MODIFICATION OF BACTERIAL CELLULOSE WITH CITRIC ACID AND ITS EVALUATION FOR POTENTIAL APPLICATION IN BONE TISSUE REGENERATION

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DEDICATION

This thesis is dedicated to my late father, who taught me that the best kind of knowledge to have, is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time.

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In the name of Allah, the beneficent and the merciful. All praises are to Him, the sustainer of the universe in which all knowledge belongs to Him. He who raised the living from the dead and the dead from the living. He raised the souls of mankind from its ultimate ignorance to a symbolic state of knowledge

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ABSTRACT

Bacterial cellulose (BC) is an advanced biocompatible polymeric biomaterial with a wide range of biomedical uses, including tissue engineering scaffolds and wound dressings. The main barriers to employing BC in tissue engineering (TE) were the collapse phenomena (the inability to reabsorb water after dehydration) and poor cell adhesion. This research focuses on modifying the nata-de-coco-based BC through thermal crosslinking with citric acid (CA) monohydrate in the absence of a catalyst as the first phase. This is to enhance the BC's biomineralization ability and biocompatibility for application as a bone tissue scaffold. Morphological, physicochemical, and mechanical characterizations of the modified BC were done by means of scanning electron microscopy (SEM), attenuated total reflectance Fourier transformed infrared (ATR-FTIR) spectroscopy, x-ray diffraction (XRD), energy-dispersive x-ray (EDX), thermal gravimetric analysis (TGA), swelling rate (SR), water contact angle (WCA) and tensile analyses. The second phase of the work explored the hydroxyapatite (HA) biomineralization potential of the MBC via a biomimetic synthesis in simulated body fluid (SBF). Selected modified BC (MBC) samples were immersed in SBF and incubated at 37 ℃ in a water bath for 1, 7, 14, and 21 days. Biomineralized samples (BMBC) were freeze-dried and characterized by means of field emission scanning electron microscopy (FE-SEM), ATR-FTIR, XRD, TGA, and wet samples for compressive modulus. The third phase was the evaluation of the biological responses of the BMBC scaffolds to human fetal osteoblast cells. MTS (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reagent and trypan blue dye were employed for cell viability and cytotoxicity while glutaraldehyde fixation was used to evaluate the cell attachment. The finding shows the emergence of ester bond associated FTIR peaks and additional crystalline XRD peaks on all MBC samples which were evidence of the CA crosslinking on the BC. The MBC samples have shown potential antibacterial activity against some bacterial species at certain concentrations based on the disc diffusion technique (DDT) and minimum inhibitory concentration (MIC) assays. Antioxidant activity evaluation has also revealed some radical atom scavenging activity of the MBC in 1-diphenyl-2-picrylhydrazyl (DPPH) solution. Samples showing the best HA nucleation were tested *in vitro* for cell viability, cytotoxicity, and attachment. Osteoblast cell proliferation and attachment on the BMBC samples after 3, 5 and 7 days of culture were the proof of its biocompatibility. Based on the *in vitro* study results presented here, it is apparent that the developed BMBC scaffold is bioactive and biocompatible; thus, it can be considered as a potential alternative for bone tissue engineering application.

