

**INVESTIGATION OF THE CYTOTOXICITY OF  
CASSAVA  
MICROFIBER-GELATIN COMPOSITE SCAFFOLD**

**BY**

**PORTIA NANA ADJOA PLANGE**

**(ID. NO: 10525304)**

**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON  
IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF  
MPHIL BIOMEDICAL ENGINEERING DEGREE**



**JUNE, 2022**

## DECLARATION

I, **Portia Nana Adjoa Plange**, do hereby declare that except for the references which have been duly cited, the entire work presented in this thesis, titled “**Investigation of the cytotoxicity of cassava microfiber-gelatin composite scaffold**” was solely conducted and written by me, and that, this thesis has never been presented either in part or in whole for any degree in this University or elsewhere.



21/06/ 2022

.....  
Portia Nana Adjoa Plange (10525304)

.....  
Date

(Student)

This thesis has been submitted for examination with our approval as supervisors.



23/06/2022

.....  
Prof. Elsie Effah Kaufmann

.....  
Date

(Principal Supervisor)



23/06/2022

.....  
Dr. Jocelyn Kofi Brobbey

.....  
Date

(Co-Supervisor)



23/06/ 2022

.....  
Dr. Anastasia Rosebud Aikins

.....  
Date

(Co-Supervisor)

## ABSTRACT

Cellulose fiber-reinforced composite scaffolds have recently become an interesting target for Biomedical Engineers and Biomaterials Researchers for Tissue Engineering (TE) applications. This has led to the exploration of cellulose from diverse sources such as tunicates, bacteria and especially from plants. Cassava bagasse, which is a fibrous solid residue obtained after the extraction of cassava and soluble sugars, has recently been explored as a potential source of cellulose, and has been successfully used to enhance the mechanical properties of gelatin scaffold for TE purposes. However, there is lack of knowledge on the biocompatibility of this fabricated scaffold, limiting its potential to be considered at the research level as a biomaterial for TE purposes. This study provided knowledge on the cytotoxicity of the scaffold by using HEK 293 and MDA MB 231 cell lines. The tests performed were according to ISO standards for checking *in vitro* cytotoxicity of medical devices- extraction and direct contact tests. Scaffold prepared with gelatin only, and cells cultured on well plates with no scaffold were used as the controls. Extracts obtained from the samples were exposed to the cells and analyzed after 24 h and 48 h of incubation, using optical and fluorescence microscopy. Additionally, cells were seeded directly on the samples and analyzed after day 1, 3 and 5 using tetrazolium-based colorimetric assay. Results obtained for HEK 293 cells demonstrated enhanced cell viability and little/no changes in cell morphology. However, there was a decline in cell viability and changes in cell morphology for MDA MB 231 cells. These results suggest that the presence of the fiber in gelatin is not cytotoxic to HEK 293 cells and can be considered for TE purposes when using normal cells. On the contrary, the presence of the fiber in gelatin is cytotoxic to MDA MB 231 cells and may not be considered for TE purposes such as 3D tumor cell studies that require the growth of cancer cells. However, further studies are required to explore the use of the cassava bagasse for its anti-cancer cell properties as demonstrated in this study.

## **DEDICATION**

I dedicate this thesis to the Almighty God, my family, and my mentors.



## ACKNOWLEDGEMENTS

I am most grateful to God for his strength and wisdom throughout my graduate study. My deepest gratitude goes to my supervisors, Prof. Elsie Effah Kaufmann, Dr. Anastasia Rosebud Aikins and Dr. Jocelyn Kofi Brobbey (University of Ghana) for their invaluable support, encouragement, mentorship, and making time to carefully read through my thesis countless times for errors, and their insightful contributions to finalize my thesis.

I would also want to express my heart-felt gratitude to Prof. Gordon Awandare (Director, WACCBIP) for sponsoring this project work and to Prof. Osseo-Asare (The Pennsylvania State University) and his colleagues for sponsoring my education. Many thanks to the Department of Biochemistry, Cell and Molecular Biology, University of Ghana for allowing me access to their laboratories and equipment, with special thanks to Dr. Isawumi Abiola for freeze drying my samples and Mr. Srinivasan for access to the microscopy room.

My sincerest appreciation also goes to members of the Aikins Lab (Department of Biochemistry, Cell and Molecular Biology), especially Samuel Mensah Baffoe and Jessica Kugblenu, for making time to tutor me on cell culture techniques and their encouragement which helped me through my project. Many thanks to my colleague, Temitayo Samson Ademolue (Department of Biochemistry, Cell and Molecular Biology) for helping me with the fluorescent microscopy imaging.

Special thanks to Dr. Godwin Amenorpe of Biotechnology and Nuclear Agricultural Research Institute (BNARI), Ghana Atomic Energy Commission (GAEC) for providing us with cassava tubers for this research, and also to Michelle Oti-Bronya and Miracle Ndego (Department of Biomedical Engineering) for assisting me with the cassava extraction process. Many thanks to Mr. Solomon Katu (Department of Biomedical Engineering) for allowing access to the lab during my cassava fiber extraction process.

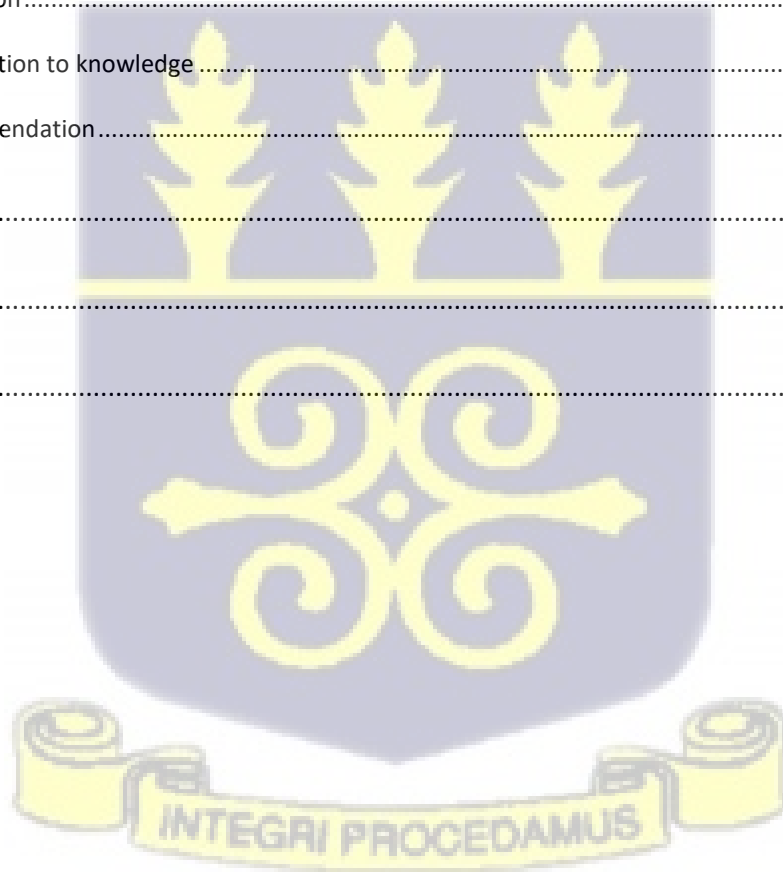
## TABLE OF CONTENT

### Contents

DECLARATION.....	ii
ABSTRACT.....	iii
DEDICATION .....	iv
ACKNOWLEDGEMENTS .....	v
TABLE OF CONTENT.....	vi
LIST OF FIGURES.....	ix
LIST OF TABLES .....	xi
LIST OF ABBREVIATIONS.....	xii
<b>CHAPTER 1 .....</b>	<b>1</b>
INTRODUCTION .....	1
1.1 Problem Statement .....	4
1.2 Significance of Study .....	5
1.3 Research Questions.....	5
1.4 Hypothesis.....	6
1.5 Research Aims and Objective.....	6
1.6 Organization of Dissertation .....	6
<b>CHAPTER 2 .....</b>	<b>7</b>
LITERATURE REVIEW.....	7
2.1 Tissue Engineering Overview .....	7
2.2 Scaffolds .....	9
2.2.1 Scaffold Fabrication.....	11

2.2.2 Scaffold Biomaterials.....	12
2.3 Gelatin.....	14
2.4 Cellulose.....	18
2.5 Cassava Fiber Cellulose.....	20
2.6 <i>In Vitro</i> Cytotoxicity Test.....	23
2.6.1 MTT Assay.....	25
2.6.2 Live/Dead Staining Assay.....	26
2.6.3 Trypan Blue Exclusion Dye Assay.....	27
2.6.4 Cell Choice for <i>In Vitro</i> Test.....	28
2.6.5 <i>In Vitro</i> Tests Done on Composite Scaffolds.....	28
<b>CHAPTER 3.....</b>	<b>31</b>
METHODOLOGY.....	31
3.1 Cassava Tubers.....	31
3.2 Reagents.....	31
3.3 Cassava Tuber Preparation and Fiber Isolation.....	32
3.4 Synthesis of Cassava Microfiber-Gelatin Scaffold.....	33
3.5 Cell Culture.....	35
3.6 Sample Sterilization.....	36
3.7 Cell Proliferation Test.....	36
3.8 Scaffold Surface Morphology Imaging.....	39
3.9 Cytotoxicity Extraction Test.....	39
3.9.1 Cell Morphology Analysis:.....	40
3.9.2 Live/Dead Staining.....	42
3.10 Data Analysis.....	43
<b>CHAPTER 4.....</b>	<b>45</b>
RESULTS AND DISCUSSION.....	45
4.1 Scaffold Surface Morphology.....	45

4.2 Extraction Test Results .....	46
4.2.1 Cell Morphology Analysis .....	46
4.2.2 Live/Dead Staining Assay.....	51
4.3 MTT Assay Results.....	55
4.3.1 HEK 293 .....	55
4.3.2 MDA MB 231 .....	58
4.3.3 Comparison of MTT Assay Results for MDA MB 231 and HEK 293 cells .....	61
<b>CHAPTER 5 .....</b>	<b>63</b>
SUMMARY, CONCLUSION AND RECOMMENDATION.....	63
5.1 Summary .....	63
5.2 Conclusion.....	63
5.3 Contribution to knowledge.....	65
5.4 Recommendation.....	66
APPENDIX.....	67
.....	68
REFERENCES.....	69



## LIST OF FIGURES

<i>Figure 1. Schematic diagram of tissue engineering methods (Belleghem et al., 2020) .....</i>	<i>9</i>
<i>Figure 2. Denaturation of collagen to produce gelatin (Bello et al., 2020). .....</i>	<i>15</i>
<i>Figure 3. Structure of lignocellulosic biomass with hemicellulose, cellulose and lignin (Alonso et al., 2012). .....</i>	<i>19</i>
<i>Figure 4. Images of fiber extraction process, (a) cassava soaked in water; (b) cassava soaked after 14 days; (c) extracted cassava fiber; (d) drying of fiber in oven. ....</i>	<i>33</i>
<i>Figure 5. Pictorial representation of scaffold fabrication process. ....</i>	<i>34</i>
<i>Figure 6. Image of Labconco Freezone Freeze dryer and freeze dried samples. ....</i>	<i>35</i>
<i>Figure 7. Setup of Spectrophotometer. ....</i>	<i>38</i>
<i>Figure 8. Pictorial representation of MTT assay to determine cell proliferation. ....</i>	<i>39</i>
<i>Figure 9. Setup of Zeiss Stereo Microscope. ....</i>	<i>39</i>
<i>Figure 10. Pictorial representation of the extraction method. ....</i>	<i>40</i>
<i>Figure 11. Setup of Optika Microscope (Italy) equipped with an Optikam B9 Digital Camera. ....</i>	<i>41</i>
<i>Figure 12. Setup of ZEISS Axio Vert A1 Inverted Phase Fluorescence Microscope. ....</i>	<i>43</i>
<i>Figure 13. Images of scaffold surface morphology of (a) GELCAS and (b) GEL only. ....</i>	<i>45</i>
<i>Figure 14. Morphological changes of HEK 293 observed under an inverted light microscope (100 x magnification) after exposure to extract of samples, scale bar (5 <math>\mu</math>m). ....</i>	<i>47</i>
<i>Figure 15. Morphological changes of MDA MB 231 cells observed under an inverted light microscope (100 x magnification) after exposure to extract of samples, scale bar (5 <math>\mu</math>m) ....</i>	<i>49</i>
<i>Figure 16. Optical microscopy images of cells after exposure to sample extract at 400x magnification, scale bar (5 <math>\mu</math>m); a- mitotic division of cells, b- cell disruption, c- formation of apoptotic bodies, d- cellular shrinkage. ....</i>	<i>50</i>

Figure 17. Fluorescence microscopy images depicting live cells stained green with Calcein-AM solution and dead cells stained red with Propidium Iodide solution for HEK 293 cells after exposure to sample extracts, scale bar (5  $\mu$ m). .....52

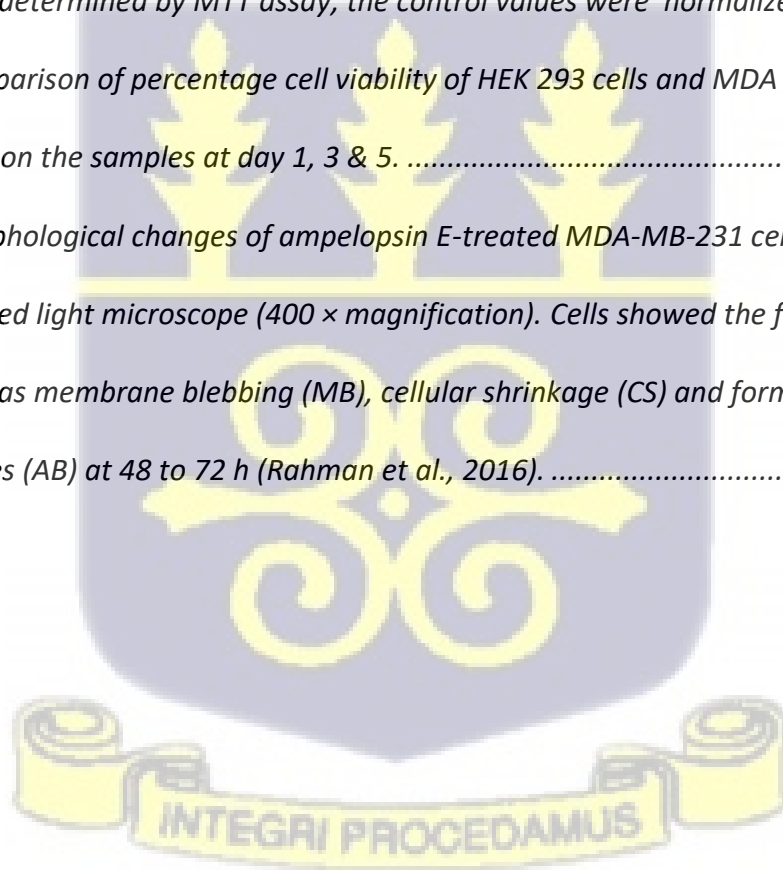
Figure 18. Fluorescence microscopy images depicting live cells stained green with Calcein-AM solution and dead cells stained red with Propidium Iodide solution for MDA MB 231 cells after exposure to sample extracts, scale bar (5  $\mu$ m). .....54

Figure 19. Percentage cell viability after 1, 3, and 5 days of culture of HEK 293 cells on the samples as determined by MTT assay; the control values were normalized to 100 %. .....56

Figure 20. Percentage cell viability after 1, 3, and 5 days of culture of MDA MB 231 cells on the samples as determined by MTT assay; the control values were normalized to 100 %. ...59

Figure 21. Comparison of percentage cell viability of HEK 293 cells and MDA MB 231 cells seeded directly on the samples at day 1, 3 & 5. ....62

Figure 22. Morphological changes of ampelopsin E-treated MDA-MB-231 cells observed under an inverted light microscope (400  $\times$  magnification). Cells showed the features of apoptosis such as membrane blebbing (MB), cellular shrinkage (CS) and formation of apoptotic bodies (AB) at 48 to 72 h (Rahman et al., 2016). .....68



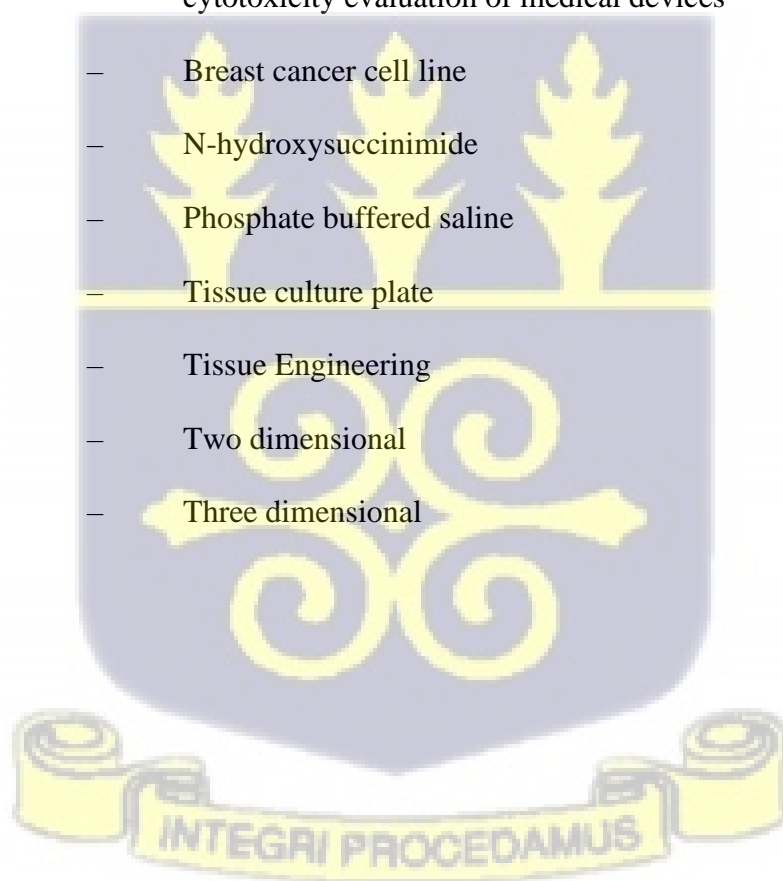
## LIST OF TABLES

<i>Table 1. Qualitative morphological grading of cytotoxicity of extracts (ISO, 2009) .....</i>	<i>41</i>
<i>Table 2. Raw data of MTT assay for HEK 293 cells at day 1, 3 &amp; 5.....</i>	<i>67</i>
<i>Table 3. Raw data of MTT assay for MDA MB 231 cells at day 1, 3 &amp; 5.....</i>	<i>67</i>



## LIST OF ABBREVIATIONS

ANOVA	–	Analysis of Variance
EDC	–	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
ECM	–	Extracellular matrix
GELCAS	–	7 % (w/v) cassava microfiber-gelatin composite scaffold
GEL only	–	3 % (w/v) gelatin scaffold
GAEC	–	Ghana Atomic Energy Commission
HEK 293	–	Human Embryonic Kidney cell line
ISO 10993-5	–	International Standard Organization standard for <i>in vitro</i> cytotoxicity evaluation of medical devices
MDA MB 231	–	Breast cancer cell line
NHS	–	N-hydroxysuccinimide
PBS	–	Phosphate buffered saline
TCP	–	Tissue culture plate
TE	–	Tissue Engineering
2D	–	Two dimensional
3D	–	Three dimensional



## CHAPTER 1

### INTRODUCTION

Accidents, injuries, and some peculiar diseases result in large tissue loss. Previously, the health sector relied solely on tissue transplants in such cases (Chandra et al., 2020; Shafiee & Atala, 2017). However, this conventional way of restoring lost tissues is adding to the death toll of victims of these unfortunate circumstances due to factors such as mismatch in donor patient numbers (Furth & Atala, 2014). To help address this problem, the emergence of a multidisciplinary field that applies engineering and life sciences principles to produce biological substitutes that restore, sustain or enhance tissue function has been in existence for some time now; Tissue Engineering (Mallick & Cox, 2013; O'Brien, 2011). Subsequently, over 4.5 million reconstructive surgical procedures are performed annually worldwide, improving the lives of patients who would have otherwise become incapacitated or died (Bellegem et al., 2020; Guerado & Caso, 2017; Sarkar et al., 2013).

The widely accepted framework used in this field of engineering to conceptualize what constitutes “biological substitutes” is the Tissue Engineering Triad, which is made up of cells, biomaterials for scaffolds and exogenous bioactive factors. In general, the Tissue Engineering (TE) approach involves at least two of the three constituents of the triad depending on the type of tissue loss (Dzobo et al., 2018; Sharma et al., 2019). However, most approaches require the use of scaffolds to regenerate or create new tissues due to damaged extracellular matrix (ECM) (Hosseinkhani et al., 2014; Sidhu, 2020; Zhao et al., 2018).

The ECM, which is nature's scaffold, is a complex network of structural and fibrous proteins, glycosaminoglycans and proteoglycans, responsible for regulating cell migration, communication, proliferation, differentiation, adhesion, and signaling (Frantz et al., 2010; Theocharis et al., 2016). Without the ECM, cells would not be able to grow into the required tissue and therefore the importance of artificial reconstruction of the ECM in Tissue

Engineering (B.-S. Kim et al., 2011; Y. Kim et al., 2016; Walma & Yamada, 2020). Thus, scaffolds (artificial ECM) serve as a template for the formation of tissues and are normally seeded with cells and sometimes growth factors, or subjected to biophysical stimuli by using a bioreactor; a device or system that applies various types of mechanical or chemical stimuli to cells (Chan & Leong, 2008; P. X. Ma, 2004). These cell-seeded scaffolds are then either cultured *in vitro* or *in vivo* (Howard et al., 2008; Seif-Naraghi & Christman, 2012).

An ideal scaffold for TE should be biocompatible, biodegradable, adequately porous with high pore interconnectivity for proper tissue integration and vascularization and finally possess adequate mechanical strength to ensure integrity (Eltom et al., 2019a; Hollister, 2005; Jones, 2005; Kazimierczak & Palka, 2019). Achieving these parameters depend greatly on the biomaterials used and their processing techniques (Garg & Goyal, 2014; Hinderer et al., 2015; Shick et al., 2019). As a result, a lot of research is ongoing to come up with suitable biomaterials that can support various types of tissue growth. For years, scaffold design has made sole use of polymers (natural or synthetic) as the primary biomaterial (Chaudhari et al., 2016; He & Benson, 2017; Khan et al., 2015b; Kohane & Langer, 2008; Kramschuster & Turng, 2012). However, no one - type polymer can give rise to all these desirable characteristics. As a result, a lot of research is ongoing to come up with suitable biomaterials that can support various types of tissue growth by incorporating the desired scaffold characteristics.

Scientists and Engineers have resolved to forming composites of both natural and synthetic polymers to compensate for the shortcomings of both kinds of polymers (Aslam Khan et al., 2021; Davis & Leach, 2008; Egbo, 2020; Ramphul et al., 2017; Shapira et al., 2016). Cellulose fiber-reinforced composite scaffolds have recently become an interesting target for Biomedical Engineers and Biomaterials Researchers for TE applications (Müller et al., 2006; Novotna et al., 2013). This is because cellulose is the most abundant biopolymer on earth with excellent renewability (Poletto & Ornaghi Jr, 2015; Van De Ven & Godbout, 2013). It can be derived

from bacteria, tunicates, and plants. It has a densely-packed glucan chain structure which improves its mechanical strength to support cellular structures and introduce various surface modifications (Baghaei & Skrifvars, 2020; Dutta et al., 2019). Another advantage about cellulose use is its ability to be sustained especially in this era of creating an eco-friendly and green world(Hickey & Pelling, 2019).

Diverse cellulose types and modifications are currently under extensive scientific research, and most importantly their biocompatibility and bioactivity are being tested with various cell types (Credou & Berthelot, 2014 ; Miyamoto et al., 1989). To ensure uniformity in the biocompatibility test, International Standards Organization (ISO) has developed standard guidelines for the biological evaluation of different categories of medical devices (Assad & Jackson, 2019). For *in vitro* cytotoxicity tests under the ISO 10993-5, there are three main categories namely, extract test, direct contact test and indirect contact test. In all these tests, the means of evaluating cytotoxicity is by assessing cell damage through morphological means, measuring specific aspects of cellular metabolism and also measuring cell proliferation (ISO, 2009; M. O. Wang et al., 2013;(Abd El-Aziz et al., 2021). Without satisfying the biocompatibility property, the scaffold cannot be used (Li et al., 2015).

Research done by Larbie et al. (2012) in Ghana suggested cassava fiber as a potential biomaterial. The results obtained from their research showed insignificant cytotoxic effect of de-starched cassava fiber granules and fine powder on human peripheral blood mononuclear cells (PBMC), together with little or no significant change in the composition of Simulated Body Fluid when cassava fiber samples were immersed in it (Larbie et al., 2012). Diabor et al. (2019), then explored cassava fiber as another source of cellulose fiber for Tissue Engineering application. Considering the surging interest in cellulose fiber- reinforced composites, Diabor et al. (2019), experimented on the use of cassava microfiber to reinforce gelatin. Gelatin is a known polymer biomaterial that has been widely used for scaffold fabrication in TE due to its

biocompatibility, biodegradability, availability, and relatively cheaper cost. However, it has poor mechanical properties and poor hydrolysis resistance, which limits its application for implants in TE (Choi & Cha, 2019).

The fiber was extracted from cassava bagasse which is considered as waste. Per their findings, they concluded that combining cassava microfiber with gelatin to form a composite scaffold had significant effect on the microstructure, morphological and mechanical properties of the scaffold. The scaffold fabricated was highly porous with surface porosity ranging between 84% and 90 %, with an average interconnected pore size of  $36.29 \pm 12.23 \mu\text{m}$ . Out of the various cassava microfiber composites, the 7 % (w/v) fiber load composite scaffold recorded a maximum compressive strength of  $0.292 \pm 0.02 \text{ MPa}$ , roughly eight (8) times higher than the pure gelatin scaffolds. It also enhanced the Young's modulus of pure gelatin scaffolds from  $(0.308 \pm 0.03 \text{ MPa})$  to  $(1.308 \pm 0.03 \text{ MPa})$  - approximately four times higher. All these characteristics indicated that the cassava microfiber - gelatin composite scaffold will be appropriate for cell seeding and growth for tissue culture. However, further research needs to be done on this 7 % (w/v) fiber load gelatin scaffold to test for cytotoxicity of the composite. Therefore, this study focused mainly on testing for the cytotoxicity of this scaffold, using both the extraction test method and direct contact method. The extraction test method analyzed the effect of the extract of the scaffold on the cell's morphology and viability, while the direct method was used in determining if cells seeded directly on the scaffold will proliferate or not (ISO, 2009). Findings of this research will go a long way to help provide more information on cassava microfiber as a potential biomaterial for scaffold enhancement when reviewing new materials at the research and development levels.

