# DEVELOPMENT AND CHARACTERIZATION OF CHITOSAN-CELLULOSE NANOFIBER HYDROGEL SCAFFOLD FOR TISSUE ENGINEERING APPLICATION

**HO YUE CHENG**

**UNIVERSITI SAINS MALAYSIA**

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**SCHOOL OF MATERIALS AND MINERAL RESOURCES ENGINEERING**

**UNIVERSITI SAINS MALAYSIA**

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By

**HO YUE CHENG**

**Supervisor: Prof. Madya Ir. Dr. Zuratul Ain Abd Hamid**

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## DECLARATION

I hereby declare that I have conducted, completed the research work and written the dissertation entitled ‘Development and Characterization of Chitosan-Cellulose Nanofiber Hydrogel Scaffold for Tissue Engineering Application’. I also declare that it has not been previously submitted for the award of any degree and diploma or other similar title of this for any other examining body or University.

Name of Student: Signature:

Date:

Witness by

Supervisor: Signature:

Date:

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## LIST OF SYMBOLS

| | |
|---|---|
| $A_1$ | Area of crystalline region |
| $A_2$ | Total area under the graph |
| kBr | Potassium Bromide |
| h | Hour |
| H0 | Pure high molecular weight chitosan scaffold |
| H10_1 | 10 w/w% CNF high molecular weight chitosan scaffold |
| M0 | Pure medium molecular weight chitosan scaffold |
| M10_1 | 10 w/w% CNF high molecular weight chitosan scaffold |
| $S_{eq}$ | Equilibrium swelling ratio |
| v/v% | Volume Percent |
| $W_f$ | Equilibrium swollen weight |
| $W_i$ | Initial weight |
| w/w% | Weight percent |
| $Zn(NO_3)_2$ | Zinc Nitrate |
| λ | Wavelength of radiation |
| % | Percentage |
| °C | Degree Celcius |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 2-D | 2-Dimensional |
| 3-D | 3-Dimensional |
| CNF | Cellulose Nano-Fiber |
| CS | Chitosan |
| DLP | Digital Light Printing |
| DNA | Di-ribonucleic Acid |
| ECM | Extracellular matrix |
| EMA | European Medicine Agency |
| FDA | Food and Drug Administration |
| FTIR | Fourier Transformed Infrared |
| g | gram |
| G | Giga |
| HCS | High Molecular Weight Chitosan |
| MCS | Medium Molecular Weight Chitosan |
| min | Minutes |
| mL | Milli-Liter |
| Pa | Pascal |
| PAA | Poly(acrylic acid) |
| PDMAEMA | poly(dimethylaminoethyl methacrylate) |
| PEG | Poly(ethylene glycol) |
| PLA | Poly(lactic acid) |
| PLG | Poly(lactide-co-glycolide) |
| PMAA | Poly(methacrylic acid) |
| rCL | Regenerated Cellulose |
| SLA | Stereolithography |
| TPA | Texture Profile Analysis |
| UTM | Universal Testing Machine |
| XRD | X-Ray Diffraction |
| γ-PGA | Poly(γ-glutamic acid) |

**PEMBANGUNAN DAN PENYIFATAN KITOSAN-NANOFIBER SELULOSA HIDROGEL SCAFFOLD UNTUK KEGUNAAN KEJURUTERAAN TISU**

## ABSTRAK

Kitosan (CS), sebagai perancah hidrogel semula jadi yang menunjukkan kekuatan mekanikal yang tidak mencukupi, telah terbukti menjadi batasan utama untuk aplikasi kejuruteraan tisu seperti kejuruteraan tisu tulang. Dalam kajian ini, batasan tersebut ditangani dengan penggabungan nanofiber selulosa (CNF) (0%, 5%, 10%, 15%, 20% dan 25 $W_{CNF}/W_{CS}$%) dalam berat molekul sederhana (MCS) dan berat molekul tinggi CS (HCS) perancah hidrogel. Analisis spektroskopi inframerah fourier transformasi (FTIR) mendedahkan bahawa interaksi antara CNF dengan MCS dan HCS adalah secara fizikal dan bukannya kimia tanpa mengubah struktur kimianya, yang sesuai untuk mengekalkan ciri biokompatibel kedua-dua CS dan CNF. Walaupun tanpa interaksi kimia, kesan pengukuhan CNF pada MCS dan HCS dari segi sifat mekanikal terbukti melalui analisis profil tekstur dan ujian mampatan, di mana penggabungan CNF menghasilkan peningkatan kekerasan, ketahanan dan tekanan mampatan pada pemuatan CNF yang optimum. Untuk sifat pembengkakan hydrogel, penggabungan CNF ke dalam perancah CS menyebabkan nisbah pembengkakan keseimbangan berkurang. Hasil yang diperolehi juga menunjukkan bahawa HCS menunjukkan kekerasan dan tekanan mampatan yang lebih tinggi berbanding dengan MCS pada setiap pemuatan CNF. Walau bagaimanapun, nisbah pembengkakan keseimbangan siri HCS lebih rendah berbanding dengan MCS.

**DEVELOPMENT AND CHARACTERIZATION OF CHITOSAN-CELLULOSE NANO-FIBRILS HYDROGEL FOR TISSUE ENGINEERING APPLICATION**

## ABSTRACT

Chitosan (CS), as a naturally derived hydrogel scaffold which exhibit insufficient mechanical strength, has been proven a major limitation for tissue engineering application such as bone tissue engineering. In this study, the limitation was tackled by incorporation of cellulose nanofiber (CNF) (0%, 5%, 10%, 15%, 20% and 25 $W_{CNF}/W_{CS}$%) in medium molecular weight (MCS) and high molecular weight CS (HCS) hydrogel scaffold. The Fourier Transform Infrared (FTIR) analysis revealed that the interaction between CNF with both MCS and HCS is physical rather than chemical without altering their chemical structure, which is ideal to maintain the biocompatible characteristic of both CS and CNF. Although without chemical interaction, the reinforcement effect of CNF on MCS and HCS in terms of mechanical properties was proven through texture profile analysis and compression test, in which the incorporation of CNF results in the enhancement of hardness, resilience and compression stress at optimum CNF loading. In case of swelling properties, the incorporation of CNF into CS scaffold cause reduced maximum swelling ratio. The results obtained also revealed that HCS exhibit higher hardness and compression stress compared to that of MCS at any CNF loading. However, the maximum swelling ratio of HCS series is lower as compared to that of MCS.