ABSTRAK

Selulosa bakteria (BC) ialah biomaterial polimer bioserasi yang inovatif dengan pelbagai aplikasi yang luar biasa dalam bioperubatan, seperti perancah kejuruteraan tisu dan pembalut luka. Halangan utama untuk menggunakan BC dalam kejuruteraan tisu (TE) adalah disebabkan oleh fenomena. Penyelidikan ini memberi tumpuan kepada pengubahsuaian BC berasaskan *nata-de-coco* melalui pemautan silang terma dengan asid sitrik (CA) monohidrat tanpa kehadiran mangkin sebagai fasa pertama. Ini adalah untuk meningkatkan keupayaan biomineralisasi BC dan biokompatibiliti untuk aplikasi sebagai perancah tisu tulang. Pencirian morfologi, fizikokimia dan mekanikal BC yang diubah suai telah dilakukan melalui mikroskopi elektron imbasan (SEM), pengurangan jumlah pantulan-Inframerah pengubah Fourier (ATR-FTIR), pembiasan sinar-X (XRD), sinar-X penyebaran tenaga (EDX), analisis termogravimetrik (TGA), kadar pembengkakkan (SR), sudut sentuhan air (WCA) dan analisis tegangan. Fasa kedua dalam kajian ini telah meneroka potensi biomineralisasi hidroksiapatit (HA) MBC melalui sintesis biomimetik dalam cecair badan simulasi (SBF). Sampel MBC yang terpilih telah direndam dalam SBF dan diinkubasi pada suhu 37 °C dalam air selama 1, 7, 14, dan 21 hari. Sampel yang telah di biomineralis (BMBC) telah dikeringkan secara beku dan dicirikan dengan kaedah mikroskop elektron pengimbasan pelepasan medan (FE-SEM), ATR-FTIR, XRD, TGA, dan sampel basah untuk modulus mampatan. Fasa ketiga ialah penilaian tindak balas biologi perancah BMBC terhadap sel osteoblas janin manusia. Reagen MTS (3-[4,5-dimetiltiazol-2-il]-2,5 difenil tetrazolium bromida) dan pewarna biru trypan digunakan untuk daya maju sel dan sitotoksisiti manakala penetapan glutaraldehid digunakan untuk menilai perlekatan sel. Kemunculan puncak FTIR yang berkaitan dengan ikatan ester dan puncak XRD kristal tambahan pada semua sampel yang diubah suai (MBC) membuktikan pemautan silang CA pada BC. Sampel MBC telah menunjukkan potensi aktiviti antibakteria melawan beberapa spesies bakteria pada kepekatan tertentu, berdasarkan teknik resapan cakera (DDT) dan ujian kepekatan perencatan minimum (MIC). Penilaian aktiviti antioksidan juga telah mendedahkan beberapa aktiviti penghapusan atom radikal MBC dalam larutan 1-difenil-2-pikrilhidrazil (DPPH). Sampel yang menunjukkan nukleasi HA terbaik telah diuji secara *in vitro* untuk daya maju sel, sitotoksisiti dan perlekatan. Percambahan sel *osteoblas* dan lekatan pada sampel BMBC selepas di kultur selama 3, 5 dan 7 hari adalah bukti biokompatibilitinya. Berdasarkan keputusan kajian *in vitro* yang dibentangkan di sini, adalah jelas bahawa perancah BMBC yang dibangunkan adalah bioaktif dan bioserasi maka, ia boleh dianggap sebagai alternatif yang berpotensi untuk aplikasi kejuruteraan tisu tulang.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BC	-	Bacterial cellulose
TE	-	Tissue engineering
CA	-	Citric acid
HA	-	Hydroxyapatite
BTE	-	Bone tissue engineering
ATR-FTIR	-	Attenuated total reflectance
XRD	-	X-ray diffraction
EDX	-	Energy dispersive x-ray
TGA	-	Thermogravimetric analysis
WCA	-	Water contact angle
SR	-	Swelling rate
MBC	-	Modified bacterial cellulose
BMBC	-	Biomineralized modified bacterial cellulose
DDT	-	Disc diffusion technique
MIC	-	Minimum inhibitory concentration
SBF	-	Simulated body fluid
FE-SEM	-	Field emission scanning electron microscope
DMEM	-	Dulbecco's modified eagle medium
CDMEM	-	Complete Dulbecco's modified eagle medium
FBS	-	Fetal bovine serum
hFOB	-	Human fetal osteoblast
DPPH	-	1-diphenyl-2-picrylhydrazyl
MHA	-	Mueller Hinton agar
TBDE	-	Trypan Blue Dye Exclusion

LIST OF SYMBOLS

°C	-	Degree Celsius
Et	-	Young's modulus
σm	-	Tensile strength
εb	-	Elongation at break
nm	-	Nanometer
CrI	-	Crystallinity index
kV	-	Kilovolt
MPa	-	Mega Pascal
GPa	-	Giga Pascal
g	-	Gram
g/L	-	Gram per liter
mg	-	Milligram
mL	-	Milliliter
L	-	Liter
µl	-	Microliter
µm	-	Micrometer
cm	-	Centimetre (s)
min	-	Minute (s)
v/v	-	Volume per volume
w/v	-	Weight per volume
w/w	-	Weight per weight
β	-	Beta
α	-	Alpha
Θ	-	Theta
cm^{-1}	-	Per centimeter
M	-	Molar
Å	-	Angstrom
µg	-	Micro gram

