UNIVERSITI PUTRA MALAYSIA

SYNTHESIS AND OPTIMIZATION OF PALMITOYL-CHITOSAN NANOPARTICLES AS A POTENT DUAL ANTIOXIDANT CARRIER WITH HYDROPHOBIC AND HYDROPHILIC COMPOUNDS

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By

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September 2022

Chair : Associate Professor Siti Nurul Ain binti Md. Jamil, PhD
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Chitosan nanoparticles (CNP) is a potent delivery vector for hydrophilic pharmaceuticals as it is biodegradable and biocompatible. In this study, it was synthesized by a non-toxic and simple ionic gelation method that successfully produced cationic, monodispersed spherical particles with an average size of 60.5 ± 2.1 nm and a polydispersity index (PDI) of 0.19 ± 0.02. The carrier was dual encapsulated with antioxidants to diversify CNP ability as it is normally being used for single encapsulation. Hydrophobic thymoquinone (TQ) and hydrophilic L-ascorbic acid (LAA) were incorporated into the carrier to evaluate their combination effects in scavenging free radicals when delivered using a nanoparticle (NP) system. However, the encapsulation of hydrophobic TQ was marred by the hydrophilicity of chitosan (CS). Thus, palmitic acid was conjugated on the CS through hydrophobic modification, which produced palmitoyl-chitosan nanoparticles (PCNP). The amphiphilic PCNP encapsulated with TQ and LAA showed increased size, 247.7 ± 24.0 nm and PDI, 0.35 ± 0.04. It also had a positive zeta potential of 19.60 ± 1.27 mV, which would make good interactions with negatively charged cell membranes during antioxidants delivery. The encapsulation efficiencies (EE) of TQ and LAA increased to 64.9 ± 5.3% and 90.0 ± 0.0%, respectively. The antioxidants followed zero order release kinetics with a controlled release manner for about 48 h. Interaction effect between TQ and LAA loaded in the NP system was determined by classical isobologram (CI) values derived from diphenyl picrylhydrazyl (DPPH) assay. Combined TQ and LAA had CI values of less than one with lower value in the PCNP system than CNP. This indicates that the interaction between those antioxidants showed higher synergistic effects in PCNP, which enhanced DPPH radical scavenging activities. Additionally, reactive oxygen species (ROS) assay was experimented on human normal lung fibroblast cell line, MRC-5 as lungs is one of the organs with high accumulation of free radicals. About 48 h post treatment, the dual loaded TQ and LAA in PCNP showed the lowest ROS level in comparison to single loaded antioxidant and bare antioxidant delivery. The H_2O_2 radical

scavenging was influenced by both controlled release property of the PCNP system and synergy between TQ and LAA. In short, the dual loaded TQ and LAA in the hydrophobically modified PCNP had successfully depicted the capability to hold more than one compound at a time in a single CS based nanocarrier. With this advancement, more highly efficacious compounds of different polarities with poor systemic uptake could be encapsulated together in NP systems to increase their pharmaceutical efficiency.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

SINTESIS DAN PENGOPTIMUMAN NANOPARTIKEL PALMITOIL-KITOSAN SEBAGAI DWIPEMBAWA ANTIOKSIDAN YANG BERPOTENSI TINGGI DENGAN SEBATIAN HIDROFOBIK DAN HIDROFILIK

