Study on Calcium Reinforced Polymeric Hydrogel Scaffolds for Bone Tissue Regeneration

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Doctoral Thesis Summary

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Faculty of Technology

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Studie polymerních hydrogelových scaffoldů plněných vápníkem pro regeneraci kostní tkáně

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SOUHRN

Léčba nežádoucích zlomenin se potýká s celou řadou problémů jako jsou infekce či další následné vynucené chirurgické zákroky. Pro řešení těchto případů se s výhodou dají použít polymerní scaffoldy, které dokáží vylepšit regeneraci kostní tkáně. Polymerní scaffoldy mají totiž vynikající biokompatibilitu, vhodné mechanické vlastnosti a jsou dobře odbouratelné v tkáni.

Tato práce se zabývá *přípravou a charakterizací nových polymerních hydrogelových scaffoldů na bázi bakteriální celulózy (BC) plněné vápníkem, jejichž úkolem je zvýšit regeneraci poškozené kostní tkáně*. Byly připraveny scaffoldové kompozice na bázi BC a syntetických polymerů polyvinylpyrrolidonu (PVP) a polyethynglykolu (PEG) ve dvou kombinacích. V prvním případě byly použity *hydrogelové scaffoldy na bázi BC plněné/vyztužené fosfátem vápenatým (CaP)*, kde CaP byl ve formě beta-tri-fosfosfátu vápenatého (β-TCP) a hydroxyapatitu (HA) v různých koncentracích. Druhou kombinaci *tvořily hydrogelové scaffoldy na bázi BC plněné fosfátem vápenatým/uhličitanem vápenatým*, které byly připraveny *in vitro* biomineralizací *hydrogelového scaffoldu na bázi BC plněné CaP*.

Strukturální vlastnosti (fyzikálně-chemické, morfologické, mechanické a viskoelastické) naznačují, že *scaffoldy plněné CaP* mají značnou schopnost bobtnání, mají vhodnou pórovitost, a další mechanické a viskoelastické vlastnosti.

Kromě toho výsledky testů **funkčních vlastností**, zahrnující biokompatibilitu, životaschopnost buněk, interakci buněk a biomateriálů (prostřednictvím studie SEM) a expresi kostních markerů (pomocí ALP analýzy) ukázaly lepší účinnost při regeneraci kostní tkáně.

Na základě zjištěných poznatků lze hydrogelové scaffoldy na bázi BC vyztužené CaP (BC-PVP-β-TCP / HA_20: 80 a BC-PVP-β-TCP / HA_50: 50) doporučit k další analýze (např. Studiem in vivo) a případně použít navržený scaffold pro regeneraci měkkých (spongiózních) kostí.

ABSTRACT

Treatments for unwanted bone fractures have different limitations like potential infection risks and requirement of secondary surgery. Polymeric tissue engineering scaffold material can be a suitable alternative treatment device for the bone tissue regeneration due to its excellent biocompatibility, mechanical property and degradability.

This thesis reports *the preparation and characterization of novel calcium reinforced bacterial cellulose (BC) based hydrogel scaffolds for its possible application in bone tissue regeneration*. The scaffolds were developed by using a combination of natural polymer, BC and other synthetic polymers like polyvinylpyrrolidone (PVP), polyethylene glycol (PEG). The scaffolds were prepared in two forms. First, *calcium phosphate (CaP) reinforced BC based hydrogel scaffolds were prepared*, where *CaP* was used in the form of β-tri calcium phosphate (β-TCP) and hydroxyapatite (HA) in different concentrations. Second, *calcium phosphate & calcium carbonate reinforced BC based hydrogel scaffolds* were prepared through template mediated *in vitro* biomineralization of *CaP filled BC based hydrogel scaffolds*.

The ***structural properties*** (physiochemical, morphological, mechanical and/or viscoelastic characterization) indicated the *CaP reinforced scaffolds* have demonstrated significant swelling ability, suitable porosity and other mechanical and viscoelastic properties.

Furthermore, ***functional properties*** (involving the biocompatibility, cell viability, cell-biomaterial interaction and bone marker expression) indicated their better efficiency for bone tissue regeneration.

Thus, the *CaP reinforced BC based hydrogel scaffolds* (*BC-PVP-β-TCP/HA_20:80 and BC-PVP-β-TCP/HA_50:50)* are recommended for further analysis (e.g. *in vivo study*) and application in soft bone tissue (ie. cancellous bone) regeneration.

CONTENTS

1. BACKGROUND

1.1. Bone and Bone Tissue Regeneration (BTR)

1. 1. 1. Bone

Bone is one of the most important parts of human musculoskeletal system. It maintains the structural framework of the animal body [1-4].

The architecture of animal bone is comprised of various structural attributes. It is comprised of extracellular matrix, which is composed of inorganic matrix that provides the strength to the bone and organic matrix, which facilitates the bone flexibility [5-6]. The bone structure composed of the following components [1, 7]:

The adult animal body is comprised of two types of bony structures: cortical bones (80%) and trabecular/ cancellous bones (20%) [8] (Figure. 1). The cortical or compact bones are dense and contain high concentration of inorganic mineral components (like hydroxyapatite); however, it also lacks high number of blood vessels and osteocytes. On the other hand, trabecular bones are composed of porous "honeycomb" like network structures [2, 8]. These two types of bones have differential mechanical properties. The mechanical property of trabecular bone has been less compared to the compact bone due to its structural architecture and porosity [2].

