Synthesis of fish gelatin nanoparticles and their application for the drug delivery based on response surface methodology

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Abstract
Fish gelatin nanoparticle is produced using Tilapia fish skin for the first time by the two-step desolvation method. Fish gelatin is chosen for producing gelatin nanoparticles because no experiment have been done in using fish gelatin and to counter the problem associated with the use of mammalian gelatin, such as bovine spongiform encephalopathy disease. The effects of several factors on the particle size such as pH, acetone concentration, glutaraldehyde volume, stirring speed and stirring time are evaluated. Optimum conditions for the formation of gelatin nanoparticles are obtained using response surface method. Fish gelatin nanoparticles with optimum size of 198.46 ± 6.1 nm can be produced using pH of 2.45, acetone percentage of 16% (vol%), glutaraldehyde 400 µℓ, stirring speed of 600 rpm, and stirring time for 6h. The thermogram and molecular interaction of fish gelatin and fish gelatin nanoparticles are characterized using DSC and FTIR. In vitro drug release kinetic is examined using 5-fluorouracil as the model drug. The entrapment efficiency of 5-fluouracil as model drug is determined to be 40%. Fish gelatin could be used as a good alternative drug carrier for mammalian gelatin.

Keywords: fish gelatin, nanotechnology, desolvation, macromolecular drug, cross-lingking

Classification numbers: 4.02, 5.08, 5.09

1. Introduction

Gelatin nanoparticles are an excellent material for drug delivery applications due to their biodegradability [1], biocompatibility [2], ability for surface modification [3], ligand attachment for target delivery and the availability of raw materials [4, 5]. Gelatin is produced from hydrolyzed collagen extracted from bovine bone, pig skin, and fish skin. Bovine and porcine gelatins have been extensively used to fabricate nanoparticles and to encapsulate the drug. For examples, bovine gelatin has been used to encapsulate cisplatin for cancer cell treatment [6], and porcine gelatin has been studied for encapsulating amphotericin [7] and ibuprofen [8]. The encapsulation efficiency of anticancer drugs have also been investigated using mammalian gelatin for doxorubicin [9], paclitaxel [10], cisplatin [11] and fluorouracil [12].

However, the use of mammalian-origin materials in drugs and food products are limited due to religion and ethical reasons. Not only Jew and Muslim groups are forbidden from consuming porcine gelatin [13], but also not so long ago bovine spongiform encephalopathy (BSE) in Europe has raised several questions concerning the use of pig gelatin...
for gelatin application [14]. Under these circumstances, fish-origin materials provided an alternative for producing gelatin nanoparticles. Furthermore, fish gelatins are being rarely used to produce gelatin nanoparticles. Until now, fish gelatin are used for producing gelatin film [15], and nanofibre [16]. Because of that, the experiment should be done to optimize the production of gelatin nanoparticles from fish gelatin.

Several methods have been used for producing gelatin nanoparticles such as the emulsion [17], coacervation [18], self-assembly [19], and desolvation method [20]. Among those approaches the desolvation method was found to provide small particles and narrow size distribution [21]. The effects of several factors on gelatin nanoparticles size have been investigated, such as gelatin concentration, pH, and acetone volume [22–25]. However, those reports have just focused on single factor exploration, which precluded the interaction between various factors. Systematic investigations have not been carried out yet in optimizing fish gelatin nanoparticles (FGNPs) production incorporating the simultaneous effects of several significant factors. Thus, the optimization of production fish gelatin nanoparticles using response surface method should be employed.

The chosen factors for producing gelatin nanoparticles depend on the gelatin characteristic, whereas the gelatin properties also depend on their resources [26]. Thus there are differences between mammalian gelatin and fish gelatin. The specific characteristic of gelatin is the triple helical structure. The proline and hydroxyproline contents are approximately 30% for mammalian, and 22–25% for warm-water fishes such as tilapia and Nile perch [27]. These differences on fish gelatin lead to lower gel modulus and lower melting temperatures compared with mammalian gelatin. Hence the conditions required for the production of fish gelatin might vary with that for the production of mammalian gelatin.

In this study focus was to explore the potential of fish gelatin to produce FGNPs. Tilapia fish skin was used as a source of gelatin. Tilapia fish is commonly breaded in fish farms in Malaysia and hence tilapia fish skin is in abundance such as in filled production company. In this context, we aim to improve the desolvation method and to obtain optimum conditions for the production of FGNPs. The significant factors were chosen based on the one-factor-at-time method, and response surface method then was used to optimize these significant factors. The differences in thermal behavior and molecular interaction between fish gelatin and fish gelatin nanoparticles were studied using differential scanning calorimetry (DSC) and Fourier-transform infrared (FTIR) spectroscopy. The nanoparticles shape was also quantified using scanning electron microscope (SEM). These experiments allowed us to determine a reproducible formulation of small sized FGNPs with narrow size distribution.

2. Experimental

2.1. Materials

Tilapia fish gelatin (gelatin type A) with gel strength of 128.11 g bloom. Acetone, hydrochloric acid and sodium hydroxide were of analytical grade and were ordered from Sigma. Glutaraldehyde (25 vol%, grade I aqueous solution), and 5-fluorouracil (5-FU) were purchased from Sigma. Double distilled water was used for all the experiments. All chemicals were of analytical grade and used as received.

