OPTIMIZATION OF THE PROCESS EXTRACTION AND DRUG RELEASE OF FISH GELATIN NANOPARTICLES DERIVED FROM TILAPIA (*OREOCHROMIS SPP)*

BY

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ABSTRACT

Gelatin is one of the popular biopolymers used for food and pharmaceutical applications, given its capability to dissolve in aqueous environments and to form nanoparticles that enable the encapsulation of various active agents into stable products. On the other hand, coupled with these capabilities and recognition by the US Food and Drug Administration (FDA) authority, as Generally Recognized as Safe (GRAS) material, it has attracted growing interest and attention from researchers to produce gelatin nanoparticles toward encapsulating various food and pharmaceutical molecules. However, even though gelatin can be produced from mammalian and fish, it is quite challenging since all sources of gelatin nanoparticles in the market originate from mammalian gelatin, which is either bovine or porcine. Although the use of such gelatin as highlighted by Muslims, Jews, and other religious backgrounds is an issue. Furthermore, there are no publications regarding gelatin nanoparticles from fish gelatin published at this stage, which further adds to this problem. Therefore, this study aims to prepare and characterize fish gelatin nanoparticles (FGNPs) and FGNPs encapsulated with an active agent. Fish gelatin was first extracted from Tilapia fish skin employing a two-step desolvation method in producing FGNPs. The initial first desolvation step was optimized to obtain consistent high molecular weight at the gelatin concentration, temperature, centrifugation speed, and centrifugation time of 9%, 45 °C, 12000 xg, and 5 min, respectively. As an outcome from this work, a new method to produce significant FGNP properties consistently was created based on this step. A second desolvation step adopting the two-step desolvation method was also optimized in which significant factors were screened using Plackett-Burman experimental design, determining that the pH, acetone percentage, and glutaraldehyde volume were the significant factors. These factors were then optimized using factorial design, indicating that FGNPs with a size of 198.46 ± 6.1 nm were produced using pH, acetone concentration, and a glutaraldehyde volume of 2.45, 16%, and 400 µl, respectively. Indeed, increasing pH and acetone concentration led to an increase in the size of particles, whereas increasing the volume of glutaraldehyde decreased the size of FGNPs. Accordingly, this work makes a valuable contribution by developing an optimized production process, thereby demonstrating the potential for the future application of FGNPs. This study has also shown that fish gelatin could be used as an alternative for mammalian gelatin for producing nanoparticles. Here, the production process of drug-loaded FGNPs was optimized in which the significant factors were screened using factorial design for encapsulation efficiency. The drug amount was also found to have a significant effect, whereas pH, acetone percentage, and the glutaraldehyde amount with stirring time was not significant concerning the encapsulation efficiency of drug-loaded FGNPs. Notably, increasing the drug amount increased encapsulation of drug-loaded FGNPs in which an encapsulation efficiency of 39%, was observed. The physicochemical characterization of the optimum formulation, as suggested, was also examined using a Scanning electron microscope (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) showing FGNPs having a smooth surface. Fourier-transform infrared diffraction (FTIR) revealed the presence of the drug in tailored FGNPs, and Powder X-ray diffraction (XRD) analysis highlighted the formation of amorphously dispersed systems having a slightly faster release profile of drug-loaded FGNPs with a biphasic

release profile. The release mechanism followed non-fickian diffusion, meaning that the release of the drug from FGNPs was governed by diffusion and erosion of the matrix.

