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Varying Formulation Parameters on Hybrid Gold Nanoparticles for Passive Targeting Chemotherapy

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Hybrid gold nanoparticles (AuNPs) hold great potential in various applications, particularly for drug delivery. However, passive targeting of these hybrid AuNPs requires specific particle sizes and encapsulation efficiency to achieve high bioavailability and competency. Varying formulation parameters highly influence particle size and encapsulation efficiency of nanoparticles. Hence, this paper investigates the influences of formulation parameters; drug (gemcitabine-GEM) and gold concentrations on the particle size and encapsulation efficiency. Hybrid AuNPs were prepared by the double emulsion method at varying GEM concentrations (2.5 – 10 mg mL^{-1}) and gold concentrations (2.5 – 10 µg mL^{-1}). The final product of hybrid AuNPs was characterised using the Malvern Zetasizer Nano ZS and UV-Vis, and the results were evaluated using statistical analysis. The best formulation was obtained at 2.5 mg mL^{-1} of GEM concentration and 5 µg of AuNPs concentration; in this condition, the hybrid gold nanoparticle achieved 287.47 ± 18.7 nm in particle size; 0.25 ± 0.09 of PdI value; -23.3 ± 0.20 mV of surface charge, encapsulation efficiency of GEM (78.0 ± 0.35 %) and encapsulation efficiency of AuNPs (63.57 ± 0.99 %). Higher GEM concentration reduces encapsulation efficiency because its hydrophilic nature leads it to quickly be distributed into the outer aqueous phase, resulting in lower encapsulation. While AuNPs show a concentration-dependent pattern in the formulation required. In conclusion, varying drug and gold concentrations in the nano-formulation can obtain specific physicochemical characteristics and maximum encapsulation efficiency of GEM and gold nanoparticles suitable for drug delivery.

Keywords: Polymer concentrations; particle size; encapsulation efficiency; preparation methods; nanoparticles

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Gemcitabine (GEM) is one of the effective wide-spectrum anti-cancer drugs that has been used as the main treatment for advanced metastatic cancer and an adjuvant therapy to surgical and radiotherapy. However, the short half-life of GEM reduced its efficacy in treating cancers. This leads to developing a drug delivery system or a carrier to prolong the circulation time and efficiently deliver GEM to target sites. Nanocarriers have shown the ability to enhance the selectivity of anti-cancer drugs and reduce systemic toxicity thus reducing the adverse side effects experienced by patients [1, 2].

Gold nanoparticles (AuNPs) have been regarded as one of the most promising multifunctional nano-carriers owing to their therapeutic and diagnostic ability which is also termed theranostic. However, the solid core of AuNPs makes the conjugation of the drug on the surface susceptible to degradation thus changing the pharmacokinetics profile of the drugs [3]. Several characteristics in successfully designing a nanocarrier include the ability to accumulate and penetrate at the target site. While appropriate particle sizes are crucial to ensure nanocarrier system success, monodispersed distribution and the surface properties of the nanocarrier also play a vital role in improving drug delivery. This is because, in this scope of the study, we focused on passive targeting through the EPR effect that is largely influenced by the particle size ideally in a

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range between 10 - 300 nm to avoid kidney filtration and elimination by macrophage cells [4].

The emulsion method is one of the simplest to encapsulate hydrophobic and hydrophilic drugs [5] and was used in this paper to fabricate hybrid gold nanoparticles. In this paper, formulation parameters of gold and drug concentrations were studied to determine their effects on particle size and encapsulation efficiency of the hybrid AuNPs.

EXPERIMENTAL

Chemicals and Material

Poly Lactide-co-Glycolide, PLGA (lactide:glycolide 65:35, Mw 40,000-75,000, Sigma Aldrich, USA), poly (ethylene glycol) bis amine, PEG-diamine (Mw 3400, Sigma Aldrich, USA), anhydrous methylene chloride (>99.8%, Sigma Aldrich, USA), dicyclohexycarbomide (DCC) (Mw 206.3, Merck, USA), N-hydroxysuccinimide (NHS) (Mw 115.06, Nacalai tesque, PLT Scientific, Malaysia), Diethyl ether (Merck, USA), Tween 80 (Merck, USA), Methanol (analysis grade, R&M Chemical, Malaysia), Acetone (analysis grade, R&M Chemicals, Malaysia). Gold nanoparticles (Nanosphere, 50 nm, 44.5 µg mL^{-1}, Cytodiagnostics, Canada). Anti-cancer drug of gemcitabine (Mw 263.20, 99.39%, Alfa Aesar, USA).