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Diseases, injuries, and trauma were the significant causes of tissue damage and degeneration that often require treatments to speed up the regeneration, repair and/or replacement of the damaged tissue [1, 2]. Among the previously established treatments methods, tissue/organ transplantation and regeneration were the most efficient [3]. Unfortunately, these methods (autograft, allograft, and xenograft) were all challenged by some drawbacks such as patient to patient rejection and cross-infection risk [4-6]. Limited donor availability is another challenge, necessitating the quest for an alternative treatment option to complement the prevailing situation. Tissue engineering (TE), often synonymous with regenerative medicine (RM), a multidisciplinary approach covering a broad range of life sciences and engineering areas tend to address these issues [7, 8].

Tissue engineering is an interdisciplinary field of study combining the knowledge of biology, biochemistry, clinical medicine, material and pharmaceutical sciences, and engineering to understand biological functions and develop substitutes able to replace, restore, maintain, and/or improve an impaired biological system [8-10]. It is a multistep process involving the use of cells seeded on a three-dimensional (3D) carrier material (the scaffold) with appropriate growth factors [4] to mimic the extracellular matrix (ECM). The success of TE is tightly connected to an appropriate scaffold that enables easy cell attachment and adequate energy transfer for the cells to proliferate and differentiate [9, 11, 12]. TE has long been a promising tool for repairing/restoring the function of different tissues, organs, and systems such as skin, bone, cartilage, nervous system, vascular system, urinogenital, and gastrointestinal tissues [5]. Bone tissue engineering (BTE) is an essential aspect of TE and a promising alternative to the traditional treatment methods for critical bone defects due to trauma,

infection, and tumor resection. It relies mainly on a bioactive scaffold with sufficient mechanical integrity to tolerate the bone remodeling process [9, 13].

Advancement in material science and engineering has led to unveiling the potential application of polymeric biomaterials as scaffolds for TE due to their physicochemical and material properties. Polymeric biomaterials have attracted much attention here, owing to their tunable properties able to resemble the ECM of a native tissue [14, 15], such as biodegradability, biocompatibility, and cell adhesive ability [8]. Bacterial cellulose (BC) is one of the explored polymeric biomaterials in BTE [16, 17] due to its fascinating properties such as excellent tensile strength, high purity, degree of polymerization, and crystallinity index [18]. While a native BC lacks sufficient bioactivity, and osteoconductivity as BTE scaffold, its hydroxyapatite (HA) composite was found to support *in-vitro* osteoblast cell attachment, proliferation, and alkaline phosphatase (ALP) expression [19-21]. Composite scaffolds of HA with other polymeric biomaterials have also been reported to support cell attachment, proliferation, and differentiation [22-24]

Hydroxyapatite (HA), having the chemical formula $(Ca_{10}(PO_4)_6(OH)_2$, is an inorganic calcium phosphate mineral found in bone with a Ca/P ratio between 1:6 and 1:5. HA is also said to constitute almost 50% (by weight) of the bone [7, 25]. It is a well-known mineral for developing bioactive scaffolds for BTE due to its outstanding osteoinductive, osteoconductive, and cell adhesive potentials [26, 27]. Compositing BC with HA was also found to enhance the BC's bioactivity [20]. However, the nonuniform dispersibility and low HA nucleation due to insufficient functionality on the BC's surface is still a challenge [28]. To overcome this, researchers explore the multifunctional potential of citric acid (CA) to tune the BC's surface chemistry for better HA nucleation and enhanced cell attachment [29-32].

CA is one of the organic acids enlisted as generally regarded as safe (GRAS) by the US food and drug administration (FDA) [33, 34]. Owing to its three carboxylic (COO-) groups and a single hydroxyl (-OH) group, CA can participate actively in hydrogen bonding interaction with OH-polymers and transform them into reactive functional polymers known as citrate-based-biopolymers (CBBs) through crosslinking

reaction [30, 35]. CBBs are advantageous in TE for the pendent chemistry that accorded them the diverse biological and material characteristics such as antioxidant, antimicrobial, and bio-adhesive properties [36]. They can further be conjugated with other bioactive molecules such as proteins and vitamins through post-polymerization reaction to suit a specific application [37]. CA has long been used as a modifier on different polymeric biomaterials via crosslinking, including the BC [38-42].

