Understanding the significance variables for fabrication of fish gelatin nanoparticles by Plackett-Burman design

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Abstract. The aim of this experiment is to screen and to understand the process variables on the fabrication of fish gelatin nanoparticles by using quality-design approach. The most influencing process variables were screened by using Plackett-Burman design. Mean particles size, size distribution, and zeta potential were found in the range 240 ±9.76 nm, 0.3, and −9 mV, respectively. Statistical results explained that concentration of acetone, pH of solution during precipitation step and volume of cross linker had a most significant effect on particles size of fish gelatin nanoparticles. It was found that, time and chemical consuming is lower than previous research. This study revealed the potential of quality-by-design in understanding the effects of process variables on the fish gelatin nanoparticles production.

1. Introduction
Gelatin is an excellent materials for production nanoparticles that have been described well in the literature [1], [2]. Gelatin is obtained from hydrolysis of collagen. Furthermore gelatin considered as GRAS or generally regarded as safe by FDA (food drug administration) [3]. Gelatin is also the cheap materials because it’s abundant, readily available, high nutrition value, biocompatible, and biodegradable [3]. Due to the characteristic of gelatin, it has been successful encapsulated different type of drug, such as antibiotics [4], bioactive extract [5], and anti-cancer [6]. The origin gelatin in the market was from porcine skin and bovine bone. Because of that, almost gelatin nanoparticles have been produced from mammalian gelatin. However, the application of mammalian gelatin was not suitable for some group, such as Jews prefer Kosher and Muslim prefer Halal [7]. Alternative source such as fish gelatin nanoparticles would be fulfill the requirement of some groups.

Mainly, mammalian gelatin nanoparticles was produced by several method, such as emulsion [8], coacervation [9], self-assembly [10], and two-desolvation method [11]. In this experiment, two-step desolvation method was applied because this method could produce small size nanoparticles with narrow size distribution [3]. However, due to lower gelling point, lower gel strength, and lower melting point of fish gelatin [12], the preparation of fish gelatin nanoparticles will be different compare to mammalian gelatin nanoparticles. In order to fabricate the fish gelatin nanoparticles with small size, the fabrication variables need to be screen and optimize.

In two-step desolvation method, many formulation parameters affect the quality of gelatin nanoparticles. Less chemical consuming and short production time in the process production were
became the main purpose, and as well as the ability of the process production brings the product to commercialization. Because of that, quality by design (QbD) was initiated by FDA [13]. QbD requires better understanding on phenomenon in each process production in order to improve their performance. One of the common methods is design of experiment. Design of experiment was the precise technique to know the most significant variables in the pharmaceutical development. Jones et al., [14] explains that the design of experiment have been used to define the objective of investigation, decide the nature of variable and response, and had a minimum number of experimental run. Usually experimental design divides to two step, the first step to screen the significant factors (Plackett-Burman design or Factorial design), the second step to optimized the significant factors (usually by Central composite design or Box-Behnken) [15].

This research focused on the potential of fish gelatin in producing gelatin nanoparticles by two-desolvation method. Plackett-Burman design was applied to screen the effect of various process variables. The selected process variables were pH of solution, concentration of acetone, volume of glutaraldehyde, stirring speed, and stirring time. The effects of the variables on particles size were analyzed. The particles size distribution and shape of fish gelatin nanoparticles was also studied using Zeta sizer and FESEM.

2. Experimental procedures

2.1. Materials
Fish gelatins were fabricated by our research group using suggested method by Jamilah [12]. The fish gelatins were stored in the 4 °C fridge before further use. Others chemicals were purchased from Sigma Aldrich such as Acetone and glutaraldehyde grade 1 (25% vol% aqueous solution). Deionized waters (18.2 mΩ cm) were used trough experiment.

2.2. Preparation of fish gelatin nanoparticles
Fish gelatin nanoparticles were prepared by using two-step desolvation method that introduced by Coester [16]. This method divided on two steps; the first step is fractionation and the second step is precipitation step. Precipitation step was the main focus in this current experiment. About 0.9 g fish gelatin was dissolved in 10 ml deionized water with constant stirring (600 rpm) and constant heating (45 °C) until clear solutions were received. Fish gelatin solutions then were transferred to falcon tube and were added with 10 ml acetone. The mixed solutions were centrifuged for 5 min in constant speed (12000 g). The supernatants were immediately discarded and the precipitated were re-dissolved in 10 ml deionized water. The pH of precipitate solution was adjusted using 0.1 M HCl until reach certain number of suggested design. The solutions then were added with acetone and were cross-linked using glutaraldehyde solution. The solutions were stirred with specified hour. The fish nanoparticles colloids were centrifuged for 30 min at 14000 rpm. The precipititates were stored at fridge until further use.