Conjugation of PLGA-PEG

The PLGA-PEG conjugation was prepared according to Klippstein et al., 2015 with some modifications [6]. The terminal carboxylic (~COOH) of PLGA was covalently conjugated to the amino group of (H_2N-PEG-NH_2) using the DCC/NHS coupling chemistry. Briefly, PLGA-COOH (250 mg, 0.0062 mmol) together with N-hydroxysuccinimide (NHS) (5.75 mg, 0.05 mmol) and dicyclohexyl carbodiimide (DCC) (10.31 mg, 0.05 mmol) were dissolved in anhydrous methylene chloride (DCM) (PLGA/NHS/DCC stoichiometry molar ratio: 1:8:8). The reaction mixture was stirred under nitrogen atmosphere for 24 h at room temperature. After the reaction, the solution was precipitated with 2 ml ice-cold diethyl ether to obtain a white viscous material washed with methanol and dried under vacuum overnight. Next, activated PLGA (PLGA-COOH) (250 mg, 0.0062 mmol) and PEG (106.25 mg, 0.03 mmol) were each dissolved in 1 mL DCM before PLGA-COOH was added dropwise to PEG bis-amine and stirred under nitrogen atmosphere for 24 h at room temperature to form a 1:5 stoichiometric molar ratio of PLGA-COOH: H_2N-PEG-NH_2. After that, an ice-cold diethyl ether will be added to give off a white precipitate of PLGA-NH-PEG-NH_2. The precipitated product will be washed with cold methanol and dried under a vacuum overnight. PLGA-PEG conjugation was characterised by Fourier transform infrared (FT-IR) spectroscopy (Spectrum Two, Perkin Elmer, USA).

Preparation of Hybrid Gold Nanoparticles (AuNPs)

Hybrid gold nanoparticles (AuNPs) were prepared by double emulsion solvent evaporation method [7]. 25 mg PLGA-PEG was dissolved in 5 mL acetone (organic solution). Next, GEM was prepared in distilled water at varied GEM concentrations of 2.5, 5 and 10 mg mL^{-1} (aqueous solution). 1 mL of AuNPs solution (2.5, 5 and 10 µg mL^{-1}) was added dropwise to aqueous solution and stirred for 30 min. Subsequently, the organic solution was added to the aqueous solution and emulsified for 30 s at 20 amp under an ice bath using an ultrasonicator (QSonica 700, QSonica LLC, USA). For the second emulsion, 20 ml of 0.2 % Tween 80 was added and emulsified again for another 1 min at 40 amp, under ice. The final formed double emulsion was stirred overnight at room temperature at 350 rpm under a fume hood to facilitate the complete evaporation of acetone. Next, the nano-particles were centrifuged (MiniSpin 5452, Eppendorf, Germany) in 2 mL centrifuge tubes at 10 000 rpm for 30 min at 4 ℃. Finally, the nanoparticles were washed with distilled water, lyophilised and kept in a −30 ℃ freezer prior use.

Nanoparticle Physicochemical Characterisation

Characterisation of particle size, zeta potential and polydispersity index (PDI) of hybrid nanoparticles was conducted using Zetasizer Nano ZS (Malvern Instrument, Worcestershire, United Kingdom). The hybrid AuNPs were diluted in deionised water (1:10) before measurements. Measurements were performed in triplicate.