خلاصة البحث

الجيلاتين هو أحد البوليمرات الحيوية المنتشر استعمالها في الاستخدامات الغذائية والصيدلانية. قدرتها على الذوبان في البيئات المائية وقابليتها لتشكيل الجسيمات النانوية تمكنها من تغليف العوامل النشطة المختلفة في شكل منتجات مستقرة. من ناحية أخرى، إلى جانب هذه القدرات فقد زاد الاعتراف بها من قبل إدارة الغذاء والدواء الأمريكية كمواد آمنة من اهتمام الباحثين في إنتاج جزيئات نانوية للجيلاتين وتغليف الجزيئات الغذائية والصيدلانية. وعلى الرغم من قدرة الثدييات والأسماك على إنتاج الجيلاتين، فإنه من السلبيات كون جميع مصادر الجزيئات النانوية الجيلاتينية في السوق هي جيلاتين الثدييات إما بقرية أو خنزيرية، حيث يعتبر استخدامها أحد المشاكل الرئيسية التي يواجهها المسلمون واليهود وغيرهم من الخلفيات الدينية. علاوة على ذلك فإن المؤلفات العلمية عن الجسيمات النانوية الجيلاتينية من جيلاتين الأسماك غير موجودة حتى الآن. ولذلك فإن الهدف من هذه الدراسة هو إعداد وتوصيف الجزيئات النانوية الجيلاتينية السمكية وتغليف عامل نشط بها. تم استخراج جيلاتين السمك من جلد أسماك البلطي، حيث تم استخدام طريقة التذويب ذي الخطوتين لإنتاج الجزيئات النانوية الجيلاتينية السمكية. تم تحسين خطوة التذويب الأولى للحصول على وزن جزيئي مرتفع وثابت. وجد أنه بالإمكان إنتاج جيلاتين ثابت ذو وزن جزيئي مرتفع على تركيز 9%، ودرجة حرارة 45 درجة مئوية، وطرد مركزي بسرعة 12000 دورة في الدقيقة ولمدة 5 دقائق. وبهذا تم تطوير طريقة جديدة لإنتاج جزيئات نانوية جيلاتينية سمكية بتجانس عال بناء على خطوة التحسين الأولى. للمزيد من التحسين تم أيضًا تحسين خطوة التذويب الثانية لطريقة التذويب ذي الخطوتين. تم مسح العوامل المهمة باستخدام تصميم بلاكيت-بورمان، وقد تم اكتشاف أن العوامل الهامة كانت الحموضة ونسبة الأسيتون وحجم الجلوتارالدهيد. ثم تم تحسين العوامل المهمة باستخدام التصميم العاملي. وجد أن الجزيئات النانوية الجيلاتينية السمكية بحجم 198.46±6.1 نانومتر تم إنتاجها على حموضة 2.45 وتركيز أسيتون 16% وحجم الجلوتارالدهيد على 400 ميكرولتر. أدت زيادة الحموضة وتركيز الأسيتون إلى زيادة حجم الجزيئات، بينما أدت زيادة حجم الجلوتارالدهيد إلى تقليل حجم الجزيئات. قدم هذا العمل عملية الإنتاج المحسنة، مما يوفر الإمكانيات الواضحة للتطبيقات المستقبلية للجزيئات النانوية الجيلاتينية السمكية. تم الكشف أيضا أن جيلاتين السمك بإمكانه أن يكون بديلا لجيلاتين الثدييات لإنتاج الجزيئات النانوية، كما تم تحسين عملية إنتاج الجزيئات النانوية الجيلاتينية السمكية المحملة بالأدوية. تم مسح العوامل الهامة لكفاءة التغليف باستخدام التصميم العاملي، وقد وجد أن كمية الدواء فقط لها تأثير كبير في حين أن معدل الحموضة وتركيز الأسيتون وكمية الجلوتارالدهيد ووقت التحريك لم تكن ذات أهمية لكفاءة الأدوية المغلفة بالجزيئات النانوية الجيلاتينية السمكية. زيادة كمية الدواء زادت أيضا تغليف الجزيئات النانوية الجيلاتينية السمكية المحملة بالأدوية. تم ملاحظة أن كفاءة التغليف كانت بنسبة 39 ٪. للجزيئات النانوية الجيلاتينية السمكية المحملة بالأدوية. تمت دراسة التوصيف الفيزيائي والكيميائي للصيغة المقترحة المثلى. أظهر مجهر المسح الإلكتروني، والمجهر الإلكتروني النافذ، ومجهر القوة الذرية أن سطح الجزيئات النانوية الجيلاتينية السمكية كان أملسا. كشف تحليل مطياف فورييه عن

وجود عقار في الجزيئات النانوية الجيلاتينية السمكية المصممة. أظهر تحليل حيود الأشعة السينية بالمسحوق تشكل أنظمة مشتتة بشكل غير متبلور. تمت ملاحظة سلوك إطلاق أسرع بقليل في الجزيئات النانوية الجيلاتينية السمكية المحملة بالأدوية مع سلوك إطلاق ثنائي الطور. اتبعت آلية الإطلاق الانتشار غير الفيكياني، مما يعني أن إطلاق الدواء من الجزيئات النانوية الجيلاتينية السمكية كان محكومًا بانتشار وتآكل المصفوفة.