2.3. Design of experiment
These experiments were focused on the screening of the significant variables that involve in precipitation step or in second step from two-step desolvation method. In this experiment experimental design (DOE) using Plackett-Burman design was applied. Plackett-Burman (PB) design was applied because less time consuming and less chemical consuming. Plackett-Burman design involved relatively less runs experiment (12 runs) to screen huge number of variables resulting critical and good degree of accuracy compared to factorial design [17]. PB matrix was generated by using Minitab® 16. The expression of linear equation was developed and shown in equation 1 below.

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + \cdots + b_nX_n
\]  

(1.0)

Where \( Y \) is the dependent variable or response, \( b_0 \) is the constant and \( b_1, b_2, \ldots, b_n \) are the coefficient of the variables \( X_1, X_2, X_3, X_4, X_5, \ldots, X_n \) (representing the effect of each factor ordered within -1, +1).
In this PB matrix were involved twelve experimental runs including five independent variables, one dummy variable and one dependent variable. Each experimental run was carried triplicate. Table 1 shows the list of the independent variables and table 2 describes experimental run matrix. Before experimental design, the chosen variables (five variables) and levels have been made base on previous researcher and preliminary research [18]–[25]. The selected independent variables were pH of the fish gelatin solution ($X_1$), acetone concentration ($X_2$), volume of glutaraldehyde ($X_3$), stirring speed ($X_4$), and stirring time ($X_5$). One dependent variable was fish gelatin nanoparticles size. The results were evaluated by statistical analysis using Analysis of variance (ANOVA). Means variable plot were also establish to analyzed the most significant variables.

**Table 1.** Experimental variables and level of Plackett-Burman design.

| Variables                  | Symbol | Units | Experimental value |
|----------------------------|--------|-------|--------------------|
| pH                         | $X_1$  | 1.5   | 5.5                |
| Percentage of Acetone      | $X_2$  | % (v/v)| 15                 |
| Volume of Glutaraldehyde   | $X_3$  | µl    | 100                |
| Stirring Speed             | $X_4$  | rpm   | 300                |
| Stirring Time              | $X_5$  | hour  | 3                  |

2.4. *Particles size measurement and FESEM measurement*

The fish gelatin nanoparticles size was measured by dynamic light scattering (DLS) at 90° in 10 mm diameter cells with a Malvern (Zen3600, UK). About 300 µl nanoparticle colloid was mixed to 30 ml of deionized water. The mixtures were sonicated at 60 Amp for 20 min. The size and particles distribution were analyzed. The polydispersity index (PdI) is ranging from 0.0 to 1.0. The surface charge of nanoparticle was measured using Zeta potential Malvern.

Shape, size, and surface morphology of fish gelatin nanoparticles were visualize using Field Emission Scanning Electron Microscope (FESEM) (JEOL, JSM 6700F Model) at an accelerating voltage of 10 kV. Prior to visualization, fish gelatin nanoparticles were mounted on small adapter and was sputter with a thin layer of gold. The adapter was then placed in the chamber.

3. **Results and Discussion**

3.1. *Impact of fabrication parameters on fish gelatin nanoparticles size*

Table 2 depicts the experimental results of twelve run from five independent variables. The statistical analysis were applied to this results in order to quantify the most influence significant variables on the fabrication fish gelatin nanoparticles by using Minitab® 16 software. The behavior of the individual variables to particles size were also analyzed by alternatively treated at the highest and lowest level. While others variables were kept constant at center levels.

Figure 1 shows the plot of main effect of each variable on the particles size. This figure not only depicts the effect of respective processing variables, but also shows the positive or negative trend of each variable in studied range. It can be seen that pH of solution and percentage of acetone had significant positive effects, while volume of glutaraldehyde had a significant negative effect on the particles size. This statement describes that small size fish nanoparticles could be produced at low pH solution, low percentage of acetone and huge volume of glutaraldehyde. This result agrees with previous works [19]–[21], that small size gelatin nanoparticles were produced bellow isoelectric point, low concentration of acetone and high volume of glutaraldehyde. It is because isoelectric point of fish gelatin had same range of with mammalian gelatins that have been used at previous work. The isoelectric point of fish gelatin is around pH 6-9 [12], [26].
Furthermore, we found fish gelatin nanoparticles could be produced below pH 3.5 while optimum pH to produce mammalian gelatin was at pH 3.5. Producing fish gelatin nanoparticles within isoelectric point would produce large-scale of gelatin nanoparticles, due to the electrostatic attraction [21]. Decreasing the pH solution leads to protonate the side chain amino acid and create the stable suspension [21]. High concentration of acetone creates large aggregate [19]. Moreover, high concentration of glutaraldehyde forced the particles become harder [27]. In other hand, short or long time stirring of gelatin solution had insignificant effect for production small nanoparticles.

