SYNTHESIS, CHARACTERIZATION AND EVALUATION OF IMMUNOMODULATORY, ANTICANCER, ANTIBACTERIAL AND WOUND HEALING ACTIVITIES OF ALGINATE NANOPARTICLES LOADED WITH *Heterotrigona itama* HONEY

MOHAMMAD AHMED ISSA ALHATAMLEH

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by

MOHAMMAD AHMED ISSA ALHATAMLEH

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LIST OF SYMBOLS

R^2	Coefficient of determination in a linear regression model
y	Dependent variable in a linear regression model
x	Explanatory variable in a linear regression model
ζ	Zeta potential (surface charge)
η	Solvent viscosity
R	Solute radius

LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
AgNPs	Silver nanoparticles
AHR	Aryl hydrocarbon receptor
AKT	Protein kinase B
ALDH	Aldehyde dehydrogenase
ALG	Alginate
AMPK	AMP-activated protein kinase
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
AuNPs	Gold nanoparticles
Bcl-xL	B-cell lymphoma-extra large
Ca^{2+}	Free calcium
$CaCl_2$	Calcium chloride
CCL1	Chemokine (C-C motif) ligand 1
cDNA	Complementary DNA
CDs	Cluster of differentiation
COX	Cyclooxygenase
CXCL9	Chemokines (C-X-C motif) ligand 9
DCs	Dendritic cells
DLS	Dynamic light scattering
DOX	Doxorubicin
DPBS	Dulbecco's phosphate-buffered saline
DPPH	α, α-diphenyl-β-picrylhydrazyl
DPX	Dibutylphthalate polystyrene xylene
DSC	Differential scanning calorimetry
EE	Encapsulation efficiency
EGF	Epidermal growth factor
EM	Electron microscopy
ERKs	Extracellular signal-regulated kinases
ESR	Estrogen receptor
FDA	Food and drug administration

FTIR	Fourier-transform infrared spectroscopy
g	Gram
GAE	Gallic acid equivalents
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Graphene oxide
GOS	Guluronate oligosaccharide
H&E	Hematoxylin and eosin
HA	Humic acid
H-ALG-NPs	Honey-loaded alginate nanoparticles
HDL	High-density lipoprotein
HMF	Hydroxymethylfurfural
HO-1	Heme oxygenase-1
IFNs	Interferons
IHC	International Honey Commission
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK1	Interleukin-1 receptor associated kinase 1
IκB	IkappaB kinase
JNK	c-Jun N-terminal kinase
kcal	Kilocalorie
kg	Kilogram
kV	Kilovolt
LC	Loading capacity
LC	Liquid chromatography
LPS	Lipopolysaccharides
LXRs	Liver X receptors
M1	Type 1 activated macrophage
M2	Type 2 activated macrophage
mA	Milliampere
MAPK	Mitogen-activated protein kinases
mAU	Milli-absorbance units
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
MDSCs	Myeloid-derived suppressor cells

MD2	Myeloid differentiation protein 2
mg	Milligrams
MIC	Minimum inhibitory concentration
MIPs	Macrophage inflammatory proteins
MS	Mass spectrometry
MSKHS	Malaysian Standard for *Kelulut* Honey Specification
MT	Masson's trichrome
MTB	Mycobacterium tuberculosis
mTOR	Mechanistic target of rapamycin
MTT	Microculture tetrazolium
Na_2CO_3	Sodium carbonate
NAG	N-acetylglucoseamine
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NPs	Nanoparticles
Nrf2	Nuclear factor erythroid 2-related factor 2
OD	Optical density
ox-LDL	Oxidized low-density lipoproteins
PARP	Poly(ADP)-ribose polymerase
PBMCs	Peripheral blood mononuclear cells
PDGFA	Platelet derived growth factor subunit A
PDI	Polydispersity index
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLGA	Poly (lactic-co-glycolic acid)
PMA	Phorbol-12-myristate-13-acetate
PPARs	Peroxisome proliferator-activated receptors
PPARs	Peroxisome proliferator-activated receptors
PTL	Parthenolide
QE	Quercetin equivalents
qPCR	Quantitative polymerase chain reaction

RIPK	Receptor interacting protein kinase
ROS	Reactive oxygen species
RPM	Revolutions per minute
RSA	Radical scavenging activity
SBH	Stingless bee honey
sBV	Sweet bee venom
SD	Standard deviation
SEM	Scanning electron microscopy
SiO_2	Silicon dioxide
STAT	Signal transducer and activator of transcription
Ta	Tantalum
TA	Tannic acid
TEM	Transmission electron microscopy
TFC	Total flavonoid content
TGA	Thermogravimetric analysis
TGF-β	Transforming growth factor-β
Th1	T-helper 1
TiO_2	Titanium dioxide
TLRs	Toll-like receptors
TPC	Total phenolic content
UV-VIS	Ultraviolet-visible
vD3	1α, 25-dihydroxyvitamin D3
VEGF	Vascular endothelial growth factor
V-rGO	Vanillin-functionalized graphene oxide
XRD	X-ray diffraction
ZnO	Zinc oxide

LIST OF APPENDICES

Appendix A *H. itama* Hives and Honey Harvesting

Appendix B Physicochemical Analysis of Honey by Mérieux NutriSciences

Appendix C Characterization of H-ALG-NPs with Varying Amounts of SBH

Appendix D Animal Ethics Approval

SINTESIS, PENCIRIAN DAN PENILAIAN AKTIVITI IMUNOMODULATORI, ANTIKANSER, ANTIBAKTERIA DAN PENYEMBUHAN LUKA OLEH NANOPARTIKEL ALGINAT YANG DIMUATKAN DENGAN MADU *Heterotrigona itama*

ABSTRAK

Penyelidikan ini bertujuan untuk menghasilkan nanopartikel alginat (ALG-NPs) yang dimuatkan dengan madu kelulut (SBH), dan untuk menilai keupayaan ALG-NPs (H-ALG-NPs) memodulasi tindak balas imun, menghalang proliferasi dan migrasi sel kanser, mempercepatkan penyembuhan luka dan menghalang pertumbuhan bakteria dibandingkan dengan SBH mentah dan ALG-NPs bebas. Komponen utama madu, sifat fizikokimia dan antioksidannya telah dianalisis. H-ALG-NPs telah direka menggunakan teknik pautan silang-ionik. Saiz dan cas H-ALG-NPs ditentukan oleh penyerakan cahaya dinamik, dan ia dicirikan menggunakan TEM, SEM, FTIR, XRD, DSC dan TGA. Aktiviti penghapusan radikal (DPPH), kecekapan pengkapsulan (EE) dan kapasiti pemuatan (LC) H-ALG-NPs juga ditentukan. Aktiviti kesitotoksikan dan anti-migrasi H-ALG-NPs telah diuji terhadap sel A549 kanser peparu, MCF-7 kanser payudara, MDA-MB-231 kanser payudara, U87 glioblastoma dan sel fibroblast kulit normal. Kesan H-ALG-NPs padaekspresi mRNA dari *TNF-α, IL-10, IL-6, IL-1β, IL-8* dan *IRAK1* gen dalam sel ini diukur menggunakan qPCR. Ekspresi mRNA pada gen-gen ini dan *TGF-β, TLR4, IRAK1, FOXP3, TFRC, PGE2, IDO1, CD73, CD39, COX1* serta *COX2* gen juga diukur dalam makrofaj yang dibezakan THP-1 yang dirawat dengan H-ALG-NPs. Aktiviti penyembuhan luka oleh H-ALG-NPs dinilai pada model tikus, manakala aktiviti antibakteria dinilai terhadap *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus* dan *Bacillus cereus* dengan kaedah MIC. Ciri-ciri