2.2. Production of gelatin nanoparticles

FGNPs were produced using the two-step desolvation method with slight modifications [28]. The first step is the fractionation of low molecular weight (LMW) and high molecular weight (HMW) of gelatin. The second step is the precipitation step to produce nanoparticles. As the second step or precipitation step is crucial for the formation of nanoparticles, the focus was on the optimization of this step.

The fractionation step started by dissolving 0.9 g fish gelatins in 10 ml distilled water under constant heating (45°C) and stirring (600 rpm) until a clear solution was achieved. Acetone was used as co-solvent to precipitate the high molecular weight. The percentage composition of acetone was calculated with respect to the total volume of the mixture (100 ml). About 10 ml acetone was added to the gelatin solution and was centrifuged at 12000 g for 5 min. The HMW fraction was obtained in the precipitate, and LMW fraction was in the supernatant solution. The supernatants containing LMW fractions were discarded. The HMW gelatin was dissolved again with 10 ml distilled water. The precipitation step begins with adjustment of pH of gelatin solution to the desired value by adding 0.1 M HCl and NaOH. FGNP were produced by adding 16 ml acetone to HMW gelatin solution and 400 µl of glutaraldehyde solution (25 vol%). The nanoparticles were centrifuged and washed three times. The acetones were removed by evaporation in a water bath at 45°C temperature. The nanoparticles were stored in temperatures of 3°C–5°C for further research.

2.3. Design of experiment

The precipitation step in the production of FGNPs was optimized sequentially. The screening for significant factors was done using the one-factor-at-a-time (OFAT) method. On the basis of previous work, the effects of parameters like pH, acetone concentration, glutaraldehyde volume, stirring speed, and stirring time on the size of FGNPs were studied. The levels of factors are summarized in table 1. The experiments were done in triplicates. The results were given as mean ± standard deviation.

| No | Factor                          | Level |
|----|---------------------------------|-------|
| 1  | pH                             | 1     | 2     | 3     | 4     |
| 2  | Concentration of acetone (%)    | 1.5   | 2.5   | 3.5   | 4.5   |
| 3  | Volume of glutaraldehyde (µl)   | 300   | 475   | 650   | 825   |
| 4  | Stirring speed (rpm)            | 150   | 300   | 450   | 600   |
| 5  | Stirring time (h)               | 3     | 6     | 12    | 18    |
Table 2. Independent variables and their corresponding levels of second step FGNPs preparation for FCCD.

| Factor | Variable                  | Level  |  |  |  |
|--------|---------------------------|--------|  |  |  |
|        |                           | −1     | 2.5 | 1  | 5  |
|        | pH                        | 1.5    | 2.5 | 3.5 |
|        | Concentration of acetone (% v/v) | 5 | 15 | 25 |
|        | Volume of glutaraldehyde (µℓ) | 300 | 475 | 650 |

deviation (SD) of three independent experiments and statistically analyzed by ANOVA, followed by the Tukey test to compare the different nanoparticle batches.

The significant factors were optimized using response surface methodology (RSM) based on three levels of face centered central composite design (FCCD) (table 2). The levels of parameters were selected based on the OFAT results. Mean particle sizes of FGNPs were used as the response variable. A total of 18 experimental runs including six center points were generated by design-expert version 7.0 software (State-Ease Inc., Minneapolis, MN). The runs were carried out in triplicates.

2.4. Determination of isoelectric point of fish gelatin

Isoelectric point of fish gelatin was identified according to preferred method [29]. In brief, about 10 ml of 1% (w/v) fish gelatin was prepared in distilled water and pH was adjusted with 0.1 M HCl or 0.1 M NaOH. The isoelectric point of fish gelatin was determined by the measuring the turbidity as identified through the measured maximum intensity at 360 nm in UV-vis spectrum [29, 30].

2.5. Loading of fish gelatin nanoparticles with drug

5-fluorouracil (5-FU) was chosen as a model drug because it has been used as the major chemotherapeutic agent. 5-FU loaded FGNPs were prepared by adding 5-FU directly to fish gelatin solution at the precipitation step.

2.6. Determination of particle size and zeta potential

Mean particle size and zeta potential of FGNPs were determined using Zeta Sizer Nano Malvern (Zen 3600, UK). The mean diameters and polydispersity index (PI) values were obtained at 90° angle in 10 mm diameter cells. Each measurement was conducted in triplicates.

2.7. Physiochemical characterization

The experiment of FTIR was made on lyophilized samples of fish gelatin and fish gelatin nanoparticles using Perkin Elmer spectrum FTIR over a diamond crystal. Small amount of samples (±1 mg) was placed in the diamond crystal and the FTIR spectra were recorded in the range of 4000–400 cm−1. The results were plot between transmittance (%) and wavenumber (cm−1).