### **1.1 Problem Statement**

Cellulose obtained from different sources are being explored daily for the enhancement of the properties of scaffolds for TE purposes. Cassava fiber which is also an alternate source for

cellulose has been studied to obtain its mechanical properties in order to consider it as a prospective biomaterial for Tissue Engineering purposes. Diabor et al. (2019) studied the cassava fiber and found that it had good material properties, the addition of the fiber to gelatin changed the microstructure, morphology, and mechanical properties of the gelatin scaffold significantly. These properties indicated that the 7 % (w/v) cassava microfiber- gelatin scaffold can support cell seeding and growth in tissue culture, yet there is lack of information on the viability of cells when in contact with this scaffold. Without this vital information, tissue engineers may not consider the use of cassava fiber as a potential biomaterial for tissue engineering unless such biocompatibility tests are done to prove that it will not be toxic to biological systems.

### **1.2 Significance of Study**

Ghana and Africa as a whole have a lot of local materials that have not been explored yet to see their potential use as biomaterials for biomedical purposes. Having characterized cassava fiber to obtain its material properties is a step in the right direction to help put Ghana on the globe when it comes to biomaterials. Going further to predict the biocompatibility of the cassava microfiber- gelatin scaffold will go a long way to contribute to the Tissue Engineering field to be specific. Availability of this information will guide tissue engineers on whether or not to use this material depending on the purpose they want it to function. Aside that, making use of cassava bagasse which would have otherwise gone waste or polluted the environment is very remarkable.

### **1.3 Research Questions**

1. Will the extracts of the 7 % (w/v) cassava microfiber-gelatin scaffold affect the morphology and growth of the cells?
2. Can the 7 % (w/v) cassava microfiber-gelatin scaffold support cell proliferation/growth?

3. Will there be enhanced cell proliferation in the cassava microfiber-gelatin than in pure gelatin scaffold?

#### **1.4 Hypothesis**

(a) The presence of the cassava microfiber in the gelatin scaffold will lead to enhanced cell proliferation with no effect on cell morphology.

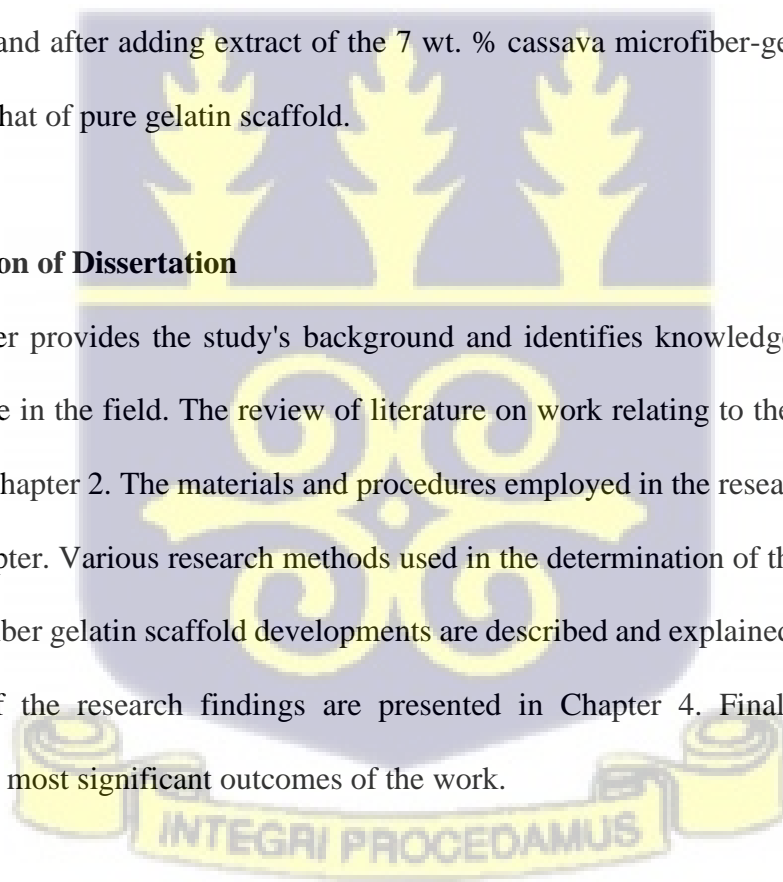
#### **1.5 Research Aims and Objective**

This research aims to study the cytotoxicity of 7 wt. % cassava- microfiber gelatin scaffold for potential use in tissue regeneration.

The main objective is to analyze effect of the 7 wt. % cassava microfiber-gelatin scaffold on cell growth and morphology of cells after culturing the seeded cells on the scaffold for specified time durations and after adding extract of the 7 wt. % cassava microfiber-gelatin scaffold, in comparison to that of pure gelatin scaffold.

#### **1.6 Organization of Dissertation**

The first chapter provides the study's background and identifies knowledge gaps in certain related literature in the field. The review of literature on work relating to the current study is the subject of Chapter 2. The materials and procedures employed in the research are presented in the third chapter. Various research methods used in the determination of the cytotoxicity of cassava-microfiber gelatin scaffold developments are described and explained. The results and explanations of the research findings are presented in Chapter 4. Finally, Chapter five summarizes the most significant outcomes of the work.



## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Tissue Engineering Overview

The term “tissue engineering” was first proposed by the attendees of the National Science Foundation (NSF) sponsored meeting in 1988 and defined as “the application of the principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationship in normal and pathological mammalian tissues and the development of biological substitutes for the repair or regeneration of tissue or organ function” (Chapekar, 2000). The true emergence of tissue engineering as a medical field began in the early 1990s when Langer and Vacanti defined tissue engineering as “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain or improve tissue or organ function” (Langer & Vacanti, 1993).

Over the years, there has been an increasing number of accidents, injuries and disease occurrence that lead to large tissue loss. Tissue or organ transplantation has been the conventional therapy to treat these patients (Chandra et al., 2020). However, limitations in compatible and available donors has become an obstacle to this approach (Beyar, 2011). The consistent increase in transplant waiting list versus the decline in organ donors necessitated the development of other novel methods to curb this challenge (Furth & Atala, 2013). This led to the emergence of the tissue engineering field with a primary goal of solving the critical shortage of donor organs through *in vitro* fabrication of functional biological structures (Mandrycky et al., 2017). Thus, this is an interdisciplinary field that integrates engineering, materials science, biology, chemistry and medicine to develop biological substitutes that restore, maintain or regenerate organs and tissues in the body (Chen & Liu, 2016).

Through this field, over 4.5 million reconstructive surgical procedures are performed annually worldwide, improving the lives of patients who would have otherwise become incapacitated or died (Guerado & Caso, 2017). The tissue engineering trio, which consists of cells, biomaterials for scaffolds, and exogenous bioactive factors/biomolecules, is a generally acknowledged umbrella utilized in this engineering field to define what constitutes “biological substitutes.” (Howard et al., 2008). Cells used for TE are either stem cells or mature cells. Stem cells are generally known to have the ability to differentiate into any cell type depending on the environment they are subjected to, while mature cells are already differentiated functional cells specific to a particular tissue, e.g. fibroblasts, epithelial cells, endothelial cells (Yamzon et al., 2008).

Biomolecules are also able to trigger or mediate crosstalk between cells and their immediate microenvironment and any other molecular signalling mechanism needed for maintaining cell growth. Hormones, cytokines, growth factors, ECM molecules, cell surface molecules, and nucleic acids are examples of biomolecules (Mitchell et al., 2016 ; Gattazzo et al., 2014). In most instances, they are embedded in the extracellular environment (scaffold/biomaterial) as they release signals from there to coordinate the cellular processes taking place (Sarkar et al., 2013). The scaffold on the other hand is known as the artificial extracellular matrix on which the cells organize and restore function to damaged tissues. The biomaterials used in designing these scaffolds need to mimic the natural 3-D extracellular matrix environment as much as possible in order to ensure cell growth (Nikolova & Chavali, 2019).

There are generally 3 classifications of the methods used in the clinical implementation of tissue engineering: acellular grafts, cellular grafts and cell therapy. These classified methods are based on different combinations of the tissue engineering triad and the type of tissue loss of the patient determines the method to be used (Bellegem et al., 2020b). Acellular grafts as the name suggest is a method that is devoid of any cellular components, with this method

synthetic or donor-derived biomaterials platforms (scaffolds) are implanted in the patient's body to support and promote tissue regeneration by facilitating revascularization (Feinberg, 2012). This method is mostly used in cases where the patient has their extracellular matrix severely damaged, e.g. used for burn management. Cellular grafts on the other hand make use of both cells and scaffolds, cells derived from patient or donors are placed on a scaffold to cellularize and mature before implanting in the patient. This method is mostly used when both cells and extracellular matrix are damaged, e.g. used for bone formation. Cell therapy also uses only cells without any scaffold, for this method cells derived from the patient or donors are harvested and grown *in vitro* and then injected into the patient's defective site (Dzobo et al., 2018; Chen & Liu, 2016). Figure.1 below gives a pictorial view of the various methods. Among the three major components of tissue engineering, scaffolds are used in most cases, these scaffolds have certain key requirements that they must be able to meet in order to qualify for use.

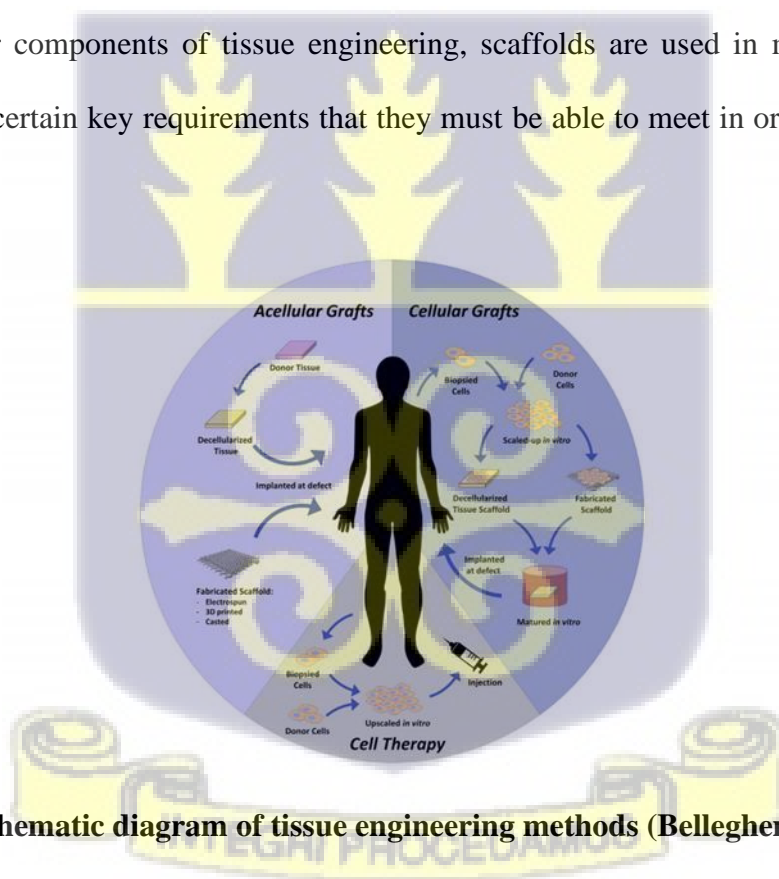


Figure 1. Schematic diagram of tissue engineering methods (Bellegem et al., 2020)

## 2.2 Scaffolds

As explained previously, scaffolds happen to play a key role in tissue engineering owing to the fact that in most cases patients suffer from damaged extracellular matrix (ECM). Scaffolds act

as the artificial ECM and serve as platforms that provide the required environment for engineering tissue and organs (Chandra et al., 2020). Thus, the term scaffold refers to the 3D biomaterial that has been fabricated prior to the addition of cells. Like the natural ECM, scaffolds also need to provide functional and structural support to the cells and their composition also varies depending on the cell or tissue in question (O'Brien, 2011). The ECM is made up of 2 main macromolecules: proteoglycans and fibrous proteins. Tissues have different ECM properties depending on the abundance of fibrous proteins such as collagen and elastin. For example for bone tissue engineering, high mechanical properties (stiffness) are required and vice versa for skin tissue engineering (Asimeng et al., 2020). Despite the variation in composition, generally an ideal scaffold needs to have suitable mechanical properties and processability, have interconnected pore structure and high porosity, be biodegradable and most importantly be biocompatible (Eltom et al., 2019; Loh & Choong, 2013). For manipulation *in vitro* and *in vivo*, scaffolds must have acceptable mechanical characteristics. For instance, for hard tissue regeneration the compression modulus ranges from 10-1500 MPa, while soft tissues range from 0.4 – 350 MPa. Using a different mechanical property can lead the cells to express a different phenotype or even lead to cell death (Chan & Leong, 2008 ; Stevens et al., 1975). Furthermore, the scaffold should not collapse when being handled or during a patient's normal activities. To ensure proper diffusion of nutrients, oxygen and extracellular fluid in and out of the matrix in order to support cell growth, the porosity and pore interconnectivity play a major role (Karande & Agrawal, 2008). The matrix needs to be 50-90 % porous with a minimum pore diameter of 100  $\mu\text{m}$  and uniform pore distribution through the structure in order to support cell attachment and interaction (Hollister, 2005).

However, one of the major challenges faced by tissue engineers is fabricating a scaffold with an optimum balance between porosity and mechanical strength, since mechanical strength and porosity are inversely proportional (Collins et al., 2021; Bahraminasab, 2020). Scaffolds also

need to be biodegradable, thus after implanting the scaffold into the patient, the scaffold's degradation rate must match the rate at which the cells are producing their own natural ECM, this way the scaffold will be able to maintain structural integrity within the body and eventually breakdown leaving the newly formed tissue to take up the mechanical load (Sultana, 2013; Amoabediny et al., 2011; L. Wang et al., 2020). Since scaffolds are targeted for commercial and clinical use, they must have the ability to be processed into different shapes, reproducible on a large scale, have the ability to be sterilized and stored with minimum fabrication cost. Most importantly they must be biocompatible, without satisfying this property they cannot be used as scaffolds for tissue engineering. In most cases the biocompatibility depends more on the material used in the fabrication of the scaffold (Bitar & Zakhem, 2014; Nardo et al., 2017). Thus, the scaffold must provide a satisfactory response to the host tissue without causing cytotoxic or immune response and its by-products after degrading should not produce inflammatory or harmful reactions to the patient as well. It should be able to allow cells adhere and proliferate without causing harm to the cells (O'Brien, 2011; Hussein et al., 2016; Dolcimascolo et al., 2019). All these properties or requirements for scaffolds are dependent on the material used and the fabrication technique used. Therefore, a lot of research is ongoing to discover new materials and enhance the fabrication techniques for better scaffold design (Haider et al., 2020).

### **2.2.1 Scaffold Fabrication**

Engineers and scientists have come up with different fabrication techniques for the design of porous scaffolds with the right mechanical strength and physico-chemical features required for tissue engineering. Some of the fabrication techniques include solvent casting, particulate leaching, gas foaming, polymer sponge replication method, phase separation, electrospinning, freeze drying and additive manufacturing also known as 3D printing (Haider et al., 2020; Eltom

et al., 2019). One of the simplest fabrication techniques known as freeze drying was used in this project and thus will be discussed in detail below.

**Freeze drying** is a dehydration procedure in which a substance is frozen to an extremely low temperature and then the surrounding pressure is reduced to allow the frozen water to sublime. First, a polymeric solution containing the required polymer and solvent is created. The solution is retained in order for polymerization to occur. By reducing the temperature of the solution in a negative pressure environment, the solvent from the resultant solution is separated. The solvent evaporates, leaving a solid polymeric scaffold structure with porous networks behind. This approach does not necessitate the use of porogens to create porosity in the scaffold structure and the pH of the solution and freezing rate can be used to monitor the structure's porosity. However, it is time demanding and the pore sizes created are usually quite small (Brougham et al., 2017; Fereshteh, 2018).

### **2.2.2 Scaffold Biomaterials**

In 1976, the first consensus conference of the European Society for Biomaterials (ESB) defined a biomaterial as 'a nonviable material used in a medical device, intended to interact with biological systems'; however the current definition is a 'material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body' (O'Brien, 2011). This shows how much biomaterials have evolved over the years and transitioned from just interacting with the body to having an influence on biological processes for tissue regeneration. Scaffolds are known as artificial extracellular matrix on which the cells organize and restore function to damaged tissues (Furth & Atala, 2013).

The biomaterials used in designing these scaffolds need to mimic the natural 3-D extracellular matrix environment as much as possible in order to ensure cell growth, adhesion, migration and differentiation (Collins et al., 2021). In some cases, these materials are also used as vehicles to supply nutrients, bioactive factors and drugs that aid in directing specific tissue growth.

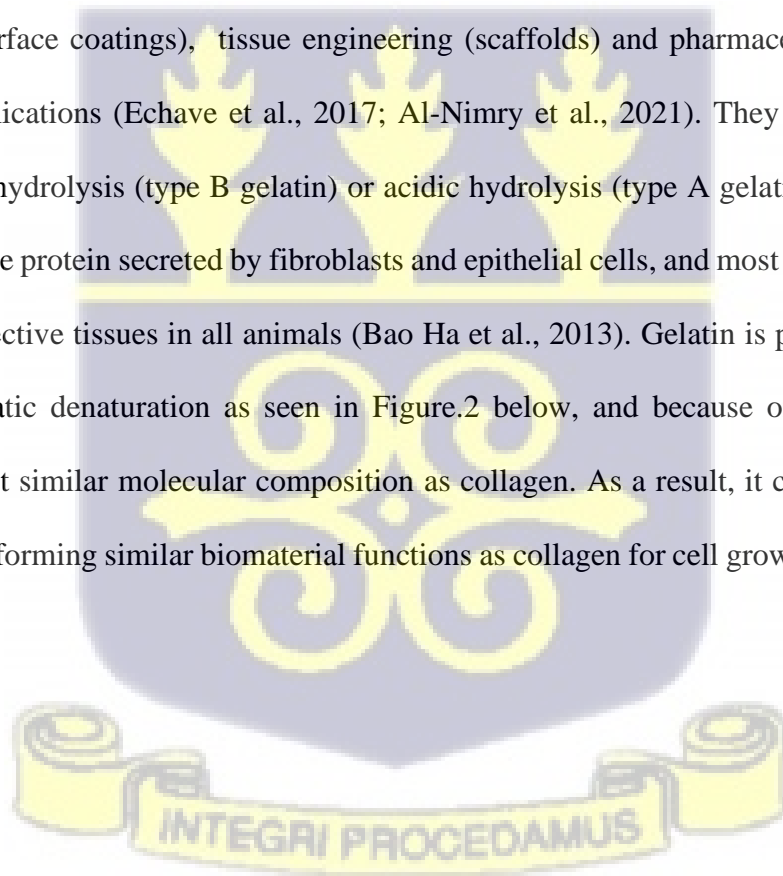
Synthetic polymers and natural polymers are the two types of biomaterials utilized to make scaffolds, each with its own set of benefits and drawbacks (Basu et al., 2010). Synthetic polymers are known to have established structures and tunable properties, can be produced in huge quantities and possess longer shelf time. The ability to manipulate their chemical composition, molecular weight or crystallinity to produce a desired property for a specific application makes them attractive for scaffold design (Dhandayuthapani et al., 2011). When used for scaffold development, they tend to have controlled degradation, porosity and mechanical properties suitable for the tissue being regenerated. However, their composition is not similar to the ECM, hence they lack cell adhesion sites, have reduced bioactivity and require some form of chemical modification in most cases to support cell growth. Some examples of synthetic polymers widely used for TE are polylactic acid (PLA), polyglycolic acid (PGA), and polylactic-co-glycolic acid (PLGA) (Place et al., 2009; Dhandayuthapani et al., 2011). Natural polymers on the other hand are made up of long chains of covalently bonded nucleotides, amino acids, or monosaccharides, making them similar to the ECM found in humans. The presence of biomolecules in them also makes them bioactive with biomimetic surfaces and biocompatible, leaving behind little or no toxic by-products after degrading (Sofi et al., 2018; Ebhodaghe, 2021).

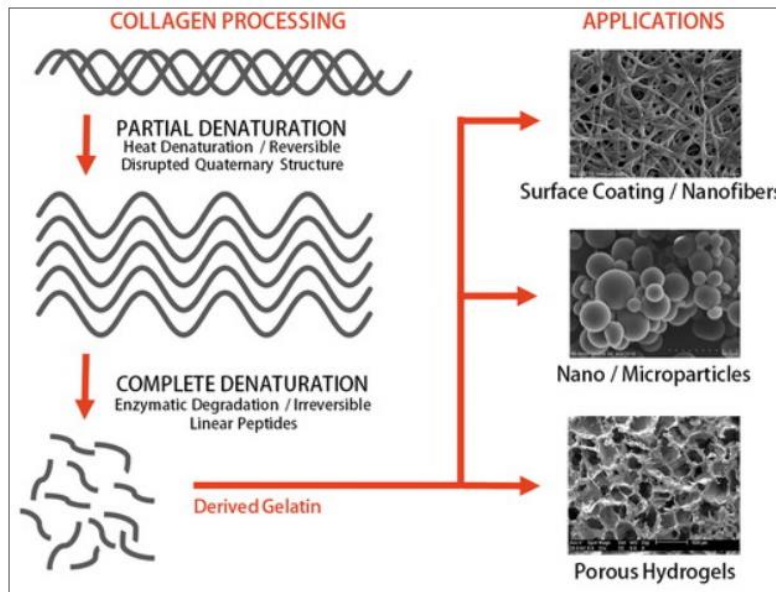
However, unlike synthetic polymers they have reduced tuneable properties, weak mechanical strength, uncontrollable degradation rate, and possible microbial contamination (i.e., endotoxins) when not purified properly after their extraction from their source. Some examples of natural polymers widely used are collagen, gelatin, silk fibroin, chitosan, cellulose, hyaluronic acid, and alginate. Over the past decade, synthetic polymers have been mostly considered for hard tissue regeneration while natural polymers are mostly considered for soft tissue regeneration until composites were explored (Reddy et al., 2021). Seeing that both natural and synthetic polymers can compensate each other in terms of their drawbacks,

scientists and engineers have resorted to the formation of composite materials for optimized functional scaffold development (Davis & Leach, 2008). Hence, the selection of the biomaterials for a particular tissue engineering project is very crucial, as the materials together with the fabrication process determine the properties of the scaffold (Deb et al., 2018). In the next paragraphs, gelatin and cellulose biomaterials will be discussed in detail since they were used in this project.

### 2.3 Gelatin

Gelatin is a well-known protein based natural polymer made up of about 85-92 % proteins, mineral salts and water and has been exploited for cosmetic, food (gelling agent, emulsifier), cell culture (surface coatings), tissue engineering (scaffolds) and pharmaceutical (capsules, ointments) applications (Echave et al., 2017; Al-Nimry et al., 2021). They are produced by either alkaline hydrolysis (type B gelatin) or acidic hydrolysis (type A gelatin) of collagen, a fibrous insoluble protein secreted by fibroblasts and epithelial cells, and most abundant in skin, bone and connective tissues in all animals (Bao Ha et al., 2013). Gelatin is produced through heat or enzymatic denaturation as seen in Figure.2 below, and because of that it has less organization but similar molecular composition as collagen. As a result, it can easily replace collagen by performing similar biomaterial functions as collagen for cell growth *in vitro* (Bello et al., 2020).





**Figure 2. Denaturation of collagen to produce gelatin** (Bello et al., 2020).

Besides, it is readily available, it is cheaper, more highly soluble than other ECM proteins, and can be extracted from many sources such as fish, pig skin, insects and cattle bone. Additionally, several studies have indicated that the varied sources have little or no significant effect on its biocompatibility. In general, they do not induce toxicity, antigenicity or have any adverse effects on cells (Echave et al., 2017).

The type of gelatin determines the characteristics such as the amino acid composition, the gel strength, the isoelectric point and charge. Gelatin type A for instance has higher gel strength, exhibits a positive charge at neutral pH, with more glycine and proline present. Gelatin type B has an isoelectric point ranging between 4.8 and 5.4 with a negative charge at a neutral pH (Gelatin Manufacturers Institute of America, 2012). They are characterized by high content of amino acids such as proline, glycine and hydroxyproline, the repeating sequences of these amino acids are responsible for the triple helical structure of gelatin and its ability to form gels (Van Vlierberghe et al., 2014 ; Aramwit et al., 2015). It is known as a promising material for tissue engineering scaffold development and also as drug delivery vehicle due to its biocompatibility, biodegradability, low antigenicity, cost effectiveness, abundance, chemical

similarities to the ECM in native tissues, and accessible functional groups that allow chemical modifications with other biomaterials or biomolecules. (Bello et al., 2020).

Notably, differences in the collagen source and preparation technique lead to chemical heterogeneity and complex physical characteristics. Therefore, in choosing gelatin as a biomaterial for any application, these factors need to be considered: the type of gelatin; the relative molecular weight also known as the 'bloom' (this determines the gelling capacity and gel strength); the purpose of the experiment; and the crosslinking method (Echave et al., 2017; Rose et al., 2014).

In spite of its desirable properties, gelatin scaffolds have low mechanical strength, are sensitive to enzymatic degradation, have reduced solubility in concentrated aqueous media, and possess poor hydrolysis resistance leading to instability when implanted. Applications such as controlled drug release, cell differentiation and wound healing that require longer periods of time become challenging when using gelatin-based materials only since the scaffolds do not last (Choi & Cha, 2019). In order to address these limitations, gelatin scaffolds are stabilized by crosslinking the material or by forming gelatin- polysaccharide composites.

For crosslinking methods there are three main types: use of physical methods such as treatment with dehydrothermal and ultraviolet light; use of chemical agents like glutaraldehyde (GTA), carbodiimides (EDC & NHS), and genipin (GP); and use of enzymes like transglutaminase, tyrosinases, and horseradish peroxidases (N. Reddy et al., 2015). The physical crosslinking method avoids potentially toxic compounds but have relatively low efficiency since they only form mainly non-covalent bonds between the polymeric chains. Chemical crosslinking methods on the other hand, are able to form more covalent bonds, resulting in much more stable scaffolds, making them a preferable method. Under chemical crosslinking methods, there are 2 main types: non-zero length type cross linkers which react with amino or carboxyl groups of amino acids and end up incorporated into the material's (gelatin) network structure,

glutaraldehyde (GTA) and genipin are considered the most used non-zero length cross-linkers; whereas zero length type activate carboxyl groups to directly react with the amino acids present on the adjacent gelatin chain (Hu et al., 2019 ; Bhattacharjee & Ahearne, 2021).