Encapsulation Efficiency (EE) (%)

The encapsulation efficiency (EE) (%) of the hybrid's AuNPs was determined using a Spectrophotometer (UV-1800, Shimadzu, Japan). 1 mL of formulated hybrid AuNPs were centrifuged for 60 minutes at 10,000 rpm. 150 µL of the supernatant were dissolved in 3 mL distilled water. Quantification of GEM and AuNPs was determined by conducting a standard curve of GEM and AuNPs. The absorption spectrum of the formulation was measured at 268 nm (GEM) and 529 nm (AuNPs). The EE (%) can be calculated using the equation [8];

$$\text{Encapsulation Efficiency (EE \%)} = \frac{\text{Encapsulated drug or AuNPs}}{\text{Amount added in the formulation}} \text{ x } 100\ \%$$

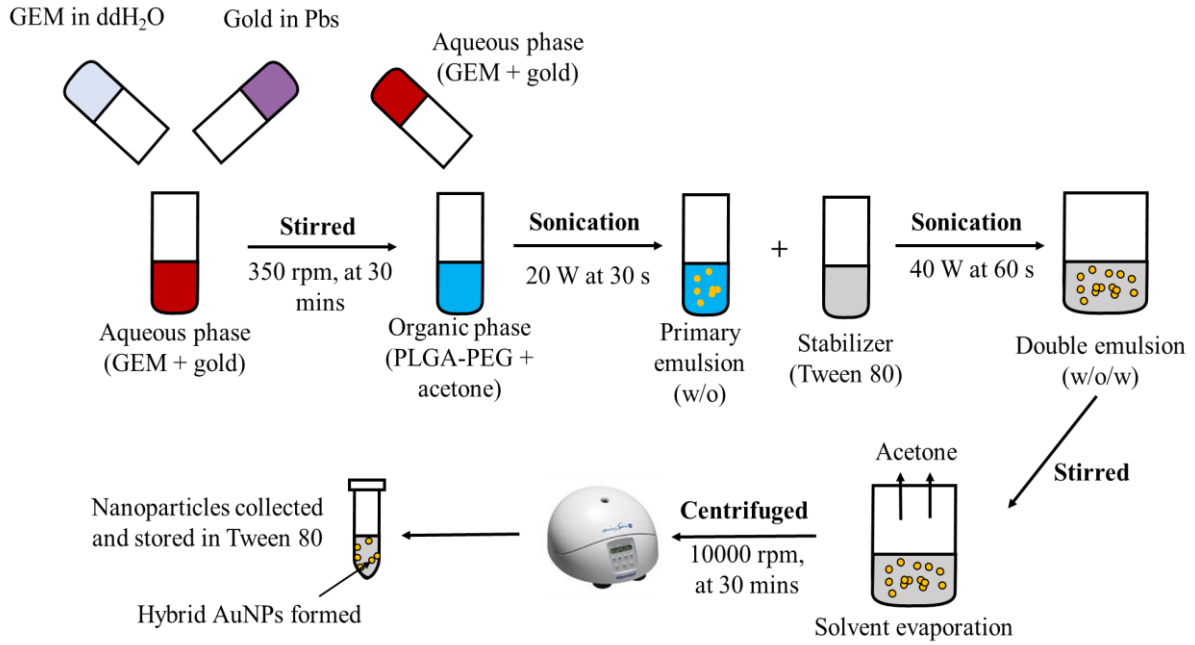

Figure 1. Preparation of Hybrid AuNPs via double emulsion method.

Data Analysis

Statistical analysis was carried out on particle size, polydispersity index (PdI), zeta potential and encapsulation efficiency using a statistics software package (SPSS (ver. 25). One-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to analyse the parameters. Data were expressed as means of standard errors. Differences were considered statistically significant at p-value ≤ 0.05.

RESULTS AND DISCUSSION

Conjugation of PLGA-PEG

Figure 2 shows the FTIR spectra of the a) PLGA b) PEG and c) PEG: PLGA. The FTIR peaks of PLGA showed bands such as -CH, -CH_2, CH_3 stretching (2995 cm^{-1} and 2946 cm^{-1}, carbonyl C=O stretching (1747 cm^{-1}), and -OH stretching (3507 cm^{-1}) while the peaks for PEG described -CH stretching (2883 cm^{-1}), the C-H bending vibration at 1454 cm^{-1} and 1359 cm^{-1} and -O-H and C-O-H stretching at 1278 and 1102 cm^{-1}. The successful conjugation of PLGA-PEG-NH2 was determined by the FTIR peaks at 1740 cm^{-1} indicating the C=O stretching in PLGA. Peaks between 2800 and 3000 cm^{-1} could be attributed to the C-H stretching vibration correlated to methylene groups in the PEG chain. In addition, peaks of 1664 cm^{-1} and 1535 cm^{-1} correspond to the amide bond.