The thermal behaviour of fish gelatin and fish gelatin nanoparticles was obtained using a differential scanning calorimeter (DSC-60, Shimadzu, Japan). The samples were prepared using aluminium pans and empty aluminium pan was used as a reference. About 5 mg of samples were sealed and heated from 20 °C to 300°C at a rate of heat flow of 10°C min⁻¹.

2.8. Morphologies characterization by scanning electron microscopy

Field emission scanning electron microscopy (FESEM JEOL, JSM 6700F Model) was used to observe the size and shape of FGNPs. Briefly, a small amount of dry FGNPs was mounted on aluminum plates and, pasted with double sided copper tapes. Then the samples were sputtered with a thin layer of gold and placed on the packet chamber at an accelerating voltage of 10kV.

2.9. Transmission electron microscopy

Transmission electron microscopy (TEM) of fish gelatin nanoparticles was performed using a Philips Tecnai F 20.2 µℓ of FGNP sample was dropped on the copper TEM grid and air dried for 2 h. The morphology data was carried at an accelerating voltage of 200kV.

2.10. Drug content and in vitro drug release

UV-vis spectrophotometer was used to determine the concentration of 5-FU in the FGNPs. About 5 mg of dried FGNP loaded with 5-FU were dispersed in 5 ml of phosphate-buffered saline (PBS) (pH 7.4) at room temperature (23°C ± 2°C), containing 2.5 mg trypsin. After 6 h of digestion, the samples were diluted to 25 ml and filtered using 0.22 µm filters. The absorbance was measured at λmax = 265 nm using Sartorius-Stedim VivaSpec UV-vis spectrophotometer. A calibration curve was prepared with different 5-FU concentrations in PBS (the presence of gelatin and trypsin had no effect on the absorbance intensity). Unloaded FGNPs were used as a blank. The entrapment efficiency (EE) was calculated using following equation

\[ EE(\%) = \frac{\text{total amount of drug added}-\text{amount of free drug present in supernatant}}{\text{total amount of drug added in formulation}} \times 100. \] (1)

In vitro release of 5-FU was determined using the method previously described [25]. A weight of 5 mg of FGNPs was dispersed in 25 ml of PBS (pH 7.4). About 2 ml sample was withdrawn at defined time intervals and was centrifuged for 20 min at 10000 g. Then 1 ml aliquots were withdrawn from the supernatant and added back to the original solution. The pellets were redispersed in 1 ml of PBS and added to the
original solution to keep the particle concentration constant. The amount of 5-FU in the supernatant was quantified using a UV-vis spectrophotometer. All the experiments were repeated three times and the average values along with the errors were calculated.

2.11. Calculation of release kinetics

In vitro 5-FU release behavior from FGNPs was studied using DDSolver utilizing data up to 60% cumulative release [31]. DDSolver is the supplement program in microsoft excel which used nonlinear least-squares curve fitting by minimizing the sum of square differences between the observed and the predicted drug dissolution values at time intervals \( t \), with the best model parameters [32]. Five models were used in this experiment namely zero order, first order, Higuchi, Weibull, and Korsmeyer-Peppas. The DDSolver also calculates several parameters allowing the statistical fitness evaluation of the model. The most appropriate model to fit the dissolution data should give the highest value of adjusted coefficient of determination \( R^2_{\text{adj}} \), smallest value of akaike information criterion (AIC), and the largest value of model selection criterion (MSC).

Exponent \( n \) of the Korsmeyer-Peppas model gives information about the release mechanism of the drug according to following equation

\[
Q_t = k t^n, \tag{2}
\]

where \( Q_t \) is a fraction of drug released at the time \( t \), \( k \) is the release rate constant and \( n \) is the release exponent. The \( n \) value is used to characterize different release for cylindrical shaped matrices. If \( n \leq 0.45 \) it is a Fickian diffusion, if \( n = 0.85 \) it is a case II transport, which is related to polymer matrix relaxation and swelling. If \( 0.45 < n < 0.85 \) it corresponds to an anomalous transport, resultant from the combination of both mechanisms and if \( n > 0.85 \) it is a super case II transport [33].

![Figure 1. Effect of different pH levels to nanoparticles size, while acetone concentrations at 40% (vol%), glutaraldehyde volume at 350 \( \mu \)ℓ, stirring speed at 600 rpm and stirring time at 6 h. Significant differences by student’s t-test are marked by asterisk, \( p < 0.05 \).](image1.png)

![Figure 2. UV-vis absorption (at 360 nm) of fish gelatin aqueous (1% w/v) at different pH.](image2.png)

3. Results and discussion

3.1. Selection of significant variables

3.1.1. Effects of pH. Figure 1 shows the effects of pH on the size of FGNP. The smallest nanoparticles were found to form at pH 2.5. Decreasing the pH produced small nanoparticles until a pH of 2.5 (\( p \leq 0.05 \)). No significant increase in the particle size was observed if the pH was below 2.5.

The pH value of gelatin solution before the precipitation step is shown at pH of 4.6. In the precipitation step, pH was adjusted to be below the isoelectric point because the isoelectric point of fish gelatin was determined at pH 6 (figure 2). As shown in figure 1, the nanoparticle size increased as the pH is near to the isoelectric point. The lowering of pH leads to protonation of the fish gelatin molecules.