madu adalah konsisten dengan piawaian IHC-Codex dan Malaysia, dan ia mempunyai aktiviti antioksidan yang baik. H-ALG-NP sfera mempunyai saiz 312 ± 4.32 nm dan cas permukaan -21.2 ± 0.29 mV. FTIR, XRD, DSC dan TGA mengesahkan pemuatan madu dalam ALG-NPs. H-ALG-NPs mempunyai EE yang tinggi sebanyak 84.74% ± 1.42 dengan LC sebanyak 23.12% ± 0.26, manakala IC_{50} DPPH menunjukkan ketinggian yg ketara daripada SBH mentah ($p > 0.00001$). Ujian MTT menunjukkan ketoksikan yang jauh lebih tinggi terhadap sel kanser oleh H-ALG-NPs berbanding madu, dan sebaliknya untuk fibroblas ($p \leq 0.001$). Ujian gores menunjukkan aktiviti anti-migrasi yang jauh lebih tinggi oleh H-ALG-NPs terhadap sel kanser A549 ($p \leq 0.001$), MCF-7 dan MDA-MB-231 ($p \leq 0.0001$) berbanding dengan madu. Ekspresi beberapa gen yang berkaitan dengan keradangan dan keradangan dalam sel kanser (i.e., MCF-7, MDA-MB-21. A549 and U87), fibroblas dan makrofaj THP-1 menunjukkan perbezaan yang ketara apabila dirawat dengan H-ALG-NPs berbanding dengan madu. Paling penting, pencarian ini mencadangkan bahawa H-ALG-NPs tapi bukan madu mentah, mungkin untuk mengurangkan hasil proinflamasi signal, membenarkan pelepasan anti-inflamasi signal, dan mengaktifkan M2 makrofaj. Luka yang dirawat dengan H-ALG-NPs menunjukkan kadar aktiviti penyembuhan yang lebih baik daripada madu mentah. H-ALG-NPs mempamerkan aktiviti antibakteria 4 kali ganda lebih kuat daripada madu mentah terhadap bakteria Gram-positif dan Gram-negatif. Kesimpulannya, hasil kajian mencadangkan bahawa H-ALG-NPs telah meningkatkan aktiviti antioksidan, imunomodulator, antikanser, antibakteria dan aktiviti penyembuhan luka. Kajian ini menunjukkan bahawa H-ALG-NPs boleh dihasilkan dengan teknik kos efektif dengan sifat terapeutik yang memberangsangkan.

SYNTHESIS, CHARACTERIZATION AND EVALUATION OF IMMUNOMODULATORY, ANTICANCER, ANTIBACTERIAL AND WOUND HEALING ACTIVITIES OF ALGINATE NANOPARTICLES LOADED WITH *Heterotrigona itama* HONEY

ABSTRACT

This research aimed to fabricate alginate nanoparticles (ALG-NPs) loaded with stingless bee honey (SBH), and to assess the ability of the honey-loaded ALG-NPs (H-ALG-NPs) to modulate immune responses, suppress cancer proliferation and migration, accelerate wound healing and inhibit bacterial growth compared to crude SBH and free ALG-NPs. The major components of honey and its physicochemical and antioxidant properties were analyzed. The H-ALG-NPs were fabricated by an ionic-cross-linking technique. The size and charge of the H-ALG-NPs were determined by dynamic light scattering, and they were characterized using TEM, SEM, FTIR, XRD, DSC and TGA. The DPPH radical scavenging activity, encapsulation efficiency (EE) and loading capacity (LC) of the H-ALG-NPs were also determined. The cytotoxicity and anti-migration activity of the H-ALG-NPs were *in vitro* tested against A549 lung cancer, MCF-7 breast cancer, MDA-MB-231 breast cancer, U87 glioblastoma and normal dermal fibroblast cell lines. The effects of the H-ALG-NPs on the mRNA expressions of *TNF-α, IL-10, IL-6, IL-1β, IL-8* and *IRAK1* genes in these cell lines were measured by qPCR. The mRNA expressions of these genes along with *TGF-β, TLR4, IRAK1, FOXP3, TFRC, PGE2, IDO1, CD73, CD39, COX1* and *COX2* genes were also measured in THP-1 differentiated macrophages treated with the H-ALG-NPs. The wound-healing activity of the H-ALG-NPs was evaluated in a mouse model, while their antibacterial activity was evaluated against *Escherichia coli, Klebsiella*

pneumonia, Staphylococcus aureus and Bacillus cereus by the MIC method. The properties of honey were consistent with the IHC-Codex and Malaysian standards, and it had good antioxidant activity. The spherical H-ALG-NPs had a size of 312 ± 4.32 nm and -21.2 ± 0.29 mV surface charge. The FTIR, XRD, DSC and TGA confirmed honey loading within the ALG-NPs. The H-ALG-NPs had a high EE of 84.74% ± 1.42 with an LC of 23.12% ± 0.26, while the IC_{50} of DPPH was significantly higher than crude SBH ($p > 0.00001$). The MTT assay showed significantly higher toxicity against cancer cell lines by the H-ALG-NPs compared to honey, and vice versa for fibroblasts ($p \leq 0.001$). The scratch assay showed significantly higher anti-migration activity by the H-ALG-NPs against A549 ($p \leq 0.001$), MCF-7 and MDA-MB-231 ($p \leq 0.0001$) cancer cells compared to honey. The expressions of several inflammatory and inflammatory-related genes in cancer cell lines (i.e., MCF-7, MDA-MB-21, A549 and U87), fibroblasts and THP-1 macrophages were significantly modulated upon treatment with the H-ALG-NPs compared to honey. Importantly, the findings suggest that the H-ALG-NPs, but not crude honey, may tend to reduce the output of pro-inflammatory signals, promotes the release of anti-inflammatory signals, and activates M2 macrophages. Wounds treated with the H-ALG-NPs showed superior healing activity than crude honey. The H-ALG-NPs exhibited 4-fold stronger antibacterial activities than crude honey against the Gram-positive and Gram-negative bacteria. In conclusion, the results suggested that the H-ALG-NPs have enhanced antioxidant, immunomodulatory, anticancer, antibacterial and wound healing activities. This study indicates that the H-ALG-NPs can be fabricated simply by a cost-effective technique with promising therapeutic properties.

CHAPTER 1

INTRODUCTION

1.1 Background

Honey is a sweet substance naturally produced by bees. This flavorful fluid substance is collected from the floral nectar; the secretions of aphid family members as they feed on plant sap (e.g., honeydew) or the secretions of parts of the living plant (Pita-Calvo & Vázquez, 2018; White, 1978). Honey is predominantly produced through the beekeeping of two main genera; Apis (European/Western bees or honeybees) and Meliponinae (stingless bee). Each kind of beekeeping has its distinct characteristics and is domesticated for honey production and agricultural pollination purposes (Moritz *et al.*, 2005). To date there are more than 40 species of stingless bee were identified as honey producers (Crane, 1992; Michener, 2013). These species are distributed in the tropical and subtropical regions (Rattanawannee & Duangphakdee, 2019). However, the distribution of stingless bee honey (SBH) is still lower compared to the more common honey produced by Apis bee species, mainly *Apis mellifera* (Abd Jalil *et al.*, 2017).

