) (C-C olefinic stretching) and around 1600 cm\(^{-1}\) (C-C aromatic double bond stretching). Those specific absorption bands were remain present in the final conjugate with a slight shift indicating the formation of resveratrol conjugation in the final FA-AuNp-Rsv conjugate. These result also correspond with findings on other research [16].

2.1.3. Size, distribution and zeta potential measurements

Particle size of the AuNp, PVA stabilized AuNp and final conjugate FA-AuNp-Rsv was measured by dynamic light scattering (DLS) method and it was found out to be 1.8 nm, 76.0 nm and 86.9 nm respectively. These results was reasonable considering an increase in particle size is due to attachment of several molecules on the surface. In the beginning of the step, it was also noted that AuNp produced without the addition of PVA result in polydispersity index (PDI) value at 0.481 indicating a more polydispersed particles, while that of stabilized with PVA result in almost uniform size with PDI at 0.178. This result was indicating that the use of PVA could lower the degree of polydispersity in the fabrication of gold nanoparticles.

Zeta potential of FA-AuNp-Rsv conjugate was found to be -29.0 mV and it serves as a good proof of nanoparticle’s stability in aqueous medium. This value indicates that the conjugate were stable due to sufficient and acceptable electrostatic repulsion. These results were later also supported by stability testing of the conjugate in different media. Zeta potential is also one of the important value to determine the stability of metal nanoparticles. The surface charge around the nanoparticles could suspend the repulsion between particles and thus avoid aggregation, and so far zeta potential around -30 mV is considered suitable to maintain stability [17].

| Table 1. Absorption, maximum wavelength and FWHM value of AuNp stabilized with different amount of PVA. |
|---|---|---|---|
| Formulation code | PVA (mg) | \(\lambda_{\text{max}}\) | (Å) | FWHM |
| F1 | 25 | 526.74 | 0.772 | 156.78 |
| F2 | 12.5 | 527.99 | 0.705 | 173.71 |
| F3 | 2.5 | 527.07 | 0.599 | 614.22 |
| F4 | 0 | 528.90 | 0.779 | 207.2 |

Figure 1. Colour changed from PVA stabilized AuNp (a) and 4Atp-AuNp conjugate (b).
2.2. Entrapment efficiency

The amount of Rsv in FA-AuNp-Rsv conjugate were measured indirectly by measuring the free Rsv present in the filtrate of nanoconjugate using HPLC method. Standard curve of free Rsv obtained were $y = 120854 x + 113973$, ($R = 0.9987$). Furthermore by measuring the sample triplicate an average concentration of 0.0397 mg/ml (39.78 ppm) were measured. Thus this result revealed that about $79.57 \pm 0.18\%$ of resveratrol was conjugated to gold nanoparticles conjugate. This result is higher from other experiment by kumar et al in which about 20% of resveratrol was successfully conjugated with gold nanoparticles [17].

2.3. Cytotoxicity Test

In this research, we were trying to increase the effectiveness of resveratrol as an anti cancer agent against breast cancer by the use of AuNp with folate residues as a homing device. MCF-7 cell line has been used as a breast cancer model cell to study cytotoxic effect of various compounds. Joshi et al studied the enhancement of chloroquine conjugated to gold nanoparticles (GNP-Chl) against MCF-7 cell line to portray it’s efficacy against breast cancers [13]. The presence of folate receptors on MCF-7 cell line makes it a perfect candidate in this study since our carrier use folic acid as an introducing agent.
Figure 4. (a) Concentration vs % inhibition curve of FA-AuNp-Rsv and Rsv, (b) IC50 comparison between FA-AuNp-Rsv and Rsv.