The lowest pH value to produce the smallest FGNPs was at 2.5. This result is slightly different compared to most mammalian gelatin. Previous studies on porcine gelatin have indicated that the pH value to produce nanoparticles in small sizes was 3.25 [34]. This might be due to the difference on negatively charged amino acid contents in the gelatin molecule. The fish gelatins have more negatively charged amino acids than mammalian gelatin; hence fish gelatin needs a more acidic pH for smaller nanoparticle formation compared to mammalian gelatin. Gelatin is sequences of amino acids. Fish gelatin molecule contains ~14% of negatively charged glutamic acid and aspartic acid, ~7% of positively charged lysine and arginine, ~8% of the chain hydrophobic amino acids (leucine, isoleucine, methionine, and valine) and ~40% of glycine [21] while mammalian gelatin contains ~13% of positively charged, ~12% of negatively charged and ~11% of hydrophobic chain. This difference in the amino acid composition affects the solubility characteristic of fish gelatin in water molecules and the processing parameters in the production of FGNPs [27].

3.1.2. Effects of acetone concentration. Acetone is used as a co-solvent in the production of FGNPs. Figure 3 shows the
effects of acetone concentration on the FGNPs size. It can be seen that increasing acetone concentration lead to increase in nanoparticle size whereas 15% (vol%) acetone produced smallest nanoparticle fish gelatin \( p \leq 0.05 \). Similar trends were observed in the previous studies \[35, 36\]. This has been attributed to the role of acetone molecules in disturbing the interaction between water and gelatin molecules resulting in gelatin molecule aggregation. Interestingly, tilapia fish gelatin requires lesser acetone to produce nanoparticles compared to mammalian gelatin. Tilapia fish gelatin used less than 15% acetone whereas mammalian gelatin needs around 70% of acetone \[37, 38\]. This is also due to the difference on amino acid content.

Acetone have been chosen as a desolvating agent because acetone is highly miscible in water and prevents denaturation of gelatin compared to ethanol \[39\]. In addition, acetone produced small sized gelatin nanoparticles with lower polydispersity index compared to ethanol \[40\].

The addition of acetone to the gelatin solution is to change the solubility character of the gelatin molecule. In the water environment, the hydrophilic amino acids existing on the surface interact with the polar water molecules forming hydrogen bonds, while the hydrophobic amino acids remain inside that gelatin in the core. The water molecules surrounded the hydrophilic section of gelatin, and stabilized the gelatin molecule structures by hydrogen bonds.

When acetones are added to the gelatin solution, the solubility of gelatin will begin to decrease and become insoluble until a certain acetone concentration is reached. This is because of the decrease in the amount of hydrogen bond that available to interact with the hydrophilic amino acids of gelatin \[40\]. Decreasing the hydrogen bonds destabilized the individual gelatin molecules leading to their aggregation. Hence, we can say that the aggregation is the rearrangement of the interaction between non-polar sections of gelatin to stabilize the gelatin molecule.

3.1.3. Effects of glutaraldehyde as crosslinking agent. The effect of glutaraldehyde volume on the size of the FGNPs was shown in figure 4. It can be seen that increasing the volume of glutaraldehyde during production of FGNPs decreased the size of the FGNPs initially \( p \leq 0.05 \). However, further addition of glutaraldehyde volume increased the FGNPs size. Glutaraldehyde is soluble in water and categorized as a hydrophilic cross-linking agent \[21\]. Glutaraldehyde works by hardening the particle through the crosslinking of amino acids.
acid chains [41]. Initial addition of glutaraldehyde volume enhances the formation of smaller size nanoparticles by crosslinking the intra amino acid bonds. However, further increase in glutaraldehyde facilitates the formation of inter-molecular linkages among the nanoparticles, thereby increasing the nanoparticle size at high concentrations. Thus, the nanoparticle sizes are controlled by the delicate balance between inter and intra-molecular bonds among the gelatin molecules [40].

3.1.4. Effects of stirring speed. As shown in figure 5, the nanoparticle size is found to decrease as the stirring speed increased from 150 rpm to 600 rpm which is the maximum allowable stirring speed in our experimental setting. However, no significant effect \((p \geq 0.05)\) of the stirring speed on the nanoparticle size was observed. The increase in stirring speed lead to increase in shearing energy to break the large particles into smaller particles, which could prevent huge agglomeration, but further increase of the stirring rate, does not have a significant impact in decreasing the particle size [42, 43]. Thus, the stirring speed should be maintained at 600 to produce smaller nanoparticle sizes.

3.1.5. Effects of stirring time. Figure 6 depicts the effects of stirring time on nanoparticles size. It shows that 6h of stirring time produced smaller nanoparticles compared to 12h of stirring time. This demonstrates that further increase in stirring time led to increase in particle size, which might be attributed to agglomeration due to enhancement in kinetic energy with an additional input of energy [44]. However, the variation in stirring time shows insignificant differences to the response \((p \geq 0.05)\). Hence, 6h was chosen and kept constant in the optimization design.