Oleh

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Nanopartikel kitosan (CNP) ialah vektor yang berpotensi untuk penghantaran farmaseutikal hidrofilik kerana ia boleh terbiodegradasi dan biokompatibel. Dalam kajian ini, ia telah disintesis dengan kaedah pengegelan ionik yang tidak toksik dan ringkas yang berjaya menghasilkan zarah sfera bersifat kationik, monodispersi dengan saiz purata 60.5 ± 2.1 nm dan indeks polidispersiti (PDI) 0.19 ± 0.02. Pembawa itu didwikapsul dengan antioksidan untuk mempelbagaikan keupayaan CNP kerana ia biasanya digunakan untuk pengkapsulan tunggal. Hidrofobik timokuinon (TQ) dan hidrofilik L-asid askorbik (LAA) telah dimasukkan ke dalam pembawa untuk menilai kesan gabungan mereka dalam sistem nanozarah (NP). Walau bagaimanapun, keberkesanan enkapsulasi hidrofobik TQ berkurang disebabkan oleh kitosan (CS) yang bersifat hidrofilik. Oleh itu, asid palmitik telah dikonjugasi pada CS melalui pengubahsuaian hidrofobik, yang menghasilkan nanopartikel palmitoil-kitosan (PCNP). PCNP amfifilik yang dikapsulkan dengan TQ dan LAA menunjukkan peningkatan saiz, 247.7 ± 24.0 nm dan PDI, 0.35 ± 0.04. Ia juga mempunyai potensi zeta positif sebanyak 19.60 ± 1.27 mV, yang akan membuat interaksi yang baik dengan membran sel bercas negatif semasa penghantaran kargo. Kecekapan enkapsulasi (EE) TQ dan LAA masing-masing meningkat kepada 64.9 ± 5.3% dan 90.0 ± 0.0%. Pelepasan antioksidan tersebut mengikuti kinetik pelepasan sifar dengan cara pelepasan terkawal selama kira-kira 48 jam. Kesan interaksi antara TQ dan LAA yang dimuatkan dalam sistem NP ditentukan oleh nilai *classical isobologram* (CI) yang diperoleh daripada ujian diphenyl picrylhydrazyl (DPPH). Gabungan TQ dan LAA mempunyai nilai CI kurang daripada satu dengan nilai yang lebih rendah dalam sistem PCNP daripada CNP. Ini menunjukkan bahawa interaksi antara antioksidan tersebut menunjukkan kesan sinergistik yang lebih tinggi dalam PCNP, yang meningkatkan aktiviti penghapusan radikal DPPH. Di samping itu, ujian spesies oksigen reaktif (ROS) telah dieksperimenkan pada sel fibroblas paru-paru manusia, MRC-5 kerana paru-paru adalah salah satu organ yang mempunyai

pengumpulan radikal bebas yang tinggi. Kira-kira 48 jam selepas rawatan, TQ dan LAA yang telah didwikapsulkan dalam PCNP menunjukkan tahap ROS yang paling rendah berbanding dengan penghantaran antioksidan yang dikapsulkan secara tunggal dan antioksidan tanpa pengkapsulan. Penghapusan radikal H_2O_2 dipengaruhi oleh pelepasan terkawal sistem PCNP dan sinergi antara TQ dan LAA. Ringkasnya, TQ dan LAA yang didwikapsulkan dalam PCNP yang diubah suai secara hidrofobik telah berjaya menggambarkan keupayaan untuk memegang lebih daripada satu sebatian pada satu masa dalam satu pembawa nano berasaskan CS. Dengan kemajuan ini, lebih banyak lagi sebatian yang sangat mujarab yang terdiri daripada kekutuban berbeza tetapi mempunyai penyerapan sistemik yang lemah boleh dikapsulkan bersama dalam sistem NP untuk meningkatkan kecekapan farmaseutikal mereka.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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A549	Adenocarcinomic human alveolar basal epithelial cell
ABTS	2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)
ACA	Anthranilic acid
AFM	Atomic force microscopy
AIDS	Aquired immunodeficiency syndrome
$Asc^{-\bullet}$	Vitamin C radical
ATCC	American Tissue Culture Collection
ATR	Attenuated total reflectance
BBB	Blood brain barrier
BPA	Bisphenol-A
CGA	Chlorogenic acid
CGM	Complete growth media
CI	Classical isobologram
CLZ	Clotrimazole
CM-H_2DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
CNP	Chitosan nanoparticles
CNP-LAA	L-ascorbic acid encapsulated chitosan nanoparticles
CNP-TQ	Thymoquinone encapsulated chitosan nanoparticles
CNP-TQ-LAA	Thymoquinone and L-ascorbic acid encapsulated palmitoyl-chitosan nanoparticles
COVID-19	Coronavirus disease
CS	Chitosan

CS MS	Chitosan master solution
CS WS	Chitosan working solution
DCF	Dichlorofluorescein
DDA	Degree of deacetylation
DFO	Deferoxamine mesylate
DLS	Dynamic light scattering
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	Diphenyl picrylhydrazine
DS	Diclofenac sodium
DX	Dexamethasone
EC_{50}	Half maximal effective concentration
EE	Encapsulation efficiency
EFV	Efavirenz
FBS	Fetal bovine serum
FESEM	Field-emission scanning electron microscopy
FITC	Fluorescein-5-isothiocyanate
FRAP	Ferric-reducing ability of plasma
FTIR	Fourier Transform Infrared
GSH	Glutathione
H_2DCF	Dicholorodihydrofluorescein
H_2DCFDA	2',7'-dicholorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid

HIV	Human immunodeficiency virus
HRTEM	High-resolution transmission electron microscopy
L929	Normal fibroblast cell line
LAA	L-ascorbic acid
MRC-5	Human normal lung fibroblast cell line
mRNA	Messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCs	Nanocapsules
NDDSs	Nano-drug delivery systems
NHS	N-hydrosuccinimide
NO	Nitric oxide
NP	Nanoparticles
$O_2^{\bullet -}$	Superoxide
OH•	Hydroxyl radical
$ONOO^-$	Peroxynitrate
ORAC	Oxygen radical absorption capacity
PBS	Phosphate buffered saline
PCA	Protocatechuic acid
PCL	Polycaprolactone
PCNP	Palmitoyl-chitosan nanoparticles
PCNP-LAA	LAA encapsulated palmitoyl-chitosan nanoparticles
PCNP-TQ	TQ encapsulated palmitoyl-chitosan nanoparticles

PCNP-TQ-LAA	Thymoquinone and L-ascorbic acid encapsulated palmitoyl-chitosan nanoparticles
PCS	Palmitoyl-chitosan
PDI	Polydispersity index
PEG	Polyethylene glycol
PLA	Polylactic acid
PLGA	Polymer poly (lactic-co-glycolic acid)
PSD	Particle size distribution
PTZ	Pentylenetetrazole
QD	Quantum dots
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute-1640
SARS-CoV-2	Coronavirus
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TNBS	2,4,6-Trinitrobenzenesulfonic acid
$TO^{\bullet}$	Vitamin E radical
TOH	Vitamin E
TPP	Tripolyphosphate
TPP WS	Tripolyphosphate working solution
TQ	Thymoquinone
TR146	Buccal carcinoma cell line

TRPM2	Transient receptor potential melastatin 2
WS	Working solution

CHAPTER 1

INTRODUCTION

1.1 Research background and outline

Multicompound supplementation involves intake of more than one compound to supplement certain vitamins and minerals that are not gained through diets, emending nutritional deficiencies (Colombo et al., 2020; Gheorghiade & Nodari, 2013). It could be the consumption of vitamin C with zinc, vitamin D with calcium and vitamin A with omega 3. A study revelead that consumption of phytonutrients in the right combination as supplements were proven to strengthen the immune system, indirectly acts as an altenative to COVID-19 prevention (Mrityunjaya et al., 2020). In addition, before the release of vaccines, COVID-19 was managed by the usage of multivitamins, minerals and probiotics as adjuvant therapy along with antiviral medicines (Islam et al., 2021). Moreover, combination of multi micronutrients (especially vitamin C, D and zinc) assisted in enhancing the immune function and reducing infection risk (Gombart et al., 2020). Nonetheless, it surely requires discipline to stick to the routine and it is more challenging to travel with a list of supplements to be brought. Taking that as a problem to be solved; in this study, chitosan nanoparticles was synthesized to contain two antioxidants of different polarities, hydrophobic thymoquinone and hydrophilic L-ascorbic acid; to determine its combinational effect along with efficiency in scavenging free radicals. Up to now, there are very few studies related to antioxidants combination, especially in a single carrier.

Hydrophobic thymoquinone (TQ), an active ingredient in *Nigella sativa* or known as black seed, has excellent antioxidant, anticancer and anti-inflammatory properties (El-far et al., 2018). Studies reported its potent antioxidative function in attenuating neurotoxic effect and oxidative stress in the nervous system, as an impact of exposure to arsenic (Kassab & El-Hennamy, 2017). Hydrophilic L-ascorbic acid (LAA) on the other hand is a powerful antioxidant that can assist in reducing cell damage by scavenging free radicals due to the presence of enediol moiety (Salkić & Selimović, 2015; Tian et al., 2009). With no reported antagonistic effects, TQ and LAA are suitable to be used together. Additionally, the utilization of TQ and LAA had attenuated bisphenol-A, BPA (a polycarbonate plastic that is used to make most bottles) - induced hepatorenal toxicity and oxidative stress in adult male albino rats as a result of synergistic effects of TQ and LAA (Alla & Alla, 2017). However, their effectiveness as an agent in nanoparticles (NP) system has not been unfolded since most of the reported NP were used to encapsulate a single drug. Therefore, this study utilized NP as carriers to hold two antioxidants of opposite polarities, hydrophobic TQ and hydrophilic LAA, which could show better therapeutic performance than single application.