APPROVAL PAGE

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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All praise and thanks be to Allah, the lord of the worlds. May the peace and blessings of Allah be upon Prophet Muhammad, his family and his companions. Thank to Almighty Allah for giving me strength and the ability to complete this research work.

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LIST OF ABBREVIATIONS

A	absorbance
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
AFM	Atomic Force Microscopy
BSE	Bovine Spongiform Encephalopathy
BSA	Bovine Serum Albumin
°C	degree(s) Celcius
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimeter
DL	Drug Loading
dW	distilled water
EE	Encapsulation Efficiency
FDA	Food and Drug Agency
FE-SEM	Field Emission Scanning Electron Microscope
FGNPs	Fish Gelatin Nanoparticles
FTIR	Fourier Transform Infra-Red
Fig	Figure
GA	Glutaraldehyde
GPNs	Gelatin Nanoparticles
GRAS	Generally Regarded As Safe
HMW	High Molecular Weigh
HPLC	High Performance Liquid Chromatography
IIUM	International Islamic University Malaysia
IR	Infra-Red
kDa	kilo Dalton
K_p	release constant
LMW	Low Molecular Weigh
M	Molar
MW	Molecular Weight
NPs	Nanoparticles
PBS	Phosphate Buffer Saline
PCS	Photon Correlation Spectroscopy
PdI	Poly dispersity Index
RSM	Response Surface Methodology
RT	Retention Time
SA	Salicylic Acid
SD	Standard Deviation
SEM	Scanning Electron Microscope
t	time
T	Temperature
TEM	Transmission Electron Microscopy
TGA	Thermal Gravimetric Analysis
USA	United Stated of America
UV	Ultraviolet
W	Weight

W/O	Water-in-oil
XRD	X-ray Diffraction

LIST OF SYMBOLS

%	percentage
µg	microgram
µl	microliter
µmol	micromole
µg/ml	microgram per mililiter
M	molarity
g	gram
g/g	gram per gram
g/l	gram per liter
h	hour
l	liter
ml	milliliter
°C	Degree Celsius (temperature)
v/v	volume per volume
w/v	weight per volume
rpm	rotations per minute

CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

The use of fishery waste such as fish skin as the source of gelatin has been associated with many advantages. In particular, one of its potentials lies in the properties of biodegradability and low toxicity, thus representing itself as a good raw material (Elgadir et al., 2013; Ismail & Abdullah, 2016). More importantly, its sourcing and collection from fishery wastes are easy; for example, Malaysia's frozen tilapia fillet exports to the European Union were increased by 13% in 2014, followed by Indonesia and Vietnam (Fish, 2015).

Gelatin is a polymer material produced from hydrolyzed collagen, which is extracted mostly from bovine bone, pigskin, and fish skin. Previous studies have thus far been limited towards the use of mammalian gelatin for the production of gelatin nanoparticles and drug encapsulation. For examples, bovine gelatin has been incorporated to encapsulate metformin in treating bone defects (Shahrezaee et al., 2018), while studies on porcine gelatin have been conducted for anti-microbial (Kirar, Thakur et al., 2018) and anti-tubercular licorice extract encapsulation (Viswanathan et al., 2018). However, no report has been released till date regarding the production of gelatin nanoparticles made of fish gelatin.

It should be noted that the usage of materials of mammalian origin in drugs and food products is limited due to religious and ethical reasons. Beyond the forbidden consumption of porcine gelatin among Jewish and Muslim groups (Sadowska etal., 2003), the emergence of Bovine Spongiform Encephalopathy (BSE)

in Europe decade ago has raised several questions concerning the use of pig gelatin for relevant applications (Pang et al., 2017). Under such circumstances, materials of fish origin serve as an alternative for producing gelatin nanoparticles. Furthermore, fish-based gelatin is rarely employed to produce gelatin nanoparticles, wherein it is geared for the production of gelatin film (Hosseini et al., 2016), and nanofibre (Kwak et al., 2017) till date. As such, it is advisable to carry out experimentation for the purpose of gelatin nanoparticles manufacturing from fish gelatin and further optimize the production process.