From the screening results, factors with p-value of less than 0.05 were significant on the response, and were selected for further optimization. Results showed that pH, acetone concentration and glutaraldehyde volume have significant effects on producing smaller nanoparticle sizes, while stirring speed and stirring time gave insignificant effects. The optimum levels of these significant factors (pH, acetone concentration,
Figure 7. Response surface plots for mean nanoparticles size of FGNPs showing interaction between (a): acetone concentration (v/v) and pH, (b): glutaraldehyde volume (µL) and pH, and (c): glutaraldehyde volume and acetone concentration.
and glutaraldehyde volume) were further determined by a response surface method.

3.2. Optimization by response surface methodology

The optimization process was conducted using response surface method (RSM). Three factors namely pH solution ($X_1$), acetone concentration ($X_2$) and glutaraldehyde volume ($X_3$) were chosen as independent factors, whereas mean particles size was a dependent factor. The other two factors namely the stirring speed and stirring time were kept constant at 600 rpm and 6 h. All experimental designs were conducted in triplicates. The result of the response surface method is shown in table 3. It can be seen that the mean particles size varied from 190 nm to 276 nm. The small particle was produced at the center point of the design.

A suitable polynomial equation involving the main individual effects and interaction factors were selected based on estimation of several statistical parameters provided by the Design of Expert® software. Equation (3) depicts a multiple regression analysis of the experimental data for mean particles size, where $X_1$ is pH, $X_2$ is acetone concentration, and $X_3$ is volume of glutaraldehyde. All parameters used in the polynomial equations were found to be statistically significant ($p < 0.05$).

$$\text{Mean particle size (nm)} = 203.97 - 5.94X_1 - 4.98X_2 + 9.00X_1 - 12.72X_1X_2 - 5.47X_2X_3 + 10.02X_1^2 + 9.98X_2^2 + 10.66X_3^2 + 8.95X_1X_2X_3 - 7.734X_1X_2 . \quad (3)$$

The statistical significance of the model equation was evaluated by the F-test for analysis of variance (ANOVA), which showed that regression is statistically significant at 95% ($p < 0.05$) confidence level. The value of $p$ less than 0.05 indicates that the model terms are also significant. The lack of fit $p$-value larger than 0.05 implies that the lack of fit is non-significant, which means the model is strong enough with less noise. In the response surface design, ANOVA was also used to determine the significant contribution of main variables and their interactions on the response variables.

It can be seen from table 4, pH ($X_1$), concentration of acetone ($X_2$), glutaraldehyde volume ($X_3$), cross product contribution ($X_1X_2$, $X_2X_3$), quadratic contribution $X_1X_2X_3$, and $X_1^2X_3$ were significant. The regression equation obtained from ANOVA depicted that the $R^2$ (multiple correlation coefficient) was at 0.9195 (a value $> 0.75$ indicates fitness of the model). This results estimates that if the fraction of overall variation in the data is accounted by the model, thus the model is capable of explaining 91% of the variation in response. The adjusted $R^2$ is 0.8949 and the predicted $R^2$ is 0.8959; these values near to 1.0 indicate that the model is good. The ‘adequate precision value’ at 23.266 also explains that the model is good.

The response surface plots were constructed by plotting the mean particle size as a response on the z-axis against any two independent variables, while other variables were kept at their optimal levels. This plot is to determine the optimal levels of each variable for production FGNPs (figure 7).

The formulations of variables were then optimized for response of mean particle size. The optimum values of the variables were obtained by numerical analysis using design expert software and based on the criterion of desirability. Afterwards, a new run of production FGNPs with the predicted levels were conducted to confirm the validity of the
optimization procedure. The optimized variables were found at pH 2.45, acetone percentage at 16% (v/v) and glutaraldehyde volume at 400 µℓ.

Figure 7(a) represents multiple interactions between pH and acetone to FGNP size. It can be seen that around 15% concentration of acetone was required to produce small nanoparticles at pH 2.5, while a slightly higher concentration of 20% acetone was required to produce small nanoparticles at pH 1.5. A decrease in pH led to the increase of interactions between the positive charges in the gelatin molecule with water ions [34]. This condition requires high acetone concentration to interrupt the water ion interaction. Thus increasing acetone concentration means increasing the effects of interruption between the hydrogen bond in the gelatin molecule. Because in low pH the hydration of network in the gelatin molecules were strong and more intensive [45].

Figure 7(b) shows multiple interactions between the pH and the volume of glutaraldehyde. Around 400 µℓ of volume of glutaraldehyde was needed to produce small sizes of FGNP at pH 2.5. As we can see at pH 2.5, the particle size decreased when glutaraldehydes were added from 300 µℓ to 400 µℓ. Then, the particle size becomes huge by increasing glutaraldehyde volume. It can be concluded that low glutaraldehyde volume was required to produce small nanoparticle sizes at low pH, moreover slightly higher volumes of glutaraldehyde were used to produce small particles at high pH. This is because at high pH, a huge volume of glutaraldehyde would congregate the gelatin nanoparticles from inside the gelatin molecule instead of making new interactions outside the molecule. However, in low pH, just a little volume of glutaraldehyde was interacted to produce small size nanoparticles, and further increments of glutaraldehyde will make a new interaction to intra-molecular produce large nanoparticle.