Nanoparticles (NP) are rapidly emerging to improvise the limitations of conventional delivery. They possess many advantages for drug delivery applications such as large surface areas for more efficient and specific delivery with high potential of having a controlled release system (Garg et al., 2019). By using NP, the encapsulated drugs can be retained longer for more effective reaction and one can suffice less dosage of drug(s), therefore, fewer side effects. NP is a type of carrier that is generally made up of a polymer and a crosslinker. In this study, ionic gelation, which involves crosslinking reactions of oppositely charged cationic chitosan polymer and polyanionic sodium tripolyphosphate crosslinker was implemented to synthesize chitosan nanoparticles, CNP (Mitchell et al., 2020; Mohammed et al., 2017).

Chitosan (CS) has been remarked as an auspicious natural polymer to make NP for the delivery of compounds, drugs and genes (Belgamwar et al., 2017; Cao et al., 2019). It originates from the deacetylation of chitin, presents in the exoskeleton of crustaceans like crabs and prawns (Delmar & Bianco-Peled, 2016). Additionally, its biodegradable and biocompatible properties eliminate the potential of CNP to get accumulated once consumed (Pang et al., 2017). Moreover, the positively charged CNP supported with mucoadhesive properties facilitated the delivery of therapeutics by promoting penetration across tight junctions of the epithelium (Mohammed et al., 2017). The cationic features of CNP enhanced its interaction with the negatively charged mucous membrane (Boegh & Nielsen, 2015; Shafabakhsh et al., 2020). Furthermore, with high content of amine moieties, CS is found to be highly modifiable. Modification purposes vary from studies. In this study hydrophobic modification was conducted to improve encapsulation of hydrophobic compound, TQ since CS is originally hydrophilic. The process was conducted by conjugating hydrophobic palmitic acid into CS, prior to the encapsulation step. By having specific hydrophobic sites on CS, the hydrophobic TQ can be held better for more efficient encapsulation and release. Hydrophilic encapsulation on the other hand was easy as the CS itself is hydrophilic.

This study discusses CNP system for co-delivery of hydrophobic TQ and hydrophilic LAA. The combination of different polarities of antioxidants in a single nanocarrier is expected to increase the encapsulation efficiency (as it is proven to show synergistic effects that provided stability (Español et al., 2016; Naderinezhad et al., 2017)), provide controlled release kinetic and efficient radical scavenging activities. With good interactions between these antioxidants as well as the right polymer for the carrier, promising results are close to reality. Positive reciprocities are observed throughout, especially from the diphenyl picrylhydrazine (DPPH) and reactive oxygen species (ROS) *in vitro* antioxidant studies. The concentrations of TQ and LAA (in the dual loaded PCNP-TQ-LAA system) needed to reduce half of the free radicals' volume, EC_{50} is predicted to lessen compared when delivered individually (in single loaded PCNP system, PCNP-TQ and PCNP-LAA) as a result of synergistic effects.