# CHAPTER 1

# INTRODUCTION

## 1.1 Research Background

Tissue engineering is a biomedical engineering discipline which combine the concept of biology with engineering to create tissues, cellular products or biological scaffold outside the body to realise the repair or replacement of tissues within the patient body. This can be achieved by the implantation of biocompatible scaffold materials which incorporated with suitable cells isolated from donor tissue (François and Martin, 2003; McClelland and Randall, 2005). In simple words, tissue engineering make use of knowledge in physics, chemistry and biology to explore effective biological material or scaffold to better manage the repair of tissues within the body. These tissue engineering scaffold provides a medium for cell delivery or give growth factors to the damaged site and an appropriate "template" for new tissue formation throughout the 3D construct of scaffold (Dawson et al., 2011). Ultimately, the development in tissue engineering aimed to address the critical gap between the growing number of patients on the waiting list for organ transplantation due to end-stage failure and the limited number of donated organs available (Furth and Mark, 2014).

Recent developments in tissue engineering have led to an increased interest in enhancing structural and compositional components of the scaffold. Accordingly, extensive studies have elucidated the significance of three-dimensional (3D) constructs, namely their morphological resemblance to the structure of the native extracellular matrix (ECM) (Maharjan et al., 2021). Biomaterials for tissue engineering must have controlled surface chemistry, porosity, and biodegradability in order to promote optimal cell adhesion, migration, and deposition of endogenous

extracellular matrix materials by the cells. According to Dawson et al., (2011), additional criteria for a bone tissue engineering scaffold are pertinent to the utility of the scaffold in the clinic. In this context, the scaffold should: (1) meet FDA approval; (2) allow cost-effective manufacture; (3) be amenable to sterilization; (4) facilitate easy handling without extensive preparatory procedures; (5) ideally, be radiographically distinguishable from newly formed tissue; and (6) allow minimally invasive implantation.

With regards to the rapid development of tissue engineering, a biological scaffold, namely hydrogel, has drawn the attention of scientist due to some of its unique properties such as high porosity, high water retention capacity without solubilizing the scaffold, and similar physical properties of natural tissue that enable to mimic the natural ECM. Hydrogel can be prepared from either synthetic or naturally occurring material. Naturally derived hydrogel poses superior cell response, cell adhesion, greater inherent biocompatibility, desirable biodegradability and can be degraded in vivo. In addition, nature-derived materials are usually inexpensive and renewable.

Among naturally derived hydrogel material, chitosan emerged as potential candidate material for tissue engineering application. Derived from chitin, chitosan is a linear natural carbohydrate biopolymer derived from chitin with a structural similarity to glycosaminoglycans of the extracellular matrix (ECM) implicated in cell–cell adhesion (Rodríguez et al., 2019). In terms of chemical structure, it is a natural polysaccharide with outstanding properties in line with excellent biodegradability, biocompatibility, and antimicrobial activity (Sultankulov, 2019). The hydroxyl group present in chitosan macromolecules give hydrophilicity to chitosan which promotes cell adhesion, proliferation, and differentiation of different types of cells and the

polycationic nature of CS at a mildly acidic condition allows immobilization of negatively charged enzymes, proteins, and DNA for gene delivery (Saranya, 2011).

## 1.2 Problem Statement

In contrary with excellent hydrophilicity, biocompatibility and biodegradability, the weakness of chitosan hydrogel scaffold is also obvious, which is lack of mechanical strength compared to common synthetic scaffold material such as poly(lactide-co-glycolide) (PLG) which is mechanically strong. In order to overcome this limitation, scientist has trying hard to incorporated different type of crosslinker in chitosan hydrogel system, in order to promote the formation of crosslinking, either chemically or physically to strengthen the mechanical properties of the hydrogel. Thus, the incorporation of fillers or crosslinker into the hydrogel matrix become an essential process in order to widen the application of chitosan-based hydrogel scaffold.

In terms of chemical crosslinker, the incorporation of glutaraldehyde, diisocyanate, ethylene glycol and tripolyphosphate has been discovered by scientists (Dimida et al., 2015; Ferreira et al., 2019). However, these chemical crosslinker are proven more or less exhibit certain level of toxicity as reported by Spear et al., which discover the cytotoxic of glutaraldehyde which may impair the biocompatibility of a chitosan hydrogel system. Mi et al., (2000) discovered another excellent naturally derived chemical crosslinker, genipin has been successfully utilized to crosslink chitosan hydrogel system. Moreover, Sung et al., (1999), reported that genipin is 5000–10000 times less cytotoxic than glutaraldehyde and this make it suitable to be used in biomedical application. Anyway, even though low, it is believed that genipin crosslinked chitosan scaffold still exhibit certain level of cytotoxicity which may not be suitable for long term application. Also, genipin do not have advantages in terms of

pricing as it costs around MYR 660.00 for only 25 mg at Sigma Aldrich official online store (www.sigmaaldrich.com).

In case of physical crosslinker, nanocellulose based material have been broadly used as filler materials to strengthen the mechanical property of the hydrogel scaffolds. Other than being a reinforcing fillers, nanocellulose also act as a physical crosslinker which does not alter the chemical structure of chitosan. Also, since nanocellulose is naturally derived, it exhibit advantages in terms of low cytotoxicity, high biocompatibility and renewable (Hickey & Pelling, 2019). Nanocellulose based filler could be divided in 3 types, namely nanofiber, nanowhisker and nanocrystal (Wang and Dong, 2018). Among 3 types of nanocellulose, very few studies have been reported on the use of CNF for reinforcement purposes (Maharjan et al., 2021). In fact, as a nanofiber, CNF has high aspect ratio, low density, high elastic modulus, excellent mechanical strength and able to give better reinforcement at the bending region for the application which involve bending. Besides, CNF can form strong physical entanglements and networks in the composite materials, thus leading to the improved mechanical properties. Other than that, CNF is also a cost-effective reinforcing element which available at $19/ gram at Alibaba (www.alibaba.com).

In this project, CNF selected as a reinforcing fillers for the CS hydrogel scaffold. CNF is a biopolymer whose structure contains highly crystalline regions with inter intramolecular hydrogen bonds that makes CNF appealing for preparing appropriate bio-based hydrogels (W. Deng et al., 2021). In addition, the research of Saltz and Kandalam (2016) has been revealed that that CNF forms a percolated network connected by the hydrogen bonds that can provide proper dispersion in a polymer matrix, so increasing the amount of CNF in hydrogel matrices could improve the mechanical and biological properties of Chitosan/CNF hydrogels.