Multiple interactions between glutaraldehyde volume and acetone concentration are shown in figure 7(c). This picture depicted that around 440 µℓ of glutaraldehyde was used to produce small sizes of FGNP at pH 2.5. Under these optimal variables, the predicted mean particles size calculated by software was 201.8 nm, and the observed experimental value after average was 198.46 ± 6.1 nm. The results confirmed that the model is valid by the error of experiment which is quite close to about 1% error and indicated that the results are in good agreement with the predictive value.

The particle sizes of fish gelatin nanoparticles prepared in the present work are different from those of mammalian origin gelatin [46, 47]. The difference in particle size can be explained by the bloom number of its source. Previous studies using mammalian gelatin showed that gelatin nanoparticles have been produced with size at 160 nm using type A gelatin bloom number 300 [46]. Different bloom numbers give different particle sizes, with larger bloom number yielding smaller particle size [47].

From the results, the small nanoparticles were produced in the interactions between pH, acetone concentration and glutaraldehyde volume. At a high pH, the intermolecular bonding is more extensive than intramolecular, while in low pH intramolecular bonding has the highest effect. The collision of molecules increases the effects of molecular bonding between each other. In addition, in high pH conditions, high volumes of glutaraldehyde were used to produce small particles, because the high volume glutaraldehyde will enforce the bonding in the molecule (intramolecule).

The DSC thermographs of fish gelatin and fish gelatin nanoparticles are presented in figure 8. The thermograph of fish gelatin (curve a in figure 8) shows the presence of two...
endothelial events which are glass transition temperature \( (T_g) \) and melting temperature \( (T_m) \) at 98 °C and 220°C, consistent with the literature value for gelatin [48]. For fish gelatin nanoparticles \( T_g \) and \( T_m \) are observed as endothermic peak around 81°C and 220°C, respectively. The first of endothermic peak represents the energy consumed during vaporizing the water present in the matric and association of the glass transition of α-amino acid blocks in the polypeptide chain [49, 50]. It was found that the \( T_g \) of fish gelatin is higher than its fish gelatin nanoparticles, these results suggested that thermal stability of fish gelatin is stronger than fish gelatin nanoparticles. The lower temperature of glass transition in the fish gelatin nanoparticles indicated that higher water bound in the structure because the increasing of polymer free due to the transformation of nanoparticles [51].

Figure 9 shows the FTIR spectra of fish gelatin and fish gelatin nanoparticles. The FTIR analysis was used to evaluate and to compare the chemical structural between fish gelatin and fish gelatin nanoparticles. The spectrum showed characteristic bands at approximately 3260 cm\(^{-1}\) (amide A, N–H stretching vibrations of NH\(_2\)), 2920 cm\(^{-1}\) (amide B, C–H stretching), 1640 cm\(^{-1}\) (amide I, C=O stretching), 1540 cm\(^{-1}\) (amide II, N–H bending), 1440 cm\(^{-1}\) (CH\(_2\) bending), and 1180 cm\(^{-1}\) (amide III, C–N and N–H Stretching) [52, 53]. The similar results on mammalian gelatins were also found by Hoseini et al [15], Dixit et al [6], and Sarmah et al [54]. In general, the result shows similar FTIR spectra for fish gelatin and fish gelatin nanoparticles but different intensity. Furthermore, the ratio of intensity of Amide I could be used to observe the loss of secondary structure and formation of random coil [55]. The expense of secondary structure and formation of random coil was related to the increasing of amide I intensity [55]. It is because of the preparation step for producing fish gelatin nanoparticles which used temperature of 45°C to dissolve the gelatin. The protein start to lost its triple structure as the temperature rich to 30°C [55]. TEM image (figure 10) shows that the fish gelatin nanoparticles are spherical and have a homogeneous size distribution in the range 24 to 80 nm.

### Table 5. Fitting of release kinetic model to 5-FU release data for FGNP (particle size 196 nm).

| Model                  | Parameters | \( R^2 \) adjusted | AIC      | MSC      |
|------------------------|------------|---------------------|----------|----------|
| Zero                   | \( k = 12.47 \pm 0.35 \) | 0.84 ± 0.01        | 32.13 ± 0.50 | 0.81 ± 0.03 |
| First                  | \( k = 0.19 ± 0.01 \) | 0.96 ± 0.00        | 24.96 ± 0.43 | 2.24 ± 0.08 |
| Higuchi                | \( k = 26.67 ± 0.76 \) | 0.98 ± 0.00        | 18.55 ± 0.80 | 3.53 ± 0.13 |
| Korsmeyer-Peppas       | \( k = 23.53 ± 0.97 \) | 0.98 ± 0.00        | 20.98 ± 0.75 | 3.04 ± 1.09 |

\( k \) is constant; \( n \) is release exponent Korsmeyer-Peppas model, \( R^2 \) is correlation coefficient, AIC is akaike information criterion, MSC is model selection criterion.