Over the last decade, research on cellulose production using microorganisms has intensively been conducted to provide an alternative for plant cellulose [43, 44]. Bacterial cellulose exhibits higher purity compared to plant cellulose, as it contains neither hemicellulose nor lignin. Moreover, a small amount of time is needed to synthesize BC, compared to plant cellulose, which takes a more extended period to grow and mature. These features make BC an attractive material for a wide range of applications, including biomedicine. Nata-de-coco, a jelly desert of Philippines origin, is a pure and cheapest form of bacterial cellulose (BC) produced through the fermentation of coconut water with unique physicochemical properties all within the range of those reported for pure bacterial cellulose [45-47]. Owing to this, nata-de-coco-based BC can serve as a promising model for exploring BC's application potentials in areas such as biomedicine, where high material purity is a fundamental demand.

Attempts have been made to develop a simple, efficient, and green method to fabricate bio-functional TE compliant BC scaffolds and implants possessing the needed biocompatibility, bioactivity, and mechanical strength. Many of these methods aimed to incorporate the commercial HA on the BC surface or synthesize it in simulated body fluid (SBF). However, these methods were challenged by the low HA nucleation and nonuniform dispersion that may be associated with limited reactivity of the BC's surface [48, 49]. Furthermore, the collapse phenomenon (inability to reabsorb water after dehydration) associated with the native BC [42] is another concern that needs to be addressed for the BC to fit better BTE scaffold.

Here, a nata-de-coco-based BC was surface-modified through CA crosslinking reaction for enhanced HA biomineralization. The modified BC (MBC) was

characterized based on physical, chemical, mechanical, and morphological properties. Antibacterial activity and antioxidant (radical scavenging activity) of the MBC were analyzed through disc diffusion technique (DDT), minimum inhibitory concentration (MIC), and 1-diphenyl-2-picrylhydrazyl (DPPH) assay, respectively. HA nucleation was initiated and evaluated on the MBC's surface through the cheap and straightforward SBF immersion method. Finally, the modified biomineralized BC (BMBC) biological activity was assessed on human fetal osteoblast cell lines for potential application in BTE.

1.2 Problem Background

Bone regeneration at the fracture site is a complex process involving a series of intracellular and extracellular signaling pathways to ensure a continuous osteoinduction and osteoconduction that leads to a complete ossification of a new bone [50, 51]. The inherent regenerative ability of a bone falls limited when there is a severe injury to the bone due to trauma or tumor [1, 2]; thus, alternative treatment options are mostly needed. For many decades, grafting techniques, autografts, allografts, and xenografts were the gold standards that later fall short due to limited bone donors, possible risk of an immune response, and infection risk at the graft site [6]. The strategies employed in TE and RM using stem cells often seeded on polymeric biodegradable scaffolds proved a remarkable potential for correcting damaged and/or diseased organ or tissue [4].

Thermoplastic materials have, in this respect, attracted the attention of biomedical engineers. Although approved by the Food and Drugs Administration (FDA), thermoplastics lack some essential features of a suitable TE scaffold. Poly(glycol-sebacate), the first biodegradable elastomer reported by Wang *et al.*, 2002 [52], was later found to be limited due to its hash polymerization conditions and low mechanical strength [37]. Notably, some of the synthetic scaffold materials have also suffered certain drawbacks like the presence of toxic chemicals and limited controllability of structure and properties [53], which may be undesirable for TE application.

In bone tissue engineering, polymer-based biomaterials of high purity and excellent properties that can be turned to simulate the three-dimensional (3D) architecture of the ECM of a native bone have attracted the researcher's attention. In this respect, BC's suitability is due to its unique properties, such as good mechanical strength, biocompatibility, biodegradability, microporosity, and tunable surface chemistry. These, with ease of mouldability into different shapes and structures, made BC a promising BTE scaffold material. Despite its advantages, BC has not been investigated much in BTE [9] although extensively used in other biomedical applications such as artificial blood vessels, wound dressings, specialty membranes, and artificial skin [25].