On the other hand, the effect of molecular weight of chitosan on the properties of resulting scaffold also could not be underestimate. This could be supported by the research of Chen et al., (1996) which discovered that, the tensile strength of chitosan membrane produced increasing proportionally with the molecular weight of chitosan used. However, no study has been carried out to investigate the effect of molecular weight of chitosan to the resulting mechanical and physical properties of hydrogel nanocomposites reinforced with CNF.

In fact, the extend of reinforcement of chitosan hydrogel is limited with the incorporation of nanofibril. It is true that the increasing loading of CNF could results in the improvement in mechanical properties of chitosan-CNF hydrogel as reported by the research of Spagnol et al., (2012) and Doench et al. (2019). However, there must be an optimum loading for CNF to reinforce the mechanical properties of hydrogel, in which when the optimum loading is exceeded, the mechanical properties will drop drastically. Also, according to the recent research of Maharjan et al., (2021), the increasing loading of CNF have results in the enhancement of mechanical properties. However, their research also revealed that the stiffness of the hydrogel increases and the water absorption of chitosan-CNF hydrogel reduced from 2471.28% to 2027.76% when the CNF loading increase from 0.05 g/mL to 0.10 g/mL. The decreasing in water absorption is actually an unfavourable drawback which may reduce cell viability.

In this case, it is possible to overcome the mentioned issue by monitoring the molecular weight of chitosan since molecular weight is one of the important parameters which could alter the physicochemical and biological properties of resulting chitosan-CNF hydrogel in order to seek for a dynamic balance between water absorption and mechanical properties. Therefore, throughout the experiment, we hope to study the feasibility of monitoring the properties of resulting hydrogel through

molecular weight of chitosan and investigate the how different molecular weight hydrogel respond to different loading of CNF.

The type of interaction between CNF and CS has been studied by using FTIR. To confirm the effect of CNF to the resulting chitosan scaffold (medium and high molecular weight), the tuneable structure and properties such as swelling properties and of the CS-CNF hydrogel were characterized. In addition, the mechanical properties of the resulting hydrogel also compared through Texture Profile Analysis (TPA) by using texture analyser and compression test by using universal testing machine (UTM).

## 1.3 Research Objectives

The main purpose of this study is to develop a chitosan-CNF hydrogel scaffold (medium molecular weight and high molecular weight) and investigate the effect of CNF loading on the physical and mechanical properties resulting hydrogel with different molecular weight for tissue engineering application. The specific research objectives are as follow:

i. To investigate the effect of different molecular weight chitosan (medium and high molecular weight) to the final mechanical and physical properties of resulting hydrogel scaffold.

ii. To investigate the effect of different loading of nano-fibers (CNF) to the final mechanical and physical properties of hydrogels scaffold.

## 1.4 Thesis Outline

There are total five chapter in this thesis as listed below:

- Chapter 1: Introduction to the research background, problem statement, research objectives and thesis outline.

- Chapter 2: Literature reviews on the hydrogel, CS and CNF together with their various system and application, tissue engineering, bioink, 3D bioprinting, variation in molecular weight of commercially available CS.
- Chapter 3: Explanation on the details of the raw materials, instruments and software, sample preparation, characterization and the overall research flow chart involved in this research.
- Chapter 4: Detailed discussion of the experimental data and results obtained.
- Chapter 5: Summary of this research and suggestions for future work.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction to Hydrogel

Hydrogels, which categorised as a crosslinked polymeric materials, are good examples of a potential and fast developing material which improves the society's benefits and motivates researchers to go beyond the boundaries to explore the application of hydrogel in various field. Initially hydrogels ware a simple inert material but quickly developed to complex stimuli-responsive in their properties, such as volume, pH value, temperature and magnetic field. Recently, stimulus-responsive hydrogels have attracted much attention because of their versatility and a broad range of applications (Cheng et al., 2019). These advanced materials will be eventually used in tissue engineering, wound healing, sensors, contact lenses and drug delivery system (Andre et al., 2015).

A hydrogel is a three-dimensional network made up of chemical or physical cross-linking of individual hydrophilic polymer chains that are capable to swell in water and hold a large amount of water while maintaining its structure at the same time as illustrated in Figure 2.1. Chemical cross-links allow the resulting hydrogel 3D scaffold to exhibit higher mechanical strength and equip the gels with elastic behaviour. It is because the linkage form through formation of strong covalent bonding between two adjacent polymer macromolecular chains. In contrast, physical crosslink only allows weak interaction in between two distinct molecular chain because the linkage mainly rely on molecular entanglement and non-covalent interactions such as weak van der Waals force, hydrogen bonding and ionic interactions which provide cohesion force to build up the entire scaffold and providing viscoelastic behaviour to the scaffold. The

two classes of hydrogels result in very different properties at both the nano- and macro-scale (Christopher, 2020).

With the present of 3D scaffold, hydrogel usually poses ability to hold water. The water content of a hydrogel material is at least 10% of its total weight or volume. The presence of hydrophilic groups such as -$NH_2$, -COOH and -OH has contributed to the hydrophilicity of the network since these functional groups are capable of form hydrogen bonding with water molecules. Due to large water content, a hydrogel material poses significant degree of flexibility which anatomical similarity to humans and behave very similar to natural tissue (Ferreira et al., 2019; Sutapa, 2016).

Hydrogels have numerous attractive features for use as tissue scaffolds. They are composed of hydrophilic polymer chains, which are either synthetic or natural in origin. The structural integrity of hydrogels scaffolds greatly dependent on type of crosslink formed between polymer chain, either physical interactions, chemical interactions or both (Jeanie and David, 2003). Hydrogels used in these applications are typically biocompatible and biodegradable, and a majority of them possess specific cell binding sites that are desirable for cell attachment, spreading, growth, and differentiation. Also, and can be delivered in a minimally invasive manner (Jeanie and David, 2003). All the mentioned the characteristics are vital to make hydrogel a designed material for medical applications and tissue engineering (Gungor-Ozkerim et al., 2018). Commercially available hydrogel can be divided into synthetic hydrogel and naturally- derived hydrogel (Zheng et al., 2020).

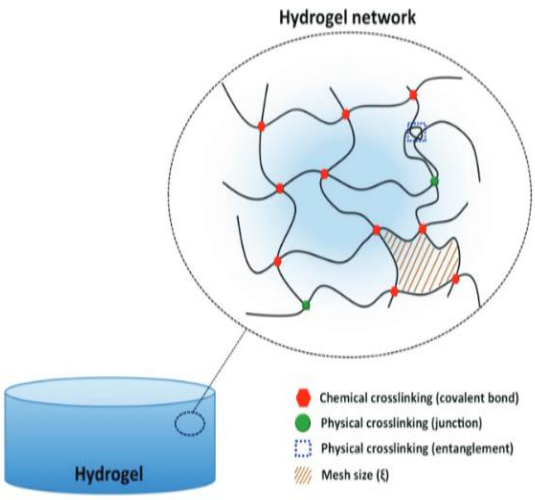


Figure 2.1: Schematic diagram of different type of crosslinking in a hydrogel scaffold (Andre et al., 2015).