Nonetheless, unlike non-zero length cross-linkers, they do not end up incorporated in the gelatin network. The most well-known and used non-zero length crosslinker is 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDC), it is most commonly utilized to crosslink polysaccharides and proteins. EDC reacts with molecules containing free carboxylic and amine groups to form an intermediate that reacts with primary amino groups. To further stabilize the amine-reactive intermediate formed by EDC, N-hydroxysuccinimide (NHS) is added to the reaction to significantly increase its cross-linking efficiency. Gelatin cross-linked with EDC and NHS has produced matrices with good resistance to enzymatic degradation and enhanced mechanical properties (Skopinska-Wisniewska et al., 2021; B. Ma et al., 2014 ; Krishnakumar et al., 2019).

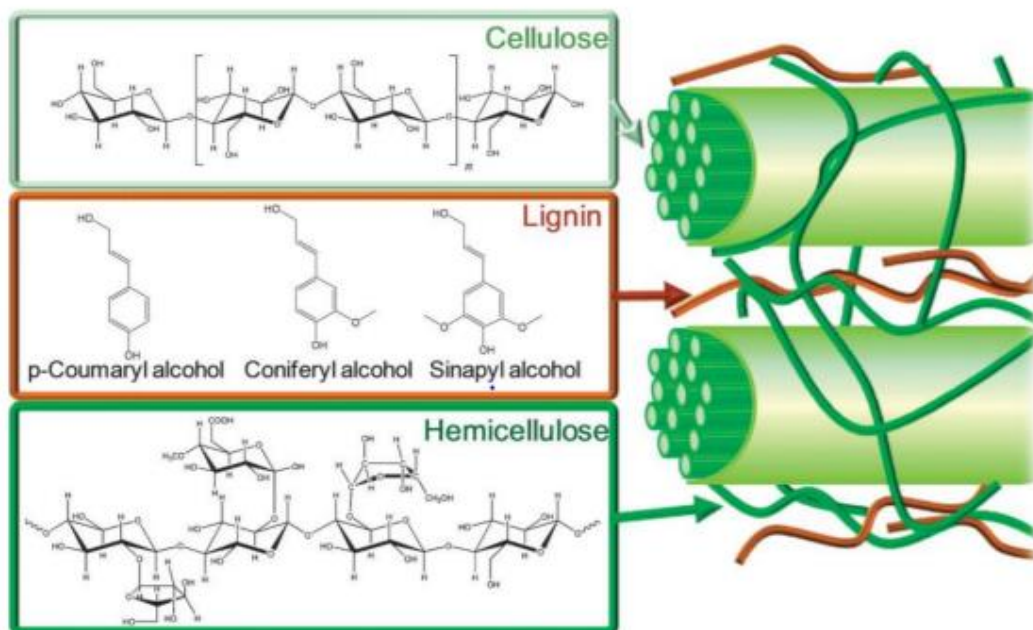
Gelatin-polysaccharide composites are made from the combination of gelatin with polysaccharides such as chitosan, cellulose, hyaluronic acid, dextran and carrageenan. Covalent bonds that are formed via the chemical interaction between carbohydrates (polysaccharides) and proteins (gelatin) tend to resemble the proteoglycans in the native ECM and mostly lead to hydrogel formation (Afewerki et al., 2019). Gelatin-polysaccharide hydrogels tend to absorb about 100 times of water than their dry mass can, and this property makes them a suitable *in vitro* platform/matrix for culturing cells, favouring cell adhesion, growth, infiltration, and tissue vascularization due to their hydrophilic nature (Asiyanbi et al., 2017). Also, the hybrid formation of these materials also results in the production of new and wide range of properties that would have otherwise been impossible to attain if only one type of material were to be used. This explains why gelatin- polysaccharide composites have been explored for diverse biomedical applications, specifically as scaffolds for tissue engineering

(Bealer et al., 2020). The presence of gelatin enhances biological performance while that of the polysaccharide enhances stability and mechanical properties (Ye et al., 2021). Moreover, since they are both green materials, they contribute to eco-technology and sustainable material design. One of the polysaccharide materials that have gained much interest lately is cellulose. Since they bring on board diverse and versatile biochemical and biophysical characteristics depending on the source of origin (Seddiqi et al., 2021). They will be discussed in depth in the proceeding paragraph.

## 2.4 Cellulose

Cellulose is the most abundant polysaccharide on earth and most common organic compound. It is a linear polysaccharide made up of 15,000 D-glucose residues linked by  $\beta$ -(1 $\rightarrow$ 4)-glycosidic bonds with intermolecular hydrogen bond that determines its stiffness and rigidity (Mikshina et al., 2013). It is typically found in nature in lignocellulosic biomass in the form of microfibrils in the cell walls of wood and plants as seen in Figure 3 below. Other sources include algae tissues, synthesized by bacteria and in the membrane of epidermal cells of tunicates (Seddiqi et al., 2021). It features an intricate multi-level structure made of bundles/aggregates of superfine fibrils, the fibrils are composed of repeating crystalline domains and amorphous domains with a cross-sectional dimension ranging from 2 to 20 nm depending on the source (Baghaei & Skrifvars, 2020; Kumar Gupta et al., 2019).





**Figure 3. Structure of lignocellulosic biomass with hemicellulose, cellulose and lignin** (Alonso et al., 2012).

In the past, cellulose has been extensively used in the industry especially for paper and cardboard production. However, with the recent increasing demand on bio-based and eco-friendly materials for scientific research purposes, there has been a sudden shift of attention to cellulose-based materials (Yaradoddi et al., 2020; Anne, 2011). This is because, cellulose is abundant and can be easily produced, has diverse and versatile biochemical and biophysical characteristics owing to the different sources from which they can be obtained, they are biocompatible, have good mechanical properties and are biodegradable (Kalia et al., 2011). Since their properties can be manipulated to obtain the desired property, they have been considered for tissue engineering. For instance, plant-based and wood-based cellulose have crystallinity ranging from 40-60%, while the other sources show higher crystallinity, this property influences the stiffness and structure of the cellulose (Sorieul et al., 2016). Courtenay et al. (2018), reviewed applications of cellulose-based composites that have been used as scaffolds to improve the regeneration of different tissues. The different cellulose types were

grouped into bacterial cellulose, nanocrystalline cellulose, microfibrillated cellulose, and cellulose derivatives (Courtenay et al., 2018). In a study conducted by Ramphul et al. (2019), cellulose obtained from sugar cane bagasse was used to form a composite scaffold with polylactide. The presence of cellulose enhanced the mechanical properties of the scaffold and increased the cell viability as well. Friend et al. (2019), also obtained cellulose from potato peels to produce electrospun-cellulose scaffolds, which supported growth of BALB-3T3 fibroblasts cells based on results from several biocompatibility tests (Friend et al., 2019).

Plants are a desirable cellulose source mainly because they are abundant and there is a pre-existing infrastructure in the textile industries for harvesting, retting/pulping (i.e. treating and isolating micron sized cellulose particles), and product processing of cellulose. Thus, there has been a lot of research to explore different sources of plant based cellulose biomaterials for tissue engineering purpose. Plant based cellulose has been obtained from cotton, corn, wheat straw, jute, and bagasse (Lavanya et al., 2011; Pachuau, 2015). Cellulose obtained from cassava bagasse has been exploited mostly as a reinforcement filler of thermoplastic starch in order to enhance its mechanical properties and make it a better alternative to synthetic thermoplastics (Wicaksono et al., 2013). However, not much research has been done on the use of cellulose obtained from cassava bagasse as a prospective biomaterial for tissue engineering application.

## 2.5 Cassava Fiber Cellulose

Cassava, biologically known as *Manihot esculenta Crantz* is one of the important agricultural commodities in Ghana, grown primarily for food consumption and known as one of the most staple food crops in developing countries (Adjei-Nsiah & Sakyi-Dawson, 2012). Ghana is known as the third largest producer of cassava in Africa with a contribution of about 46% agricultural Gross Domestic Product (GDP) to Ghana's agricultural economy (Bayitse et al., 2017). Since it is readily available, low cost, recyclable, and biodegradable cassava has been

exploited for industrial applications besides food consumption (S. Li et al., 2017). Cassava starch and flour have been very popular in the food and textile industry. Cassava bagasse which is a fibrous solid residue obtained after the extraction of cassava and soluble sugars has also been used in other parts of the world as reinforcement material in the packaging industry for biodegradable plastics and disposables. The bagasse is known to enhance the physical, thermal, mechanical, and structural properties of these plastics (Diyana et al., 2021; Edhirej et al., 2017).

Here in Ghana, cassava bagasse left behind after the industrial use of cassava is literally disposed off as waste with no use found for it. Cassava bagasse from literature is known to be made up of (40-60 %) residual starch, (5-11 %) moisture, (15-50 %) fiber of the total residue and small amounts of lipids and proteins. What made this fiber worth exploiting is the fact that it contains no cyanide and most importantly has high natural cellulose fiber present in it, although it has hemicellulose and lignin present as well (Larbie et al., 2012).

In order to accrue some benefit from the waste left behind, Larbie et al., (2012), decided to find use for cassava fiber by exploring it for tissue engineering purposes. Although cassava bagasse was yet to find any meaningful application in biomedical engineering at the time, cassava starch and flour had been used to develop a biodegradable polymer blend and bio-composite for tissue scaffold by K. W. Kim et al., (2012). The results obtained from their findings indicated that it had good biocompatibility, good mechanical properties, and non-toxic degradation products (K. W. Kim et al., 2012).

Larbie et al., (2012) first tested the cytotoxicity of the cassava bagasse also known as de-starched cassava fiber by checking its effect on human peripheral blood mononuclear cells (PBMC) and examined any changes in the composition of simulated body fluid after immersion the fiber samples in it. After immersing the samples in PBMC, the cytotoxicity was determined by using lactate dehydrogenase test. When the cytoplasmic membrane is damaged, lactate

dehydrogenase, a stable cytoplasmic enzyme, is released into the cell culture supernatant. As a result, as the number of dead or membrane-damaged cells rises, so does the activity of the LDH enzyme in the cell culture supernatant, indicating cytotoxicity. The results obtained after performing this test indicated that the LDH concentration in the samples with the fiber fell within the non- cytotoxic range. The samples were also immersed in Simulated Body Fluid (SBF) and changes in the concentrations of elements such as Mg, Cu, Cl, Mn, Ca, Na, and K present in the fluid were determined at specific end points using Instrumental Neutron Activation Analysis (INAA). The results obtained showed that, there was no statistically significant difference between the concentration of SBF only samples and SBF with the fiber samples. These preliminary results suggested that cassava fiber in their granules or powder form had no significant cytotoxic effect and thus could be considered for biomaterial applications after extensive characterization has been done (Larbie et al., 2012).

This study now led Diabor et al. (2019) to investigate the mechanical properties, physicochemical, morphological and microstructural characteristics, and thermal degradation profiles of single elementary cellulose fibers and the central vascular fiber (“thick-core fiber”) isolated from three cassava genotypes. The results indicated no significant differences between the single elementary fiber and the thick core fiber for all three genotypes, the XRD analysis also showed similar diffraction pattern with minimal variation in the signal intensities implying that there was little or no difference in their crystalline structure. However, there was significant difference between the tensile strength and Young’s modulus of the fibers of the genotypes studied, with a particular genotype, ID4 recording the highest elastic modulus of  $336.485 \pm 130.803$  MPa and highest average tensile strength of  $7.567 \pm 3.844$  MPa. The fiber that exhibited optimum properties was further used as a reinforcement material for the fabrication of a composite gelatin scaffold for potential tissue engineering application.

Different fiber weight fractions (3%, 5% and 7 % (w/v)) were used in fabricating the cassava microfiber/gelatin composite 3- dimensional scaffold to examine the effect of the microfiber on the mechanical properties and microstructure of the scaffold. The composite scaffold formed had rougher surfaces than pure gelatin scaffolds, with about 84 – 90 % porosity and an average interconnected pore size of  $36 \pm 12 \mu\text{m}$ . the highest maximum compressive strength of  $0.29 \pm 0.02 \text{ MPa}$  which happened to be about eight (8) times higher than that of pure gelatin was recorded by the 7 % (w/v) cassava fiber/ gelatin composite. From the results obtained it was concluded that the destarched cassava fiber can be used as a reinforcement material to enhance mechanical properties of polymeric tissue engineering scaffolds. Also, the surface architecture and porosity gave an indication that the fibers have the potential to enhance cell-matrix adhesion and support cell growth (Diabor et al., 2019). Hence, it was recommended that further studies be done to test the biocompatibility (cytotoxicity) of this 7 % (w/v) cassava microfiber/ gelatin composite scaffold by seeding cells on them and performing cell viability studies to see if the presence of the fiber in the scaffold induces any negative effects on cell growth. Therefore, this study is part of a larger study to determine the suitability of cassava fiber for potential tissue engineering application. Thus, the need for a cytotoxicity testing on the scaffold fabricated by Diabor et.al. (2019).

## **2.6 In Vitro Cytotoxicity Test**

Any medical device that will end up implanted in the human body undergoes preclinical testing through a variety of *in vitro* and *in vivo* examinations in order to be approved by regulatory bodies (Myers et al., 2017). The main objective of the tests done is to evaluate the biocompatibility of the various biomaterials that are used in fabricating the device (Inayat-Hussain et al., 2008). Where “biocompatibility is defined as the material’s ability to perform with an appropriate host response in a specific application without having any negative effect

on the adjacent host cells or lead to significant scarring or otherwise elicit a response that detracts from its desired function” (Morais et al., 2010 ; W. Li et al., 2015). When it comes to tissue engineering, this particular property must be satisfied because of how delicate tissue engineered products are especially with respect to biomaterials used for fabricating scaffolds. Considering that scaffolds interact directly with cells, it is essential to ensure the materials used do not have any toxic effect on cells or the human body in general if implanted (Groth et al., 1995).

In order to ensure uniformity in biocompatibility tests, International Standards Organization (ISO) has developed standard guidelines for the biological evaluation of different categories of medical devices. Under the ISO, there are a series of biocompatibility tests such as cytotoxicity, genotoxicity, sensitization, irritation (intracutaneous reactivity), acute systemic toxicity, subacute and subchronic toxicity, implantation, hemocompatibility, pyrogenicity, chronic toxicity, and carcinogenicity as well as degradation testing that are essential in determining the biological response and inertness of a medical device (ISO, 2009). It is after performing these tests that one can be permitted to use an animal for an *in vivo* test. Therefore, these tests not only help in detecting safety and efficacy of devices but also contribute to the reduction of animals used for test procedures. For a medical device such as the scaffold, cytotoxicity is one of the essential requirements the biomaterial must pass to be considered for TE (Assad & Jackson, 2019).

*In vitro* cytotoxicity tests under the ISO 10993-5 can be categorized into three namely, extract test, direct contact test and indirect contact test. The extraction test has the samples sterilized and in the exact condition they would be in when used, an extraction vehicle that can precisely mimic its use clinically is placed on the samples. The extract is then exposed to the test cells, incubated for a minimum of 24 hours and then analyzed. With the direct contact, the samples are sterilized and the test cells seeded directly on them. The cells together with the samples are

incubated for a minimum of 24 hours and qualitative or quantitative assessment of the viability of the cells is done. Indirect contact testing on the other hand has an agar overlay separating the test cells from the sample which are then assessed after a specified incubation period (W. Li et al., 2015). Generally all these tests assess cell damage by morphological means, measure cell growth and also measure specific aspects of cellular metabolism (Assad & Jackson, 2019). The nature of the sample to be tested and how it will be used clinically are the factors that determine the type of test/s to use, in some cases the tests are combined if the need be (ISO, 2009). Fluorescence microscopy, optical microscopy, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) imaging have also been used to assess cell viability, distribution, and morphology (Vielreicher et al., 2013).

There are various forms of assay used to determine cell viability in order to predict the cytotoxicity of a biomaterial/medical device. The assays can be divided into four categories dye exclusion assays, colorimetric assays, fluorometric assays and luminometric assays. Examples of the various assays are: Dye exclusion ( Trypan blue, eosin, Congo red, erythrosine B assays); Colorimetric assays (MTT assay, MTS assay, XTT assay, WST-1 assay, WST-8 assay, LDH assay, SRB assay, NRU assay and crystal violet assay); Fluorometric assays (Live/dead staining assay, alamarBlue assay, CFDA-AM assay) and Luminometric assays (ATP assay and real-time viability assay) (Aslantürk, 2018).

Some of the most common assays are discussed below:

### **2.6.1 MTT Assay**

The MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay is one of the most widely used colorimetric techniques for assessing cytotoxicity or cell viability. . This assay assesses cell viability primarily through the functioning of the cell’s mitochondria by measuring the activity of mitochondrial enzymes such as succinate dehydrogenase. The reagent used is a yellow substrate that cleaves to the mitochondria of living cells and gets reduced by

NADH to yield dark blue formazan product (Kumar et al., 2018). The formazan product is water insoluble, hence forms a precipitate, so an organic solvent such as DMSO/isopropanol is added to dissolve it. After that, it can be quantified by light absorbance at specific wavelength (usually between 500-600 nm) using a spectrophotometer, the corresponding absorbance recorded is then directly proportional to the cell viability (Stone et al., 2009). A comparison between the untreated negative control which does not elicit any cytotoxic effect is done and if the % cell viability is equal to or more than 70 % of the negative control, the material is biocompatible (Bopp & Lettieri, 2008). This approach has been frequently used to test cell viability since it is simple to use, safe, and has good reproducibility. However, in performing this test, because of how light sensitive it is, it must be done in the dark or with as minimum light as possible to ensure accuracy in results. The general formula used in calculating the percentage cell viability is (Aslantürk, 2018), where OD is optical density:

$$\text{Percentage cell viability} = \frac{\text{Test mean OD}}{\text{Control mean OD}} * 100 \dots \dots \dots \text{equation. 1}$$

### 2.6.2 Live/Dead Staining Assay

The Live Dead assay comprises a staining solution made up of two fluorescent dyes that identify and label live and dead cells differently. The cell viability is measured by the level of intracellular esterase activity in a fluorometric assay. Intact, viable cells are labeled green by the live cell dye. It is membrane permeable and non-fluorescent until ester groups are removed by numerous intracellular esterases and render the molecule fluorescent. The dead cell dye labels cells with compromised plasma membrane red by binding to the DNA with high affinity (Riss et al., 2004). It is membrane-impermeant and therefore can only get to the nucleus through disordered areas of dead cell membrane. Once bound to DNA, it intercalates with the DNA double helix and the red fluorescence increases >30-fold. This assay is useful for rapid quantitation of cell viability and the results can be obtained by using a flow cytometer or fluorescent microscope to capture images of the stained sample. An example of the commonly

used dye is Calcein-AM for staining live cells and Propidium iodide for staining dead cells. They both work on the principle of cell membrane permeability and give off different fluorescence at different wavelengths, and can be used separately or together to assess cell viability (Atale et al., 2014). The mode of data assessment is qualitative, thus a comparison between the image for cells without treatment and the ones that have been treated with an extract of the samples is done. The more the intensity/ number of cells with green fluorescence the higher the cell viability and vice versa.

### 2.6.3 Trypan Blue Exclusion Dye Assay

In determining the number of live and/or dead cells in a cell suspension, trypan blue dye exclusion assay can be used. Trypan blue is a molecule with significant negative charge and works on the principle that live cells have intact cell membrane that keeps the dye out while dead cells do not (Stone et al., 2009). In this assay, adherent or non-adherent cells are washed and suspended in media or PBS. An amount of cell is taken from the cell suspension and mixed with the dye, after which a drop is placed on a hemocytometer, and visualized under an optical microscope to be counted manually or with an automatic cell counter. The viable cells will have a clear cytoplasm while the dead cells will have a blue cytoplasm (Kamiloglu et al., 2020). This method is widely used especially in determining cell viability before seeding cells for any experiment. This is because it is relatively fast, easy, affordable and a good measure of the membrane integrity of cells (Piccinini et al., 2017).

The percentage cell viability (%CV) can then be calculated using this formula (Strober, 2015):

$$\%CV = \frac{\text{total number of viable cells per ml of aliquot}}{\text{total number of cells per ml of aliquot}} \times 100 \dots \dots \text{equation. 2}$$

#### 2.6.4 Cell Choice for *In Vitro* Test

Generally, in performing all these viability tests to determine how biocompatible a material is, the cells that need to be used are also considered. The factors that are taken into consideration when choosing the cells are the ease of culture, availability, reproducibility, and wide range of use in cytotoxicity tests (Geraghty et al., 2014). However, the ISO-10993-5 guideline also have recommended cell lines for cytotoxicity tests of medical devices. In most cases L929 murine fibroblast cells are used, however other cell lines such as human colorectal adenocarcinoma cells (Caco-2), human keratinocyte cells (HaCaT), HeLa cells, different murine cell lines (C3H-L, Balb/3T3), human embryonic kidney cells (HEK293), and many more have also been used (Anderson, 2016; Bácskay et al., 2018; Karakecili et al., 2007).

HEK 293 cell line has been successfully used to test the cytotoxicity of bacterial cellulose/hydroxyapatite (Grande et al., 2009) and poly- $\beta$ -hydroxybutyrate (PHB) scaffold (Romo-Urbe et al., 2017). The cells have also been used for testing carbon nanotubes and some nanoparticles explored for potential use as biosensors (Kumari et al., 2018; Jeyarani et al., 2020).

#### 2.6.5 *In Vitro* Tests Done on Composite Scaffolds

With the recent interest in the fabrication of composite materials to enhance scaffold properties, new materials are constantly discovered and explored for potential tissue engineering application. For all these materials that are incorporated into already existing biomaterials such as gelatin, *in vitro* biocompatibility tests are done to ensure that the presence of the material has no negative effect on cells. Some of the composite scaffolds fabricated are discussed with a focus on their *in vitro* cytotoxicity. Xing et al., (2010), investigated the biocompatibility of a porous 3-D cellulose microfibril/gelatin composite scaffold for cell culture. The cellulose microfibril was obtained from bleached Kraft hardwood fiber sheets from a paper company. Their findings indicated that the presence of the microfibrils enhanced the mechanical property

to 75 % higher than that of gelatin only, and the fibers also provided linear pathways for the cells to attach and grow compared to gelatin where the cells had formed clusters. Thus, the presence of the fiber significantly supported human mesenchymal cell growth and ECM formation. They used MTT assay and live/dead staining assay to determine cell viability. Other assays indicated that the presence of the fiber supported cell adhesion and even preserved the multilineage differentiation of the cells. Therefore, it was concluded that cellulose from the microfiber can be considered for potential tissue engineering application.

Ramphul et al., (2017), also extracted cellulose from sugarcane bagasse and incorporated it into polylactide and polydioxanone electrospun scaffold, L929 murine fibroblast cells were used to determine the cytotoxicity. The results obtained from their MTT assay indicated higher cell viability in the composites, and SEM imaging results also showed that the cells adopted a round morphology similar to the control tissue culture plate (TCP). Additionally, plasma membrane protrusions known to be indicators of healthy cellular adhesion and interaction were present. Hence, the cellulose fiber was concluded not to be cytotoxic.

Cai & Kim, (2010), prepared and characterized bacterial cellulose/gelatin scaffold and tested its cytotoxicity using 3T3 fibroblast cells. They reported that the presence of the bacterial cellulose improved the Young's modulus from 3.65 GPa to 3.87 GPa due to the alignment of the nanofibers in the gelatin. The BC/gelatin scaffold also supported cell adhesion and proliferation after incubating the scaffolds seeded with cells for 48 h. They suggested that the scaffold is bioactive and can be used for wound dressing or TE applications. Grande et al., (2009), also reported that bacterial cellulose/hydroxyapatite composite material was able to support HEK 293 cell growth. For their experiment, they formed a thin film of the composite and seeded cells on them, however after taking the thin film out of the media, they realized most of the cells did not attach to the surface of the film but instead the TCP. That notwithstanding, they concluded that the material was not cytotoxic since cells were still able

to proliferate in the presence of the composite material. Thus, the cell-surface adhesion properties need to be improved in order to use it for TE application.

Another interesting thing to note is that, some of these scaffolds are also fabricated to support the growth of some disease cells such as cancer cells in order to provide much better biomimetic disease tissue models. Studies have shown that cellular response to drug treatment in 3-Dimensional cell culture platforms are similar to what occurs *in vivo* compared to 2D (Edmondson et al., 2014). J. Wang et al., (2018), investigated the use of novel bacterial cellulose/gelatin hydrogel as a 3-D scaffold for tumor cell culture using MDA-MB 231 cancer cell lines. They used MTT assay, SEM imaging and immunofluorescence staining to determine the cell proliferation, morphology, adhesion and infiltration of the cells. Their results indicated that the composite supported cell growth better than in the bacterial cellulose only. The cells had transformed into spherical shapes with pseudopodia bonded to the scaffolds, indicating good cell adhesion. Therefore, they concluded that the composite scaffold can be successfully used for *in vitro* cancer biology studies, clinical diagnosis and for tumor TE. Moreover, since both materials are cost effective, it can serve as an inexpensive candidate for tumor cells cultured *in vitro*. Seo & You, (2009) fabricated  $\gamma$  ray crosslinked gelatin- poly (vinyl alcohol) hydrogels and Tao et al., (2017), also added Lap nanoparticles to gelatin/carboxymethyl/chitosan to form composite scaffolds. In both cases, the incorporation of the material enhanced and supported cell growth as seen in other similar experiments. This goes to confirm that composites are really important, especially for a biomaterial like gelatin and cellulose; gelatin enhances the biological properties while cellulose enhances the mechanical properties. As seen in all cases, *in vitro* cytotoxicity tests had to be done hence the need for this particular test for the 7 % (w/v) cassava microfiber-gelatin, in order to be considered as a biomaterial for TE application also.

## CHAPTER 3

### METHODOLOGY

In this chapter, methods used in determining the cytotoxicity of the samples are described. All the experiments done were according to ISO10993-5 Standards for checking *in vitro* cytotoxicity of medical devices, which in this case is the scaffold. The extraction test method was used to obtain data for the analysis of cell growth and morphology (objective 1), while the direct method (MTT assay) was used to obtain data for the analysis of the viability of cells seeded directly on the scaffold at different time points (objectives 2 and 3). Fiber extraction process and scaffold preparation were done following the protocol of Diabor et al., (2019). The only change made to the protocol was an increase in the Gelatin percentage from 1 % (w/v) to 3 % (w/v), this was a recommendation from Diabor et al., (2019) to improve the binding effect of gelatin on the cassava microfibers. All experiments were performed in triplicates.