1.2 Research problems and approaches

The conventional delivery of antioxidant is using oral tablet, which limits the bioavailability and does not have controlled release property (Adepu & Ramakrishna, 2021; Aung et al., 2022; Milošević et al., 2011). Hence, NP are regarded as potent vehicles for efficient therapeutics transports as they hold beneficial properties such as higher bioavailability, longer cycle time and higher cell and tissues uptake (Gupta et al., 2019; Quan et al., 2015). NP also have few limitations that could be unlocked to enrich their functions. First, selection of a suitable polymer to serve as user friendly-NP. In achieving this, a biodegradable polymer should be used and it is mainly based on natural sources. CS which is derived from crustacean shells, was selected in this study as it suits the purpose best, without leaving significant toxic effects. Second, functions of each nanocarrier can be diversified if it is able to contain more than one therapeutic agent to provide a multicompound supplementation or multidrug therapy. Therefore, dual antioxidants of different polarities, hydrophobic TQ and hydrophilic LAA were encapsulated in CNP. TQ and LAA were chosen due to the synergistic effects when delivered in combination to treat pentylenetetrazole (PTZ)-induced generalized seizures (Ullah et al., 2014). Besides that, the delivery of combined TQ and LAA had increased the naturally-produced antioxidant, glutathione (GSH) and antioxidant enzyme, superoxide dismutase (SOD) in the liver of BPA-induced rats, which experienced hepatorenal toxicity and oxidative stress (Alla & Alla, 2017). Hence, antioxidative activities of combined TQ and LAA were further experimented in this study with the implementation of NP as the carrier to determine its effects in scavenging free radicals. Third, low encapsulation efficiency of hydrophobic TQ may be caused by poor solubility in the hydrophilic CS carrier. Thus, chemical modification was performed through the conjugation of palmitic acid from palmitic acid N-hydrosuccinimide (NHS) ester to the amine groups of CS, producing palmitoyl-chitosan nanoparticles, PCNP. The modification was conducted to increase the encapsulation efficiency of a poorly water-soluble compound, TQ by providing hydrophobic sites for hydrophobic-hydrophobic interactions. This could subsequently augment the therapeutic efficacies, synergistic effects and sustained release properties. In the future, the PCNP can be used for numerous combinations of hydrophobic and hydrophilic therapeutics needed in myriad illnesses treatments. Fourth, lungs are one of the sites with high accumulation of free radicals. If they are left untreated, it could lead to oxidative stress and illnesses emergence. In countering this problem, adequate antioxidants TQ and LAA can be supplied in a more effective form, which is the PCNP.

1.3 Research novelty

Incorporation of dual classes of antioxidant compounds, thymoquinone (TQ) and L-ascorbic acid (LAA) into hydrophobically modified palmitoyl-chitosan nanoparticles (PCNP) system to affect the synergistic effects. Combination of compounds or drugs may lead to the formation of additive, antagonistic or synergistic effects. However, the synergistic effect is the most wanted outcome

as it implies that the total effect of the interacted compounds is greater than the sum of the individual effects of each compound. Hence, the combination of TQ and LAA is proven to show enhanced interaction, which could later result in a more effective release and delivery for free radical scavenging. These efficiencies could be further amplified using the high surface area and modified PCNP.

1.4 Hypothesis of study

Incorporation of dual classes of antioxidant compounds; thymoquinone (TQ) and L-ascorbic acid (LAA) into hydrophobically-modified palmitoyl-chitosan nanoparticles (PCNP) system will increase the encapsulation efficiency, offer controlled release properties, affect the synergistic effects and enhance free radicals scavenging activities.

1.5 Research objectives

The aims for this research project:

1. To synthesize chitosan nanoparticles using ionic gelation method and to chemically modify it with palmitic acid;
2. To characterize hydrophobically modified palmitoyl-chitosan nanoparticles (PCNP) containing thymoquinone (TQ) and L-ascorbic acid (LAA);
3. To evaluate radical scavenging activities of thymoquinone and L-ascorbic acid by the dual loaded PCNP.

1.6 Scope of study

The study covers the synthesis of chitosan nanoparticles, CNP carrier by ionic gelation method using chitosan polymer and tripolyphosphate crosslinker. The carrier was intended to be encapsulated with dual antioxidants, hydrophilic L-ascorbic acid, LAA and hydrophobic thymoquinone, TQ. However, since one of the compounds is hydrophobic, slight modification is needed to properly accommodate it better. Thus, hydrophobic modification on the chitosan backbone will be conducted using palmitic acid NHS ester to produce palmitoyl-chitosan nanoparticles, PCNP. The proposed system is predicted to elevate the encapsulation and release efficiencies of those antioxidants. Furthermore, physicochemical characterizations on the dual loaded TQ and LAA in both unmodified CNP and modified PCNP will be performed to compare their differences and improvements. Lastly, the capability of the encapsulated NP in scavenging free radicals available in the human lung fibroblast cell line, MRC-5 will be determined by two antioxidant assays; DPPH and ROS. All this information would be essential to conclude the potentials possessed by the dual

loaded PCNP carrier in delivering and reducing the radicals through the synergistic effects that may be produced as a result of combining TQ and LAA.

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