The size between FGNPs and 5-FU loaded FGNPs also varied. The size of FGNP after validation showed to be around 198.46 ± 6.1 nm, while 5-FU loaded FGNP was at 238.02 ± 7.4 nm. It can be concluded that the loading increases nanoparticle size. The size of drug-loaded FGNPs was 20% larger than unloaded FGNPs.

### 3.5. In vitro drug release kinetics

Figure 12 shows the release behavior of 5-FU from FGNPs in PBS at pH 7.4. It can be seen at the 5 h incubation, almost 50% of 5-FU was released to medium and after 12 h of incubation, 80% release was found. From the results, two stages of 5-FU release from FGNPs were observed. The first stage is rapid release where around 50% of the drug was released in the initial 5 h. The second stage is followed by the sustained release.

The rapid release is due to the nature of the fish gelatin. As a carrier, gelatin is highly hydrophilic thus it facilitates water uptake from release medium to matrix and results in a higher initial burst release. This condition have been described by Nahar et al [47] that the release behavior of drugs from particles depend on several factors, such as the size of particles, type of the polymer carrier, swelling characteristic of the particles, nature of crosslinking agent, and nature of the drug. Another cause of initial burst release is also because of the presence of the drug attached on the surface of particles [56, 57]. In vitro drug release from FGNP is expected to be similar to type A mammalian gelatin that has similar isoelectric points. According to previous papers, this finding is similar to mammalian gelatin nanoparticles releasing amphotericin and resveratrol through initial burst in PBS at pH 7.4 [57, 58].

This experiment also revealed that FGNP has similar release kinetics to mammalian gelatin. Mammalian gelatin nanoparticles follow the Fickian mechanism in releasing ibuprofen and 5-FU to medium release [8, 12]. As shown in table 5, the goodness of fit for the various models ranked in the order: Korsmeyer-Peppas \( \approx \) Higuchi > First-order > Zero-order . The value of exponent \( n \) from the Korsmeyer-Peppas model is around 0.5 indicating that the 5-FU release from FGNP follows the Fickian diffusion.

### 4. Conclusions

FGNPs are produced with particle sizes of 191–245 nm by the two-step desolvation method using acetone as a non-solvent and glutaraldehyde as a cross linker. Three factors
on production of FGNPs such as concentration of acetone, volume of glutaraldehyde and pH are found to have significant effects on the size of the gelatin nanoparticles. The effects of the significant factors have been evaluated. Increasing pH and acetone concentration leads to increase in the particles size, while increasing volume of glutaraldehyde decreases the size of FGNP. The optimum conditions of those factors are pH 2.45, acetone percentage 16% (vol%) and glutaraldehyde at 400 µl. FGNP then was produced using those optimum conditions and was loaded with the model drug. 5-FU was used as a model hydrophilic drug loaded into FGNP. The in vitro release kinetics of 5-FU was investigated. The release of 5-FU from FGNP followed the Korsmeyer-Peppas model kinetics with Fickian mechanism. Thus it can be concluded that FGNP presented a good alternative for the delivery of hydrophilic drugs such as 5-FU.