In Malaysia, most of the local honey producer species belong to Meliponinae. In 2000, Malaysia produced only 5 percent of the country's honey needs, which is considered a trivial estimate compared with Malaysia's abundant nectar resources (Ismail & Ismail, 2018). In 2010, by providing sufficient aid, Malaysia managed to increase farm production to 284% of the country's honey needs (Ismail & Ismail, 2018). However, there was a continuous increase in honey imports, mainly from Australia, New Zealand, China and Iran (Ismail, 2012). Meanwhile, a significant and continuous decrease in honey exports was also reported from 2010 to 2017 (Ismail & Ismail, 2018). Although Malaysian SBH is cost-effective, it is facing export

restrictions due to its moisture content which is higher than the amount allowed according to the Codex Standard 12-1981 (up to 20 %) (Omar *et al.*, 2019).

Honey has religious and traditional histories from different ethnic communities worldwide in treating human diseases (Khan *et al.*, 2018). In this era, honey has proven therapeutic properties as an antioxidant, anti-inflammatory, antibacterial, antimutagenic, expedite wound healing, antidiabetic, antifungal, antitumor and antiviral (Ahmed *et al.*, 2018). It is believed that SBH is a superior promising source of biologically active compounds over western honey, and this can be attributed to the rich vegetation in the tropical and subtropical regions where stingless bees are found (Abd Jalil *et al.*, 2017).

It is well known that honey is an immune booster that improves the proliferation of T and B lymphocytes, stimulates phagocytosis, and regulates the production of vital pro-inflammatory cytokines from monocytes, such as tumor necrosis factor (TNF), interleukin 1 beta (IL-1β) and IL-6 (Abuharfeil *et al.*, 1999; Tonks *et al.*, 2003). Honey also showed anti-inflammatory activity which inhibits the expression of these pro-inflammatory cytokines (Miguel *et al.*, 2017). This dual immunomodulatory role of honey has been attributed to its content of beneficial constituents and antioxidant properties (Miguel *et al.*, 2017; Ranneh *et al.*, 2019), which prevent and manage oxidative stress. The antioxidant activity of honey is positively correlated to its content of phenolic compounds (Kek *et al.*, 2014). Moreover, it was reported that the mechanisms for anticancer activity of honey are antioxidant, anti-inflammatory, apoptotic, immunomodulatory, anti-proliferative, and providing estrogenic effects (Waheed *et al.*, 2019).

Ancient Romans, Assyrians, Egyptians, Greeks and Chinese utilized honey as a topical treatment for wounds and skin illnesses. Thus, honey is found to be the best natural wound healer as it has been targeted to clear bacteria, malodor and debridement (Hixon *et al.*, 2019). It was reported that the anti-inflammatory properties possessed by honey have prompted a remarkable improvement in wound healing (Jull *et al.*, 2015). Furthermore, honey was also found to heal rapidly infected post-operative wounds, pressure ulcers and Fournier's gangrene (Jull *et al.*, 2015). The antimicrobial activities of honey have been well studied against many bacteria and fungi (Albaridi, 2019; Israili, 2014). It was reported that different types of honey, especially SBH, have high antimicrobial activity against Gram-negative (e.g., *Escherichia coli* and *Klebsiella pneumoniae*) and Gram-positive (e.g., *Staphylococcus aureus* and *Bacillus cereus*) bacteria (Avila *et al.*, 2019).

On the other hand, recent years have witnessed the establishment of a variety of applications and approaches that utilize nanomaterials to enhance the therapeutic efficacy and bioavailability of bee products, especially honey (Bonsignore *et al.*, 2021; Tatli Seven *et al.*, 2018). To study the effectiveness of bee products in targeting the physiological sites of different diseases (especially tumors and inflammations), different types of nanomaterials (e.g., lipids and polymers) and self-nano-emulsifying drug delivery systems were used in the loading and delivery of bee products or their extracts (Fan *et al.*, 2013; Fitria *et al.*, 2021; Ilhan-Ayisigi *et al.*, 2020; Tatli Seven *et al.*, 2020; Ullah *et al.*, 2020). The bee products-incorporated nano-objects have shown higher bioactivity, bioaccessibility, and physical and chemical stability than the crude products. Further, nanotechnology applications have allowed the loading of specific compounds extracted from bee products with more predictable therapeutic effects for use in targeted therapies (Patra *et al.*, 2018). Towards keeping bee products as natural

and non-toxic therapeutics, the use of nanocarriers from natural materials has received special attention (Bonsignore *et al.*, 2021; Sharma *et al.*, 2019; Shreyash *et al.*, 2021).

Owing to their biocompatibility, biodegradability, stability, sustainability, and ability to control the release of loaded therapeutics, naturally derived polysaccharides have emerged as efficient polymeric nano-carriers (Jana *et al.*, 2021; Maiti & Jana, 2019; Nitta & Numata, 2013). Furthermore, polysaccharides have allowed for flexibility in establishing chemical modifications designed to modulate specific size and surface properties of the products (Hasnain et al., 2020b). Natural polysaccharide nanomaterials have proven to be well suited for protecting small and large molecules as well as biologics against cellular degradation and environmental hazards (Hasnain et al., 2020b). Their reactive functional groups represent a cell-adhesive polymer surface on which cells can interact and adhere through passive and active adhesion, enabling improved drug retention time, absorption, and intracellular bioavailability (Hasnain et al., 2020b).

Alginate (ALG) has been one of the most studied polysaccharides. ALGs are naturally occurring hydrophilic polysaccharides extracted from the seaweeds or obtained via bacterial biosynthesis (Hasnain et al., 2020a). Over the past few decades, several forms of ALG-based systems have been fabricated and examined for use in a variety of different pharmaceutical and biomedical applications such as controlled drug delivery, wound dressings, tissue engineering, tissue regeneration, and dental impressions (Hasnain et al., 2020a). ALG-based nanomaterials are among the most extensively characterized biopolymers used to develop targeted or localized delivery systems for therapeutic compounds, including bee products or their extracts (Abulateefeh & Taha, 2015; Homem *et al.*, 2021; Saberian *et al.*, 2021). This is due to

their desirable characteristics that include a high therapeutic-payload, targeted efficiency, pH sensitivity, capability of protection from degradation, thickening properties, gelling abilities, high availability, and relatively low cost (He *et al.*, 2020; Lee & Mooney, 2012; Sun & Tan, 2013).