1.3 Problem Statement

Numerous challenges facing bone regeneration, such as delayed fracture healing due to serious bone injuries and/or disease, have necessitated the quest for alternative treatment options. The gold standard grafting technique (autografts, allografts, and xenografts) that fall short due to limited donors, risk of immune response and infection was relieved by the new approach of TE [6]. Meanwhile, the TE approach employing polymeric biodegradable scaffolds seeded with cells is also constrained by limited bioactivity, biocompatibility, and mechanical strength. Furthermore, some scaffolds were reported to contain toxic chemicals either during fabrication or within their chemical structure, which may be released via the scaffold's degradation.

To contain these challenges, especially in BTE, non-toxic polymeric biomaterials of high purity and excellent physicochemical properties moldable to mimic the 3D architecture of the ECM of a native bone became of utmost interest. Cellulose is one of the advantaged polymers owing to its purity, a high degree of polymerization and water holding capacity, non-toxicity, and biodegradability. Although animals lack the enzyme for cellulose degradation [54], cellulose scaffold was reported to undergo a slow degradation in rat subcutaneous tissue [55]. While this may be a potential limitation for cellulose scaffolds, on the one hand, it can as well be

advantageous on the other hand considering the mechanical strength and time needed for complete bone regeneration [9, 56].

Bacterial cellulose has long been investigated as a scaffold for some TE applications such as drug delivery systems [57], wound dressings [58], vascular grafts [59], and musculoskeletal systems [28, 60]. Coupled with the ease of mouldability into diverse shapes, biocompatibility and microporosity, the unique controllable surface chemistry has made BC a polymer of choice in BTE. Conversely, the irreversible fiber collapse after drying [42, 56], limited functionality and poor cell attachment have impeded the applicability of BC scaffolds in BTE where the cells to be seeded needs an enabling space for efficient adherence, energy transfer and metabolic exchange to optimally proliferate and differentiate.

While attempts have been made to improve the bioactivity and poor cell attachment associated with cellulose scaffolds through the incorporation of HA [5, 9, 20, 61-63], the nucleation of HA is said to be dependent on the materials' surface chemistry. It is established that the hydroxyl (–OH) groups of cellulose have a very poor HA induction compared to carboxyl (–COOH) groups [48, 49]. This could be the basis for the low HA nucleation leading to poor cell attachment on the BC's surface, hence the need for further modification. Leveraging BC's tunable chemistry and the osteoconductive nature of HA, surface modification can enhance a better HA nucleation, thus, improving the poor cell attachment.

CA is an organic acid and a prominent intermediate in the tricarboxylic acid (TCA) cycle of cellular respiration reported to improve the BC's rehydration ability and fiber porosity [42]. CA was also reported to enhance the nucleation of HA [31, 64] and modulate the cellular response of some polymeric scaffolds [65]; thus, it can be used to crosslink the BC. Therefore, the CA crosslinking is expected to impart functionality on the BC's surface that can enhance homogenous nucleation of HA in SBF [25, 66]. It is also envisaged to improve the BC's physicochemical, mechanical, and biological properties, sufficient for cell attachment and proliferation.

1.4 Objectives of the Study

The following objectives were outlined to address the problems mentioned above:

(a) To modify *Nata-de-Coco-based* BC with citric acid at optimal crosslinking conditions towards enhanced HA biomineralization.

(b) To characterize the mechanical, chemical, and physical properties of the modified BC (MBC).

(c) To evaluate hydroxyapatite (HA) growth on the MBC through simulated body fluid (SBF) immersion method.

(d) To evaluate the *in-vitro* cell cytotoxicity, proliferation, and adhesion of human fetal osteoblast (hFOB) cell lines on the modified and biomineralized BC (BMBC).