#### 3.1 Cassava Tubers

Cassava root tubers, biologically recognized as *Manihot esculenta Crantz* with cloned genotype known as IITA-TMS-GAEC-140006 were harvested from the farm of the Biotechnology and Nuclear Agricultural Research Institute (BNARI), Ghana Atomic Energy Commission (GAEC) and used for this study.

#### 3.2 Reagents

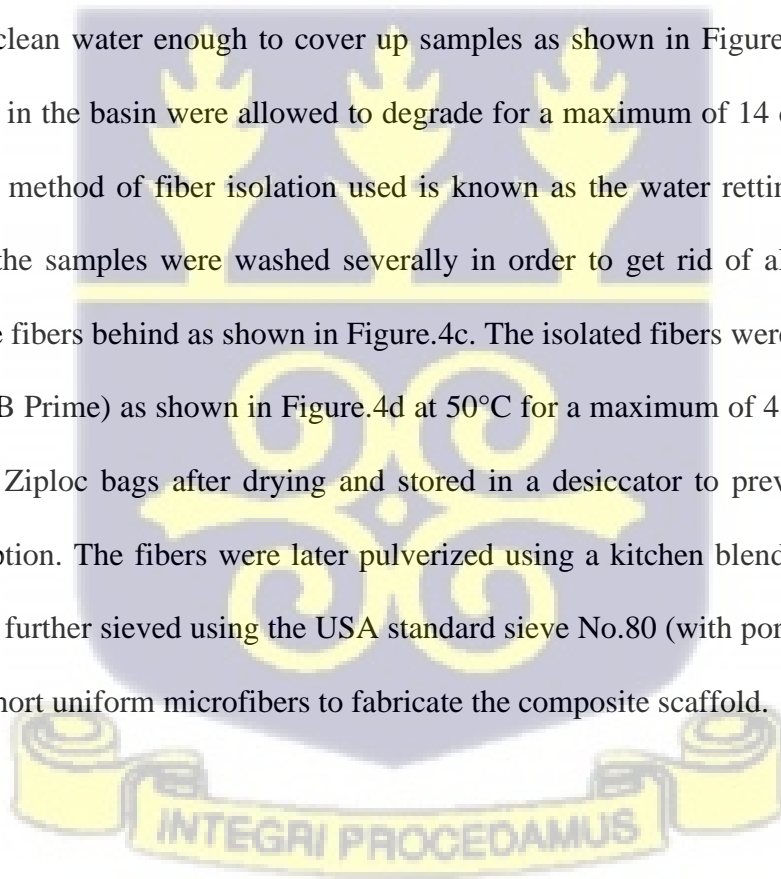
Gelatin Type B powder from bovine skin (G9391), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide, (E7750), N-hydroxysuccinimide (130672), Phosphate-buffered saline (P5368), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Live/Dead cell double staining kit, Dulbecco's Modified Eagle's Medium (DMEM), Penicillin

Streptomycin, 0.5 % Trypsin- EDTA, Fetal Bovine Serum (FBS), were purchased from Sigma-Aldrich. HEK 293 and MDA MB 231 cells were originally from American Type Culture Collection (ATCC, USA).

Serological pipettes, 96 Well TC-treated microplates, pipette tips, 60 x 15 mm style Polystyrene Petri dishes and cell culture flasks were also purchased from Sigma-Aldrich, USA.

### **3.3 Cassava Tuber Preparation and Fiber Isolation**

The cassava tubers were obtained from GAEC and washed under running water to get rid of any soil residue before transporting it to the laboratory. The cassava samples were then peeled with a kitchen knife into basins, washed several times until they were clean and then soaked in the basin with clean water enough to cover up samples as shown in Figure.4a. The cassava samples soaked in the basin were allowed to degrade for a maximum of 14 days as shown in Figure.4b .This method of fiber isolation used is known as the water retting method. After soaking them, the samples were washed severally in order to get rid of all degraded parts leaving only the fibers behind as shown in Figure.4c. The isolated fibers were then dried in an Oven (GENLAB Prime) as shown in Figure.4d at 50°C for a maximum of 48 hours. Samples were placed in Ziploc bags after drying and stored in a desiccator to prevent any form of moisture absorption. The fibers were later pulverized using a kitchen blender to form small short fibers and further sieved using the USA standard sieve No.80 (with pore opening of 180 µm) to obtain short uniform microfibers to fabricate the composite scaffold.



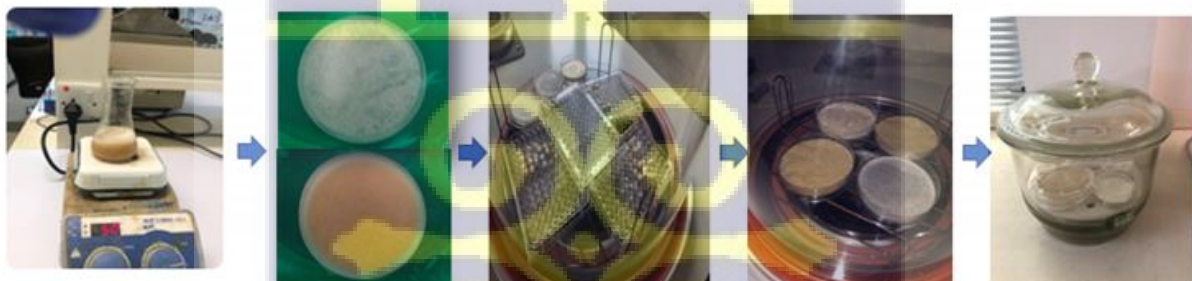


**Figure 4. Images of fiber extraction process, (a) cassava soaked in water; (b) cassava soaked after 14 days; (c) extracted cassava fiber; (d) drying of fiber in oven.**

### **3.4 Synthesis of Cassava Microfiber-Gelatin Scaffold**

Using Diabor et.al, (2019) protocol for the scaffold fabrication, three-dimensional cassava microfiber-gelatin scaffolds and pure gelatin scaffolds were fabricated by a freeze-drying technique except that 3 % (w/v) gelatin was used instead of 1 % (w/v), as recommended by Diabor et al., (2019) to improve the binding effect of gelatin and the cassava microfibers. The 3 % (w/v) gelatin solution was prepared by dissolving 1.20 g of gelatin (Type B powder from bovine skin, Sigma-Aldrich G9391) in 40 ml of distilled water (DI water) in a beaker, heated at 50 °C with stirring for 1 h using a hot plate magnetic stirrer. In preparing the composite scaffold, the same steps for the gelatin solution were repeated except that 7 % (w/v) of the cassava microfiber was weighed and added gently to the gelatin solution with continuous stirring for 1 h to ensure even distribution of the fibers in the gel. The mixed solution for both

the gelatin only and gelatin with the microfibers, was allowed to equilibrate to room temperature and then specific amounts of the crosslinking agents were added to them. An amount of 0.03 g of NHS and 0.05 g of EDC was added to each mixture and stirred using a magnetic stirrer once again but without heat for a minimum of 15 minutes to crosslink the mixture. The crosslinked mixture was then pipetted into either a 10 mm polystyrene petri dish (20 ml of mixture) or 96 well plate (150  $\mu$ l of mixture) depending on the test to be performed. The samples were neatly covered and sealed with parafilm and transferred to a 4°C fridge to allow gelation, and then later transferred to a -20 °C to form ice crystals, for 12 h each. All these procedures stated above except for preparing the mixture on the hot plate magnetic stirrer, were done under a laminar flow hood to ensure sterile conditions. The freeze drying method was used in fabricating pores in the scaffold, thus samples were lyophilized in a Labconco Freezone freeze dryer (Figure 6) for a maximum of 36 h. The samples were then stored in a desiccator until further experiments and analyzes.



**Figure 5. Pictorial representation of scaffold fabrication process.**





**Figure 6. Image of Labconco Freezone Freeze dryer and freeze dried samples.**

### 3.5 Cell Culture

Cryopreserved cells for both HEK 293 and MDA MB 231 were obtained from the Department of Biochemistry, Cell and Molecular Biology, University of Ghana (Cancer Lab\_Aikins lab). The frozen cells were thawed for < 1 minute in a 37°C water bath and quickly transferred into a falcon tube. About 5 ml of pre-warmed growth media was gently added to it and spun in the centrifuge at a speed of 700 rpm for 3 mins to separate the media from the cells. After spinning, the supernatant was pipetted off leaving the cell pellet formed at the bottom of the falcon tube. The cell pellet was then gently resuspended in 1 ml of media, transferred into a T-25 flask (Corning/Costar) containing 5 ml of pre-warmed media and placed in a cell culture incubator with 37°C and 5 % CO<sub>2</sub>. The cells were monitored under an optical microscope with media changed every 2-3 days, upon reaching 80 % confluence, the cells were sub-cultured into a T-75 culture flask to further expand their number. This was done by pipetting off the old media in the T-25 culture flask, rinsing with 5 ml PBS twice, then adding 300-500 µl of trypsin-EDTA (0.5 % trypsin, 5.3Mm EDTA) to the flask and incubating for about 3-5 mins. The cells were

then viewed under the microscope to check if they have detached, 5 ml media was pipetted into the flask, swirled and pipetted off into a falcon tube. The same procedure for the frozen cells was repeated but the spinning speed used was 1000 rpm for 5 mins. The resuspended cell pellet was then transferred into the T-75 flask. All media used for culturing the cells was prepared with high glucose Dulbecco's Modified Eagle's Medium (D-MEM) with L-Glutamine, 4500 mg/L D-Glucose, without Sodium Pyruvate supplemented with 10 % Fetal Bovine Serum, Research grade and 1 % Penicillin-Streptomycin (5,000 U/mL). All activities were performed under aseptic conditions and reagents were obtained from Sigma Aldrich.

### **3.6 Sample Sterilization**

All samples were sterilized under same conditions prior to each experiment. The samples were placed in the laminar flow hood and a specific amount of 70 % ethanol was added to the samples and left overnight in the hood to dry. For samples in the 96 well plate and the 10 mm polystyrene petri dish, 50  $\mu$ l and 1 ml of ethanol were added, respectively. Afterwards the samples were washed with PBS twice prior to cell seeding.

### **3.7 Cell Proliferation Test**

This test assessed the proliferation of cells seeded on the sterilized scaffold samples in 96 well plate via MTT assay. Cells were harvested from the T-75 culture flask after the 2<sup>nd</sup> - 3<sup>rd</sup> passage number using trypsin-EDTA, the harvested cell pellets were resuspended in 1 ml of media after centrifuging. The cell concentration and viability were then determined using Trypan blue exclusion method. The dilution factor (df) used was 10  $\mu$ l of cell suspension and 30  $\mu$ l of trypan blue staining solution pipetted and mixed together in an Eppendorf tube. A hemocytometer was used to count the cells, 10  $\mu$ l of a mixture of the stain and cells was pipetted under the glass cover slip on the hemocytometer and viewed under the microscope. The live cells in each

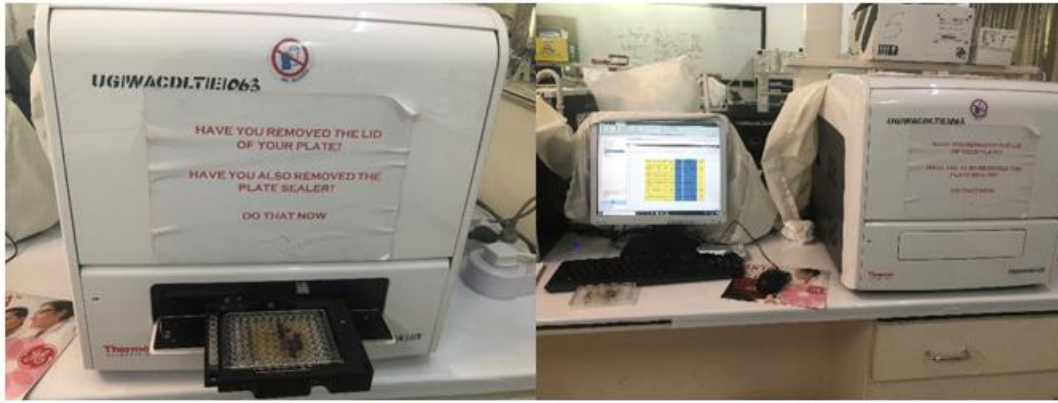
quadrant was counted manually and the average live cell was calculated and recorded. The formula used to calculate the live cell concentration is shown below:

*live cell concentration (per ml) = average live cell number  $\times$  df; ... .. equation. 3*

*where df =  $4 \times 10^4$*

After calculating the cell concentration per ml, the ratio of required cell seeding concentration was calculated from it. For the 96 well plate,  $1 \times 10^4$  cells were seeded per well and 100  $\mu$ l of media was added to each well. The cells were seeded on the sterilized samples and incubated for 3 hours prior to the addition of media, this was to allow the cells attach to the surface of the scaffolds instead of the plate. In this assay, cells cultured in the well plate without the scaffold was used as one of the controls. The samples with only media and no cells were used as the color control, this was to cater for any absorbance produced from the samples themselves. The blank used was media only with no cells to also cater for any absorbance produced from the media only, and negative control used was cells growing in the well plate without any scaffold present.

The cells seeded on the scaffold were incubated at specific time points, day 1, 3 & 5 and MTT assay was performed at these specific time points. MTT solution was prepared by weighing 0.025 g of the powder into 10 ml PBS, 20  $\mu$ l of the solution was added to the samples, the controls and blank and incubated for 4 h. After, 100  $\mu$ l of isopropanol prepared by adding 163  $\mu$ l of hydrochloric acid to 50 ml of propan-2-ol was added to each well and also incubated for 30 mins to dissolve the purple formazan precipitate formed. The absorbance of each well in the plate was determined using the spectrophotometer set at a wavelength of 590 nm as shown in Figure 7 below.



**Figure 7. Setup of Spectrophotometer.**

The percentage cell viability (% CV) was calculated from the absorbance using the formula below:

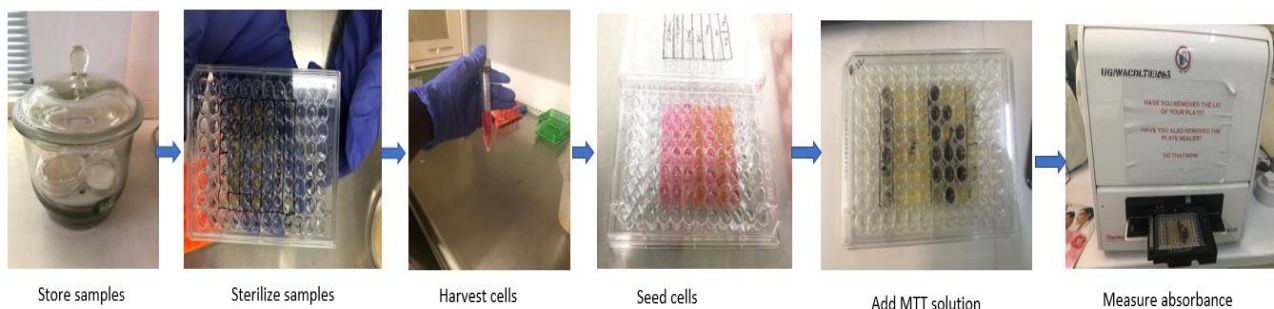
$$\% CV = \frac{\text{test absorbance} - \text{color control absorbance}}{\text{negative control absorbance} - \text{blank absorbance}} \times 100\% \dots \dots \text{equation. 4}$$

Where:

- Test absorbance = absorbance of samples with cells
- Color control absorbance = absorbance of samples without cells
- Negative absorbance = absorbance of cells with media only
- Blank absorbance = absorbance of media only

Graphs of the viability was plotted and analyzed, all experiments were performed in triplicates. MTT assay solution is light sensitive, hence all experiments were performed in the dark or with minimum light as much as possible. Figure 8 below shows a graphical representation of the experiment.





**Figure 8. Pictorial representation of MTT assay to determine cell proliferation.**

### 3.8 Scaffold Surface Morphology Imaging

This test was performed by imaging the surface morphology of the prepared scaffolds using Zeiss Stereo Microscope as shown in Figure 9 below. The freeze-dried samples in petri dishes were placed under the microscope and images were taken.



**Figure 9. Setup of Zeiss Stereo Microscope**

### 3.9 Cytotoxicity Extraction Test

This extraction test method was performed by checking the effect of the extract obtained from sterilized samples in petri dishes on the morphology and viability of the cells. After sterilizing samples and washing with PBS, a calculated amount of media was pipetted on the samples.

The surface of the device to volume ratio of the extract vehicle recommended by ISO 10993-5 (ISO, 2009) is  $125 \text{ mm}^2/\text{ml}$ , hence this was used to calculate the corresponding volume of media to be added to the samples. For a large petri dish with about 100 mm diameter, 15 ml of media was added, while for a small petri dish with about 60 mm diameter, 10 ml of media was added. The samples were covered and sealed with parafilm and incubated at  $37^\circ\text{C}$  for 24 hours, the extract from the samples at this specific time point was added to the cells.

Cells were seeded into 24 well plates at a seeding density of  $1 \times 10^5$  cells/ml and further incubated for 24 hours to ensure cells had attached and started growing before adding the extract. The media on the cells was pipetted off and washed with PBS before adding  $500 \mu\text{l}$  of the extract to the fresh media added to the cells. After adding the extract to the cells, they were incubated for 24 and 48 hours, respectively at  $37^\circ\text{C}$ . The cells were analyzed for any changes in morphology or cellular degeneration. The negative control used was the cells without any extract, and for the positive control 70 % ethanol was used as the extract. All the experiments were performed in triplicate.



**Figure 10. Pictorial representation of the extraction method.**

### 3.9.1 Cell Morphology Analysis:

After incubating the samples and controls with the extracts for 24 and 48 hours, the extract was pipetted off and the cells were washed twice with PBS to get rid of debris from the extract solution. Images of the cells were then taken with an optical microscope as shown in Figure 10 below, to check the effect of the extract on the cell morphology. Per ISO standards, Table 1

was used to qualitatively grade cytotoxic effects of the extract on the cell morphology. A comparison between the controls and the samples was done.



**Figure 11. Setup of Optika Microscope (Italy) equipped with an Optikam B9 Digital Camera.**

**Table 1. Qualitative morphological grading of cytotoxicity of extracts (ISO, 2009)**

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.
1	Slight	Not more than 20 % of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50 % of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis; not more than 50 % growth inhibition observable.
3	Moderate	Not more than 70 % of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50 % growth inhibition observable.
4	Severe	Nearly complete destruction of the cell layers.

### 3.9.2 Live/Dead Staining

The live/dead staining was to give a visual representation of the number of cells alive and the number dead after incubating the cells with the extract. This staining kit contains Calciin-AM which stained the live cells green and Propidium Iodide (PI) which stained the dead ones red. After pipetting off the extract from the cells, the cells were washed with PBS several times (4x) to get rid of any residual esterase activity. The cells were then trypsinized from the petri dish since the fluorescent microscope (ZEISS Axio Vert A1 Inverted Phase Fluorescence Microscope) used could not view the cells in the dish when stained directly. A volume of 300  $\mu$ l trypsin-EDTA was added to the 24 well plate, incubated for about 5 mins and then viewed under the optical microscope to check if the cells have successfully detached. PBS (1 ml) was added to the trypsinized cells to stop the reaction, and then the trypsinized cells were transferred into an Eppendorf tube. This was done for all the cells with their tubes labelled with the corresponding name tag. Using a micro-centrifuge, the cells in the Eppendorf tube were spun at 1000 rpm speed for 3 mins, this was repeated four times. The supernatant was pipetted off, leaving behind the cell pellet and 50  $\mu$ l of the staining solution prepared was added to the cell pellet and resuspended in it. Before adding the staining solution, the Eppendorf tubes were covered with foil and the lights were turned off since the solution is light sensitive. The mixture was then incubated at 37 °C for 15 mins prior to imaging of the cells. 20  $\mu$ l of the mixture was pipetted on a glass slide with a cover slip and then viewed using the fluorescent microscope as shown in Figure. 11 to detect the live and dead cells.

In preparing the staining solution, the producers recommended staining the cells to determine the suitable concentration of PI and Calciin-AM that can effectively stain cells, after doing this test the suitable concentration was between 1-5  $\mu$ M. Then, 10  $\mu$ l of Calciin-AM and 5  $\mu$ l of PI of the suitable concentration was added to 5 ml PBS to prepare the staining solution as recommended by the producers.



**Figure 12. Setup of ZEISS Axio Vert A1 Inverted Phase Fluorescence Microscope.**

### **3.10 Data Analysis**

The MTT assay produced continuous data in the form of the absorbance measured. Using Microsoft Excel 2016, the data were organized and the three closest absorbance values were used to calculate the percentage cell viability for each sample. The percentage cell viability was expressed as a percentage of the control value obtained for the negative control (cells without scaffold). Using the GraphPad Prism 8 Software, descriptive statistics was done to find the mean and standard deviation (SD) of the three % cell viability calculated. The mean  $\pm$  SD (represented as error bars) for each sample was plotted as bar graphs using the software.

Statistical analyzes of the results were carried out using one-way analysis of variance (ANOVA) (GraphPad Prism 8 Software) to determine if there was any statistical significance among the samples. Shapiro-Wilk test was performed to test for normality of the data before using ANOVA and all data obtained were normally distributed. Tukey's post hoc test was used to further determine if the difference is significant at  $p$  value  $< 0.05$ . The level of significance was represented by the number of '\*' displayed on the graph;  $p \leq 0.0001$ (\*\*\*\*),  $p \leq 0.0002$  (\*\*\*),  $p \leq 0.0021$  (\*\*),  $p \leq 0.0332$  (\*) and  $p > 0.05$  (ns).

Images obtained from fluorescence and optical microscopy were analyzed using ImageJ software. Table. 1 (adopted from ISO standards) was used to analyze the cytotoxicity of the images obtained from the optical microscope. Images obtained from fluorescence microscopy were analyzed by visually inspecting the intensity/number of the live (green) cells in comparison to that of the dead (red) cells. Higher live cell intensity indicated less cytotoxicity and vice versa.



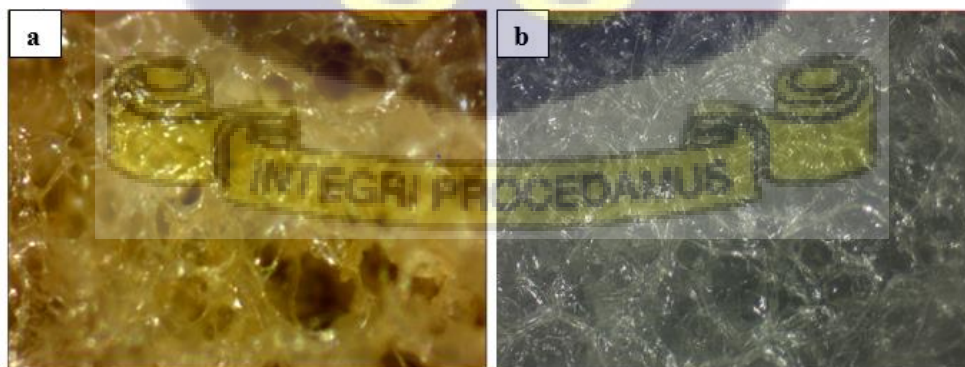
## CHAPTER 4

### RESULTS AND DISCUSSION

In this chapter the results obtained for each experiment are reported and discussed. The results obtained are in two major sections, the extraction test and the direct contact test (MTT assay). It is important to note that all tests performed were according to ISO standards for checking *in vitro* cytotoxicity of medical devices (ISO, 2009). For all the experiments done, HEK 293 cells and cancer cell line MDA MB 231 were used. The HEK 293 cells was to check if the scaffold can be considered for TE purposes using normal cells, while MDA MB 231 cancer cells was to check if the scaffold can be used for 3D tumor cell studies.

#### 4.1 Scaffold Surface Morphology

Figure 13 below shows the surface morphology of GELCAS and GEL only. GELCAS had rough surface and interconnected pores with fibers randomly dispersed in the gelatin matrix as described in Diabor's work (Diabor et al., 2019). Since the same scaffold fabrication procedure was used, it can be suggested that the scaffold properties recorded in Diabor's work are similar to this work. Seo et al., (2009) performed a similar continuation study that focused solely on analyzing biocompatibility of a scaffold they had already fabricated and characterized. Hence, the reason for not performing characterization test of the GELCAS and GEL only scaffold again, considering that this is also a continuation study.