Effect of Drug Concentration in the Formulation

Three different concentrations of GEM (2.5, 5 and 10 mg mL^{-1}) were used to prepare hybrid gold nanoparticles while the amount of polymer was kept constant (25 mg). Table 1 listed the effects of different GEM concentrations on nanoparticle characteristics; particle size, PdI, zeta potential and encapsulation efficiency (EE %). The GEM concentrations of 2.5, 5 and 10 mg mL^{-1} did not significantly ($p > 0.05$) affect the average particle size of 81.59 ± 2.96, 85.15 ± 3.67 and 84.46 ± 1.60 nm when the concentration increased from 2.5, 5 and 5 mg mL^{-1}, respectively. These results indicate the need for more replicates. The range of particle size is not directly proportional to the GEM concentration. Similarly, Aggarwal et al. found that the addition of drug concentration does not increase proportionally but exhibits the smallest particle size at certain GEM concentrations [9]. In contrast, Zhao and Qi found an increase in particle size with an increase in GEM concentration [10]. These differences are due to several factors including the PLGA-PEG and GEM constituents. Differences in PLGA copolymer, lactic acid and glycolic acid percentage can affect its molecular weight, thus influencing the interaction with GEM [11]. In addition, it could also differ due to the concentration study range, molecular weight and GEM modification [12]. Different concentrations of GEM do not significantly affect the polydispersity index (PdI) but are within the suggested range for nanoparticles (<0.3).

Increased drug concentration was shown to be inversely proportional towards the EE %, (81.35 ± 5.56, 53.88 ± 5.4 and 49.12 ± 1.5 respectively to GEM concentration of 2.5, 5 and 10 mg mL^{-1}). This is due to the hydrophilicity nature of the drug that tends to rapidly partition out into the outer aqueous phase thus higher drug amount would result in lower EE% [9]. The varying concentrations of GEM preserve the

negative surface charge at around -12 to -17 mV indicating addition of GEM does not significantly change the surface charge of the nanoparticles. Among the three different concentrations of GEM, concentration 2.5 mg mL^{-1} was found to be the best formulation as this formulation showed the smallest size of 81.59 ± 2.96 nm; 0.18 ± 0.01 of PdI value; -14.07 ± 1.238 mV surface charge and the highest EE% of GEM of 81.35 ± 5.56 %. Generally, three main characteristics to determine the physical stability of nanoparticles and the suitability of a formulation are the particle size (100-300 nm), polydispersity indices (<0.3) and zeta potential (±30 mV) [4]. Higher zeta potential values determine the physical stability while the negative surface charge helps improve the nanoparticle's internalisation compared to positively charged nanoparticles[13].