In general, several methods have been employed for gelatin nanoparticle production, such as emulsion (E. J. Lee & Lim, 2017), coarcevation (Patra et al., 2016), self-assembly (Z. Li & Gu, 2011), and desolvation (Carvalho et al., 2018). Among these approaches, the desolvation method has been found to offer small particle sizes and narrow size distribution (Sahoo et al., 2015). The production of this material is typically dependent on the factors chosen, wherein their selection is guided by the gelatin characteristics itself, which are also dictated by the resources (Ingvild J. Haug et al., 2004). Therefore, one can expect differences between gelatin made from mammalian and fish sources accordingly. For example, gelatin is specifically characterized by its triple helical structure. Moreover, its proline and hydroxyproline contents differ accordingly; mammalian sources account for 30%, while warm-water fishes such as tilapia and Nile perch log 22-25% (Muyonga et al., 2004). As a result of these differences, fish-based gelatin generates a lower gel modulus and melting temperature in comparison with its mammalian counterpart. Thus, the conditions required for fish gelatin nanoparticle production may vary to that of mammalian gelatin nanoparticle.

Accordingly, the current research aims to investigate the use of fish gelatin extracted from local fish waste (i.e. fish skin) as the main source for nanoparticle production. To this end, the two-step desolvation method is adopted and the optimized factor is selected accordingly. The process includes a look into drug encapsulation using fish gelatin nanoparticles, following which statistical methods are employed to determine the optimum processing conditions for both fish gelatin nanoparticles and drug-loaded fish gelatin nanoparticles. Then, the characteristics of both nanoparticle types are analyzed.

1.2 PROBLEM STATEMENT AND SIGNIFICANCE OF STUDY

In general, the currently employed raw materials for gelatin production are primarily sourced from mammalian skins and bones, which are either bovine or porcine in nature. According to different ethical, religious, and health reasons, the use of such gelatin in various food and non-food products are some of the main issues highlighted by Muslims, Jews, vegetarians, and people of other religious backgrounds. Hence, the demand for fish gelatin is now on the increase, which is further supplemented by aquaculture being identified as a means for increasing fish production in Malaysia and the huge resources invested for tilapia production. In this regard, fish skin is a major and expansive by-product of the domestic fish-processing industry that has systemically caused wastage and pollution, which may thus provide the sought-after source of gelatin. Furthermore, fish gelatin offers the advantages of cheap cost and easy availability. However, no report has been presented regarding the production of gelatin nanoparticles made from tilapia fish skin thus far. Hence, this effort is highly significant due to the sustainable raw material that is readily accessible and towards fortifying the Halal industry in Malaysia.

The two-step desolvation method offers a straightforward process and produces nanoparticles of the desired size (Geh et al., 2016; S.A. Khan & Schneider, 2013). Therefore, it is chosen for the production of fish gelatin nanoparticles in this study, wherein the first step of desolvation allows the removal of low molecular weight gelatin. This will allow the remaining high molecular weight gelatin to form dense and small-sized particles after cross-linking occurs (Azarmi et al., 2006; Sahoo et al., 2015). However, the experimental session is done in batch to batch dependent on each other, which will result in high molecular weight content heterogeneities after cleaving off the low molecular weight given the gelatin's tendency nanoparticles to form un-uniformly. Collectively, these findings indicate that designing improved methods for a consistently high molecular weight gelatin obtainment is sorely needed.

Furthermore, fish and mammalian gelatin both typically have the same characteristics. However, the content of imino acid in fish gelatin is less compared to mammalian gelatin, which results in its lower gelling and melting temperatures (Ingvild J. Haug et al., 2004; Muyonga et al., 2004). Moreover, previous works of relation have only focused on optimizing the process production for the second step of the two-step desolvation method in mammalian gelatin nanoparticle manufacturing. Hence, this reflects the present need for research efforts emphasizing an optimized process of fish gelatin nanoparticle production.

Besides, it is known that a successful nanoparticle system should possess a high loading capacity to reduce the amount of carrier required for administrative purposes. The benefits of fish gelatin usage for gelatin nanoparticle production by using an optimized process is undeniable. The material is associated with perks such as reduced waste from frozen fillet production, increased economic value of waste