**Figure 13. Images of scaffold surface morphology of (a) GELCAS and (b) GEL only.**

## 4.2 Extraction Test Results

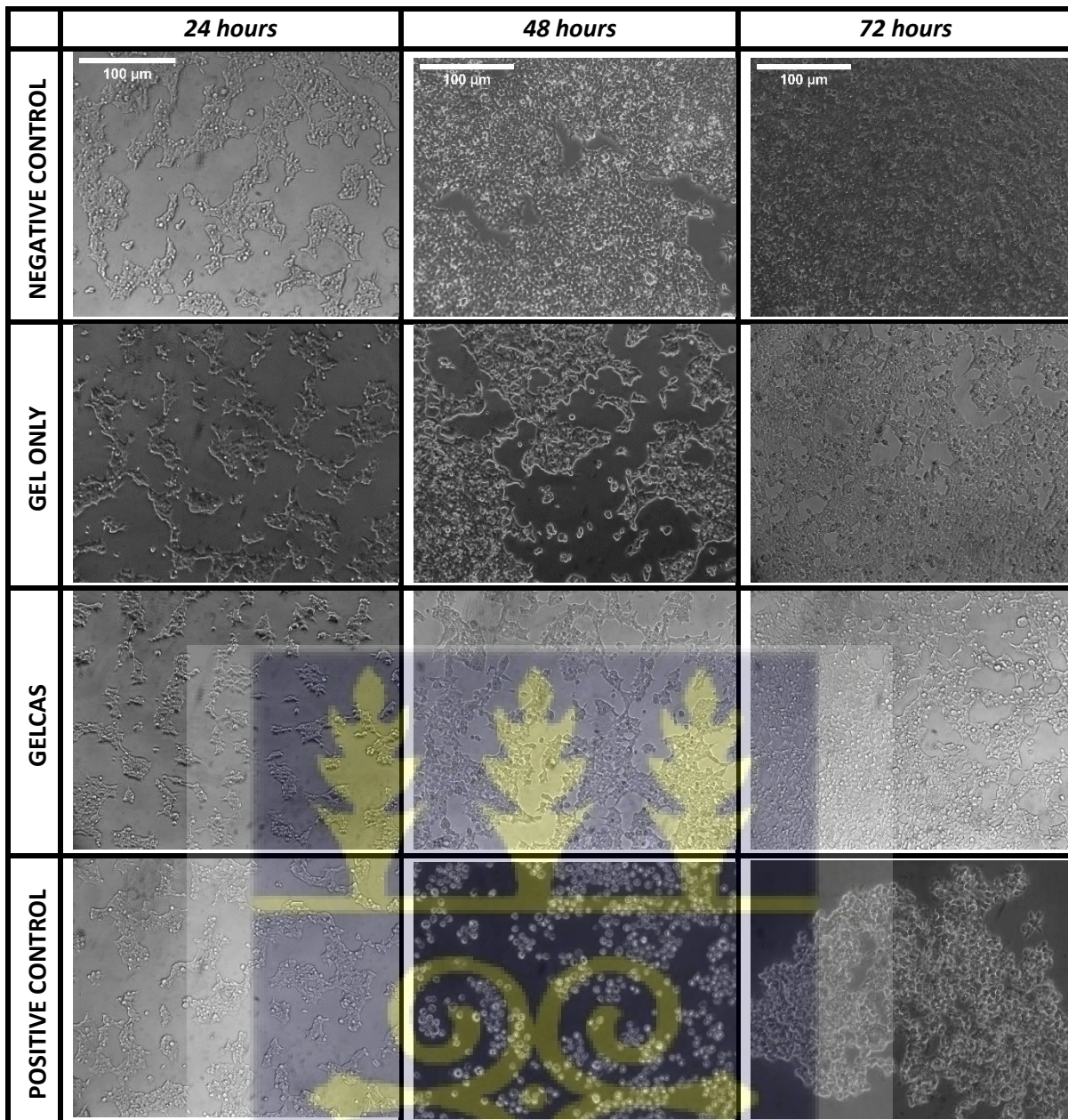
The results obtained for this test were in two parts, one was for cell morphology analysis while the other was for live/dead staining assay. All experiments were performed in triplicate.

### 4.2.1 Cell Morphology Analysis

This analysis was done to determine the effect of the extract obtained from incubating the samples with media at 37 °C for 24 h, on the morphology of both HEK 293 and MDA MB 231 cells. Per the ISO standard, the factors that were considered in this analysis were the cell shape, the state of the cell attachment to the plate, and observation of the cell growth inhibition after addition of extract (ISO, 2009). These factors were used to determine the grade of cytotoxicity using Table 1. The negative control which does not elicit cytotoxic effect, and the positive control which elicit cytotoxic effect were also used as reference points to determine the cytotoxic level, by comparing the results obtained for the test samples to them.

#### 4.2.1.1 HEK 293 Optical Microscopy Images

Figure 14 below shows captured images from an optical microscope after exposing HEK 293 cells to the extracts. Since HEK 293 cells are adherent in culture, it was observed that the cells had initial attachment and were connected to each other 24 hours after seeding in all cases. The cells are also known to appear less circular than common cell lines and tend to display dendritic processes, as seen from Figure 14 for all samples at the 24 hour time point. Thus, all the cells were in the same condition and were exhibiting healthy cellular characteristics prior to the addition of the extracts. After the addition of the extract from the GEL only and GELCAS to the cells, it was observed that at time point 48 h (24 h incubation after adding extract), the cells had increased in number, maintained their morphology, and were well attached to the well plate for both samples.



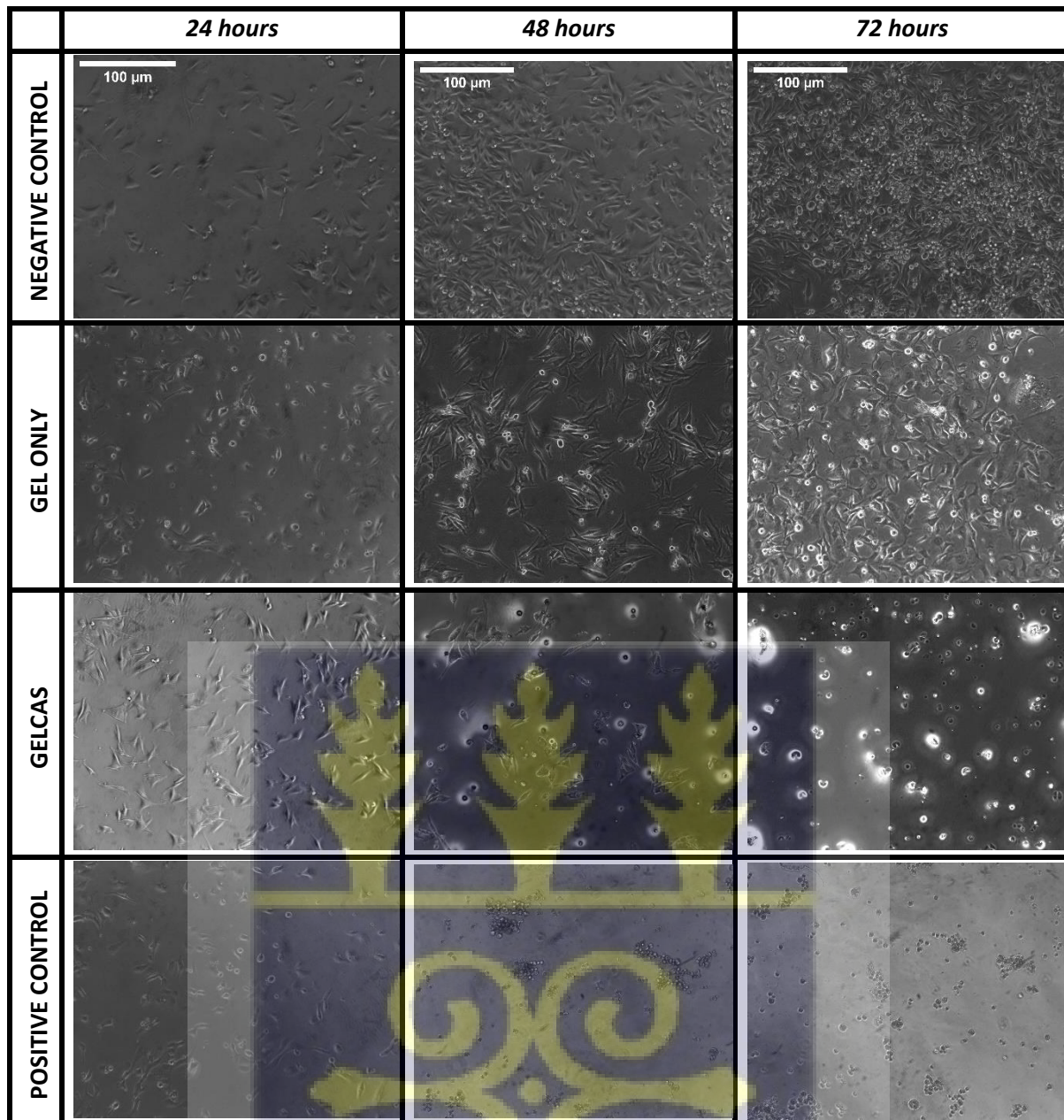
**Figure 14. Morphological changes of HEK 293 observed under an inverted light microscope (100 x magnification) after exposure to extract of samples, scale bar (100 μm).**

A similar observation was made at time point 72 h (48 h incubation after adding extract) but with a further increase in the cell number and increase in cell interconnection, indicating that the presence of extracts had little or no effect on the cell morphology or growth. This observation was expected for GEL only extract, since gelatin is a recognized biomaterial already in use for TE applications. Similarly, for the negative control which had no extract present, it was observed that there was a higher increase in cell number than in all cases as

expected. The cells for the negative control had reached approximately 70-80 % confluence at time point 48 h and increased to about 90-100 % at time point 72 h. Using this as a reference point, it can be deduced that the cell confluence for the GELCAS and GEL only was approximately 50-60 % at time point 48 h with an increase to 70-80 % confluence. Therefore, although the presence of the extract did not affect the morphology and interconnection of the cells, it had slight growth inhibition on the cells. Per the ISO standard Table 1, it can be inferred that GELCAS can be considered as Grade 1, with slight reactivity. Since both test samples had similar reactivity, it can be concluded that the presence of the cassava fiber in the gelatin had little or no significant effect on the HEK 293 cell line. Thus, the GELCAS material is not cytotoxic to HEK 293 cells. The positive control that was treated with 70 % alcohol on the other hand, gave a cytotoxic response as expected; the cells became round, losing their initial morphology and were suspended in culture instead of adhering to the well plate. After 72 h, they had clustered together and were floating in the well plate.

#### ***4.2.1.2 MDA MB 231 Optical Microscopy Images***

Figure 15 below shows optical microscopy images after exposing MDA MB 231 cells to the extracts. MDA MB- 231 are adherent cancer cells that have epithelial- like morphology, they appear phenotypically as spindle shaped cells similar to fibroblast like cells. As seen in Figure 15 below, the cells attached and were expressing their phenotype, 24 hours after seeding for all samples. Indicating that the cells were in a healthy state prior to the addition of the extract. After the addition of the extract from GEL only to the cells it was observed that the cells maintained their morphology and slightly increased in cell number at time point 48 h, with further increase in cell number at time point 72 h. Again, this was expected since gelatin has been successfully used in fabricating a 3-D scaffold for tumor cell culture using the same cell line (Wang et al., 2018).

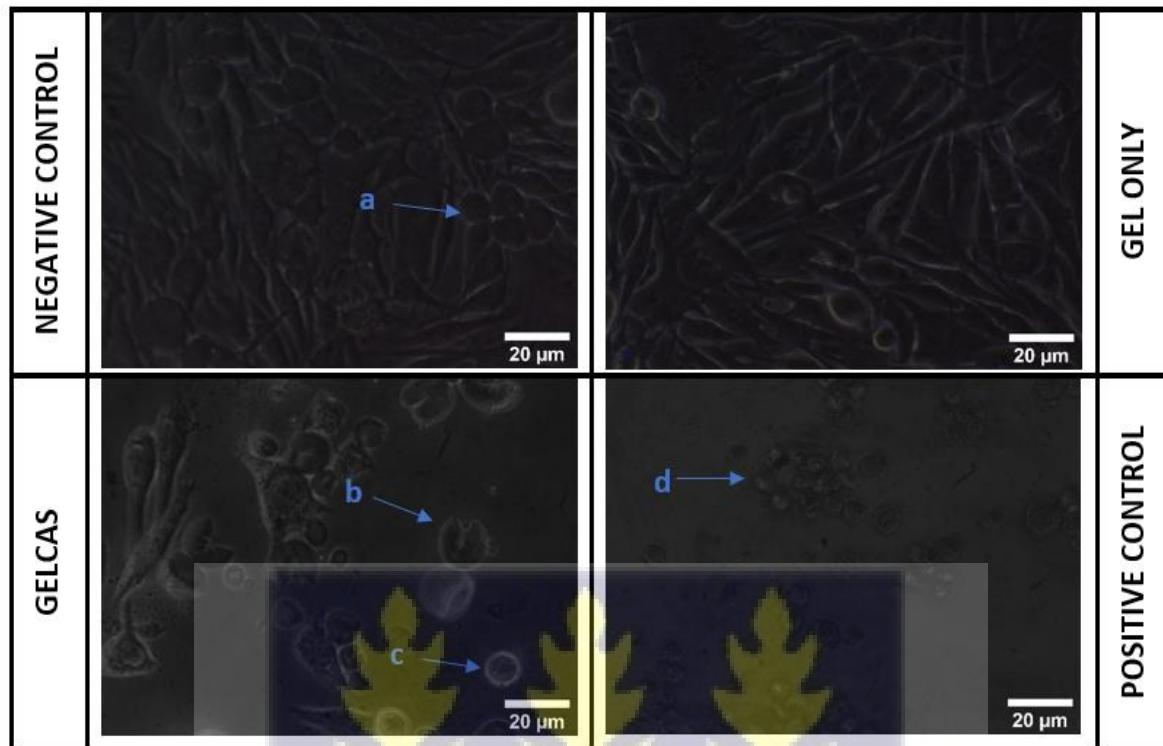


**Figure 15. Morphological changes of MDA MB 231 cells observed under an inverted light microscope (100 x magnification) after exposure to extract of samples, scale bar (100  $\mu\text{m}$ )**

Similarly, the negative control exhibited same cell characteristics as GEL only except that it had higher cell proliferation than the GEL only, for all the different time points, similar to the results for HEK 293 cells.

Contrary to the expectation for GELCAS, the cells were rather observed to be changing morphology from their spindle shape to circular shapes. The positive control also had the cells shrinking and losing their morphology as well, as expected because of the 70 % alcohol. To

better understand and see more clearly what was going on, images of the cells after exposure to the sample extracts were taken at (400x) magnification as seen in Figure 16 below.



**Figure 16. Optical microscopy images of cells after exposure to sample extract at 400x magnification, scale bar (20 µm); a- mitotic division of cells, b- cell disruption, c- formation of apoptotic bodies, d- cellular shrinkage.**

The negative control and GEL only had similar cell morphology, with some of the cells undergoing mitosis as seen from Figure 16. On the other hand, the cells exposed to GELCAS extract did not look healthy, they rather looked like the cell membrane had been disrupted (Figure 16b) and did not have similar mitotic division ongoing as seen in the negative control (Figure 16a) and GEL only. From literature this change indicated that the cells were undergoing apoptotic cell death (Rahman et al., 2016), meaning the presence of the material from GELCAS had a negative effect on the cells by inducing their death. After 72 hours, majority of the cells showed apoptotic features such as cellular shrinkage and apoptotic bodies (Brown et al., 2014). These features exhibited by the cells, were also similar to cancer cells after treatment with some potent compounds that are being investigated as anti-cancer agents. For instance, ampelopsin

E, a compound isolated from *Dryobalanops*, was reported to exhibit cytotoxic effect on MDA MB 231 by inducing apoptosis and cell cycle arrest (Rahman et al., 2016). The morphological changes reported after the treatment in varied concentrations are similar to the results obtained in Figure 16 for GELCAS and can be seen in appendix (Figure 22).

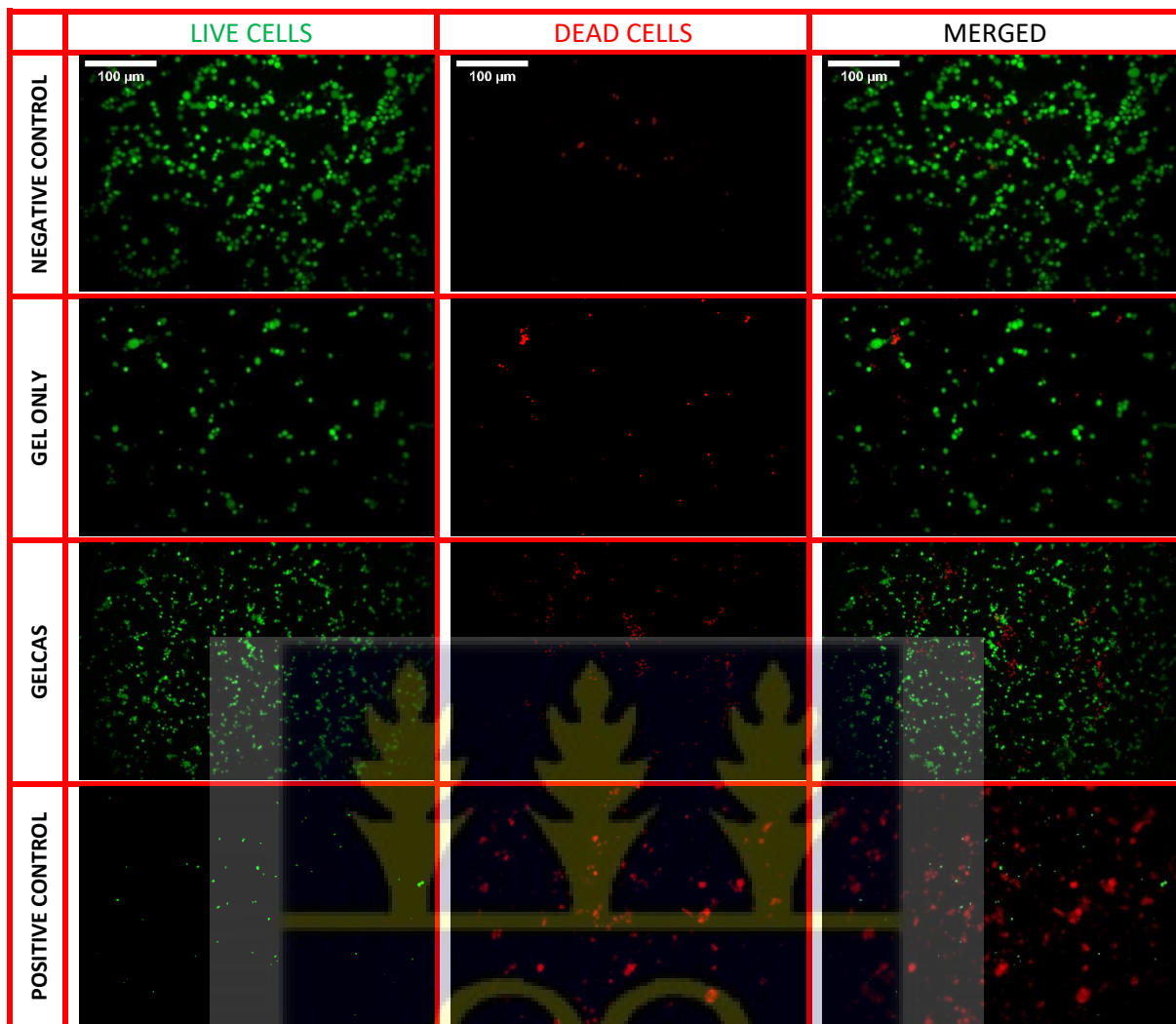
Per ISO standard Table 1, it can be inferred that the effect of GELCAS extract on the cells is between grades 3-4, moderate/severe with some similarities to the positive control. Therefore, GELCAS can be said to be cytotoxic to MDA MB 231 cancer cells per the results obtained. This is an interesting finding, considering that the extract was not toxic to the HEK 293 cells but to the cancerous MDA MB 231. However, unlike the positive control where the mode of cell death is usually classified as cell necrosis, as a result of the imbalance in cell environment due to the presence of the alcohol (Hoek et al., 2002; Plante et al., 2013), the mode by which the GELCAS extract caused the cells to die is not known. Hence, further studies will need to be done in order to understand the mechanism of cell death, that way it can also be exploited as a potential anti-cancer agent. Also, a co-culture of both normal and cancer cells can be done to ascertain that the GELCAS extract has preferential cytotoxic effect on the cancer cells only.

#### **4.2.2 Live/Dead Staining Assay**

This study was done to show the number of cells alive and number of cells dead and to further confirm the effect of the extracts on cell viability. This test was done for both HEK 293 and MDA MB 231 cells, and the intensity of the live cells versus the dead cells was used in determining the cytotoxicity level.

##### **4.2.2.1 HEK 293 Fluorescence Microscopy Images**

Figure. 17 below shows fluorescence microscopy images of the HEK 293 cells after exposure to the extracts from the samples. Separate images of live only and dead only were taken and then merged to fully represent both live and dead cells.



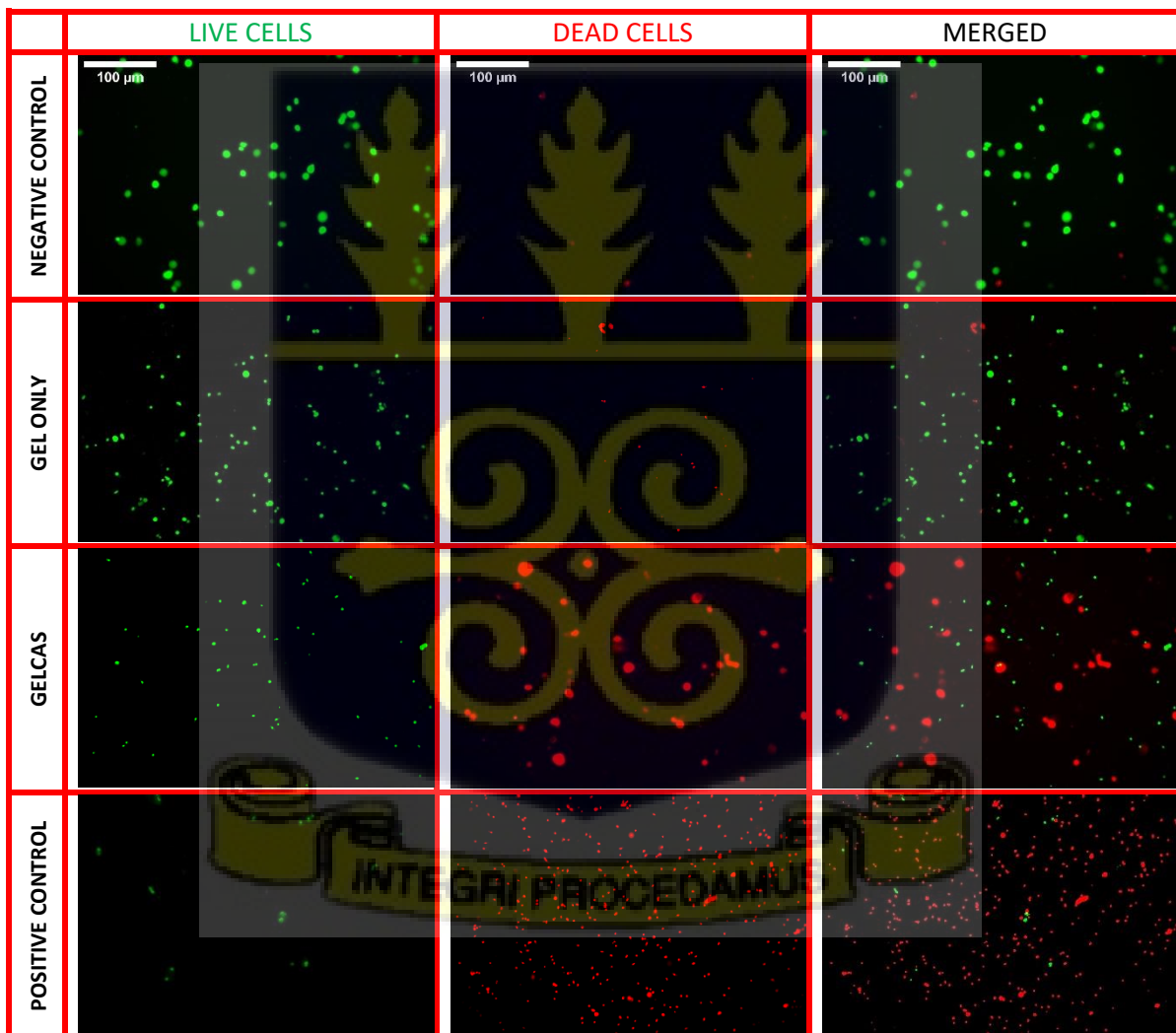
**Figure 17. Fluorescence microscopy images depicting live cells stained green with Calcein-AM solution and dead cells stained red with Propidium Iodide solution for HEK 293 cells after exposure to sample extracts, scale bar (100  $\mu\text{m}$ ).**

As seen in Figure 17, there was higher intensity of live cells for GELCAS, GEL only and the negative control, with the least intensity observed for the positive control. This result is in agreement with the results presented in Figure 14, where the cells maintained their morphology for all samples with the exception of the positive control. Again, the intensity for dead cells was highest in the positive control, followed by GEL only and GELCAS, with the least intensity observed in the negative control as expected. The slightly higher intensity for dead cells supports the slight inhibition of cell growth in GELCAS and GEL only samples as

presented in Figure 14. Therefore, it can be concluded from these results that GELCAS is not cytotoxic to HEK 293 cells, hence the presence of the cassava fiber in the gelatin had little or no significant effect on the cells. This could be attributed to the fact that cellulose is generally known to be biocompatible, thus its presence in the fiber had no toxic effect on the cells (Mikshina et al., 2013).

#### 4.2.2.2 MDA MB 231 Fluorescence Microscopy Images

Figure 18 below shows fluorescence microscopy images of the MDA MB 231 cells after exposure to the extracts from the samples.



**Figure 18. Fluorescence microscopy images depicting live cells stained green with Calcein-AM solution and dead cells stained red with Propidium Iodide solution for MDA MB 231 cells after exposure to sample extracts, scale bar (100  $\mu$ m).**

For the MDA MB 231 cells, the intensity of live cells for the GEL only was higher than that of the GELCAS, whereas the intensity for dead cells was higher in GELCAS than in GEL only. This indicates that, the presence of cassava fiber in gelatin resulted in an increase in the death of the cancer cells, contrary to what occurred in the HEK 293 cells. Although this result was not expected, the difference in results may be attributed to the presence of other components such as hemicellulose and lignin attached to the cellulose (Sorieul et al., 2016) present in the fiber. Since cellulose is generally known to be biocompatible and has been used to support even cancer cell growth (Kumar Gupta et al., 2019).

According to Wang et al.,(2015), some lignin-carbohydrate complexes formed as a result of chemical bonds between cellulose, hemicellulose and lignin are known to exhibit anti-proliferative properties at the cellular level. They extracted two lignin-carbohydrate complexes from the chaga mushroom (*Inonotus obliquus*), and examined their cytotoxicity against cancer cells. Their findings indicated that the extracts had anti-tumoral activity on the cells as expected. However, the mechanism by which cell apoptosis was induced was mostly by inhibiting NF- $\kappa$ B, a protein transcription factor responsible for turning on the gene expression that prevents cancer cells from undergoing cell apoptosis. This particular protein transcription factor exists in an inactive form in normal cells, but is active in cancer cells (Wang et al., 2015). Considering that the same extract from GELCAS resulted in the death of the MDA MB 231 cancer cells while supporting HEK 293 cell growth, it could be that the mechanism of cell death may be similar to what was reported by Wang et al., (2015).