Owing to their ability to mimic the native extracellular matrix that supports cells by providing structural and biochemical conditions for cell attachment, proliferation, migration, and differentiation, ALGs are extensively involved in wound healing and tissue engineering applications (Kalva *et al.*, 2021; Nosrati *et al.*, 2021; Sun & Tan, 2013). Currently, there are several types of ALG-based dressings that are commercially available for various types of wounds (e.g., Algicell™ and AlgiSite M™) (Aderibigbe & Buyana, 2018). Also, ALGs are able to modulate innate immune defense mechanisms. For example, through the NF-κB pathway, the stimulatory effect of ALG seems to involve in activation of macrophages present in large numbers in wound granulation tissue, playing a vital role in accelerating the wound healing process by promoting and resolving inflammation, removing dead cells and enhancing cell proliferation and tissue restoration (Krzyszczyk *et al.*, 2018; Yang & Jones, 2009). ALG has a stimulatory effect on monocytes, increasing the production of inflammatory cytokines, such as TNF, IL-6, and IL-1, which are necessary for intracellular signaling and thus for orchestrating the wound-healing process in some situations (Eming *et al.*, 2007; Kulseng *et al.*, 1996; Thomas *et al.*, 2000). Moreover, ALGs possess other desired properties including their antioxidant, antibacterial, and anti-lipid peroxidation capacities, as well as preserving original characteristics such as flavor and color (Alves *et al.*, 2021).

In cancer research, ALG-based nanomaterials emerged based on their potential to reduce the side effects of anti-cancer chemotherapeutic agents and enhance their efficacy in targeting and penetrating the target cells. They can also be used to overcome chemotherapeutic resistance, which could result from several intracellular pathways of drug uptake and a higher accumulation of drugs inside cancer cells (Alfarouk *et al.*, 2015; He *et al.*, 2020). For instance, the *in vitro* assays on breast cancer cells indicated that the involvement of ALG resulted in dose and time-dependent cytotoxic activity, as well as a superior sustained release of doxorubicin (DOX) (Katuwavila *et al.*, 2016).

1.2 Thesis Statement

Despite exhibiting its tremendous medicinal properties, honey has still been abandoned and disregarded in the modern pharmaceutical era and is only classified under complementary medicine. Although it is a rich source of a wide range of beneficial constituents, the utility of SBH in the context of medicinal and therapeutic properties is still not well discovered compared to honey by honeybees. It is important to utilize the unique physiochemical characteristics of SBH to increase the demand and market value of this honey via different methods. Since the size of total SBH production has become larger compared to the market and export demands, not only in Malaysia but globally, it is crucial to conduct research studies to explore the biological and therapeutic potentials of SBH in order to move it to the pharmaceutical industry.

Honey faces multiple challenges when directly applied in therapeutic applications, such as stickiness, limited skin permeation, restricted route of administration, and complexities in implementing targeted therapy. Therefore, nanotechnology offers promising approaches to overcome these challenges and unlock

the full therapeutic potential of honey. One such approach is the utilization of ALG nanoparticles (NPs) as a carrier for honey. ALG NPs provide a sustainable and green option for nanoformulation due to their biocompatibility and biodegradability. By choosing ALG NPs as the carrier for honey, we ensure that our therapeutic approach remains natural, minimizing the use of synthetic or potentially harmful materials. In addition, the encapsulation of honey within ALG NPs offers several advantages. By leveraging the unique properties of ALG NPs as a carrier for honey, we can address the above challenges. This approach not only overcomes these limitations but also aligns with our commitment to sustainability and eco-friendly practices, as ALG NPs offer a natural and green option for nanoformulation. Furthermore, ALG NPs can facilitate sustained and prolonged release of honey's bioactive compounds, leading to prolonged therapeutic effects. ALG NPs can also provide a protective microenvironment for honey's bioactive components, shielding them from degradation, thereby enhancing their overall bioavailability and therapeutic potential.

After knowing the huge potentials of ALG, for enhancing the therapeutic capabilities of SBH, it would be promising to synthesize SBH-loaded ALG-based nanomaterials and study their characteristics, biological potentials and therapeutic effects. This novel product may deserve considerable attention as a promising pathway for industrial and therapeutic applications. Moving this approach from research to industry could play an important role in the innovative development of stingless beekeeping and other associated industries in tropical countries. This could also help the beekeepers to increase and sustain their income, and promote SBH as a promising future commodity. To the best of our knowledge, this is the first research to develop a nanoformulation for the delivery of SBH or its compounds.

1.3 Objectives

1.3.1 General Objective

The objective of this research was to fabricate a novel class of ALG-NPs loaded with Malaysian honey (by *Heterotrigona itama* bees) and to assess the ability of these NPs to modulate the immune responses, inhibit the proliferation and migration of different cancer cell lines, accelerate wound healing and inhibit bacterial growth compared to crude honey and free ALG-NPs.

1.3.2 Specific Objectives

1. To evaluate the physicochemical (mineral content, sugar content, moister content, pH and colour intensity) and antioxidant (TPC, TFC and DPPH) properties of the Malaysian *H. itama* honey.

2. To synthesize and characterize ALG-NPs loaded with *H. itama* honey (H-ALG-NPs) in terms of particle size, charge, morphology, chemical interactions, thermal stability, loading capacity, encapsulation efficacy, and antioxidant activity.

3. To determine the immunomodulatory activity of the H-ALG-NPs on THP-1 differentiated macrophage, A549 lung adenocarcinoma, MCF-7 and MDA-MB-231 breast cancer, U87 glioblastoma and normal fibroblast cell lines based on the alterations in the mRNA expression of inflammatory response genes (*in vitro*).

4. To determine the cytotoxicity and anti-migration activity (wound healing) of the H-ALG-NPs against A549, MCF-7, MDA-MB-231, U87 and normal fibroblast cell lines (*in vitro*).

5. To assess the wound-healing activity of the H-ALG-NPs in a model of BALB/c mouse excisional wound based on wound closure percentage and histopathological examination (*in vivo*).

6. To determine the antibacterial activities of the H-ALG-NPs against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and two Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumonia*) by means of minimum inhibitory concentration test (*in vitro*).

1.4 Research Hypothesis

We hypothesized that the process of loading honey into ALG-NPs is applicable and would result in the production of NPs with desirable characteristics. Owing to their nano-size, modified surface charge and novel physicochemical properties, the H-ALG-NPs would have superior biological effects and therapeutic possibilities over crude honey. This was expected to be resulted from increased surface area for interaction with cells, improved cellular uptake, and controlled release of encapsulated honey's phytochemicals.

More specifically, we hypothesized that the mRNA expression patterns of inflammatory genes in THP-1 macrophages, selected cancer cell lines and normal fibroblasts would be different upon treatment with the H-ALG-NPs compared to crude honey. This was attributed to the ability of H-ALG-NPs to modulate gene expression or alter cellular responses through specific interactions with target cells or cellular signaling pathways. Furthermore, the H-ALG-NPs would have higher cytotoxicity and anti-migration activity against selected cancer cell lines compared to crude honey, and vice versa for fibroblasts. This was highly expected as literature on the potential of

ALG-NPs-based therapies to improve drug delivery to cancer cells showed that did not only enhance cellular uptake, but also induce targeted cytotoxic effects.

We also hypothesized that the H-ALG-NPs would close excisional wounds in mice significantly earlier compared with crude honey. H-ALG-NPs may enhance skin permeation, controlled release, stimulation of cellular migration, and modulation of the wound microenvironment, as well as their vital role in creating moist wound environment and minimizing bacterial infections. Lastly, the H-ALG-NPs would have superior antibacterial activities against Gram-negative and Gram-positive bacteria over crude honey. Modification of surface charge by H-ALG-NPs can cause targeted disruption of bacterial cell walls leading to inhibition of bacterial growth as well as prevention of bacterial adhesion and accumulation. Indeed, it is worth mentioning that ALG oligosaccharides and TA possess slightly low antioxidant properties, which can further contribute to the therapeutic effects described in the above hypothesis.