### 2.1.1 Synthetic Hydrogels

Synthesised hydrogel often poses excellent reproducibility and chemically consistent compared to some of the uncertainties encountered when using naturally derived biomaterials. The physical and chemical properties of synthetic hydrogels are modifiable according to the desire application owing to the control over the chemical and biological functional group presentation. This could be achieved by altering the cross-linking chemistry during hydrogel formation. Such hydrogels can be functionalized with immobilized biological elements that accommodate natural interaction between cells and extra-cellular matrix (ECM) (Laura et al., 2019). However, the drawback of using synthesized hydrogel is also significant. Synthesized hydrogels often show inadequate bioactivity compared with nature-derived hydrogels. The example of commonly synthesized hydrogels are poly(lactic acid) (PLA), poly(ethylene glycol) (PEG), poly(γ-glutamic acid) (γ-PGA) and poly (ε-caprolactone) diacrylate (Cheng and Chen, 2017; Kim et al., 2021).

### 2.1.2 Naturally Derived Hydrogels

According to Zheng et al. (2020), naturally derived hydrogels are extracted from organism's polysaccharides and proteins. Unlike synthesized hydrogels, naturally-derived hydrogels such as chitosan, alginate, gelatin agarose, collagen, hyaluronan and fibrin have superior cell response and cell adhesion, highly hydrated and can be degraded *in-vivo* (Tonda-Turo et al., 2020; Lim et al., 2019; Misbhak et al., 2019). More importantly, nature-derived materials are inexpensive and renewable and can be used as cell-laden hydrogel bioinks (Gungor-Ozkerim et al., 2018). However, these have a few limitations, for example, high degradation rate, complex purification process, and poor mechanical properties. Among the previously mentioned naturally-derived hydrogels, Hidaka et al., (2021) reported that chitosan has attractive features such as antimicrobial activity, biodegradability, and biocompatibility. Although the sources of naturally derived materials are far more constrained than synthetic polymers, the highest proportion of bioink studies have concentrated on natural polymers.

## 2.2 Chitosan Hydrogel

Chitosan (CS) is a linear polysaccharide that consists of randomly distributed β -(1–4) linked D-glucosamine and N-acetyl-D-glucosamine as shown in Figure 2.2. CS is approved by Food Drug Administration (FDA) and European Medicines Agency (EMA) for use in wound dressing and as vaccine adjuvant, and tissue engineering. CS is classified under Chemical Responsive Hydrogel which are sensitive to pH changes of external environment similar to poly(acrylic acid) (PAA), poly(methacrylic acid) (PMAA), and poly(dimethylaminoethyl methacrylate) (PDMAEMA). These polymers are widely used in the preparation of hydrogel.

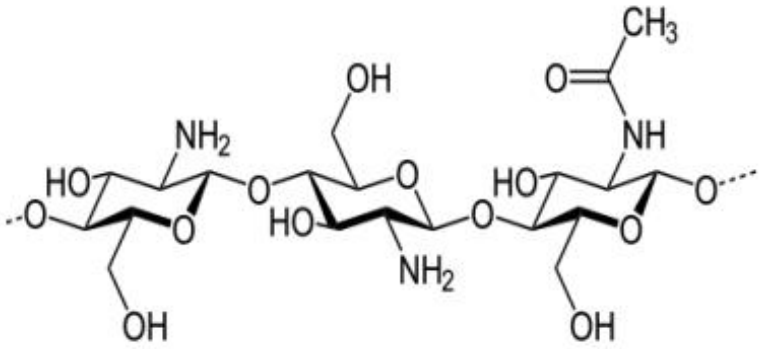


Figure 2.2: Chemical structure of commercially available chitosan.

According to Mantha et al., (2019), the pH-sensitive nature of CS allow it to combine with thermoresponsive crosslinker to produce dual-stimuli responsive hydrogel which respond to localized conditions of pH and temperature in the human body. The pH-responsive characteristic allows good promising of CS to be utilized in various tissue engineering application, such as controlled drug delivery and incorporation of bioactive agents. Chitosan-poly acrylic acid hydrogels have been tested for colon specific drug delivery. Early in 2010, the research of Gong et al. had successfully developed a chitosan-based hydrogel for specific drug delivery at human colon by using Chitosan and acrylic acid grafted chitosan through the utilization of N, N'-methylene-bis-(acrylamide) as a crosslinker. In their research, it is proven that the combination between biodegradability of chitosan by colonic normal flora along with pH sensitivity of the polyacrylic acid segment make this system a potential suitable carrier for gradual drug release in release colonic region.

Other than drug delivery, chitosan scaffolds also pose high potential to be used for various type of tissue regeneration application such as skin, bone, cartilage, skin and even nerves (Ahmadi et al., 2015). However, in terms of nerve regeneration in nerve tissue engineering, the treatment for central nervous system disorder is still a challenging issue since nerve cells exhibit low regeneration ability. Thus, the utilization

on CS scaffold in nerve treatment should be used in combination with neural stem cells as mentioned in the research of Li et al., (2012).

According to the research Zargar et al., (2015), chitosan are useless products of the crabbing and shrimp canning industry. The processing of crustacean shells mainly involves the removal of proteins and the dissolution of high concentration of calcium carbonate in crab shells. Final chitosan is deacetylated in 40 % sodium hydroxide for 1–3 h at 120 °C to produce about 70 % deacetylated chitosan. The chitin and chitosan processing includes:

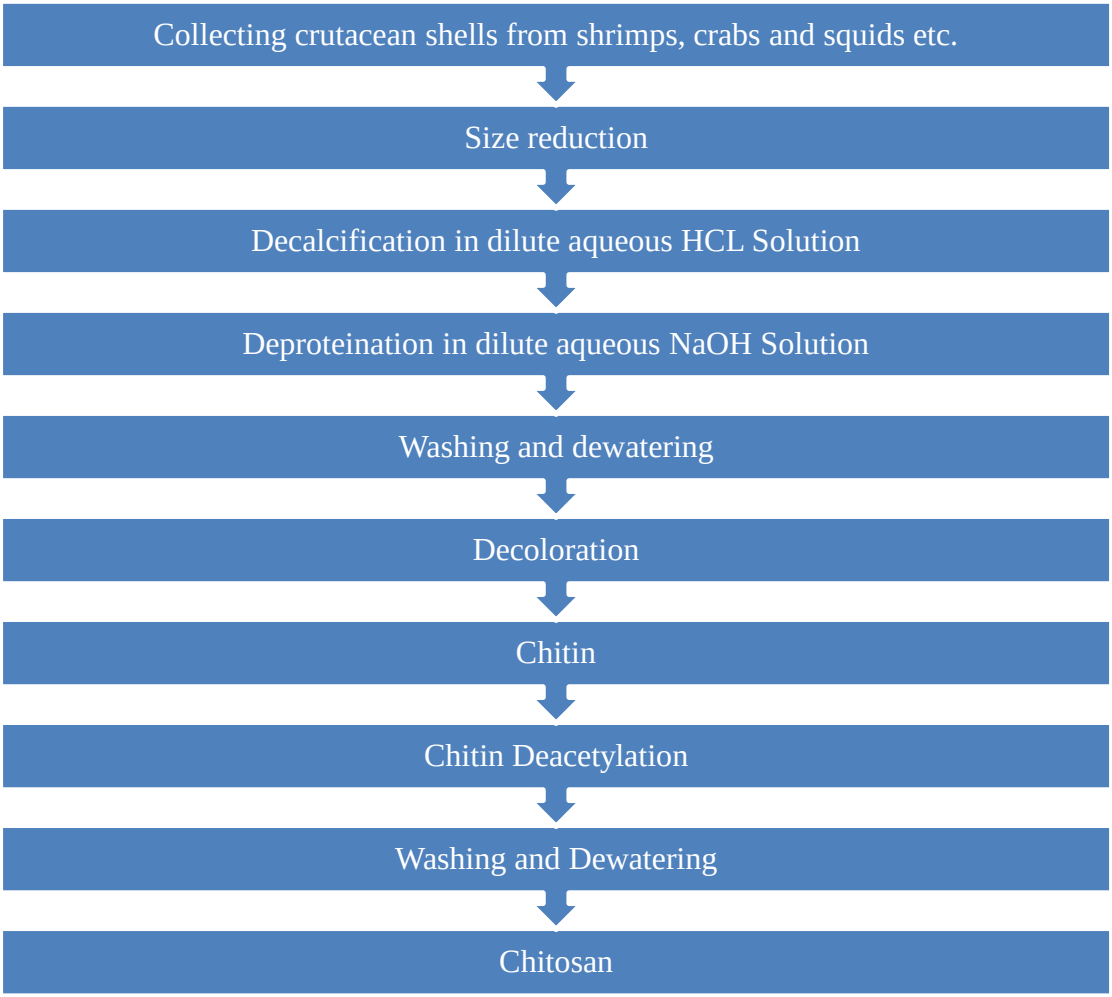


Figure 2.3: Processing steps to obtain chitosan from crustacean shells (Zargar et al., 2015).

### 2.2.1 Advantages and Limitations of Chitosan

This polymer is highly sustainable, cheap and easy to find. It can be derived naturally from chitin shells of shrimp, shellfish and other crustaceans via chemical processing which . As reported by Laura et al., (2019), it is one of the most well-studied biomaterials for biomedical applications, including neural tissue engineering and regenerative medicine. In addition, Moura et al., (2007) stated that chitosan exhibits good ability in film and gel forming excellent ability to produce three-dimensional scaffolds. Besides, chitosan also approved by US Food and Drug Administration (FDA) to be used medical wound dressing because of its excellent antibacterial and antifungal characteristic (Zheng et al., 2020).

However, the scaffold structure of chitosan is relatively weak and unstable with lower mechanical properties in comparison to synthetic hydrogel. Chitosan based hydrogels reach high viscosities with optimal printability, but they are not stable in physiologic conditions, if not crosslinked. Thus, it is necessary to crosslink the chitosan hydrogel in order to modified and stabilize so that it last long enough over a desired period of time. A far stronger and more extensive intermolecular interaction is able to achieve through chemical cross-linking processes which link the molecular chain of hydrogel through covalent bonding. The presence of covalent crosslinked structure enables improvement on the properties to the resulting hydrogel. Other than that, chemically induced cross-linking under physiological conditions produces a relatively stable network corresponding to a more predictable degradation behaviour. In brief, crosslinking is necessary to enhance mechanical strength and chemical stability, porosity and aqueous swelling features of chitosan-based materials (Moura et al., 2015).

### 2.2.2 Ways to Overcome Limitation

#### 2.2.2(a) Various Crosslinking Agents

One approach to overcome the low mechanical properties of Chitosan hydrogel scaffold is by crosslinking reaction through various crosslink agents. In this case, Dimida et al., (2015) had reported a few types of reagents have been proven effective for the covalent crosslinking of chitosan such as glutaraldehyde, diisocyanate, ethylene glycol and tripolyphosphate. For instance, Ferreira et al., (2019) had successfully crosslinked chitosan hydrogen with the incorporation of glutaraldehyde as the primary crosslinking agent. Author also found that the reaction is more efficient when using catalysts such as zinc nitrate ($Zn(NO_3)_2$). Even though the incorporation of these crosslinking agent is claimed to exhibit low cytotoxicity, but unfortunately, studies at earlier 1980 had revealed that these synthetic crosslinking reagents used are all more or less cytotoxic and may impair the biocompatibility of a chitosan hydrogel system (Speer et al.,1980). Therefore, we may conclude that the toxicity of synthetic cross-linking agents presents a major drawback in the use of hydrogel in biomedical application, such as injectable matrices or as in situ-forming polymer scaffolds, because their seepage into body fluids can be harmful, even at low concentrations. Hence, it is desirable to provide a naturally derived crosslinking reagent to which has low cytotoxicity and more biocompatible to crosslink the hydrogel for biomedical applications (Moura et al., 2015).

To solve the biocompatibility issue of hydrogel crosslinked by this issue, a new naturally derived crosslinking agent which is genipin is introduced. Earlier in 2000, three scientists namely Fwu-Long Mi, Hsing-Wen Sung and Shin-Shing Shyu had attempted to synthesis and characterize a novel chitosan-based network crosslink by naturally occurring crosslinker. Fortunately, they successfully synthesis chitosan-based

hydrogel crosslinked by a naturally derived reagent, which is genipin. It has been reported that genipin is readily react with amino acids or proteins to form dark blue pigments which make genipin a suitable reagent for the crosslinking of chitosan-based hydrogel. According to Sung et al., (1999), genipin is 5000–10000 times less cytotoxic than glutaraldehyde which is an extremely suitable crosslink agents to be used in the synthesis of hydrogel for medical application.