Also plant extracts or compounds such as phenolics, alkaloids, polysaccharides and glycoproteins, lectins, tannins and lignins have been reported to selectively induce apoptosis

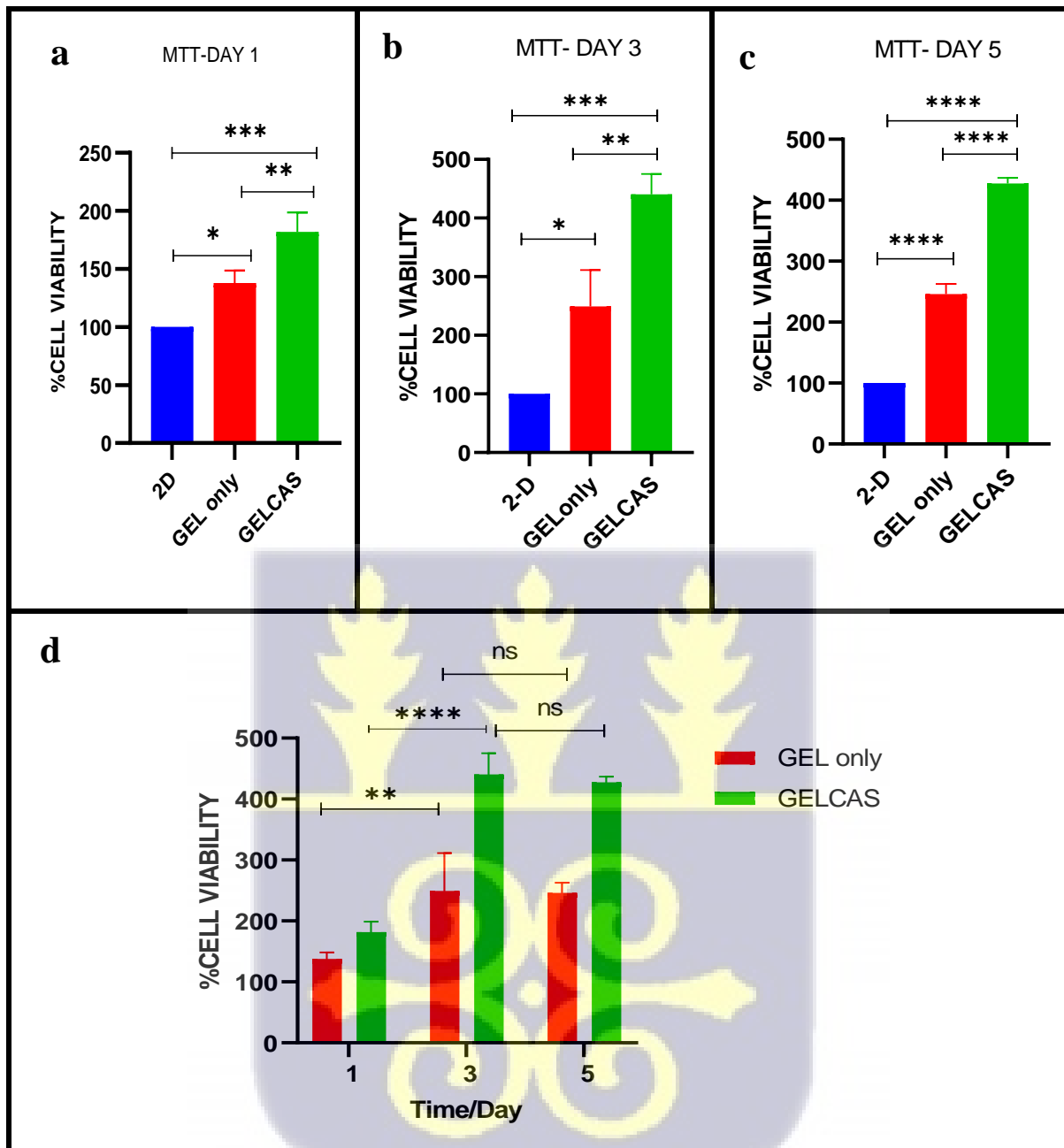
in neoplastic cells instead of normal cells (Safarzadeh et al., 2014). However, more studies need to be done to verify this hypothesis. Aside that, ethanol cassava extracts have also been investigated for their cytotoxic effect against different cancer lines and they all showed promising results as potential anti-cancer agents (Widiastuti et al., 2018; Sadudee, S., 2017; Rahmat et al., 2004). Therefore, cassava in itself may possess anti-proliferative properties against cancer cells.

### **4.3 MTT Assay Results**

MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. This colorimetric assay is based on the reduction of a yellow tetrazolium salt to purple formazan crystals by metabolically active cells. The higher the absorbance, the higher the cell viability (Kamiloglu et al., 2020). Hence, this test was done to determine the viability of cells seeded directly on the scaffold, as this reflects better, the use of the scaffold in a clinical setting. Also, the results obtained from this test assisted in further confirming the results obtained from the extraction method test. Similar to the extraction test, both HEK 293 and MDA MB 231 cells were used for this test. The raw data used in plotting the graphs for both cell lines are represented in a tabular form in the appendix.

#### **4.3.1 HEK 293**

The mean absorbance of the negative control was calculated and used as the reference point for 100 % cell viability at all the respective time points, since cells are generally known to proliferate well on TCP. The cell viability for all the samples (GEL only and GELCAS) was expressed as a percentage of the negative control value. The percentage cell viability (CV) was calculated from the measured absorbance (AB) obtained from the spectrophotometer reading using equation. 1. Figure 19 below shows a graphical representation of the percentage cell viability for HEK 293 cells.



**Figure 19. Percentage cell viability after 1, 3, and 5 days of culture of HEK 293 cells on the samples as determined by MTT assay; the control values were normalized to 100 %.**

As seen in Figure. 19a, b and c, after culturing the cells on samples at the different time points ( day 1,3 and 5), GELCAS exhibited higher cell viability than the GEL only at all the time points. Statistical analysis done indicated that, the difference in the cell viabilities was significant for all the days with all p values < 0.05. This suggests that, the presence of the

cassava microfiber in gelatin had a positive effect on cell growth as expected. The enhanced cell growth in GELCAS compared to GEL only could be due to the differences in structure of the scaffolds reported by Diabor et al. (2019). Considering that the same protocol used by Diabor et al. (2019) in fabricating the scaffold was used in this work, it can be expected that there will be similarity in the structure of the scaffold made by Diabor et al. (2019) and the one fabricated in this work. In his work he indicated that, the GELCAS scaffold had short discontinuous cassava fibers randomly dispersed in the gelatin matrix, and the surface morphology appeared much rougher compared to that of GEL only. From literature, these properties are known to support cell binding to the surface and serve as contact guidance for directing and spacing cells to grow along the fibers (Xing et al., 2010). This is expected to reduce the cluster growth of cells, preventing cells from competing for nutrients and ending up dying as may have been the case in GEL only. Again, when cells are able to adhere strongly to the scaffold, it permits multilayered cell formation, since there is a larger surface area for them to attach and grow as proposed by Xing et al. (2010).

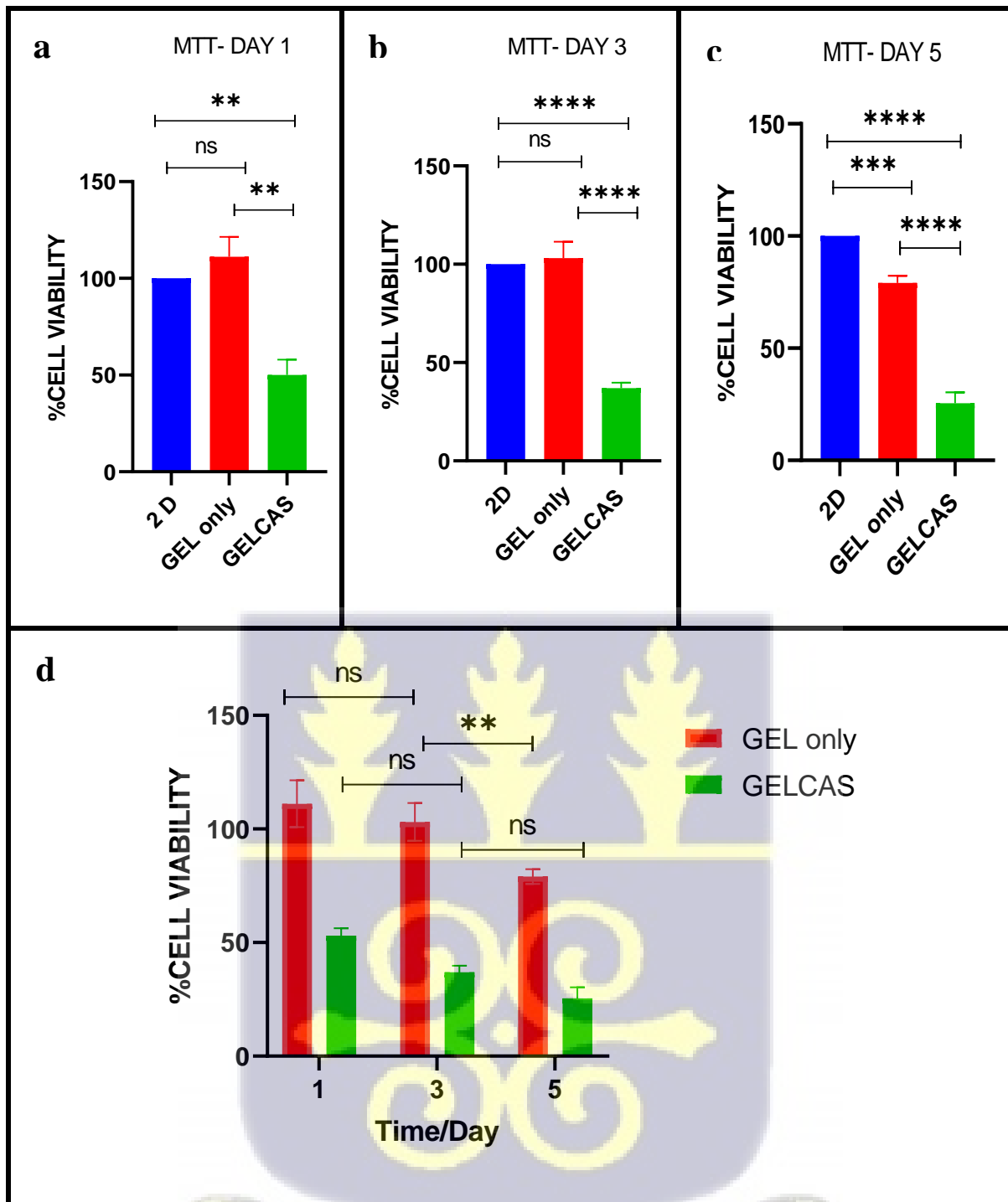
The results obtained are also comparable to similar composite scaffolds, found in literature that also recorded higher cell viability in cellulose-enhanced composite scaffolds. Huang et al. (2016), recorded higher cell viability in gelatin enhanced with partially sulfated cellulose, while Ramphul et al. (2017), reported a similar trend for their cellulose enhanced PLLA-cellulose composite. Xing et al. (2010), also very similar to this work, recorded higher cell viability in cellulose enhanced gelatin than in gelatin only. All these suggest that the presence of cellulose in composite scaffolds, promotes the growth of cells. Also, both 3D samples (GEL only and GELCAS) had higher cell viability than the negative control which had cells growing on a 2-D platform, further supporting the fact that 3D platforms have multiple layers for cell attachment. Hence, can provide more room for cell attachment, unlike the negative control which allows cell growth in one layer. From Figure 18c, it can be observed that although there

was a significant difference between day 1 and day 3, with regard to cell viability for both samples, there was no statistically significant difference in the viability between day 3 and 5. This means that, there was not much cell growth after day 3 and this could be due to the reduction in the inner cross section porosity of the scaffold as reported by Diabor et al. (2019). Thus, the reduction in the porosity may have negatively affected the growth of the cells within the matrix (Deb et al., 2018), preventing them from proliferating further as expected. In conclusion, the deduction made from results obtained from this test is that GELCAS is not cytotoxic to HEK 293 cells, in agreement with the results obtained from the extraction test.

#### 4.3.2 MDA MB 231

The mean absorbance of the negative control was calculated and normalized to 100 % cell viability at all the respective time points, since cells are generally known to proliferate well in TCP. The cell viability for all the samples (GEL only & GELCAS) was expressed as a percentage of the negative control value. The percentage cell viability (CV) was calculated from the measured absorbance (AB) obtained from the spectrophotometer reading using equation. 1. Figure 20 below represents the graphical representation of data obtained from the MTT assay for MDA MB 231 cells.

The percentage cell viability calculated from Table 4 (appendix) generally indicated that GELCAS had the least cell viability at all-time points as compared to the other samples. From Figure.20a, b and c, the trend of GEL only having the highest cell viability, followed by the negative control was consistent throughout. Although GEL only recorded the highest cell viability in all cases, there was a reduction in cell viability from  $111.1 \pm 10.34$  recorded on day 1, to  $79.07 \pm 3.28$  recorded on day 5.



**Figure 20.** Percentage cell viability after 1, 3, and 5 days of culture of MDA MB 231 cells on the samples as determined by MTT assay; the control values were normalized to 100 %.

This was unexpected, however the reduction could have been as a result of cells inability to form aggregates or cluster due to the structure of the gel, this is known to reduce cancer cell proliferation and metabolic activity (Labuschagne et al., 2019).

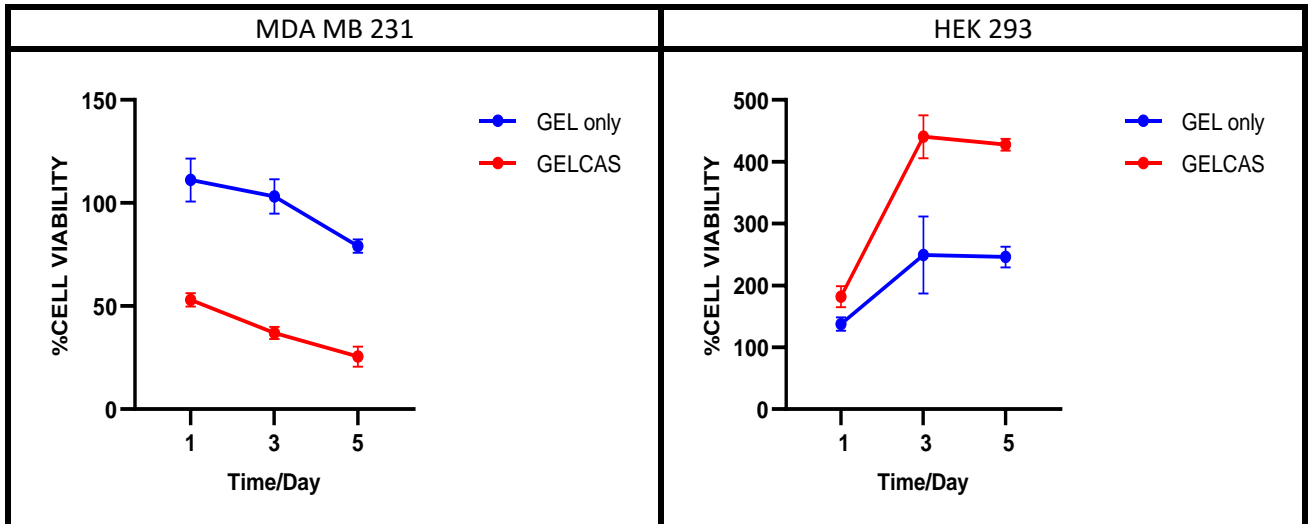
The difference between percentage cell viability of GEL only and GELCAS was also statistically significant for all the time points, indicating that the presence of the fiber did not support the growth of the MDA MB 231 cancer cells unlike it did for HEK 293 cells. This result was unexpected because from literature, Wang et al., (2018), fabricated bacterial cellulose/ gelatin scaffold and investigated its potential use as a 3D scaffold for tumor cell culture by seeding MDA MB- 231 cells on it. Their findings indicated that the cells significantly attached, proliferated and differentiated, making it an ideal *in vitro* culture scaffold for tumor cells. Ul-Islam et al., (2019), also fabricated bacterial cellulose/chitosan scaffold for potential application in the diagnosis of ovarian cancer, similarly the scaffold supported the cell growth of the ovarian cancer cells (A2780). Therefore, the presence of cellulose can be said to have little or no negative effect on the cell viability of cancer cell lines, but rather supports their growth. This happened to be contrary to the results obtained in this study.

From Table 4 (appendix), there is a gradual decline in the cell viability of the MDA MB 231 cells cultured in the GELCAS scaffold from  $50.13 \pm 7.89$  to  $25.48 \pm 4.88$ . This decline may not be associated with the structure of the scaffold since the same scaffold has been able to support HEK 293 cell lines. In addition to that, MDA MB 231 cell diameter is averagely 17- 18  $\mu\text{m}$  in the same range as HEK 293 cells, hence the pores in the scaffold can allow cell movement (Connolly et al., 2020). Taking into consideration how fast cancer cells in general proliferate, it was expected that there would be even higher cell viability than that of HEK 293, but clearly that was not the case. Since, the presence of cellulose from literature so far has been seen to support cancer cell lines (Wang et al., 2018), it can be inferred that the other components present in the cassava fiber may be a contributing factor to the decline recorded, since the fiber was not chemically treated to obtain pure cellulose.

Lignin is known to be the second most abundant polymer after cellulose and forms 30 % of the lignocellulosic biomass (Chen, 2014). Current studies are focusing on the use of lignin and its derivatives as promising candidates for anti-cancer applications. Lignin is also being explored as a delivery vehicle for enhanced cancer therapy (Vinardell & Mitjans, 2017). A novel alkaline-lignin-conjugated-poly (lactic-co-glycolic acid) (L-PLGA) nanoparticle drug delivery system (NPDDS) to improve the efficacy of GDC-0623, a targeted cancer therapy, was recently studied. The result obtained indicated that, L-PLGA NPs loaded with GDC-0623 NPs demonstrated a nearly 50% reduction in cell numbers compared to free GDC-0623 and PLGA-GDC NPs when tested *in vitro* (Byrne, 2020). Another study showed that Fe<sub>3</sub>O<sub>4</sub>-LNPs at concentration of 250 µg/mL showed an increased toxicity towards MDA-MB-231, MCF-7, and EA.hy926 cell lines, and the Fe-LNPs (250 µg/mL) were also toxic against MDA-MB-231 cells (Figueiredo et.al, 2017). All these suggest that the presence of the lignin in the fiber may have resulted in the decline in cell viability. The results obtained from this MTT assay result further validates the extraction test result, indicating that GELCAS is cytotoxic to the growth of MDA MB 231 cells.

#### 4.3.3 Comparison of MTT Assay Results for MDA MB 231 and HEK 293 cells

Figure. 21 below compares cell viability results for the two cell lines used in this experiment. It can be concluded from the graphs that compared to GEL only, GELCAS enhanced the growth of HEK 293 cells negatively affected cell growth of cancerous MDA MB 231 cells. These results are very interesting as they suggest that the presence of the cassava fiber in gelatin is not cytotoxic to HEK 293 cells but to MDA MB 231 cancer cells. Thus, the hypothesis that the presence of the cassava microfiber in the gelatin scaffold will lead to enhanced cell proliferation with no effect on cell morphology, can be accepted for HEK 293 cells but rejected for MDA MB 231 cancer cells.



**Figure 21. Comparison of percentage cell viability of HEK 293 cells and MDA MB 231 cells seeded directly on the samples at day 1, 3 & 5.**



## CHAPTER 5

### SUMMARY, CONCLUSION AND RECOMMENDATION

#### 5.1 Summary

This study was conducted to investigate the cytotoxicity of 7 % (w/v) cassava microfiber gelatin composite scaffold, by studying its effect on cell viability and cell morphology, in accordance with ISO standards for *in vitro* cytotoxicity testing of medical devices. Cassava microfiber was isolated and used in fabricating the composite scaffold via freeze-drying methods. Optical microscopy and fluorescence microscopy were used in examining changes in cell morphology and determining the ratio of live/ dead cells using Calcein AM and Propidium Iodide staining solutions, after the cells had been exposed to extracts from the samples. The results obtained were compared to gelatin scaffold only and to cells cultured on TCP with no scaffold.

Cells were also seeded directly onto the fabricated scaffolds and cell viability analyzed at different time points after seeding (Day 1, 3&5) using MTT assay. The % cell viability was calculated using the measured absorbance and graphs were plotted to compare the results obtained. Statistical analysis was performed ( $p < 0.05$ ) on the results obtained to determine if the presence of the cassava microfiber influenced the cell viability.

#### 5.2 Conclusion

The following conclusions can be drawn from the study:

1. Results obtained from the cytotoxicity extraction test suggested that, the presence of the cassava fiber had insignificant effect on the cell morphology and cell viability of HEK 293 cells. There was no change in cell morphology, and there was good cell adhesion to the well plate even after incubating the cells with the extracts for 48 h. The cells were able to

maintain their morphology and proliferate in the presence of the extract, although there was a slight inhibition of cell growth when compared to the cells without the extract. Fluorescence microscopy images obtained further validated the optical microscopy results, as there was higher live cell intensity than dead cell intensity after adding the extract to the cells. Both GEL only and GELCAS scaffolds exhibited similar results, suggesting that the presence of the fiber had no significant effect of the HEK 293 cell morphology and the cells' ability to proliferate.

2. MTT assay results for HEK 293 cells seeded directly on the scaffolds also indicated higher cell viability in GELCAS than in GEL only. There was significant increase in % cell viability from  $181.9 \pm 16.88$  to  $440.4 \pm 34.71$  for GELCAS, whereas GEL only increased from  $137.7 \pm 10.84$  to  $249.3 \pm 62.13$  from day 1 to 3, respectively. However, there was a decline in viability from day 3 to 5 in all cases, although the difference was not statistically significant ( $p > 0.05$ ). The higher cell viability in GELCAS than the pure gelatin scaffold indicated that, the cassava fiber is not toxic to cell growth of HEK 293, and thus can be considered as a reinforcement material to enhance cell growth in tissue engineering scaffolds.
3. The cytotoxicity extraction test conducted for MDA MB 231 cancer cells suggested that, the presence of the cassava fiber in gelatin had negative effect on the cell morphology and viability, contrary to the results obtained for HEK 293. The cell morphology changed from their spindle shape to circular shapes, after the cells were exposed to the extract from GELCAS, whereas that of GEL only maintained the morphology and cell growth. Fluorescence microscopy images indicated higher intensity of live cells for GEL only than

GELCAS, while GELCAS had higher intensity of dead cells, confirming that the GELCAS was toxic to cancer cells.

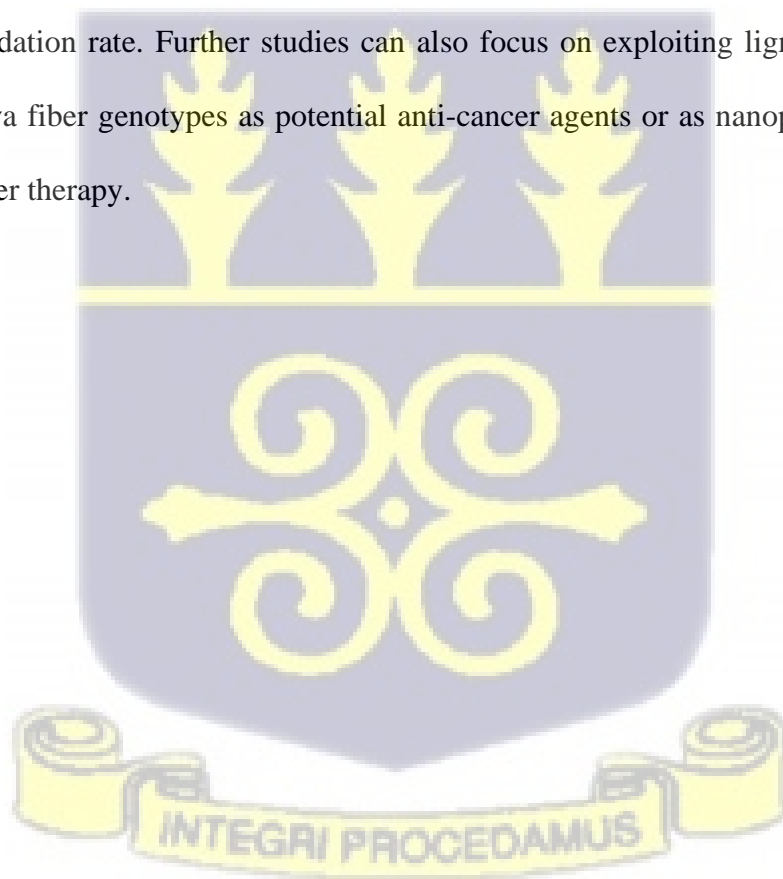
4. MTT assay results for MDA MB 231 cells seeded directly on the scaffolds also indicated higher cell viability in GEL only than in GELCAS. The difference between the cell viability of GEL only and GELCAS was statistically significant ( $p < 0.05$ ) at all the time points. There was a decline in cell viability of GELCAS from  $50.13 \pm 7.89$  to  $25.48 \pm 4.88$  from day 1 to 5. This suggests that GELCAS did not support the growth of the cells and the presence of the fiber in gelatin is cytotoxic to MDA MB 231 cells.
5. GELCAS influenced the two cell types studied differently; it supported the growth of HEK 293 cells, while inducing the death of MDA MB 231 cancer cells. Presence of other components such as lignin in the cassava fiber could be a contributing factor to the death of cancer cells. The incorporation of the fiber significantly enhanced HEK 293 cell growth. Since GELCAS (7 % (w/v) fiber/gelatin composite) is not cytotoxic to HEK 293 cells, it can be considered as a reinforcement material for gelatin for tissue engineering purposes.

### 5.3 Contribution to knowledge

This study was able to achieve the objectives set out, by providing in depth knowledge on the cytotoxicity of the 7 % (w/v) cassava microfiber gelatin composite scaffold, by assessing its effect on cell viability and cell morphology. The study provided interesting findings that suggest that, the presence of the fiber in gelatin is not cytotoxic to HEK 293 cells but cytotoxic to MDA MB 231 cancer cells. Therefore, these findings could help inform biomaterials engineers and materials researchers about the cytotoxicity of this material when considering it as a reinforcement material for tissue engineering purposes. It can also be potentially considered for bone tissue engineering. Materials researchers may also consider exploring the material as a potential anti-cancer agent.