In order to test the enhancement of resveratrol potency in conjugate, we compare the effect of various concentration FA-AuNp-Rsv conjugate and free Rsv on MCF-7 cell line using MTT assay. Cells were treated with various concentrations (5, 10, 20, 40, 80 and 160 µM) of resveratrol in FA-AuNp-Rsv conjugate and compared with that of bare resveratrol and incubated for 24 hours. The cells response were determined as a percent of inhibition calculated using the MTT assay. As it was shown in Figure 4(a), the inhibition of FA-AuNp-Rsv conjugate by every dose was higher than that of free Rsv. The conjugate also gave inhibition nearly twice higher than bare resveratrol in every concentration. This result is reasonable and can be explained in two ways. The first is that metal nanoparticles has been proved to possess a cytotoxic activity thus can act synergistically with anti-cancer agents [18,19]. Secondly folic acid on FA-AuNp-Rsv conjugate can act as a helper that increase the amount of conjugate presence on the MCF-7 cells surface through binding with folate receptors and by that maximize the drugs that enter the cells.

Furthermore, the IC50 value then calculated from linear curve of log concentration vs Normalized inhibition (%) using Graphpad Prism 5 software. It was found that FA-AuNp-Rsv gave lower value (21.28 ± 1.04 µM) than Rsv (49.94 ± 1.06 µM). Furthermore, two-way anova analysis showed that there were significant differences between each inhibition result for all concentration samples (P< 0.05). There were also statistical differences between FA-AuNp-Rsv and Rsv group (P< 0.05). Thus we can conclude from our data that resveratrol's potency has been enhanced using gold nanoparticles bearing folate residues as a carrier. In earlier study by Kim et al, treatment of resveratrol gave an induced condensation and nuclear fragmentation on the observed MCF-7 cells and suggested that apoptosis induction as the main mechanism of inhibitory activity [20].

3. CONCLUSION

Resveratrol conjugated to gold nanoparticles decorated with folate has been successfully synthesized and tested for their cytotoxicity activity in this study. The further characterization data showed that the conjugation was successful, well dispersed and stable. Also the potency of resveratrol in the final nanoconjugate was higher than that of resveratrol alone. It was concluded from this research that the conjugation resveratrol to gold nanoparticles decorated with folic acid could elevate the effectiveness of resveratrol as an alternative to cancer therapy. These results could be a valuable informations for another enhancement of low bioavailability compounds.

4. MATERIALS AND METHODS

4.1. Materials

Chloroauric acid (HAuCl4) solution, sodium borohydride (NaBH4), polyvinyl alcohol/PVA (Mw 67000), 4-Aminothiophenol (4ATP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC),
N-hydroxysuccinimide (NHS), folic acid and resveratrol were purchased from Sigma–Aldrich, USA. Ethanol, deionised water, acetonitril, and all other reagents and solvents were of analytical grade.

4.2. Synthesis of PVA stabilized AuNp

In short a three different AuNp solution were prepared to study effect of stabilizers concentration. Briefly, a solution of 0.625 ml HAuCl₄ 0.01 M were dissolved and stirred in deionized water. Shortly after, an ice cold 0.1 ml of freshly prepared NaBH₄ 0.1 M solution were added to the solution and the stir continued for 15 minutes. The colour rapidly changed from pale yellow to ruby red indicated a formation of gold nanoparticles. After that, a series of aqueous solution containing varied amount of PVA added to those three different AuNp solution. The PVA stabilized AuNp were obtained and kept for next studies.

4.3. Synthesis of FA-AuNp Conjugate

FA was conjugated with AuNp through 4Atp as linker. 4Atp functionalized AuNp was prepared according to the method present in literature [21]. In the first step, a varied ethanolic solution of 4Atp (0.3 M) that has been prepared before was added drop wise to the stirred solution of AuNp (10 ml). The solution then stirred for about an hour to complete the conjugation. The colour of the solution changed from ruby red to purple blue indicated that the attachment has taken place to form 4Atp-AuNp conjugate.