It is notable that the low cytotoxicity of genipin is attributed to its derivation from natural. The research works from Mi et al., (2000) mentioned that genipin is an aglucone of geniposide, a component of the Chinese medicine which may be isolated from the fruits of Gardenia jasminoides Ellis. According to Dimida et al., (2015), genipin is commonly manufactured by using ß-glucosidase and is extracted by a direct chemical procedure or by a microbiological process involving Peniccilium nigricans that produces ß-glucosidase, which in turn hydrolysed geniposide into aglycone genipin. It is also reported that genipin is widely used in variety of pharmacological activities such as choleric action and inhibition of gastric acid secretion. The crosslink mechanism of genipin are shown in Figure 2.4.

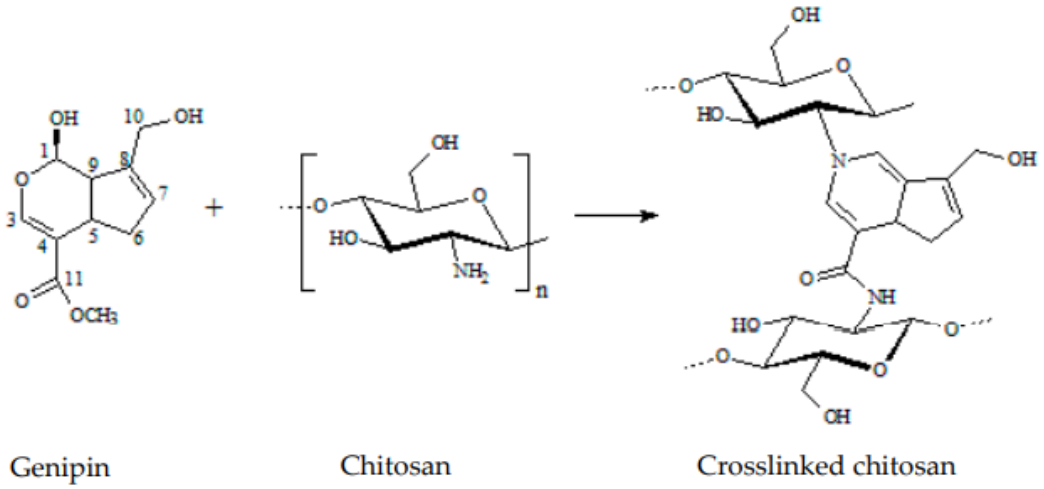


Figure 2.4: Crosslink mechanism of genipin with chitosan (Pomari et al., 2019).

The success of Mi et al., (2000) to synthesis genipin crosslinked chitosan hydrogel which has significantly higher biocompatibility compared to other synthetic crosslinking agent has drawn attention of other researchers to develop chitosan-based hydrogel for biomedical application. Although chitosan hydrogel poses high biocompatibility and improved strength through crosslinking agents, however, the mechanical strength poses by scaffold is still way to weak for some application such as human cartilage replacement.

### 2.2.2(b) Reinforcement through Nanocellulose Based Fillers

Then, many studies have added nanoparticles as reinforcing fillers to their matrices in order to improve the mechanical properties and further explore the potential of chitosan hydrogel in various applications. Wei et al., (2021) reported that some structures and properties of hydrogel or aerogel scaffolds can be reinforced by the incorporation of nanocellulose based fillers due to its biocompatibility, biodegradability, hydrophilic and excellent mechanical properties which make nanocellulose suitable to be used for biomedical application. According to the research of Pisani et al. (2020), the incorporation of nanocellulose poses great influence on the Young's modulus, compressive Young's modulus, swelling rate, gel time, surface morphology, porosity, the antibacterial properties of scaffolds, and can regulate the balances among these properties in order to promote the proliferation rates of cells and the biorecognition sites of the scaffolds (Pisani et al., 2020).

Nanocellulose is able to be derived from abundant naturally occurring resources such as cotton and rice husk. In recent years, nanocellulose has received growing worldwide attention because of its extraordinary supramolecular structure and exceptional properties (W. Deng et al., 2021). Donius et al., (2014) has stated that, nanocelluloses is commonly range from rod-like highly crystalline cellulose

nanocrystals to longer and more entangled cellulose nanofibers, commonly denoted as cellulose nanocrystal (CNC) and cellulose nanofibril (CNF), in which both of them are naturally-derived fillers. CNF, as a cost-effective reinforcing element, can form strong physical entanglements and networks in the composite materials, thus leading to the improved mechanical properties.

Fiber-reinforced hydrogels can be engineered to mimic many biological tissues as they present similar microstructural and mechanical properties, and functionality (Doench et al., 2019). Thus, naturally-derived fillers reinforced hydrogel have great potential for tissue engineering and biomedical application.

To utilize CNF as a reinforcing filler for hydrogels, isolation of CNF from cellulose with linear β-1,4- linked D-glucopyranose units, is in an unavoidable issue. Since hydrogels can be used in biomedical areas, the biocompatibility of CNF plays a vital role besides the reinforcement of hydrogels. Cellulose nanofibrils or nanofibers (CNF) have been an interesting biopolymer and becoming a keen focus of researchers during the last decade due to outstanding characteristics such as their good stiffness/ elastic modulus (30-50 GPa), high strength 2-6 GPa, availability, renewable origin and high biocompatibility since it is a naturally-derived fiber (Ismail et al. 2019; Usov et al. 2015).

The ability of CNF molecular chain to form inter and intra-molecular hydrogen bonding are the main contributors to its extraordinary mechanical properties. According to Popescu, (2017), cellulose macromolecule consists of two different ends: one reducing end which consists of a free hemiacetal (aldehyde) at the C1 position, another end is non-reducing contain a d-glucose unit with a free hydroxyl group at the C4 position. Each β-glucopyranose units consist of three hydroxyl groups at C6 (primary alcohol), C2 and C3 (secondary alcohols) (Popescu, 2017). These hydroxyl group allow

cellulose macromolecules to form intramolecular hydrogen bonds and intermolecular hydrogen bonds as illustrated in Figure 2.5. The intramolecular hydrogen bonding contribute to the stiffness of the polymer chain, while the intermolecular hydrogen bonding allow the linear polymers to aggregate and form fibres (Kalia et al., 2011; Moon et al., 2011).