CHAPTER 2

LITERATURE REVIEW

This chapter provides a thorough review of literature relevant to the current study, with a focus on the topics of ALG-based nanomaterials and the medicinal properties of honey, particularly SBH. To highlight these areas, two review articles were produced from this chapter and published in highly-regarded journals (refer to LIST OF PUBLICATIONS). Therefore, the topics that have been comprehensively reviewed and discussed in the published articles will not be extensively repeated herein too.

The chemistry and physicochemical properties of ALGs (e.g., non-toxic, biodegradable and biocompatible), along with the fabrication methods of ALG-based nanomaterials, were well described in our recently published review article titled "Applications of Alginate-Based Nanomaterials in Enhancing the Therapeutic Effects of Bee Products". The biomedical and pharmaceutical applications of ALG-based nanomaterials were also discussed with a particular emphasis on the findings of studies that introduced ALG-based nanomaterials for the delivery of bee products (e.g., honey, propolis and bee product) or their extracts. This article was published by *Frontiers in Molecular Biosciences* (Q2, IF = 6.113) (Al-Hatamleh *et al.*, 2022). Moreover, detailed information about stingless bees and the medicinal properties of their products, particularly the potential of SBH, was provided in our published review article titled "Antioxidant-Based Medicinal Properties of Stingless Bee Products: Recent Progress and Future Directions". This article was published by *Biomolecules* (Q2, IF = 6.064) (Al-Hatamleh *et al.*, 2020).

On the other hand, the THP-1 cell line was specifically chosen as a model for studying the potential immunomodulatory effects of H-ALG-NPs. Since this is the first

time a combination of honey and ALG is being examined on this cell line, it is therefore crucial to have a comprehensive understanding of this model and any previous findings related to this study. Additionally, this model has not been previously addressed in any of our published review articles.

2.1 Alginate

ALGs are anionic polysaccharides derived from brown seaweeds (algae). ALGs are linear unbranched polymers consists of two monomeric units: β-(1→4)-linked D-mannuronic acid (M) and α-(1→4)-linked L-guluronic acid (G) (Abka-Khajouei *et al.*, 2022). These two monosaccharides (i.e., M and G) are connected by glycosidic linkages, and the proportion of M and G units in the polymer chain can vary depending on the source of the ALG (Dodero *et al.*, 2020). There are three forms of segments of the polymer blocks in ALG. The first form is homopolymeric blocks; these blocks consist of repeating units of either G or M and have a higher degree of uniformity in their chemical properties. The second is copolymeric blocks; these blocks consist of alternating segments of G and M units and are characterized by their heterogeneous chemical properties. The third is random copolymeric blocks; these blocks consist of random distribution of G and M units present (Figure 2.1) in small amounts and have a lower degree of uniformity in their chemical properties (Al-Hatamleh *et al.*, 2022).

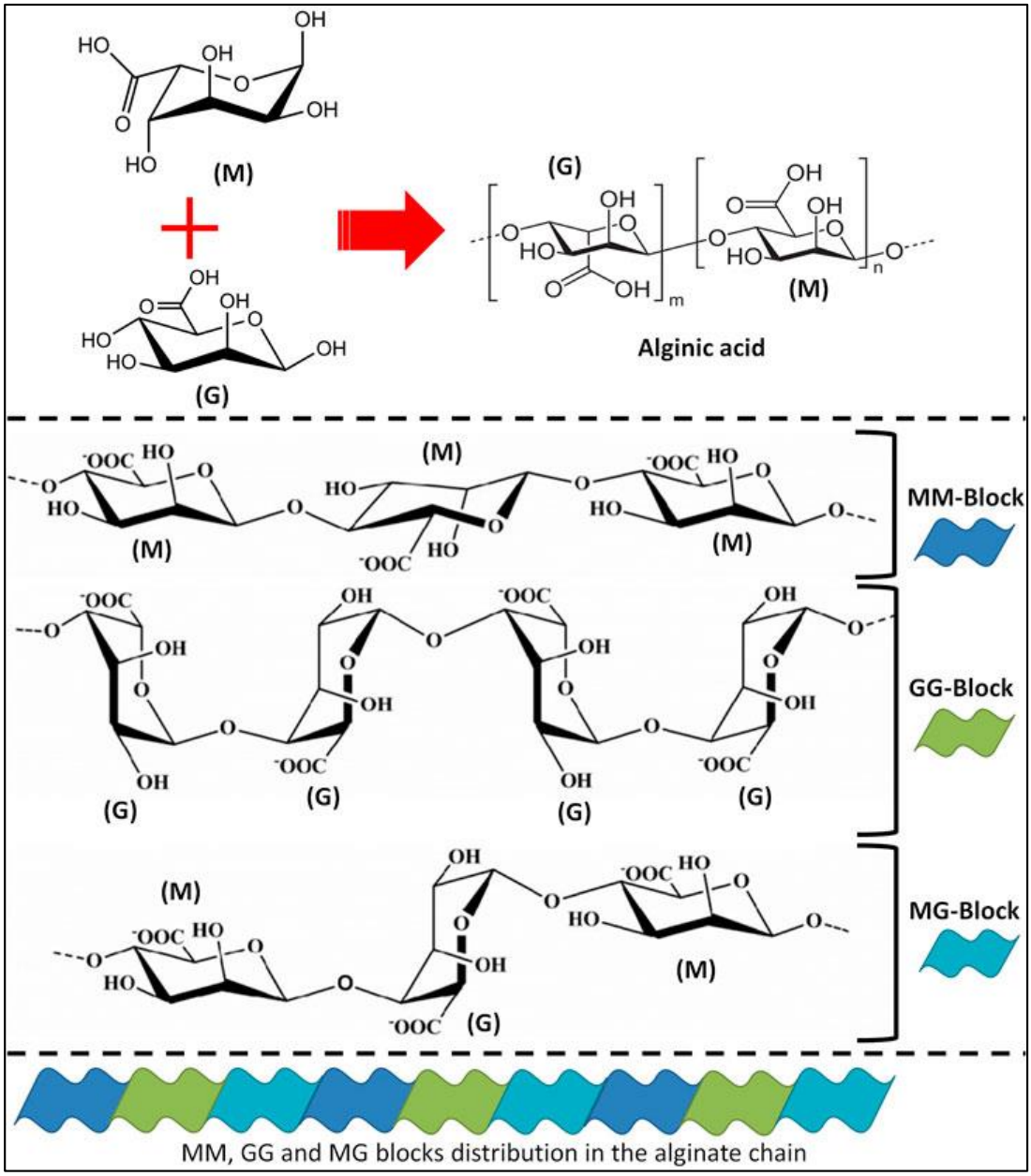

Figure 2.1 The structural details of ALG.
(M) refers to D-mannuronic acid and (G) refers to L-guluronic acid.

The most common ALG structure is a copolymeric block and it contribute significantly to the gel-forming properties of ALG (Lee & Mooney, 2012). The proportion of these blocks in ALG can vary depending on the source of the ALG, and it will affect the properties of the ALG and its applications. For example, ALGs with a higher proportion of G units will form stronger gels than those with a higher proportion of M units (Rosiak *et al.*, 2021). When ALG is mixed with divalent cations (e.g., calcium ions), the G units in the ALG chain form cross-links with the cations, creating a three-dimensional network of polymer chains that can trap water and form a gel, the resulted structure is termed as "egg-box" structure because it resembles the shape of an egg carton (Abka-Khajouei *et al.*, 2022) (Figure 2.2).