1.5 Scope of the Study

Within the scope of this study, a nata-de-coco-based BC was modified for BTE application. A multifunctional modifier of OH-polymers CA was used at different concentrations in a simple hydrothermal crosslinking reaction to produce the modified BC (MBC). The resultant MBC samples were subjected to morphological, chemical, physical, and mechanical characterization through SEM, FTIR, XRD, WCA, SR, TGA, and tensile analysis. Antibacterial, antioxidant, and *in-vitro* degradation properties of the MBC samples were also assessed. Selected MBC samples were then subjected to HA nucleation study by SBF immersion method to produce a biomineralized, modified BC (BMBC). Samples were then characterized through FTIR, XRD, TGA and compressive strength. The study is however limited to *in-vitro* testing of the BMBC, where human fetal osteoblast (hFOB) 1.19 (ATCC® CRL 11372™) cell lines were employed to evaluate the cytotoxicity (MTS assay),

proliferation, and attachment in complete Dulbecco's modified eagle medium (CDMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

1.6 Significance of the study

In line with the sustainable development goals, using green and efficient technology to develop products, equipment, and systems are a step ahead to address the numerous global challenges in different areas of human endeavor, including health and medicine. Biotechnological innovations emerged to attract much of researchers' interest due to their potential of preserving natural resources and minimizing the unnatural entities' adverse effects on human lives. The strategic and innovative research measures through which biopolymers are alternatively employed to replace or reduce synthetic polymers' demand have led the to many scientific discoveries. Biomaterials modified with CA, known as citrate-based-biomaterials (CBBs), are among the numerous alternatives to the synthetic polymers used in many TE applications.

BTE seeks a porous, biocompatible, mechanically compliant, and bioactive scaffolding material and BC is said to only lack sufficient bioactivity due to low HA-inducing functional groups. It is an eco-friendly biomaterial with superior chemical and material properties that can be tuned through a modification to suit a specific application purpose. Therefore, the CA crosslinking here addresses the major impediments (collapse phenomenon and limited functionality) limiting BC's application as a BTE scaffold. While the improved water absorption rate is vital for BTE scaffold material, the additional carboxylic (COO-) groups also enhance the uniform HA nucleation, which subsequently improves the BC's bioactivity and cell adhesive ability. Furthermore, nata-de-coco-based BC preferred in this study is to explore the cheapest and purest BC from the easiest large scale production method. This is expected to save time and reduce the high cost incurred in small scale laboratory BC production.

1.7 Thesis Organization

The thesis was organized to contain five (5) chapters for easy comprehension by readers. Chapter 1 was designed to introduce the background, objectives, scope, and significance of the research. Chapter 2 captures the literature review of the important aspect of the study that comprises of a general overview on cellulose, bacterial cellulose, characterization techniques, and application, especially in biomedical field. Chapter 3 covers the general description of methods used in addressing the 3-phases of the research. Phase 1 describes the methods employed in crosslinking modification and characterization of the samples succeeded by *in-vitro* biosynthesis and characterization techniques used for HA biomineralization in phase 2 and biocompatibility evaluation methods in phase 3. Chapter 4 covers the major research outcomes from the crosslinking modification and characterization, HA biosynthesis and characterization of bacterial cellulose., to the cell cytotoxicity, proliferation, and adhesion testing of the biomineralized BC on hFOB cell lines. The conclusion of the research findings and recommendation for future work were presented in Chapters 5.

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LIST OF PUBLICATIONS

1. **Salihu, R.**; Foong, C.Y.; Abd Razak, S.I.; Kadir, M.R.A.; Yusof, A.H.M.; Nayan, N.H.M. (2019) Overview of inexpensive production routes of bacterial cellulose and its applications in biomedical engineering. *Cellulose Chemistry and Technology*, 53, 1–13. **(Q2, IF: 1.467)**.

2. **Salihu, R.**; Razak, S.I.A.; Zawawi, N.A.; Kadir, M.R.A.; Ismail, N.I.; Jusoh, N.; Mohamad, M.R.; Nayan, N.H.M. (2021) Citric acid: Agreen cross-linker of biomaterials for biomedical applications. *European Polymer Journal*, 146, 110271. **(Q1, IF: 4.598)**.

3. **Salihu, R.**; Ansari, M.N.M.; Abd Razak, S.I.; Ahmad Zawawi, N.; Shahir, S.; Sani, M.H.; Ramlee, M.H.; Wsoo, M.A.; Mohd Yusof, A.H.; Nayan, N.H.M.; et al. (2021) Catalyst-free crosslinking modification of nata-de-coco-based bacterial cellulose nanofibres using citric acid for biomedical applications. *Polymers*, 13, 2966. **(Q1, IF: 4.329)**.