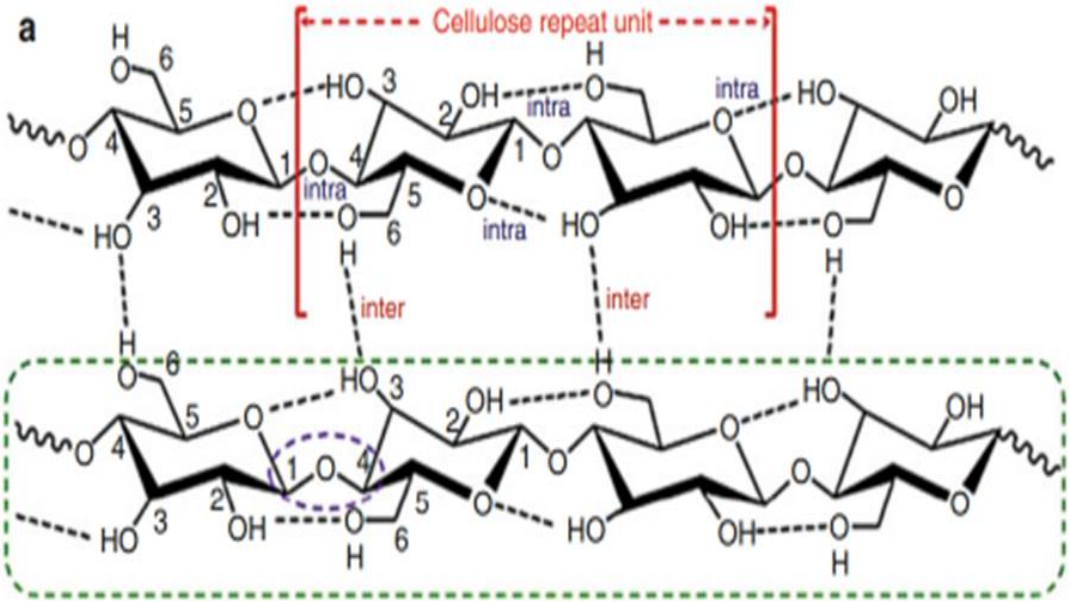


Figure 2.5: Hydrogen bonding in cellulose macromolecule (Zhang et al., 2019).

According to Bahadoran et al. (2020), CNF poses two significant advantages in comparison to other typical nano-structured materials such as carbon nanotube and graphene nanosheets as listed below:

i. Cellulose has high abundancy in natural sources which can be extracted from wood and non-wood agriculture products such as cotton and wheat. The abundancy of cellulose in natural had make it an environmental-friendly yet biocompatible material. These factors had lead cellulose-based material potential to be used in biomedical applications such as tissue engineering, wounds healing and transplant.

ii. In addition, due to the active surface of CNF, they can be functionalized by various chemical treatment for specific applications.

Lately, a study has been conducted on chitosan hydrogel reinforced with CNF for by Doench et al. (2019) for intervertebral disc tissue repair. The research found that the incorporation of CNF in chitosan hydrogel matrix had successfully enhanced the hydrogel mechanical properties. In brief, the increasing in CNF content from 0.2 wt% to 0.4 wt% had cause an enhancement of maximum elastic modulus from 0.24 MPa to 0.30 MPa. In terms of porosity, scanning electron microscopy (SEM) images reveal that the increase in CNF content yielded hydrogel of smaller pore sizes for a given chitosan concentration.

In another study from Spagnol et al., (2012) had revealed that incorporation of CNF to chitosan-grafted poly(acrylic acid) matrix also show profound effect to the swelling capacity and absorption rate of the hydrogel. The introduction of cellulose nanofibrils within the chitosan-grafted poly(acrylic acid) network greatly improved the equilibrium of water absorbency capacity and decreased the necessary time to reach the equilibrium condition. In detail, the capacity value and the rate constant of the swelling for the hydrogel without CNF were 381 $g_{water}/g_{absorbent}$ and 2.86 x $10^{-6}$ $min^{-1}$, respectively. By adding CNF, these values were increased to 486 $g_{water}/g_{absorbent}$ and 4.06 x$10^{-6}$ $min^{-1}$, respectively. Besides that, in comparison with unreinforced hydrogel, the compression strength and modulus of hydrogel with 5 wt% of CNF were increased by about 69% and 140%, respectively. Also, as the loading of CNF getting higher, the mechanical properties of hydrogel were increased continuously.

It is notable that cellulose derived nano-fillers especially CNC and CNF have been extensively used in the preparation of chitosan-based hydrogel and reported to achieve positive results in the reinforcement of the novel chitosan hydrogel. However,

no comprehensive study has been done to investigate the effect different molecular weight chitosan to the properties of resulting chitosan hydrogel nanocomposites.

### 2.2.3 Variations in Chitosan Molecular Weight

According to Sun et al., (2009), commercially available chitosan is characterized by a degree of deacetylation between 70 and 95% and a MW between 50,000 and 2,000,000 Da. In this case, the commercially available chitosan is divided into 3 different molecular weight, which is low-molecular-weight (LMW, ~40,000 Da), medium-molecular-weight (MMW, ~480,000 Da) and high molecular-weight (HMW, ~850,000 Da) chitosan. Seyfarth et al., (2008) had reported that molecular weight of the chitosan hydrogel is one of the parameters that could monitor the physicochemical and biological properties of chitosan. According to Chen et al. (1996), the tensile strength of chitosan membrane produced increasing proportionally with the molecular weight of chitosan used.

## 2.3 Tissue Engineering

The tremendous need for organs and tissue nowadays has driven the rapid development of tissue engineering. This field utilize the combination of knowledge on physics, chemistry and biology such as cell biology, biomaterials science, cell-material interactions, surface characterization and even nerve tissue engineering (Ahmadi et al., 2015; Sherbiny and Yacoub, 2013). In simple words, tissue engineering is a discipline seeks to fabricate, restore, preserve, enhance tissue functions and facilitate living replacement parts for the body. It also aims to provide a solution for organ mismatch issue, replacement of damaged organs or defective tissue which may cause by either accidents or disease (Jeanie and David, 2003; Sherbiny and Yacoub, 2013).