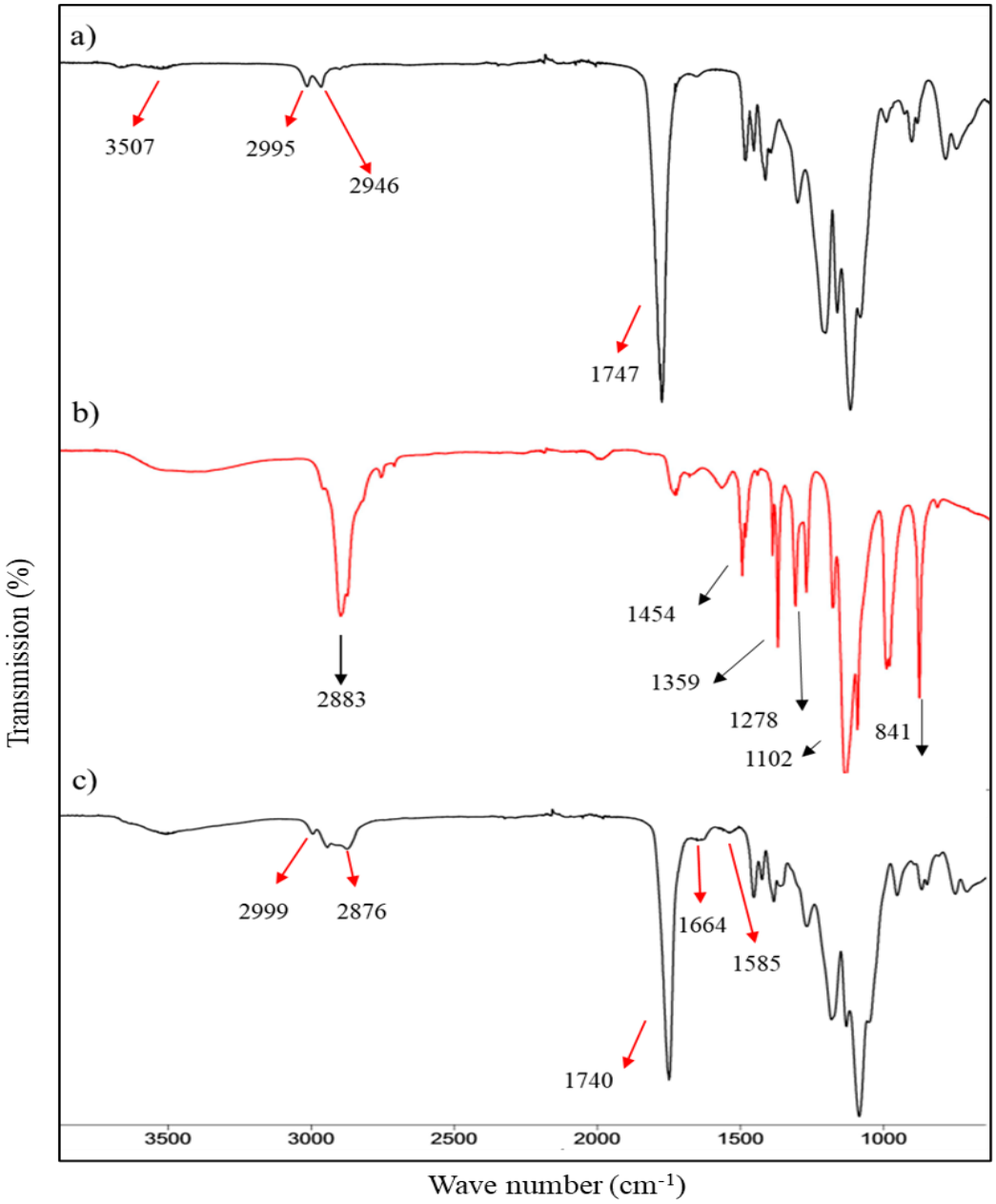

Figure 2. FT-IR spectra of a) PLGA, b) PEG and c) PEG: PLGA.

Table 1. Effects of different drug concentrations (mg mL^{-1}) on the characteristics of hybrid nanoparticles. The values are the mean ± error (SE) of triplicate experiments.

Formulation	Size	PdI	ζ (mV)	EE% GEM
PLGA-PEG (Blank)	91.36 ± 3.75[a]	0.22 ± 0.02[a]	-16.55 ± 0.78[a]	-
PLGA-PEG/GEM 2.5	81.59 ± 2.96[a]	0.18 ± 0.01[a]	-14.07 ± 1.23[a]	81.35 ± 5.56[a]
5	85.15 ± 3.67[a]	0.17 ± 0.03[a]	-12.45 ± 1.35[b]	53.88 ± 5.4[b]
10	84.46 ± 1.60[a]	0.18 ± 0.07[a]	-14.04 ± 0.65[a]	49.12 ± 1.5[c]

Table 2. Effects of different AuNPs concentrations (µg mL^{-1}) on the characteristics of hybrid nanoparticles. The values are the mean ± error (SE) of triplicate experiments.