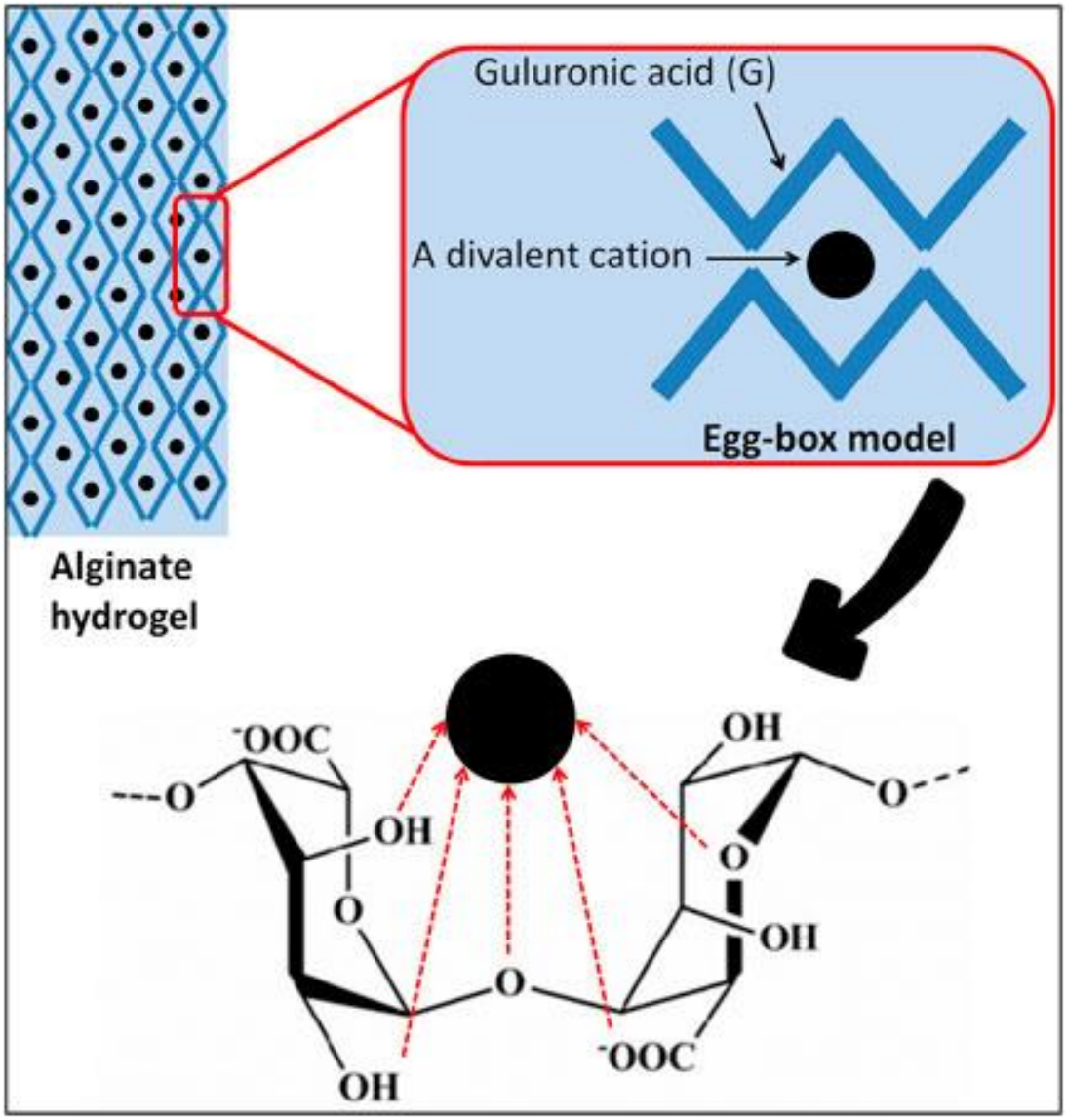

Figure 2.2 Egg-box model-based gelation of ALGs

It is also worth to mention that ALG can be modified to change its properties and make it more suitable for specific applications through different modifications other than crosslinking. For example, ALGs can be mixed with other biopolymers to create hybrid materials that combine the properties of different biopolymers. This can lead to the creation of new materials with enhanced properties that can be used in a wide range of applications (Hurtado *et al.*, 2022). In addition, ALG derivatives can be produced by introducing different chemical groups into the polymer chain, such as carboxyl, aldehyde, or ester groups. These approaches is known as derivatization, and it can be used to improve the mechanical properties of the ALG gel, or to make it more responsive to changes in pH or temperature (Putri *et al.*, 2021; Szabo *et al.*, 2020). ALG derivatives, such as propylene oxide or epichlorohydrin ethers, can be used as emulsifiers, thickeners and gelling agents (Pawar & Edgar, 2012; Putri *et al.*, 2021). Furthermore, ALG can be physically modified by methods such as lyophilization, freeze-drying, or extrusion to improve its mechanical properties, or to create new forms of ALG such as microbeads, fibers, or films. Several chemical modification methods, such as oxidation, reduction or hydrolysis, were also introduced to change the chemical properties of ALG (Banks *et al.*, 2019; Deng *et al.*, 2021; Rosiak *et al.*, 2021). Besides, ALG can be functionalized by attaching biomolecules such as proteins, peptides, enzymes or antibodies to it, which can change its properties, or can be used to target specific cells or tissues (Dalheim *et al.*, 2016; Zhang *et al.*, 2021b). Overall, these properties make ALG-based nanomaterials a versatile biopolymer that can be used in a wide range of nanomedicine applications.

2.1.1 Nanotechnology

Nanotechnology is an interdisciplinary field of science and engineering that deals with the manipulation and control of matter at the nanometer (nm) scale,

typically in the range of 1 to 100 nm. This technology has shown great potential to revolutionize a wide range of industries, from medicine and biology to engineering and materials science (Bayda *et al.*, 2019). Nanomedicine and nanobiotechnology are emerging branches of nanotechnology science that involve the application of nanotechnology to medicine and biology. Nanotechnology is the manipulation of materials at the atomic and molecular level, and it allows for the creation of nanomaterials with unique properties that are not found in bulk materials. This makes it possible to create new diagnostic and therapeutic tools for use in medicine and biology. Nanomedicine is the application of nanotechnology to diagnosis and treatment of diseases, such as in targeted drug delivery systems to specific cells or tissues, which can increase the effectiveness of the drugs while reducing side effects (Abdel-Mageed *et al.*, 2021; Khan *et al.*, 2015; Teli *et al.*, 2010). Thus, nanomedicine research is basically based on nanobiotechnology applications (Figure 2.3). Both nanomedicine and nanobiotechnology are still in the intermediate stages of development, but they hold great promise for improving human health (Al-Hatamleh *et al.*, 2019a). The small size of nanomaterials allows them to easily enter cells and tissues, which makes them useful for diagnostic and therapeutic applications. However, more research is needed to fully understand the potential risks and benefits of these technologies amidst the presence of numerous types of nanomaterials with different characteristics and physicochemical properties.