The core concept of tissue engineering are described and illustrated in Figure 2.5. An organ or tissue can be developed through isolation and incubation of the targeted cell which obtained from patient's small tissue biopsy and harvested in vitro. The harvested cells are then transferred or seeded into a suitable 3D hydrogel scaffold which mimic the ECM of the targeted tissue. According to Sherbiny and Yacoub, (2013) the selected scaffold should be able perform the key function to:

i. show active cell-biomaterial interactions which enable rapid cell growth,
ii. allow seeded cell to be deliver the seeded cells to the desired site in the patient's body,
iii. promote cell adhesion so that cell can growth and attached firmly on scaffold,
iv. permit adequate transport of gases, nutrients and growth factors to ensure cell survival, proliferation, and differentiation (poses adequate porosity), and
v. do not cause or poses negligible toxicity and inflammation in vivo,

After sufficient cell growth, the cell-loaded scaffolds are ready to be transplanted into targeted patient's parts. The transplantation can be done by either through direct injection with the utilization of medical needle, minimally invasive delivery technique, or through surgery to implant the fabricated tissue at the desired site in the patient's body.

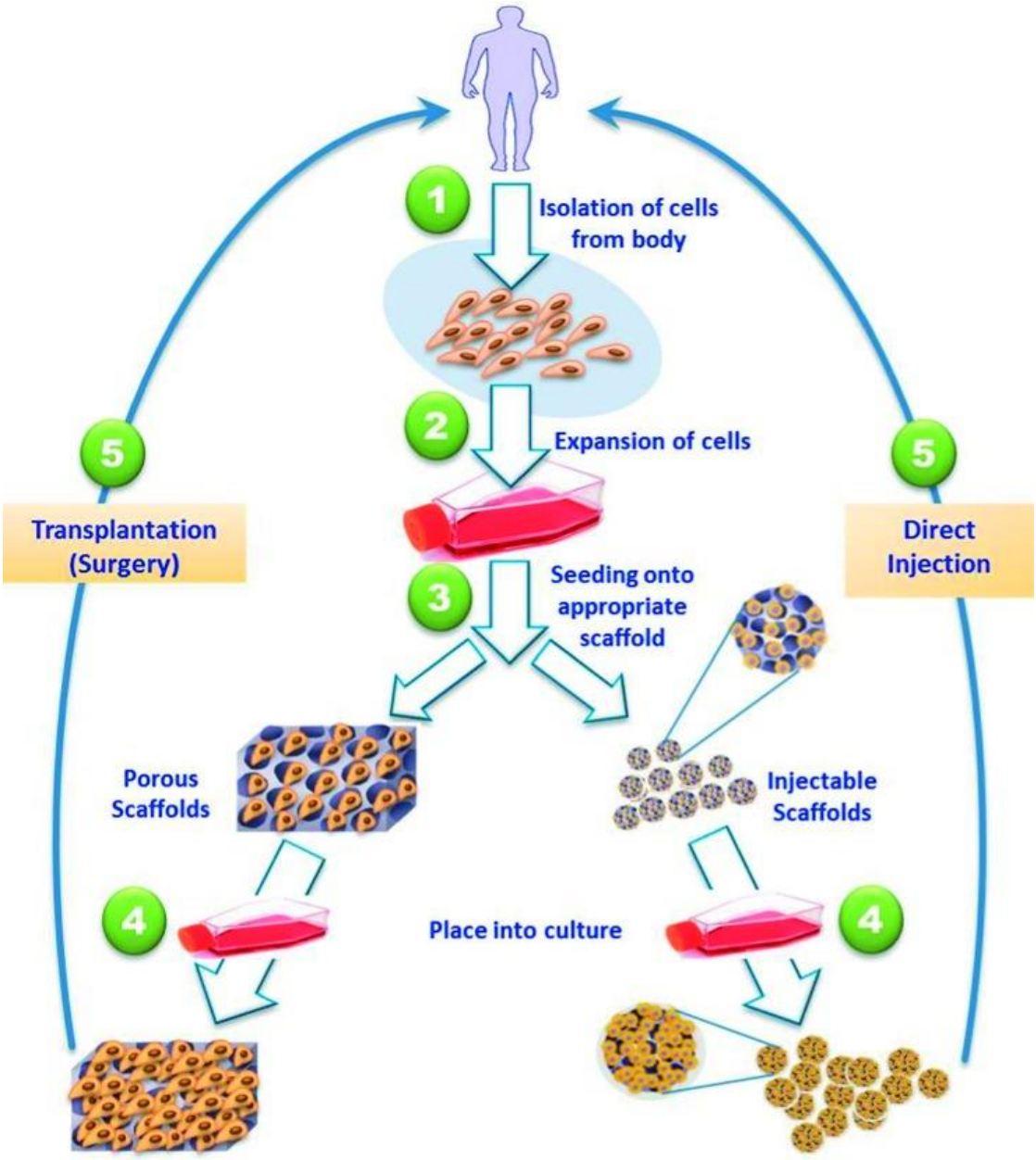


Figure 2.6: Illustration of the most common concept and approach in tissue engineering Sherbiny and Yacoub, (2013)

From the description and illustration on the basic concept of tissue engineering, we may notice that designing a scaffold with optimal characteristic as mentioned previously is a vital step for successful tissue engineering. In this case, hydrogel scaffold has received attention from scientist since last decades due to its characteristic similar to ECM of human body cell, porosity while at the same time poses adequate strength to

function as a template for cell attachment, cell growth cellular proliferation and survival.

Thus, we may conclude that the present of porous structure in hydrogel matrix is important for tissue engineering since porosity have massive impact on swelling behavior, cell adhesion and cell proliferation rate that vital to facilitate tissue growth. In this case, chitosan emerged as a suitable candidate for tissue engineering application. According to Ahmidi et al., (2015), three distinct method are available for the preparation of porous chitosan hydrogel scaffold for tissue regeneration which are freeze drying, gas foaming and salt leaching. Salt leaching method utilized the pH sensitive nature of chitosan to prepare hydrogel. This method is suitable to be used in combination with a technology which capable to prepare a hydrogel scaffold with high structural integrity, namely 3D-Bioprinting.

### 2.3.1 Bioink and 3D-Bioprinting

3D-bioprinting combines biomaterials, living cells, drugs, growth factors, and genes in a controlled and accurate manner mimicking living tissue and organs. The technique is studied with two main aims as reported by Lee & Cho, 2016; Park, Jung, & Min, (2016):

i. fabricating mini-tissue as diseases in vitro models and drug testing, and
ii. obtaining engineered tissues for implantation in the human body

In recent years, there is an increasing interest in the development of 3D complex functional architectures with appropriate biomaterials and cells, in particular with the strategy of mimicking the cellular microenvironment of native tissues. Three-dimensional printing has emerged as a powerful tool for tissue engineering, which applies additive manufacturing to bio-fabricate 3D tissue-resembling objects with a high degree of spatial organization. A layer-by-layer deposition of materials, called