FA solution (8.8 mg) was activated by addition of freshly prepared EDC (1 ml, 20 mM) and NHS (1 ml, 50 mM) in DMSO. The solution was stir for up to 3 hours to complete the activation. The solution later added to the 4Atp-AuNp conjugate to form FA-AuNp conjugate through 4Atp link. Stirring was continued for the next 2 hours to complete the reaction. An observation also conducted to characterize using spectrophotometer UV-Vis (Shimadzu) and FT-IR to confirm the attachment for each step.

4.4. Synthesis of FA-AuNp-Rsv conjugate

Resveratrol conjugation to AuNp procedure was done according to the method in literature with slight modification [17]. 1 ml solution of resveratrol (2 mM) in ethanol was added to FA-AuNp conjugate solution and kept for continues stirring for 2 hours in room temperature and the conjugate result (FA-AuNp-Rsv) was purified using a centrifuge tube filters (MWCO of 10kDa). The pellet obtained and washed several times and suspended in water for further verification.

4.5. Characterization

The absorption spectra of AuNp and conjugates are observed using UV-Vis spectroscopy (Shimadzu UV-1601). Sample solutions were put in 10 mm path length quartz cuvettes and measurement conducted in range of 200-800 nm wavelength for characteristic surface Plasmon resonance (SPR).

Fourier transform infrared analysis was performed using Shimadzu FT-IR type 8400s. Samples were freeze dried and mixed with KBr powder and measured at 400-4000 cm⁻¹ wavelength.

Particle size, distribution and zeta potential were measured on a particle size analyzer (Zetasizer Nano ZS, Malver Instruments, Ltd., UK). Samples were suspended in deionised water and placed in cell and measured at room temperature.

4.6. Entrapment efficiency

The concentration of resveratrol in FA-AuNp-Rsv conjugate were measured using high performance liquid chromatography instruments with UV-Vis detector based on methods presented literature with slight modifications [22]. The standard curve of resveratrol initially prepared using different concentration of resveratrol ranging from 1 to 50 µg ml⁻¹ and an injection of 20 µl was applied for all standards and samples. The chromatographic separation was conducted with Phenomenex C₁₈ column and detection was carried out at 306 nm. The mobile phase was a mixture of methanol: 10 mM potassium dihydrogen phosphate buffer (pH 6.8): acetonitrile (63:30:7) at a flow rate of 1 ml/min.

Encapsulation efficiency (EE) was determined by measuring the concentration of free Rsv in filtrate obtained from purification of FA-AuNp-Rsv conjugate by ultracentrifugation. EE will then be measured triplicate and calculated as below:

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EE \ (% ) = \frac{\text{Rsv initial concentration added} - \text{Rsv concentration on filtrate}}{\text{Rsv initial concentration added}} \times 100 \quad (\text{Eq. 1})
\]
4.7. Cytotoxicity test

MTT test was chosen as a method to determine the cytotoxic activity in the final step of this research. Six varied concentration ranging from 5 to 160 µM were prepared for both groups of FA-AuNP-Rsv solution and free Rsv solution. Concentration were calculated based on the concentration of resveratrol in both samples. The samples later tested against human breast adenocarcinoma cell line (MCF-7). Cells were grown using DMEM medium in 96-well plates (4500 cells in 100 µL per well), supplemented with 10% fetal bovine serum and antibiotics at 37 °C with 5% CO2. Samples were added to each well in triplicate and after 24 hours of incubation, 10 µL MTT reagent (5 mg/ml) was administered to each well and incubation was continued for the next 4 hours. Subsequently the purple formazan crystals were dissolved in ethanol and the absorbance was read at a wavelength of 595 nm. The IC50 values were determined from the dose-response curves using GraphPad 5 Prism software. Statistical two-way variance analysis also conducted using SPSS 22.0 software.

4.8. Statistical analysis

Statistical analysis were performed for all data results in cytotoxicity test using one-way variance analysis with confidence level of 95%, with SPSS 22.0 statistics software. All the experiments were performed triplicate.

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