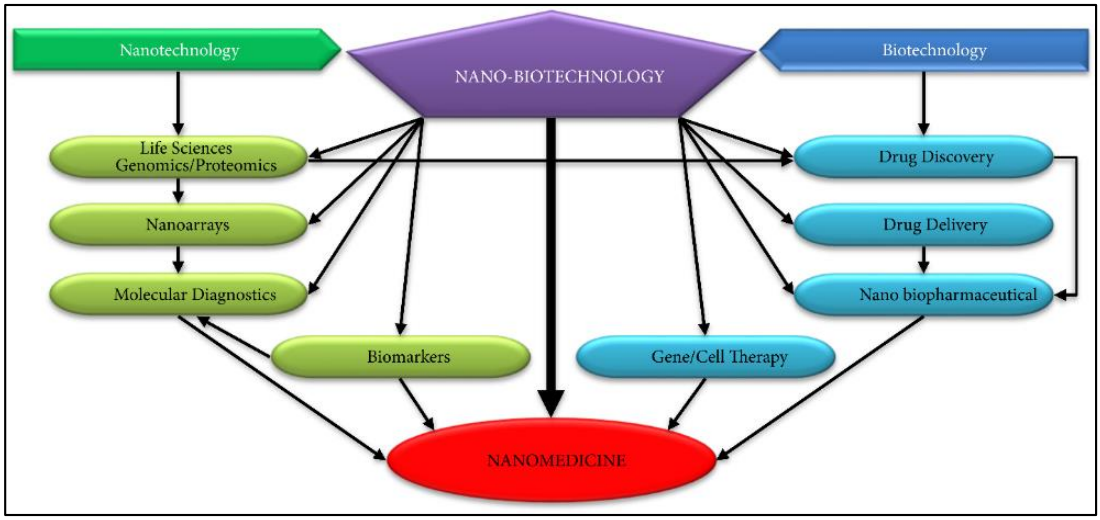

Figure 2.3 Illustration of how nanomedicine research is based on the applications of nanobiotechnology

There are several types of nanomaterials used in drug delivery, such as liposomes, polymeric nanomaterials, dendrimers and inorganic nanomaterials (gold and magnetic NPs), each with their own unique properties and advantages (Al-Hatamleh *et al.*, 2019b; De Jong & Borm, 2008; Patra *et al.*, 2018). Polymeric nanomaterials, such as polyethylene glycol (PEG) and polylactic acid (PLA), are composed of polymers, which are molecules made up of repeating units called monomers. Polymeric nanomaterials have unique properties that make them useful for a wide range of applications, particularly in the field of drug delivery (Zielinska *et al.*, 2020). One of the main advantages of polymeric nanomaterials is their biocompatibility, as well as their potential controlled release, ability to encapsulate a wide range of drugs (hydrophilic and hydrophobic), ability to protect the loaded therapeutic agents, and their ability to be functionalized with targeting moieties (e.g., antibodies or peptides) (Begines *et al.*, 2020; Grottkau *et al.*, 2013). These properties make polymeric nanomaterials powerful nano-delivery systems for improving the bioavailability and effectiveness of therapeutic agents in the human body.

Biopolymers are polymers that are produced from natural sources, such as plants, algae, microbes and animals (e.g., cellulose, starch, chitosan, hyaluronic acid, chitin and ALG) (Baranwal *et al.*, 2022; Gheorghita *et al.*, 2021). Biopolymers are biocompatible, biodegradable and renewable materials, making them involved in a wide range of applications, including in food packaging, agriculture, textiles, pharmaceutical and biomedical applications. More specifically, these biocompatible materials are widely used in drug delivery, biomedical imaging, biosensing, gene therapy, tissue engineering, wound dressings, sutures and implants (Arif *et al.*, 2022; Baranwal *et al.*, 2022; Gardikiotis *et al.*, 2022). Overall, biopolymers are currently gaining more attention as a more sustainable and safer alternative to synthetic

polymers (e.g., polyethylene and polyester), especially for nanomedicine applications. ALG is one of the most researched biopolymers in the pharmaceutical area for applications in drug delivery.

2.1.2 Nanomedicine Applications of ALG

Owing to their unique nontoxic, functional and mechanical properties, as well as their high hydrophilicity and biocompatibility, various ALG-based nano-sized delivery systems have been successfully fabricated based on various preparation techniques for different nanomedicine applications (Figure 2.4). There are several techniques that can be used to prepare ALG nanomaterials for the delivery of drugs or other small molecules (Al-Hatamleh *et al.*, 2022). To create small droplets that can be cross-linked with divalent cations, ALG must be first emulsified in an aqueous solution, dissolved in a solvent (e.g., methanol or ethanol), electrostatically charged or mechanically homogenized to create small droplets. Also, ALG can be mixed with a solution containing divalent cations and then passed through a microfluidic device to form NPs with a precise size and shape, mixed with divalent cations to form NPs by self-assembling (Choukaife *et al.*, 2020; Lopes *et al.*, 2017; Zhang *et al.*, 2021b). These techniques can be used to create ALG nanomaterials with various sizes, shapes and properties, which can be tailored for specific applications in drug delivery, tissue engineering, and other biomedical applications.

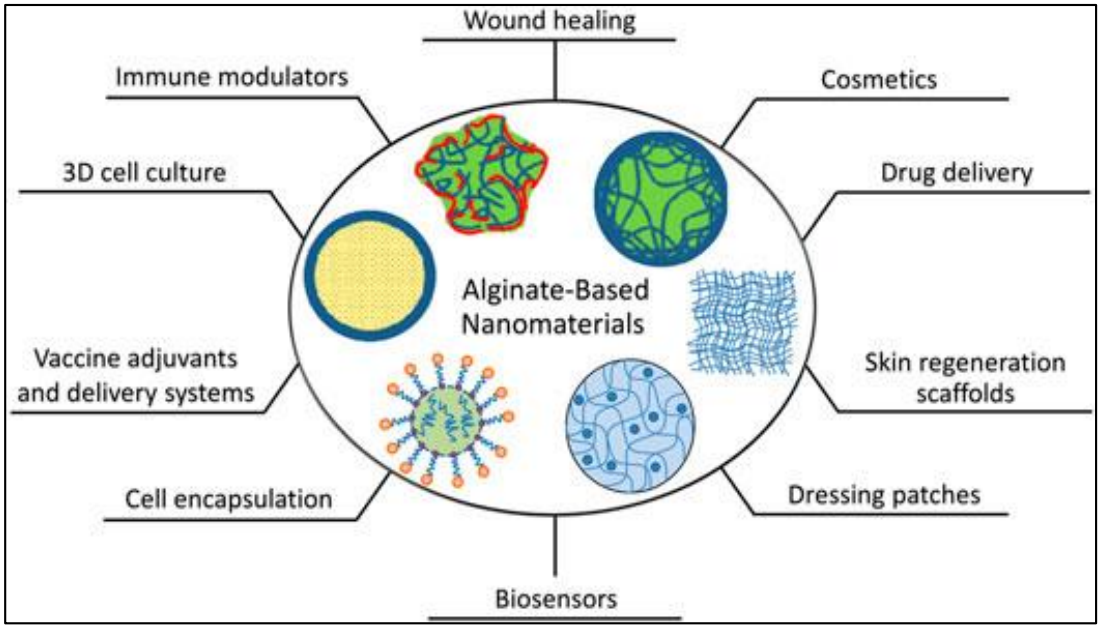

Figure 2.4 Schematics of the present and potential biomedical and pharmaceutical applications of ALG-based nanomaterials