#### 5.4 Recommendation

It is recommended that further studies be done to analyze the morphology and adhesion of cells seeded directly on the GELCAS scaffold in order to further validate the results obtained from the MTT assay. Based on the results obtained for MDA MB 231 cells, further experiments need to be done to better understand why the scaffold is toxic to the cancer cells, and the mode through which they are inducing the death of the cancer cells. Additionally, a comparative study between the untreated fiber and pure cellulose obtained from the chemically treated fiber can be done, to confirm if the presence of the other components (lignin, hemicellulose, etc.) affect the cells in any way. Since scaffolds are required to have controlled degradability, it is recommended that degradation studies are done to ensure the fabricated scaffold has an optimum degradation rate. Further studies can also focus on exploiting lignin extracts from different cassava fiber genotypes as potential anti-cancer agents or as nanoparticles for drug loading in cancer therapy.



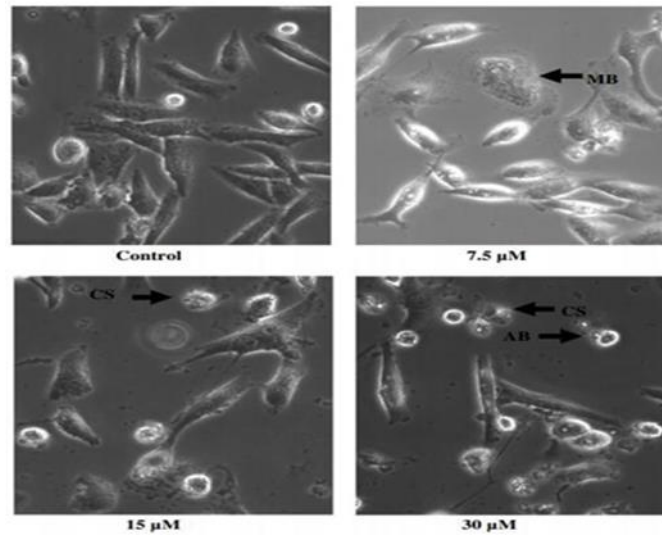
## APPENDIX

**Table 2. Raw data of MTT assay for HEK 293 cells at day 1, 3 & 5.**

DAY	NEG CONTROL		GELATIN			GELCAS		
	AB (mean ± SD)	CV (%) (mean± SD)	AB	CV (%)	CV (%) (mean± SD)	AB	CV (%)	CV (%) (mean± SD)
1	0.073±0.00	100±0.00	0.101	140.93	137.7±10.84	0.121	175.814	181.9±16.88
			0.09	125.581		0.144	168.837	
			0.105	146.512		0.126	200.93	
3	0.340±0.06	100±0.00	0.725	282.119	249.3±62.13	1.182	467.682	440.4±34.71
			0.71	288.079		0.921	401.325	
			0.447	177.616		1.177	452.185	
5	0.383±0.08	100±0.00	0.801	231.838	245.9±16.82	1.493	432.127	427.6±9.39
			0.834	241.389		1.499	433.864	
			0.914	264.544		1.44	416.787	

**Table 3. Raw data of MTT assay for MDA MB 231 cells at day 1, 3 & 5.**

DAY	NEG CONTROL		GELATIN			GELCAS		
	AB (mean ± SD)	CV (%) (mean± SD)	AB	CV (%)	CV (%) (mean± SD)	AB	CV (%)	CV (%) (mean± SD)
1	0.264±0.03	100± 0.00	0.298	112.879	111.1± 10.34	0.139	52.652	50.13±7.89
			0.318	120.455		0.109	41.288	
			0.264	100		0.149	56.439	
3	0.374±0.06	100±0.00	0.405	108.192	103.1±8.33	0.143	38.201	36.95±2.88
			0.403	107.658		0.146	39.002	
			0.350	93.5		0.126	33.659	
5	0.392±0.05	100±0.00	0.301	76.688	79.07±3.28	0.091	23.185	25.48±4.88
			0.305	77.707		0.123	31.083	
			0.325	82.803		0.087	22.166	



**Figure 22. Morphological changes of ampelopsin E-treated MDA-MB-231 cells observed under an inverted light microscope (400 × magnification). Cells showed the features of apoptosis such as membrane blebbing (MB), cellular shrinkage (CS) and formation of apoptotic bodies (AB) at 48 to 72 h (Rahman et al., 2016).**



## REFERENCES

- Abd El-Aziz, A. M., El-Maghraby, A., Ewald, A., & Kandil, S. H. (2021). In-vitro cytotoxicity study: Cell viability and cell morphology of carbon nanofibrous scaffold/hydroxyapatite nanocomposites. *Molecules*, 26(6). <https://doi.org/10.3390/molecules26061552>
- Adjei-Nsiah, S., & Sakyi-Dawson, O. (2012). Promoting cassava as an industrial crop in Ghana: Effects on soil fertility and farming system sustainability. *Applied and Environmental Soil Science*, 2012, 940954. <https://doi.org/10.1155/2012/940954>
- Afewerki, S., Sheikhi, A., Kannan, S., Ahadian, S., & Khademhosseini, A. (2019). Gelatin-polysaccharide composite scaffolds for 3D cell culture and tissue engineering: Towards natural therapeutics. *Bioengineering & Translational Medicine*, 4(1). <https://doi.org/10.1002/btm2.10124>
- Al-Nimry, S., Dayah, A. A., Hasan, I., & Daghmash, R. (2021). Cosmetic, Biomedical and Pharmaceutical Applications of Fish Gelatin/Hydrolysates. *Marine Drugs*, 19(3), 145. <https://doi.org/10.3390/md19030145>
- Alonso, D. M., Wettstein, S. G., & Dumesic, J. A. (2012). Bimetallic catalysts for upgrading of biomass to fuels and chemicals. *Chemical Society Reviews*, 41(24), 8075–8098. <https://doi.org/10.1039/C2CS35188A>
- Amoabediny, G., Salehi-Nik, N., & Heli, B. (2011). The Role of Biodegradable Engineered Scaffold in Tissue Engineering. In *Biomaterials Science and Engineering*. <https://doi.org/10.5772/24331>
- Anderson, J. M. (2016). Future challenges in the in vitro and in vivo evaluation of biomaterial biocompatibility. *Regenerative Biomaterials*, 3(2), 73–77. <https://doi.org/10.1093/RB/RBW001>
- Anne, B. (2011). Environmental-Friendly Biodegradable Polymers and Composites. In *Integrated Waste Management - Volume I*. <https://doi.org/10.5772/16541>
- Aramwit, P., Jaichawa, N., Ratanavaraporn, J., & Srichana, T. (2015). A comparative study of type A and type B gelatin nanoparticles as the controlled release carriers for different model compounds. *Materials Express*, 5(3). <https://doi.org/10.1166/mex.2015.1233>
- Asimeng, B. O., Karadag, I., Iftekhhar, S., Xu, Y., & Czernuszka, J. (2020). XRD and IR revelation of a unique g-C<sub>3</sub>N<sub>4</sub> phase with effects on collagen/hydroxyapatite bone scaffold pore geometry and stiffness. *SN Applied Sciences*, 2(8). <https://doi.org/10.1007/s42452-020-03218-8>
- Asiyanbi, T. T., Bio-Sawe, W., Idris, M. A., & Hamed, A. M. (2017). Gelatin-polysaccharide based materials: A review of processing and properties. *International Food Research Journal*, 24, 313–319.
- Aslam Khan, M. U., Abd Razak, S. I., Al Arjan, W. S., Nazir, S., Sahaya Anand, T. J.,

- Mehboob, H., & Amin, R. (2021). Recent Advances in Biopolymeric Composite Materials for Tissue Engineering and Regenerative Medicines: A Review. *Molecules (Basel, Switzerland)*, 26(3), 619. <https://doi.org/10.3390/molecules26030619>
- Aslantürk, Ö. S. (2018). In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages. In *Genotoxicity - A Predictable Risk to Our Actual World* (p. Ch. 1). IntechOpen. <https://doi.org/10.5772/intechopen.71923>
- Assad, M., & Jackson, N. (2019). Biocompatibility Evaluation of Orthopedic Biomaterials and Medical Devices: A Review of Safety and Efficacy Models. In *Encyclopedia of Biomedical Engineering* (Vols. 1–3, pp. 281–309). <https://doi.org/10.1016/B978-0-12-801238-3.11104-3>
- Atale, N., Gupta, S., Yadav, U. C. S., & Rani, V. (2014). Cell-death assessment by fluorescent and nonfluorescent cytosolic and nuclear staining techniques. *Journal of Microscopy*, 255(1), 7–19. <https://doi.org/10.1111/jmi.12133>
- Bácskay, I., Nemes, D., Fenyvesi, F., Váradi, J., Vasvári, G., Fehér, P., Vecsernyés, M., & Ujhelyi, Z. (2018). Role of Cytotoxicity Experiments in Pharmaceutical Development. In D. Nemes (Ed.), *Cytotoxicity* (p. Ch. 8). IntechOpen. <https://doi.org/10.5772/intechopen.72539>
- Baghaei, B., & Skrifvars, M. (2020). All-Cellulose Composites: A Review of Recent Studies on Structure, Properties and Applications. *Molecules (Basel, Switzerland)*, 25(12), 2836. <https://doi.org/10.3390/molecules25122836>
- Bahraminasab, M. (2020). Challenges on optimization of 3D-printed bone scaffolds. *BioMedical Engineering OnLine*, 19(1), 69. <https://doi.org/10.1186/s12938-020-00810-2>
- Bao Ha, T. Le, Minh, T., Nguyen, D., & Minh, D. (2013). Naturally Derived Biomaterials: Preparation and Application. In *Regenerative Medicine and Tissue Engineering*. <https://doi.org/10.5772/55668>
- Basu, B., Katti, D. S., & Kumar, A. (2010). Advanced Biomaterials: Fundamentals, Processing, and Applications. In *Advanced Biomaterials: Fundamentals, Processing, and Applications*. <https://doi.org/10.1002/9780470891315>
- Bayitse, R., Tornyie, F., & Bjerre, A. B. (2017). Cassava cultivation, processing and potential uses in Ghana. In *Handbook on Cassava: Production, Potential Uses and Recent Advances* (pp. 313–334).
- Bealer, E. J., Onissema-Karimu, S., Rivera-Galletti, A., Francis, M., Wilkowski, J., de la Cruz, D. S., & Hu, X. (2020). Protein-polysaccharide composite materials: Fabrication and applications. In *Polymers* (Vol. 12, Issue 2). <https://doi.org/10.3390/polym12020464>
- Belleghem, S. M. Van, Mahadik, B., Snodderly, K. L., & Fisher, J. P. (2020a). Overview of Tissue Engineering Concepts and Applications. In *Biomaterials Science* (pp. 1289–1316). Elsevier. <https://doi.org/10.1016/b978-0-12-816137-1.00081-7>
- Belleghem, S. M. Van, Mahadik, B., Snodderly, K. L., & Fisher, J. P. (2020b). Overview of

- Tissue Engineering Concepts and Applications. In *Biomaterials Science*. <https://doi.org/10.1016/b978-0-12-816137-1.00081-7>
- Bello, A. B., Kim, D., Kim, D., Park, H., & Lee, S. H. (2020). Engineering and functionalization of gelatin biomaterials: From cell culture to medical applications. *Tissue Engineering - Part B: Reviews*, 26(2), 164–180. <https://doi.org/10.1089/ten.teb.2019.0256>
- Beyar, R. (2011). Challenges in organ transplantation. *Rambam Maimonides Medical Journal*, 2(2), e0049–e0049. <https://doi.org/10.5041/RMMJ.10049>
- Bhattacharjee, P., & Ahearne, M. (2021). Significance of crosslinking approaches in the development of next generation hydrogels for corneal tissue engineering. In *Pharmaceutics* (Vol. 13, Issue 3, pp. 1–24). <https://doi.org/10.3390/pharmaceutics13030319>
- Bitar, K. N., & Zakhem, E. (2014). Design Strategies of Biodegradable Scaffolds for Tissue Regeneration. *Biomedical Engineering and Computational Biology*, 6, BECB.S10961. <https://doi.org/10.4137/becb.s10961>
- Bopp, S. K., & Lettieri, T. (2008). Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. *BMC Pharmacology*, 8, 8. <https://doi.org/10.1186/1471-2210-8-8>
- Brougham, C. M., Levingstone, T. J., Shen, N., Cooney, G. M., Jockenhoevel, S., Flanagan, T. C., & O'Brien, F. J. (2017). Freeze-Drying as a Novel Biofabrication Method for Achieving a Controlled Microarchitecture within Large, Complex Natural Biomaterial Scaffolds. *Advanced Healthcare Materials*, 6(21). <https://doi.org/10.1002/adhm.201700598>
- Brown, D. A., Yang, N., & Ray, S. D. (2014). Apoptosis. In P. B. T.-E. of T. (Third E. Wexler (Ed.), *Encyclopedia of Toxicology: Third Edition* (pp. 287–294). Academic Press. <https://doi.org/10.1016/B978-0-12-386454-3.00242-6>
- Cai, Z., & Kim, J. (2010). Preparation and characterization of novel bacterial cellulose/ gelatin scaffold for tissue regeneration using bacterial cellulose hydrogel. *Journal of Nanotechnology in Engineering and Medicine*, 1(2). <https://doi.org/10.1115/1.4000858>
- Chan, B. P., & Leong, K. W. (2008). Scaffolding in tissue engineering: General approaches and tissue-specific considerations. *European Spine Journal*, 17(SUPPL. 4), 467–479. <https://doi.org/10.1007/s00586-008-0745-3>
- Chandra, P. K., Soker, S., & Atala, A. (2020). Tissue engineering: current status and future perspectives. In *Principles of Tissue Engineering* (pp. 1–35). <https://doi.org/10.1016/b978-0-12-818422-6.00004-6>
- Chapekar, M. S. (2000). Tissue engineering: Challenges and opportunities. *Journal of Biomedical Materials Research*, 53(6), 617–620. [https://doi.org/https://doi.org/10.1002/1097-4636\(2000\)53:6<617::AID-JBM1>3.0.CO;2-C](https://doi.org/https://doi.org/10.1002/1097-4636(2000)53:6<617::AID-JBM1>3.0.CO;2-C)

- Chaudhari, A. A., Vig, K., Baganizi, D. R., Sahu, R., Dixit, S., Dennis, V., Singh, S. R., & Pillai, S. R. (2016). Future prospects for scaffolding methods and biomaterials in skin tissue engineering: A review. In *International Journal of Molecular Sciences* (Vol. 17, Issue 12). <https://doi.org/10.3390/ijms17121974>
- Chen, F.-M., & Liu, X. (2016). Advancing biomaterials of human origin for tissue engineering. *Progress in Polymer Science*, 53, 86–168. <https://doi.org/10.1016/j.progpolymsci.2015.02.004>
- Chen, H. (2014). Biotechnology of lignocellulose: Theory and practice. In *Biotechnology of Lignocellulose: Theory and Practice*. <https://doi.org/10.1007/978-94-007-6898-7>
- Choi, G., & Cha, H. J. (2019). Recent advances in the development of nature-derived photocrosslinkable biomaterials for 3D printing in tissue engineering. *Biomaterials Research*, 23(1), 18. <https://doi.org/10.1186/s40824-019-0168-8>
- Collins, M. N., Ren, G., Young, K., Pina, S., Reis, R. L., & Oliveira, J. M. (2021a). Scaffold Fabrication Technologies and Structure/Function Properties in Bone Tissue Engineering. In *Advanced Functional Materials* (Vol. 31, Issue 21). John Wiley and Sons Inc. <https://doi.org/10.1002/adfm.202010609>
- Collins, M. N., Ren, G., Young, K., Pina, S., Reis, R. L., & Oliveira, J. M. (2021b). Scaffold Fabrication Technologies and Structure/Function Properties in Bone Tissue Engineering. *Advanced Functional Materials*, 31(21), 2010609. <https://doi.org/https://doi.org/10.1002/adfm.202010609>
- Courtenay, J. C., Sharma, R. I., & Scott, J. L. (2018). Recent advances in modified cellulose for tissue culture applications. *Molecules*, 23(3). <https://doi.org/10.3390/molecules23030654>
- Credou, J., & Berthelot, T. (2014). Cellulose: from biocompatible to bioactive material. *Journal of Materials Chemistry B*, 2(30), 4767–4788. <https://doi.org/10.1039/C4TB00431K>
- Davis, H., & Leach, J. (2008). Hybrid and Composite Biomaterials in Tissue Engineering. *Topics in Multifunctional Biomaterials and Devices*, 530, 1–26. [http://www.oulu.fi/spareparts/ebook\\_topics\\_multifunctional/abstracts/davis.pdf](http://www.oulu.fi/spareparts/ebook_topics_multifunctional/abstracts/davis.pdf)
- Deb, P., Deoghare, A. B., Borah, A., Barua, E., & Das Lala, S. (2018). Scaffold Development Using Biomaterials: A Review. *Materials Today: Proceedings*, 5(5), 12909–12919. <https://doi.org/10.1016/j.matpr.2018.02.276>
- Dhandayuthapani, B., Yoshida, Y., Maekawa, T., & Kumar, D. S. (2011). Polymeric Scaffolds in Tissue Engineering Application: A Review. *International Journal of Polymer Science*, 2011, 290602. <https://doi.org/10.1155/2011/290602>
- Diabor, E., Funkenbusch, P., & Kaufmann, E. E. (2019). Characterization of Cassava Fiber of Different Genotypes as a Potential Reinforcement Biomaterial for Possible Tissue Engineering Composite Scaffold Application. *Fibers and Polymers*, 20(2), 217–228. <https://doi.org/10.1007/s12221-019-8702-9>

- Diyana, Z. N., Jumaidin, R., Selamat, M. Z., Ghazali, I., Julmohammad, N., Huda, N., & Ilyas, R. A. (2021). Physical properties of thermoplastic starch derived from natural resources and its blends: A review. In *Polymers* (Vol. 13, Issue 9, pp. 5–20). <https://doi.org/10.3390/polym13091396>
- Dolcimascolo, A., Calabrese, G., Conoci, S., & Parenti, R. (2019). Innovative Biomaterials for Tissue Engineering. In *Biomaterial-supported Tissue Reconstruction or Regeneration*. <https://doi.org/10.5772/intechopen.83839>
- Dutta, S. D., Patel, D. K., & Lim, K.-T. (2019). Functional cellulose-based hydrogels as extracellular matrices for tissue engineering. *Journal of Biological Engineering*, 13(1), 55. <https://doi.org/10.1186/s13036-019-0177-0>
- Dzobo, K., Thomford, N. E., Senthebane, D. A., Shipanga, H., Rowe, A., Dandara, C., Pillay, M., & Motaung, K. S. C. M. (2018a). Advances in regenerative medicine and tissue engineering: Innovation and transformation of medicine. *Stem Cells International*, 2018. <https://doi.org/10.1155/2018/2495848>
- Dzobo, K., Thomford, N. E., Senthebane, D. A., Shipanga, H., Rowe, A., Dandara, C., Pillay, M., & Motaung, K. S. C. M. (2018b). Advances in regenerative medicine and tissue engineering: Innovation and transformation of medicine. *Stem Cells International*. <https://doi.org/10.1155/2018/2495848>
- Ebhodaghe, S. O. (2021). Natural Polymers for Tissue Engineering Applications: Review. *Journal of Biomaterials Science, Polymer Edition*, 1–49. <https://doi.org/10.1080/09205063.2021.1958185>
- Echave, M. C., Burgo, L. S., Pedraz, J. L., & Orive, G. (2017). Gelatin as Biomaterial for Tissue Engineering. *Current Pharmaceutical Design*, 23(24), 3567–3584. <https://doi.org/10.2174/0929867324666170511123101>
- Edhirej, A., Sapuan, S. M., Jawaid, M., Zahari, N. I., & Sanyang, M. L. (2017). Effect of cassava peel and cassava bagasse natural fillers on mechanical properties of thermoplastic cassava starch: Comparative study. *AIP Conference Proceedings*, 1901. <https://doi.org/10.1063/1.5010532>
- Edmondson, R., Broglie, J. J., Adcock, A. F., & Yang, L. (2014). Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay and Drug Development Technologies*, 12(4), 207–218. <https://doi.org/10.1089/adt.2014.573>
- Egbo, M. K. (2020). A fundamental review on composite materials and some of their applications in biomedical engineering. *Journal of King Saud University - Engineering Sciences*. <https://doi.org/10.1016/j.jksues.2020.07.007>
- Eltom, A., Zhong, G., & Muhammad, A. (2019a). Scaffold Techniques and Designs in Tissue Engineering Functions and Purposes: A Review. *Advances in Materials Science and Engineering*, 2019, 3429527. <https://doi.org/10.1155/2019/3429527>
- Eltom, A., Zhong, G., & Muhammad, A. (2019b). Scaffold Techniques and Designs in Tissue

- Engineering Functions and Purposes: A Review. In *Advances in Materials Science and Engineering* (Vol. 2019). Hindawi Limited. <https://doi.org/10.1155/2019/3429527>
- Feinberg, A. W. (2012). Engineered tissue grafts: Opportunities and challenges in regenerative medicine. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, 4(2), 207–220. <https://doi.org/10.1002/wsbm.164>
- Fereshteh, Z. (2018). Freeze-drying technologies for 3D scaffold engineering. In Y. Deng & J. B. T.-F. 3D T. E. S. Kuiper (Eds.), *Functional 3D Tissue Engineering Scaffolds: Materials, Technologies, and Applications* (pp. 151–174). Woodhead Publishing. <https://doi.org/10.1016/B978-0-08-100979-6.00007-0>
- Frantz, C., Stewart, K. M., & Weaver, V. M. (2010). The extracellular matrix at a glance. In *Journal of Cell Science* (Vol. 123, Issue 24, pp. 4195–4200). <https://doi.org/10.1242/jcs.023820>
- Friend, D. F. L., Leyva González, M. E., Caraballo, M. M., & de Queiroz, A. A. A. (2019). Biological properties of electrospun cellulose scaffolds from biomass. *Journal of Biomaterials Science, Polymer Edition*, 30(15), 1399–1414. <https://doi.org/10.1080/09205063.2019.1636351>
- Furth, M. E., & Atala, A. (2013). Tissue Engineering: Future Perspectives. In *Principles of Tissue Engineering: Fourth Edition* (pp. 83–123). Elsevier Inc. <https://doi.org/10.1016/B978-0-12-398358-9.00006-9>
- Furth, M. E., & Atala, A. (2014). *Chapter 6 - Tissue Engineering: Future Perspectives* (R. Lanza, R. Langer, & J. B. T.-P. of T. E. (Fourth E. Vacanti (eds.); pp. 83–123). Academic Press. <https://doi.org/https://doi.org/10.1016/B978-0-12-398358-9.00006-9>
- Garg, T., & Goyal, A. K. (2014). Biomaterial-based scaffolds-current status and future directions. In *Expert Opinion on Drug Delivery* (Vol. 11, Issue 5, pp. 767–789). Informa Healthcare. <https://doi.org/10.1517/17425247.2014.891014>
- Gattazzo, F., Urciuolo, A., & Bonaldo, P. (2014). Extracellular matrix: A dynamic microenvironment for stem cell niche. *Biochimica et Biophysica Acta - General Subjects*, 1840(8), 2506–2519. <https://doi.org/10.1016/j.bbagen.2014.01.010>
- Gelatin Manufacturers Institute of America. (2012). Gelatin Handbook GMIA. *Gelatin Handbook*.
- Geraghty, R. J., Capes-Davis, A., Davis, J. M., Downward, J., Freshney, R. I., Knezevic, I., Lovell-Badge, R., Masters, J. R. W., Meredith, J., Stacey, G. N., Thraves, P., Vias, M., & UK, C. R. (2014). Guidelines for the use of cell lines in biomedical research. *British Journal of Cancer*, 111(6), 1021–1046. <https://doi.org/10.1038/bjc.2014.166>
- Grande, C. J., Torres, F. G., Gomez, C. M., & Carmen Bañó, M. (2009). Nanocomposites of bacterial cellulose/hydroxyapatite for biomedical applications. *Acta Biomaterialia*, 5(5), 1605–1615. <https://doi.org/10.1016/j.actbio.2009.01.022>
- Groth, T., Falck, P., & Miethke, R. R. (1995). Cytotoxicity of biomaterials - Basic mechanisms

- and in vitro test methods: A review. *ATLA Alternatives to Laboratory Animals*, 23(6), 790–799. <https://doi.org/10.1177/026119299502300609>
- Guerado, E., & Caso, E. (2017). *Challenges of bone tissue engineering in orthopaedic patients*. 8(2), 87–98. <https://doi.org/10.5312/wjo.v8.i2.87>
- Haider, A., Haider, S., Rao Kummara, M., Kamal, T., Alghyamah, A. A. A., Jan Iftikhar, F., Bano, B., Khan, N., Amjid Afridi, M., Soo Han, S., Alrahlah, A., & Khan, R. (2020). Advances in the scaffolds fabrication techniques using biocompatible polymers and their biomedical application: A technical and statistical review. *Journal of Saudi Chemical Society*, 24(2), 186–215. <https://doi.org/10.1016/j.jscs.2020.01.002>
- He, W., & Benson, R. (2017). Polymeric Biomaterials. In M. B. T.-A. P. E. H. (Second E. Kutz (Ed.), *Applied Plastics Engineering Handbook: Processing, Materials, and Applications: Second Edition* (pp. 145–164). William Andrew Publishing. <https://doi.org/10.1016/B978-0-323-39040-8.00008-0>
- Hickey, R. J., & Pelling, A. E. (2019). *Cellulose Biomaterials for Tissue Engineering*. 7(March), 1–15. <https://doi.org/10.3389/fbioe.2019.00045>
- Hinderer, S., Brauchle, E., & Schenke-Layland, K. (2015). Generation and Assessment of Functional Biomaterial Scaffolds for Applications in Cardiovascular Tissue Engineering and Regenerative Medicine. *Advanced Healthcare Materials*, 4(16), 2326–2341. <https://doi.org/10.1002/adhm.201400762>
- Hoek, J. B., Cahill, A., & Pastorino, J. G. (2002). Alcohol and mitochondria: A dysfunctional relationship. *Gastroenterology*, 122(7), 2049–2063. <https://doi.org/https://doi.org/10.1053/gast.2002.33613>
- Hollister, S. J. (2005). Porous scaffold design for tissue engineering. *Nature Materials*, 4(7), 518–524. <https://doi.org/10.1038/nmat1421>
- Hosseinkhani, M., Mehrabani, D., Karimfar, M. H., Bakhtiyari, S., Manafi, A., & Shirazi, R. (2014). Tissue engineered scaffolds in regenerative medicine. *World Journal of Plastic Surgery*, 3(1), 3–7. <http://www.ncbi.nlm.nih.gov/pubmed/25489516> <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4236978>
- Howard, D., Buttery, L. D., Shakesheff, K. M., & Roberts, S. J. (2008). Tissue engineering: Strategies, stem cells and scaffolds. *Journal of Anatomy*, 213(1), 66–72. <https://doi.org/10.1111/j.1469-7580.2008.00878.x>
- Hu, W., Wang, Z., Xiao, Y., Zhang, S., & Wang, J. (2019). Advances in crosslinking strategies of biomedical hydrogels. *Biomaterials Science*, 7(3), 843–855. <https://doi.org/10.1039/C8BM01246F>
- Hussein, K. H., Park, K. M., Kang, K. S., & Woo, H. M. (2016). Biocompatibility evaluation of tissue-engineered decellularized scaffolds for biomedical application. *Materials Science and Engineering C*, 67, 766–778. <https://doi.org/10.1016/j.msec.2016.05.068>