Formulation	Size	PdI	ζ (mV)	EE% GEM	EE% AuNPs
PLGA -PEG (Blank)	75.263 ± 1.35[a]	0.134 ± 0.02[a]	-10.7 ± 0.3[a]	-	-
PLGA-PEG/GEM	92.95 ± 1.74[b]	0.12 ± 0.01[a]	-10.63 ± 0.28[a]	-	-
PLGA-PEG/GEM/AuNPs 2.5	261.60 ± 12.30[c]	0.37 ± 0.01[b]	-23.6 ± 0.6[b]	78.0 ± 0.35[a]	38.95 ± 1.90[a]
5	287.47 ± 18.7[d]	0.25± 0.09[c]	-23.3 ± 0.20[b]	50.4 ± 1.50[b]	63.57 ± 0.99[b]
10	349.83 ± 6.0[e]	0.36 ± 0.01[d]	-19.96 ± 0.18[b]	56.13 ± 0.04[b]	63.95 ± 0.42[b]

Effect of AuNPs Concentration in the Formulation

Three different concentrations of gold nanoparticles (AuNPs) (2.5, 5 and 10 µg mL^{-1}) were used in the preparation of hybrid gold nanoparticles while the amount of drug (2.5 mg mL^{-1}) and polymer (25 mg mL^{-1}) were kept constant. Table 2 shows the effects of different AuNPs concentrations on nano-particle characteristics; particle size, PdI, zeta potential and encapsulation efficiency (EE %). For the particle size, at concentrations of 0, 2.5, 5 and 10 µg mL^{-1} AuNPs, the size drastically increases from 92.95 ± 1.74, 261.60 ± 12.30, 287.47 ± 18.7 and 349.83 ± 6.0 nm indicating concentration dependant effect which represents a linear relationship. Similarly, Xiong et al. also determined that the addition of AuNPs is directly proportional to the particle size [14]. However, at an AuNPs concentration of 10 µg mL^{-1}, the particle size has exceeded the optimum range for tumour delivery (<300 nm). The distribution of the particles in the formulation also significantly increased by the addition of AuNPs 0, 2.5, 5 and 10 µg mL^{-1}, resulting in average values of 0.12 ± 0.01, 0.37 ± 0.01, 0.25 ± 0.09 and 0.36 ± 0.01 (p <0.05).

The negatively charged citrate-coated AuNPs in the formulation help significantly increase the zeta potential value from -10.63 ± 0.28 to around -20 mV at 2.5 and 5 µg mL^{-1}, respectively. The zeta potential values determine the physical stability of nanoparticles and it varies depending on the surface charge of dispersion. The values below ± 5 mV will result in fast aggregation of nanoparticles while at around 20 mV, it exhibits short–term stability [15]. Higher zeta potential will improve the stability of nanoparticles [4]. The encapsulation efficiency of GEM is significantly the highest at 2.5 µg mL^{-1} (78.0 ± 0.35 %) while the lowest at 5 µg mL^{-1} (50.4 ± 1.50 %) suggesting the encapsulation of GEM is not concentration dependent. At AuNPs of 5 and 10 µg mL^{-1}, the EE% of GEM and AuNPs is not significantly different at values of (56.13 ± 0.04 50.4 ± 1.50 %) and (63.57 ± 0.99 and 63.95 ± 0.42) indicating 5 µg mL^{-1} is the maximum capacity of AuNPs to achieve balance encapsulation between GEM and AuNPs.

CONCLUSION

This study presents the effects of varying formulation parameters of the double emulsion method in preparing the hybrid gold nanoparticles. In conclusion, AuNPs concentrations have significantly affected the physicochemical characteristics (particle size, zeta potential and surface charge) and the encapsulation efficiency of GEM and AuNPs. The differences in GEM concentration only significantly influence the encapsulation efficiency of GEM.

Therefore, by varying drug and AuNP concentrations, appropriate nanoparticles with fitted physicochemical characteristics for drug delivery can be achieved. In addition, the therapeutic ability can be escalated as the drug encapsulated can be adjusted to the maximum capacity.

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