ALG-based nanomaterials were used as nanocarriers for a variety of drugs, molecules and compounds in advanced biomedical and pharmaceutical applications (Dodero *et al.*, 2021). ALG nanomaterials have been used for the delivery of a wide range of drugs, such as anticancer drugs (e.g., doxorubicin and cisplatin) (He *et al.*, 2020) and anti-inflammatory drugs (e.g., dexamethasone, ibuprofen and diclofenac) (Haley & von Recum, 2019). Overall, ALG nanomaterials not only improve the targeting capacity but also enhance bioavailability and biocompatibility of drugs making them more effective and safer for use in medical applications. Furthermore, they are used in the encapsulation and delivery of enzymes (e.g., lipases, proteases and glucose oxidase), proteins and peptides (i.e., insulin, growth factors and vaccines), helping to protect them from degradation with better targeting (Chai *et al.*, 2020; Kolambkar *et al.*, 2011; Sarei *et al.*, 2013). Also, ALG nanomaterials have been utilized for nucleic acid delivery, such as plasmids DNA, siRNA and miRNA, to target cells, leading to the expression of therapeutic proteins or silencing of specific genes (Alallam *et al.*, 2020; Ding *et al.*, 2017).

Moreover, ALG-based nanomaterials have also been used for the delivery of natural products such as plant extracts, essential oils and phytochemicals. Natural products are known for their medicinal properties, but they often have poor solubility, stability and bioavailability. ALG-NPs not only assist in the delivery of these products but also help to protect them from degradation, increase their solubility, and target them to specific sites in the body (Bharali *et al.*, 2011; Karim *et al.*, 2022; Watkins *et al.*, 2015). For example, various plant extracts (e.g., curcumin, resveratrol and quercetin) that have anti-inflammatory, antioxidant and anticancer properties have been successfully loaded into ALG nanomaterials for drug delivery purposes (Aluani *et al.*, 2017; Saralkar & Dash, 2017). Additionally, ALG nanomaterials have been used

for the delivery of phytochemical compounds that have various antioxidant-based medicinal properties, such as phenolics and carotenoids (Karim *et al.*, 2022). Interestingly, based on the above studies, it has been found ALG nanomaterials to be tailored to specific natural products, and their properties can be modulated to improve the bioavailability and efficacy of these natural products.

Particularly, recent years have witnessed utilization of ALG-based nanomaterials to enhance the involvement of bee products or their extracts in treatment of diseases and wound healing applications (Abasalizadeh *et al.*, 2020; Homem *et al.*, 2021; Stojkovska *et al.*, 2019; Tang *et al.*, 2019). This is due to their offering desirable characteristics that include a high therapeutic-payload, targeted efficiency, pH sensitivity, capability of protection from degradation, thickening properties, gelling abilities, bioavailability, and relatively low cost (Al-Hatamleh *et al.*, 2022). Therefore, ALG-based nanomaterials can play key roles in enhancing the therapeutic capabilities of bee products and keeping their physicochemical properties deserve a considerable attention as a promising pathway for industrial and therapeutic applications (Figure 2.5).

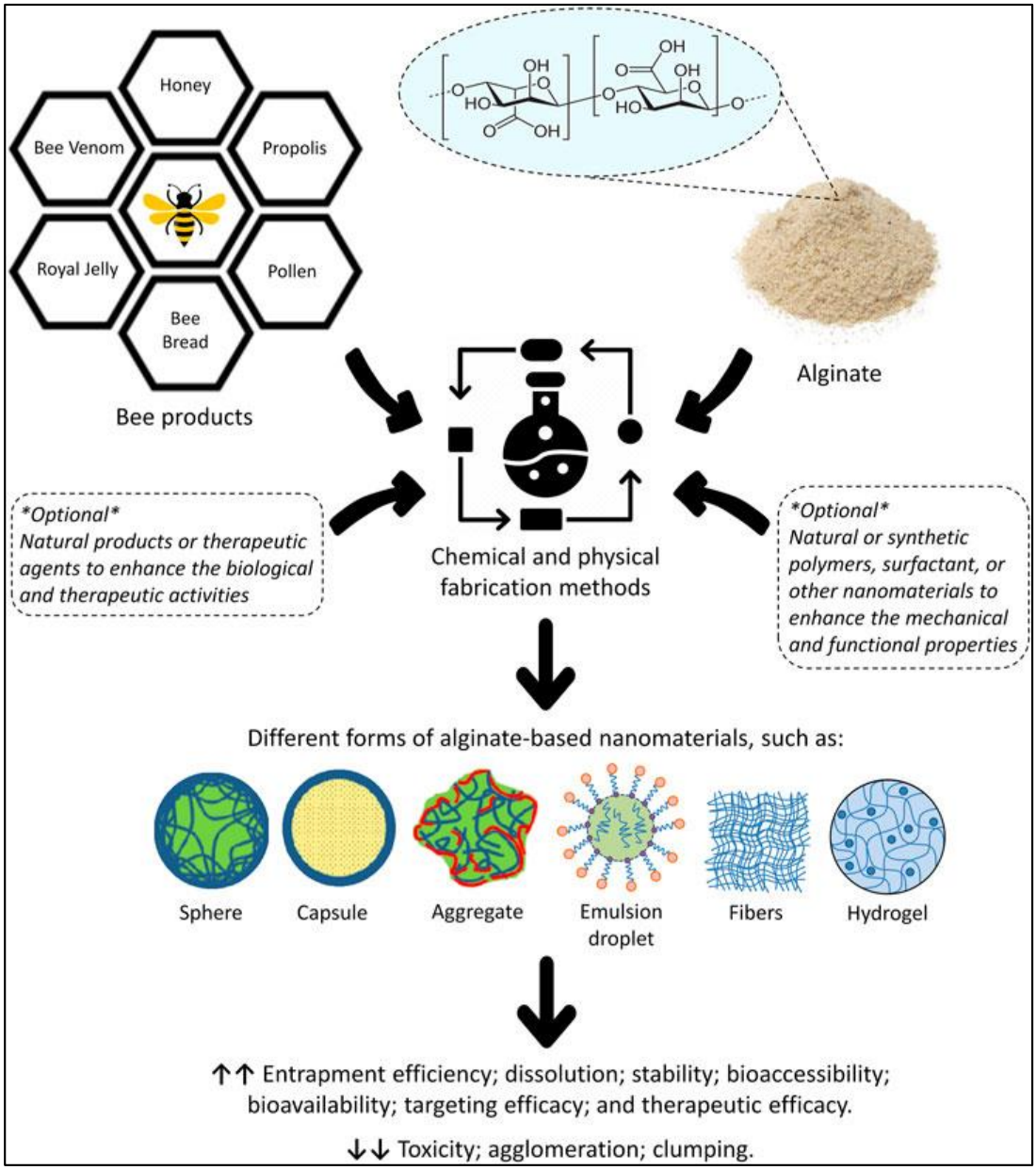

Figure 2.5 General overview of the potential role of ALG-based nanomaterials in enhancing the biological and therapeutic properties of bee products