- Inayat-Hussain, S., Rajab, N. F., & Siew, E. L. (2008). In vitro testing of biomaterials toxicity and biocompatibility. In L. B. T.-C. R. to B. Di Silvio (Ed.), *Cellular Response to Biomaterials* (pp. 508–537). Woodhead Publishing. <https://doi.org/10.1533/9781845695477.3.508>
- ISO, I. O. for S. (2009). International Standard ISO 10993-5. *Biological Evaluation of Medical Devices-Part 5: Tests for In Vitro Cytotoxicity*.
- Jeyarani, S., Vinita, N. M., Puja, P., Senthamilselvi, S., Devan, U., Velangani, A. J., Biruntha, M., Pugazhendhi, A., & Kumar, P. (2020). Biomimetic gold nanoparticles for its cytotoxicity and biocompatibility evidenced by fluorescence-based assays in cancer (MDA-MB-231) and non-cancerous (HEK-293) cells. *Journal of Photochemistry and Photobiology B: Biology*, 202. <https://doi.org/10.1016/j.jphotobiol.2019.111715>
- Jones, J. R. (2005). Scaffolds for tissue engineering. In L. L. Hench & J. R. B. T.-B. Jones Artificial Organs and Tissue Engineering (Eds.), *Biomaterials, Artificial Organs and Tissue Engineering* (pp. 201–214). Woodhead Publishing. <https://doi.org/10.1533/9781845690861.4.201>
- Kalia, S., Dufresne, A., Cherian, B. M., Kaith, B. S., Avérous, L., Njuguna, J., & Nassiopoulos, E. (2011). Cellulose-based bio- and nanocomposites: A review. *International Journal of Polymer Science*, 2011. <https://doi.org/10.1155/2011/837875>
- Kamiloglu, S., Sari, G., Ozdal, T., & Capanoglu, E. (2020). Guidelines for cell viability assays. *Food Frontiers*, 1(3), 332–349. <https://doi.org/10.1002/fft2.44>
- Karande, T. S., & Agrawal, C. M. (2008). Functions and requirements of synthetic scaffolds in tissue engineering. In *Nanotechnology and Tissue Engineering: The Scaffold*. <https://doi.org/10.1201/9781420051834.ch3>
- Kazimierczak, P., & Palka, K. (2019). *Development and Optimization of the Novel Fabrication Method of Highly Macroporous Chitosan / Agarose / Nanohydroxyapatite Bone Scaffold for Potential Regenerative Medicine Applications*. 1–14.
- Khan, F., Tanaka, M., & Ahmad, S. R. (2015). Fabrication of polymeric biomaterials: a strategy for tissue engineering and medical devices. In *Journal of Materials Chemistry B* (Vol. 3, Issue 42, pp. 8224–8249). Royal Society of Chemistry. <https://doi.org/10.1039/c5tb01370d>
- Kim, B.-S., Park, I.-K., Hoshiba, T., Jiang, H.-L., Choi, Y.-J., Akaike, T., & Cho, C.-S. (2011). Design of artificial extracellular matrices for tissue engineering. *Progress in Polymer Science*, 36(2), 238–268. <https://doi.org/https://doi.org/10.1016/j.progpolymsci.2010.10.001>
- Kim, K. W., Lee, B. H., Kim, H. J., Siroth, K., & Dorgan, J. R. (2012). Thermal and mechanical properties of cassava and pineapple flours-filled PLA bio-composites. *Journal of Thermal Analysis and Calorimetry*, 108(3), 1131–1139. <https://doi.org/10.1007/s10973-011-1350-y>
- Kim, Y., Ko, H., Kwon, I. K., & Shin, K. (2016). Extracellular Matrix Revisited: Roles in

- Tissue Engineering. *International Neurourology Journal*, 20(Suppl 1), S23–S29. <https://doi.org/10.5213/inj.1632600.318>
- Kohane, D. S., & Langer, R. (2008). Polymeric biomaterials in tissue engineering. *Pediatric Research*, 63(5), 487–491. <https://doi.org/10.1203/01.pdr.0000305937.26105.e7>
- Krishnakumar, G. S., Sampath, S., Muthusamy, S., & John, M. A. (2019). Importance of crosslinking strategies in designing smart biomaterials for bone tissue engineering: A systematic review. *Materials Science and Engineering C*, 96, 941–954. <https://doi.org/10.1016/j.msec.2018.11.081>
- Kumar Gupta, P., Sai Raghunath, S., Venkatesh Prasanna, D., Venkat, P., Shree, V., Chithananthan, C., Choudhary, S., Surender, K., & Geetha, K. (2019). An Update on Overview of Cellulose, Its Structure and Applications. In *Cellulose*. <https://doi.org/10.5772/intechopen.84727>
- Kumar, P., Nagarajan, A., & Uchil, P. D. (2018). Analysis of cell viability by the MTT assay. *Cold Spring Harbor Protocols*, 2018(6), 469–471. <https://doi.org/10.1101/pdb.prot095505>
- Kumari, P., Panda, P. K., Jha, E., Pramanik, N., Nisha, K., Kumari, K., Soni, N., Mallick, M. A., & Verma, S. K. (2018). Molecular insight to in vitro biocompatibility of phytofabricated copper oxide nanoparticles with human embryonic kidney cells. *Nanomedicine*, 13(19), 2415–2433. <https://doi.org/10.2217/nnm-2018-0175>
- Labuschagne, C. F., Cheung, E. C., Blagih, J., Domart, M.-C., & Vousden, K. H. (2019). Cell Clustering Promotes a Metabolic Switch that Supports Metastatic Colonization. *Cell Metabolism*, 30(4), 720-734.e5. <https://doi.org/10.1016/j.cmet.2019.07.014>
- Langer, R., & Vacanti, J. P. (1993). Tissue engineering. *Science (New York, N.Y.)*, 260(5110), 920–926. <https://doi.org/10.1126/science.8493529>
- Larbie, L., Hayford, C. F., & Kaufmann, E. E. (2012). Characterisation of Cassava Fibre for Use As a Biomaterial. *International Journal of Engineering Science and Technology (IJEST)*, 4(07), 3537–3545.
- Lavanya, D., Kulkarni, P., Dixit, M., Raavi, P., & Krishna, L. (2011). Sources of Cellulose and Their Applications—a Review. *Int J Drug Formul Res*, 2, 2.
- Li, S., Cui, Y., Zhou, Y., Luo, Z., Liu, J., & Zhao, M. (2017). The industrial applications of cassava: current status, opportunities and prospects. In *Journal of the Science of Food and Agriculture* (Vol. 97, Issue 8, pp. 2282–2290). <https://doi.org/10.1002/jsfa.8287>
- Li, W., Zhou, J., & Xu, Y. (2015). *Study of the in vitro cytotoxicity testing of medical devices (Review)*. 617–620. <https://doi.org/10.3892/br.2015.481>
- Loh, Q. L., & Choong, C. (2013). Three-dimensional scaffolds for tissue engineering applications: Role of porosity and pore size. *Tissue Engineering - Part B: Reviews*, 19(6), 485–502. <https://doi.org/10.1089/ten.teb.2012.0437>

- Ma, B., Wang, X., Wu, C., & Chang, J. (2014). Crosslinking strategies for preparation of extracellular matrix-derived cardiovascular scaffolds. *Regenerative Biomaterials*, 1(1), 81–89. <https://doi.org/10.1093/rb/rbu009>
- Ma, P. X. (2004). Scaffolds for tissue fabrication. *Materials Today*, 7(5), 30–40. [https://doi.org/10.1016/S1369-7021\(04\)00233-0](https://doi.org/10.1016/S1369-7021(04)00233-0)
- Mallick, K. K., & Cox, S. C. (2013). Biomaterial scaffolds for tissue engineering. In *Frontiers in Bioscience - Elite*. <https://doi.org/10.2741/E620>
- Mandrycky, C., Phong, K., & Zheng, Y. (2017). Tissue engineering toward organ-specific regeneration and disease modeling. *MRS Communications*, 7(3), 332–347. <https://doi.org/10.1557/mrc.2017.58>
- Mikshina, P., Chernova, T., Chemikosova, S., Ibragimova, N., Mokshina, N., & Gorshkov, T. (2013). Cellulosic Fibers: Role of Matrix Polysaccharides in Structure and Function. In *Cellulose - Fundamental Aspects*. <https://doi.org/10.5772/51941>
- Mitchell, A. C., Briquez, P. S., Hubbell, J. A., & Cochran, J. R. (2016). Engineering growth factors for regenerative medicine applications. *Acta Biomaterialia*, 30, 1–12. <https://doi.org/10.1016/j.actbio.2015.11.007>
- Miyamoto, T., Takahashi, S., Ito, H., Inagaki, H., & Noishiki, Y. (1989). Tissue biocompatibility of cellulose and its derivatives. *Journal of Biomedical Materials Research*, 23(1), 125–133. <https://doi.org/10.1002/jbm.820230110>
- Morais, J. M., Papadimitrakopoulos, F., & Burgess, D. J. (2010). Biomaterials/tissue interactions: Possible solutions to overcome foreign body response. *AAPS Journal*, 12(2), 188–196. <https://doi.org/10.1208/s12248-010-9175-3>
- Müller, F. A., Müller, L., Hofmann, I., Greil, P., Wenzel, M. M., & Staudenmaier, R. (2006). Cellulose-based scaffold materials for cartilage tissue engineering. *Biomaterials*, 27(21), 3955–3963. <https://doi.org/10.1016/j.biomaterials.2006.02.031>
- Myers, D. K., Goldberg, A. M., Poth, A., Wolf, M. F., Carraway, J., McKim, J., Coleman, K. P., Hutchinson, R., Brown, R., Krug, H. F., Bahinski, A., & Hartung, T. (2017). From in vivo to in vitro: The medical device testing paradigm shift. *Altex*, 34(4), 479–500. <https://doi.org/10.14573/altex.1608081>
- Nardo, T., Carmagnola, I., Ruini, F., Caddeo, S., Calzone, S., Chiono, V., & Ciardelli, G. (2017). Synthetic Biomaterial for Regenerative Medicine Applications. In G. Orlando, G. Remuzzi, & D. F. B. T.-K. T. Williams (Eds.), *Kidney Transplantation, Bioengineering, and Regeneration: Kidney Transplantation in the Regenerative Medicine Era* (pp. 901–921). Academic Press. <https://doi.org/10.1016/B978-0-12-801734-0.00065-5>
- Nikolova, M. P., & Chavali, M. S. (2019). Recent advances in biomaterials for 3D scaffolds: A review. *Bioactive Materials*, 4, 271–292. <https://doi.org/10.1016/j.bioactmat.2019.10.005>

- Novotna, K., Havelka, P., Sopuch, T., Kolarova, K., Vosmanska, V., Lisa, V., Svorcik, V., & Bacakova, L. (2013). Cellulose-based materials as scaffolds for tissue engineering. *Cellulose*, 20(5), 2263–2278. <https://doi.org/10.1007/s10570-013-0006-4>
- O'Brien, F. J. (2011). Biomaterials & scaffolds for tissue engineering. In *Materials Today*. [https://doi.org/10.1016/S1369-7021\(11\)70058-X](https://doi.org/10.1016/S1369-7021(11)70058-X)
- Pachua, L. S. (2015). A Mini Review on Plant-based Nanocellulose: Production, Sources, Modifications and Its Potential in Drug Delivery Applications. *Mini Reviews in Medicinal Chemistry*, 15(7), 543–552. <https://doi.org/10.2174/1389557515666150415150327>
- Piccinini, F., Tesei, A., Arienti, C., & Bevilacqua, A. (2017). Cell Counting and Viability Assessment of 2D and 3D Cell Cultures: Expected Reliability of the Trypan Blue Assay. *Biological Procedures Online*, 19(1), 8. <https://doi.org/10.1186/s12575-017-0056-3>
- Place, E. S., George, J. H., Williams, C. K., & Stevens, M. M. (2009). Synthetic polymer scaffolds for tissue engineering. *Chemical Society Reviews*, 38(4), 1139–1151. <https://doi.org/10.1039/b811392k>
- Plante, M. K., Arscott, W. T., Folsom, J. B., Tighe, S. W., Dempsey, R. J., & Wesley, U. V. (2013). Ethanol promotes cytotoxic effects of tumor necrosis factor-related apoptosis-inducing ligand through induction of reactive oxygen species in prostate cancer cells. *Prostate Cancer and Prostatic Diseases*, 16(1), 16–22. <https://doi.org/10.1038/pcan.2012.37>
- Poletto, M., & Ornaghi Jr, H. (2015). Cellulose - Fundamental Aspects and Current Trends. In *Cellulose - Fundamental Aspects and Current Trends*. BoD–Books on Demand. <https://doi.org/10.5772/59889>
- Rahman, N. A., Yazan, L. S., Wibowo, A., Ahmat, N., Foo, J. B., Tor, Y. S., Yeap, S. K., Razali, Z. A., Ong, Y. S., & Fakurazi, S. (2016). Induction of apoptosis and G2/M arrest by ampelopsin E from *Dryobalanops* towards triple negative breast cancer cells, MDA-MB-231. *BMC Complementary and Alternative Medicine*, 16(1). <https://doi.org/10.1186/s12906-016-1328-1>
- Ramphul, H., Bhaw-Luximon, A., & Jhurry, D. (2017). Sugar-cane bagasse derived cellulose enhances performance of polylactide and polydioxanone electrospun scaffold for tissue engineering. *Carbohydrate Polymers*, 178(July), 238–250. <https://doi.org/10.1016/j.carbpol.2017.09.046>
- Reddy, M. S. B., Ponnamma, D., Choudhary, R., & Sadasivuni, K. K. (2021). A comparative review of natural and synthetic biopolymer composite scaffolds. In *Polymers* (Vol. 13, Issue 7). <https://doi.org/10.3390/polym13071105>
- Reddy, N., Reddy, R., & Jiang, Q. (2015). Crosslinking biopolymers for biomedical applications. In *Trends in Biotechnology* (Vol. 33, Issue 6, pp. 362–369). <https://doi.org/10.1016/j.tibtech.2015.03.008>
- Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J., & Minor, L. (2004). Cell Viability Assays. *Assay Guidance Manual*.

<http://www.ncbi.nlm.nih.gov/pubmed/23805433>

- Riss, T., Niles, A., Moravec, R., Karassina, N., & Vidugiriene, J. (2004). Cytotoxicity Assays: In Vitro Methods to Measure Dead Cells. In *Assay Guidance Manual*. <http://www.ncbi.nlm.nih.gov/pubmed/31070879>
- Romo-Uribe, A., Meneses-Acosta, A., & Domínguez-Díaz, M. (2017). Viability of HEK 293 cells on poly- $\beta$ -hydroxybutyrate (PHB) biosynthesized from a mutant *Azotobacter vinelandii* strain. Cast film and electrospun scaffolds. *Materials Science and Engineering: C*, 81, 236–246. <https://doi.org/10.1016/J.MSEC.2017.07.045>
- Rose, J. B., Pacelli, S., El Haj, A. J., Dua, H. S., Hopkinson, A., White, L. J., & Rose, F. R. A. J. (2014). Gelatin-based materials in ocular tissue engineering. In *Materials* (Vol. 7, Issue 4, pp. 3106–3135). <https://doi.org/10.3390/ma7043106>
- Safarzadeh, E., Shotorbani, S. S., & Baradaran, B. (2014). Herbal medicine as inducers of apoptosis in cancer treatment. *Advanced Pharmaceutical Bulletin*, 4(SUPPL.1), 421–427. <https://doi.org/10.5681/apb.2014.062>
- Sarkar, D., Zhao, W., Schaefer, S., Ankrum, J. A., Teo, G. S. L., Pereira, M. N., Ferreira, L., & Karp, J. M. (2013). Overview of Tissue Engineering Concepts and Applications. In B. D. Ratner, A. S. Hoffman, F. J. Schoen, & J. E. B. T.-B. S. (Third E. Lemons (Eds.), *Biomaterials Science: An Introduction to Materials: Third Edition* (pp. 1122–1137). Academic Press. <https://doi.org/10.1016/B978-0-08-087780-8.00109-1>
- Seddiqi, H., Oliaei, E., Honarkar, H., Jin, J., Geonzon, L. C., Bacabac, R. G., & Klein-Nulend, J. (2021). Cellulose and its derivatives: towards biomedical applications. *Cellulose*, 28(4), 1893–1931. <https://doi.org/10.1007/s10570-020-03674-w>
- Seo, K. H., You, S. J., Heung, J. C., Chun-Ho, K., Who Kyong, L., Yoon Mook, L., Young Chang, N., & Ju Woong, J. (2009). In Vitro and In Vivo Biocompatibility of  $\gamma$ -ray Crosslinked. *Tissue Engineering and Regenerative Medicine*, 6(4), 414–418.
- Shafiee, A., & Atala, A. (2017). Tissue Engineering: Toward a New Era of Medicine. In *Annual Review of Medicine* (Vol. 68). <https://doi.org/10.1146/annurev-med-102715-092331>
- Shapira, A., Feiner, R., & Dvir, T. (2016). Composite biomaterial scaffolds for cardiac tissue engineering. *International Materials Reviews*, 61(1), 1–19. <https://doi.org/10.1179/1743280415Y.0000000012>
- Sharma, P., Kumar, P., Sharma, R., Bhatt, V. D., & Dhot, P. S. (2019). Tissue Engineering; Current Status & Futuristic Scope. In *Journal of medicine and life* (Vol. 12, Issue 3, pp. 225–229). NLM (Medline). <https://doi.org/10.25122/jml-2019-0032>
- Shick, T. M., Abdul Kadir, A. Z., Ngadiman, N. H. A., & Ma'aram, A. (2019). A review of biomaterials scaffold fabrication in additive manufacturing for tissue engineering. *Journal of Bioactive and Compatible Polymers*, 34(6), 415–435. <https://doi.org/10.1177/0883911519877426>
- Sidhu, P. (2020). Recent Advances in Tissue Engineering and Regenerative Medicine.

- Skopinska-Wisniewska, J., Tuszyńska, M., & Olewnik-Kruszkowska, E. (2021). Comparative study of gelatin hydrogels modified by various cross-linking agents. *Materials*, 14(2), 1–17. <https://doi.org/10.3390/ma14020396>
- Sofi, H. S., Ashraf, R., Beigh, M. A., & Sheikh, F. A. (2018). Scaffolds Fabricated from Natural Polymers/Composites by Electrospinning for Bone Tissue Regeneration. *Advances in Experimental Medicine and Biology*, 1078, 49–78. [https://doi.org/10.1007/978-981-13-0950-2\\_4](https://doi.org/10.1007/978-981-13-0950-2_4)
- Sorieul, M., Dickson, A., Hill, S. J., & Pearson, H. (2016). Plant fibre: Molecular structure and biomechanical properties, of a complex living material, influencing its deconstruction towards a biobased composite. *Materials*, 9(8), 1–36. <https://doi.org/10.3390/ma9080618>
- Standardization, I. O. for. (2006). ISO 10993-5 Biological evaluation of medical devices. 61010-1 © Iec:2001.
- Stevens, P., Robinson, R. L., Dyke, K. Van, & Stitzel, R. (1975). The temperature dependence of [8-3H]adenosine labelling of the perfused bovine and canine adrenal gland. *Journal of Pharmacy and Pharmacology*, 27(11), 864–865. <https://doi.org/10.1111/j.2042-7158.1975.tb10232.x>
- Stone, V., Johnston, H., & Schins, R. P. F. (2009). Development of in vitro systems for nanotoxicology: methodological considerations. *Critical Reviews in Toxicology*, 39(7), 613–626. <https://doi.org/10.1080/10408440903120975>
- Strober, W. (2015). Trypan Blue Exclusion Test of Cell Viability. *Current Protocols in Immunology*, 111(1), A3.B.1-A3.B.3. <https://doi.org/10.1002/0471142735.ima03bs111>
- Sultana, N. (2013). Scaffolds for tissue engineering. In *SpringerBriefs in Applied Sciences and Technology* (Issue 9783642348013, pp. 1–17). Springer Verlag. [https://doi.org/10.1007/978-3-642-34802-0\\_1](https://doi.org/10.1007/978-3-642-34802-0_1)
- Tao, L., Zhonglong, L., Ming, X., Zezheng, Y., Zhiyuan, L., Xiaojun, Z., & Jinwu, W. (2017). In vitro and in vivo studies of a gelatin/carboxymethyl chitosan/LAPONITE® composite scaffold for bone tissue engineering. *RSC Advances*, 7(85), 54100–54110. <https://doi.org/10.1039/C7RA06913H>
- Theocharis, A. D., Skandalis, S. S., Gialeli, C., & Karamanos, N. K. (2016). Extracellular matrix structure. In *Advanced Drug Delivery Reviews* (Vol. 97, pp. 4–27). Elsevier B.V. <https://doi.org/10.1016/j.addr.2015.11.001>
- Ul-Islam, M., Subhan, F., Islam, S. U., Khan, S., Shah, N., Manan, S., Ullah, M. W., & Yang, G. (2019). Development of three-dimensional bacterial cellulose/chitosan scaffolds: Analysis of cell-scaffold interaction for potential application in the diagnosis of ovarian cancer. *International Journal of Biological Macromolecules*, 137, 1050–1059. <https://doi.org/10.1016/j.ijbiomac.2019.07.050>

- Van De Ven, T. G. M., & Godbout, L. (2013). *Cellulose: fundamental aspects*. BoD–Books on Demand.
- Van Vlierberghe, S., Graulus, G. J., Samal, S. K., Van Nieuwenhove, I., & Dubruel, P. (2014). Porous hydrogel biomedical foam scaffolds for tissue repair. In P. A. B. T.-B. F. for T. E. A. Netti (Ed.), *Biomedical Foams for Tissue Engineering Applications* (pp. 335–390). Woodhead Publishing. <https://doi.org/10.1533/9780857097033.2.335>
- Vielreicher, M., Schürmann, S., Detsch, R., Schmidt, M. A., Buttgerit, A., Boccaccini, A., & Friedrich, O. (2013). Taking a deep look: Modern microscopy technologies to optimize the design and functionality of biocompatible scaffolds for tissue engineering in regenerative medicine. *Journal of the Royal Society Interface*, *10*(86). <https://doi.org/10.1098/rsif.2013.0263>
- Vinardell, M. P., & Mitjans, M. (2017). Lignins and their derivatives with beneficial effects on human health. In *International Journal of Molecular Sciences* (Vol. 18, Issue 6). <https://doi.org/10.3390/ijms18061219>
- Walma, D. A. C., & Yamada, K. M. (2020). The extracellular matrix in development. In *Development (Cambridge, England)* (Vol. 147, Issue 10). <https://doi.org/10.1242/dev.175596>
- Wang, J., Zhao, L., Zhang, A., Huang, Y., Tavakoli, J., & Tang, Y. (2018). Novel bacterial cellulose/gelatin hydrogels as 3D scaffolds for tumor cell culture. *Polymers*, *10*(6), 581. <https://doi.org/10.3390/polym10060581>
- Wang, L., Wang, C., Wu, S., Fan, Y., & Li, X. (2020). Influence of the mechanical properties of biomaterials on degradability, cell behaviors and signaling pathways: current progress and challenges. *Biomaterials Science*, *8*(10), 2714–2733. <https://doi.org/10.1039/D0BM00269K>
- Wang, M. O., Etheridge, J. M., Thompson, J. A., Vorwald, C. E., Dean, D., & Fisher, J. P. (2013). Evaluation of the in vitro cytotoxicity of cross-linked biomaterials. *Biomacromolecules*, *14*(5), 1321–1329. <https://doi.org/10.1021/bm301962f>
- Wang, Q., Mu, H., Zhang, L., Dong, D., Zhang, W., & Duan, J. (2015). Characterization of two water-soluble lignin metabolites with antiproliferative activities from *Inonotus obliquus*. *International Journal of Biological Macromolecules*, *74*, 507–514. <https://doi.org/10.1016/j.ijbiomac.2014.12.044>
- Wicaksono, R., Syamsu, K., Yuliasih, I., & Nasir, M. (2013). Cellulose Nanofibers from Cassava Bagasse: Characterization and Application on Tapioca-Film. *Chemistry and Materials Research*, *313*(13), 2225–2956.
- Xing, Q., Zhao, F., Chen, S., McNamara, J., DeCoster, M. A., & Lvov, Y. M. (2010). Porous biocompatible three-dimensional scaffolds of cellulose microfibril/gelatin composites for cell culture. *Acta Biomaterialia*, *6*(6), 2132–2139. <https://doi.org/10.1016/j.actbio.2009.12.036>
- Yamzon, J. L., Kokorowski, P., & Koh, C. J. (2008). *Stem Cells and Tissue Engineering*

Applications of the Genitourinary Tract. *Pediatric Research*, 63(5), 472–477.  
<https://doi.org/10.1203/PDR.0b013e31816a704a>

Yaradoddi, J. S., Banapurmath, N. R., Ganachari, S. V., Soudagar, M. E. M., Mubarak, N. M., Hallad, S., Hugar, S., & Fayaz, H. (2020). Biodegradable carboxymethyl cellulose based material for sustainable packaging application. *Scientific Reports*, 10(1), 21960.  
<https://doi.org/10.1038/s41598-020-78912-z>

Ye, J., Yang, G., Zhang, J., Xiao, Z., He, L., Zhang, H., & Liu, Q. (2021). Preparation and characterization of gelatin-polysaccharide composite hydrogels for tissue engineering. *PeerJ*, 9. <https://doi.org/10.7717/peerj.11022>

Zhao, P., Gu, H., Mi, H., Rao, C., Fu, J., & Turng, L. sheng. (2018). Fabrication of scaffolds in tissue engineering: A review. In *Frontiers of Mechanical Engineering* (Vol. 13, Issue 1, pp. 107–119). <https://doi.org/10.1007/s11465-018-0496-8>