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References

[1] Yao C H, Liu B S, Hsu S H, Chen Y S and Tsai C C 2004 J. Biomed. Mater. Res. A 69A 709
[2] Balakrishnan B and Jayakrishnan A 2005 Biomater. 26 3941
[3] Weber C, Coester C, Kreuter J and Langer K 2000 Int. J. Pharm. 194 91
[4] Weber C, Reiss S and Langer K 2000 Int. J. Pharm. 211 67
[5] Coester C, Langer K, Weber C, von Briesen H and Kreuter J 2000 Eur. J. Pharm. Biopharm. 49 393
[6] Dixin N, Vaibhav K, Pandey R S, Jain U K, Katara O P, Katyal A and Madan J 2015 Biomed. Pharmacothe. 69 1
[7] Nahar M, Dubey V, Mishra D, Mishra P K, Dube A and Jain N K 2010 J. Drug. Target 18 95
[8] Nayarayan D et al 2013 Nanomedicine 9 818
[9] Han S, Li M, Liu X, Gao H and Wu Y 2013 Colloid. Surf. B 102 833
[10] Lu Z, Yeh T K, Tsai M, Au J L and Wintjes M G 2004 Clin. Cancer Res. 10 767
[11] Ding D, Wang J, Zhu Z, Li R, Wu L, Liu B and Jiang X 2012 ACS Appl. Mater. Interfaces 4 1838
[12] Naidu B V K and Paulson A T A 2011 J. Appl. Polym. Sci. 121 3495
[13] Sadowska M, Kolodziejka I and Niecikowska C 2003 Food Chem. 81 257
[14] Kasankala L, Xue Y, Yao W, Hong S and He Q 2007 Bioreosur. Technol. 98 3338
[15] Hosseini S F, Rezaei M, Zandi M and Farahmandghavi F 2016 Chemistry 194 1266
[16] Kwak H W, Shin M, Lee J Y, Yun H, Song D W, Yang Y, Shin B S, Park Y H and Lee K H 2017 Int. J. Biol. Macromol. 102 1092
[17] Lee E J and Lim K H 2017 Bioprocess Biosyst. Eng. 40 1701
[18] Komnareddy S and Amiji M 2005 Bioconjug. Chem. 16 1423
[19] Li Z and Gu L 2011 J. Agric. Food Chem. 59 4225
[20] Carvalho J A, Abreu A S, Ferreira V T P, Gonçalves E P, Tedesco A C, Pinto J G, Ferreira-Strixino J, Beltrame Junior M and Simioni A R 2018 J. Biomat. Sci. Polym. Ed. 5063 5063
[21] Elzoghby A O 2013 J. Control Release 172 1075
[22] Khan S A and Schneider M 2013 Macromol. Biosci. 13 455
[23] Bajpai A K and Choubey J 2006 J. Mater. Sci. Mater. Med. 17 345
[24] Gupta A K and Wells S 2004 IEEE Trans. Nanobiosc. 3 66
[25] Khan S A and Schneider M 2014 Macromol. Biosci. 14 1627
[26] Haug I J, Draget K I and Smidsrod O 2004 Food Hydrocolloids 18 203
[27] Muyonga J H, Cole C G B and Duodu K G 2004 Food Hydrocolloids 18 581
[28] Coester C, Langer K, von Briesen H and Kreuter J 2000 J. Microencapsul. 17 187
[29] Hafidz R and Yaakob C 2011 Int. Food. Res. J. 817 813
[30] Patra S, Basak P and Tibarewala D N 2016 Mater. Sci. Eng. C 59 310
[31] Arifin D Y, Lee L Y and Wang C H 2006 Adv. Drug Deliv. Rev. 58 1274
[32] Zhang Y, Mao H, Zhou J, Zou A, Li W, Yao C and Xie S 2010 AAPS J. 12 263
[33] Dash S, Murthy P N, Nath L and Chowdharly P 2010 Acta Pol. Pharm. 67 217
[34] Ahsan S M and Rao C M 2017 Int. J. Nanomed. 12 795
[35] Von Storp B, Engel A, Boeker A, Ploeger M and Langer K 2012 J. Microencapsul. 29 138
[36] Zhai X 2013 Gelatin nanoparticles and nanocapsules for dermal delivery PhD Thesis Freie Universität Berlin
[37] Abozeid S M, Hauthout R M and Abou-Aisha K 2016 Colloid. Surf. B 145 607
[38] de Oliveira C A, Darío M F, Sarruf F D, Mariz I F A, Velasco M V R, Rosado C and Baby A R 2016 Colloid. Surf. B 140 531
[39] Scopes R K 2013 Protein Purification: Principles and Practice (New York: Springer)
[40] Azarmia S, Huang Y, Chen H, Mabry D, Abramson B, Roed W, Löbenberga R, Azarmii S, Huang Y and Chen H 2006 J. Pharm. Sci. 9 124
[41] Schrieber R, Herbert G and Garries H 2007 Gelatine Handbook: Theory and Industrial Practice (Weinheim: Wiley-)
[42] Yang Y Y, Chung T S and Ping Ng N 2001 Biomaterials 22 231
[43] Rahman Z, Zidan A S, Habib M J and Khan M A 2010 Int. J. Pharm. 389 186
[44] Patel J, Dhingani A, Garala K, Raval M and Sheth N 2014 Bioconj. Chem. 5 125
[45] Nurlu A G and Sarbon N M 2015 Int. Food. Res. J. 22 572
[46] Geh K J, Hubert M and Winter G 2016 J. Microencapsul. 33 1
[47] Nahar M, Mishra D, Dubey V and Jain N K 2008 Nanomedicine 4 252
[48] Eslahi N, Dadashian F, Hemmati Nejad N and Rabiee M 2014 J. Appl. Polym. Sci. 131 11
[49] Urraite-Montoya M H, Arias-Moscoso J L, Plascencia-Jatomea M, Santacruz-Ortega H, Rouzaud-Sánchez O, Cardenas-Lopez J L, Marquez-Rios E and Ezquerra-Brauer J M 2010 Bioreosur. Technol. 101 4212
[50] Langmaier S, Mokrejs P, Kolomaznik K and Mladek M 2008 Thermochim. Acta 469 52
[51] Chen S, Tang L, Su W, Weng W, Osako K and Tanaka M 2015 Food Chem. 188 350
[52] Pelton J T and McLean L R 2000 Anal. Biochem. 277 167
[53] Kong J and Yu S 2007 Acta Biochim. Biophys. Sin. 39 549
[54] Sarmah M, Bamik N, Hussain A, Ramteke A, Sharma H K and Maji T K 2015 J. Mater. Sci. 50 7303
[55] Al-Saidi G S, Al-Alawi A, Rahman M S and Guizani N 2012 Int. Food. Res. J. 19 1167
[56] Ratner B D 2004 Biomaterials Science: An Introduction to Materials in Medicine (New York: Elsevier)
[57] Kumar R, Nagarwal R C, Dhanawat M and Pandit J K 2011 J. Biomed. Nanotechnol. 7 325
[58] Ali M A, Ali M F, Giliberto E, Greco E, Mello D and Viscuso E 2016 Appl. Phys. A 122 552