| Run | pH (X₁) | Concentration of Acetone (% vol v/v) (X₂) | Volume of Glutaraldehyde (µl) (X₃) | Stirring Speed (rpm) (X₄) | Stirring Time (hours) (X₅) | Observed Particles size (nm) | Predicted Particles size |
|-----|---------|---------------------------------|---------------------------------|-----------------|-----------------|--------------------------|-------------------------|
| 1   | 5.5     | 15                              | 600                             | 300             | 3               | 275.72±0.07              | 274.54                  |
| 2   | 5.5     | 65                              | 100                             | 900             | 3               | 850.45±6.57              | 722.38                  |
| 3   | 1.5     | 65                              | 600                             | 300             | 21              | 244.49±2.05              | 264.16                  |
| 4   | 5.5     | 15                              | 600                             | 900             | 3               | 245.88±4.09              | 280.12                  |
| 5   | 5.5     | 65                              | 100                             | 900             | 21              | 882.13±3.85              | 708.95                  |
| 6   | 5.5     | 65                              | 600                             | 300             | 21              | 518.19±6.46              | 364.52                  |
| 7   | 1.5     | 65                              | 600                             | 900             | 3               | 227.85±1.07              | 283.18                  |
| 8   | 1.5     | 15                              | 600                             | 900             | 21              | 205.25±5.60              | 166.34                  |
| 9   | 1.5     | 15                              | 100                             | 900             | 21              | 191.84±3.16              | 182.09                  |
| 10  | 5.5     | 15                              | 100                             | 300             | 21              | 233.04±2.10              | 276.86                  |
| 11  | 1.5     | 65                              | 100                             | 300             | 3               | 267.34±2.76              | 293.35                  |
| 12  | 1.5     | 15                              | 100                             | 300             | 3               | 204.71±0.72              | 189.93                  |

Table 2. Plackett-Burman design matrix.

![Variables Plot for Particles size](image)

Figure 1. Main effect of variables in Plackett-Burman design of experiment on particles size.
3.2. Statistical analysis of Plackett-Burman design

In this experiment, we performed analysis of variance (ANOVA). The statistical analysis with ANOVA would produce the significance of models and regression coefficient in Plackett-Burman design. This experiment carried out by using 95% of confidence level. Means, the significance variables will be calculated based on $p$ value at 0.05. The variable would be considered as insignificant variables when $p$ value are higher than 0.05. The ANOVA results agree that pH of the solution, concentration of acetone, and volume of glutaraldehyde are the effective variable on producing small size nanoparticles, because they have lower $p$ value than 0.05. The $p$ value of model was obtained to be low value, this result representing that the regression model was also significant with a 95% confidence level. The R square of the results was at 0.86, means good correlation between observed particles size and predicted particles size. This R square also depicts that this model could explain almost 86% of the response variation. However, the $p$ value of curvature was found to be higher than 0.05. That means, the levels of variables is in slope area and need to be changed in order to choose the correct level before optimization.

The polynomial equation between particles size and independent variables was given below.

$$Y = -34.98 + 52.01X_1 + 4.22X_2 - 0.25X_3 + 0.19X_4 - 0.75X_5 - 53.84X_6$$  \hspace{1cm} (3.0)

3.3. Characterization of prepared fish gelatin nanoparticles

The fish gelatin nanoparticle was fabricated using suggested variables such as pH at 1.5, percentage of acetone at 15%, and volume of glutaraldehyde at 600 µl, briefly, while other insignificant variables were treated in the center point. Figure 2 shows the particles size distribution from zeta-sizer analysis. It can be seen that, the average particles size of fish gelatin nanoparticles was about 240±9.76 nm, and the polydispersity index was at 0.3. This fish gelatin nanoparticle had slightly difference in particles size compared with mammalian gelatin nanoparticles was produced by Kumari around 110-257 nm [1]. The morphology of fish gelatin nanoparticles was quantified using FESEM (Figure 2b). FESEM result shows fish gelatin nanoparticles had a round shape. However, some variation in size distribution was depicted in the FESEM images. This phenomenon due to the uncontrolled effect of neutralization the molecule charge during specific pH [19], [28].

![Particles size distribution of fish gelatin nanoparticles (a) and FESEM images of fish gelatin nanoparticles (b).](image)

4. Conclusion

Fish gelatin nanoparticle was successfully developed using two-step desolvation method. Plackett-Burman quality by design approach was used to screen the independent variables and to understand the effect of most significant factors for production of fish gelatin nanoparticles. From the
Plackett-Burman results, three variables such as pH solution, percentage of acetone, and volume glutaraldehyde were found to have significant effects. While other variables like stirring time and stirring speed were insignificant on response. This results means by this method, we can reduce time and energy consuming in producing fish gelatin nanoparticles. To be concluded Plackett-Burman design is an efficient method for screening the most significant factors with minimum experimental run. Additionally, studies will continue to further optimization on production of fish gelatin nanoparticles.

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