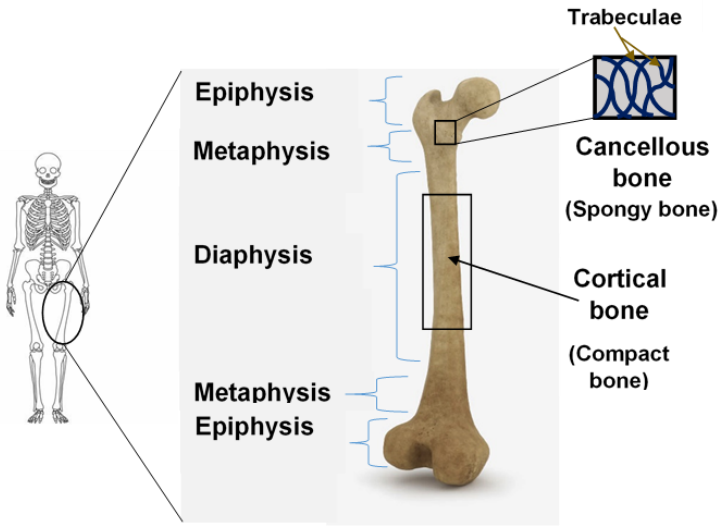


Figure 1. Cortical and cancellous bone of human long bone. (Self-representation)

1. 1. 2. Bone regeneration and Bone remodeling

a. Bone regeneration event

The bone regeneration event is a complex process (Figure. 2). However, the important events of bone fracture healing or bone regeneration indicated below[9]:

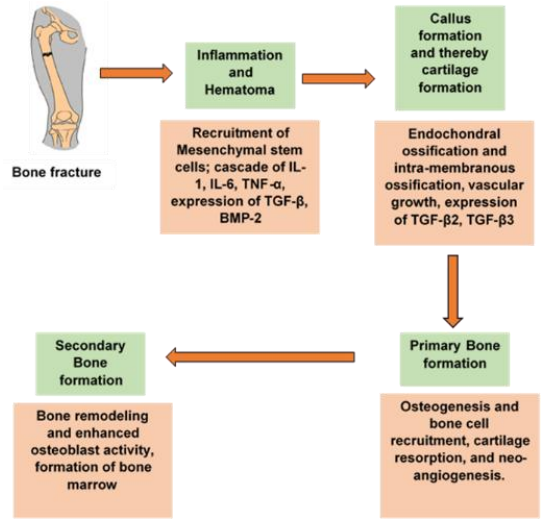


Figure 2. Bone fracture healing and bone regeneration. (Self-representation based on AI-AQI et al., 2008 [9]).

b. Bone remodeling event

Bone remodeling refers to the physiological event in which old/damaged bone is replaced by newly formed bone. In this process, osteoclast removes the damaged bone by bone resorption and new bones are formed by osteoblast. This process has 4 different stages, which are: Initiation of bone remodeling, bone resorption, bone matrix synthesis and mineralization of bone matrix[7].

1.2 Tissue engineering and its principles in BTR

1.2. 1. Tissue Engineering

"Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" [10].

Different studies and researches indicated that bone related disorders like osteoporosis caused 8.9 million fracture situations worldwide annually, with a condition where a fracture happens in every 3 seconds [11]. The existing treatment

methods include the application of bone grafts [12-13]. The bone grafts contain the following characteristics in the context of bone tissue engineering application [2, 14-15]:

I. *Osteoconductivity*

Osteoconductivity is the property of the graft/implant material, which elaborates the significant formation and growth of the bone cells on the surface or within the porous structures of the graft material.

II. *Osteoinductivity*

Osteoinductivity is the ability or property of the graft material through which the graft material induces the pluripotent stem cells to differentiate into bone-forming cell lineage.

III. *Osseo-integration*

Osseo-integration refers to the direct contact between the bone and the graft material.

The *bone grafting method* is most popular methods for tissue fracture repair. This method includes two different approaches:

a. Autologous bone grafting:

Autologous bone grafting is considered as a gold standard for the bone defect treatment. In this process, the graft is collected from one site and implanted in another site of the same host. However, different limitations like longer surgical time, potential infection, and limited quantity of the graft material are also notable. This type of bone grafts has used for the defects of cancellous, cortical bones, bone marrow etc. [2, 14, 16].

b. Allogenic bone grafting

Allogenic bone grafting involves the collection of bone graft from another person and implanted into the patient. However, the limitations of this methods are also notable like graft rejection, possible introduction of infection from donor to host etc. The allogenic grafting is used for cortical bones, cancellous and demineralized bones [16, 17].

1.2.2 Bone graft substitutes

On the other hand, different graft substitutes have been used notably for orthopedic application. The bone substitute materials which are being currently used involves: growth factor based substitute, cell based substitutes, ceramic based substitutes, polymer based substitute etc [18-19].

1.2.3. Bone graft substitutes presently in market

Different bone graft substitutes that are currently available/ in development stage**.**[20-24] Scaffold materials composed with β-TCP, HA and biphasic calcium phosphate were developed by the industrial manufacturers of various countries like *CONDUIT® TCP Granules (*DePuy Spine, USA), *MBCP® (*Biomatlante Biologic solutions, France), *Artebone* ® *(*BBS-Bioactive Bone Substitute, Finland). Additionally, scaffolds (*ACI-Maix*, Matricel, Germany) was also developed by applying collagen as a component.

1.2.4. Principle of tissue engineering (TE)

The successful implementation of tissue engineering (TE) application involves the significant interaction and functioning of scaffolds, cells, and cell signaling systems. These three components together form a "*Tissue engineering triad*" [25], which stimulates and facilitates the tissue regeneration event.

The tissue engineering strategy involves **TWO** major approaches:

a. TE construct with cell and scaffold

The cells from allogenic or autologous sources are cultured and proliferated *in vitro,* then these cells are seeded onto a scaffold matrix, which ultimately forms a cell laden structure. The cell laden structure serves as tissue engineering construct where the cells can proliferate, differentiate and thereby facilitates the formation of a regenerated tissue [25].

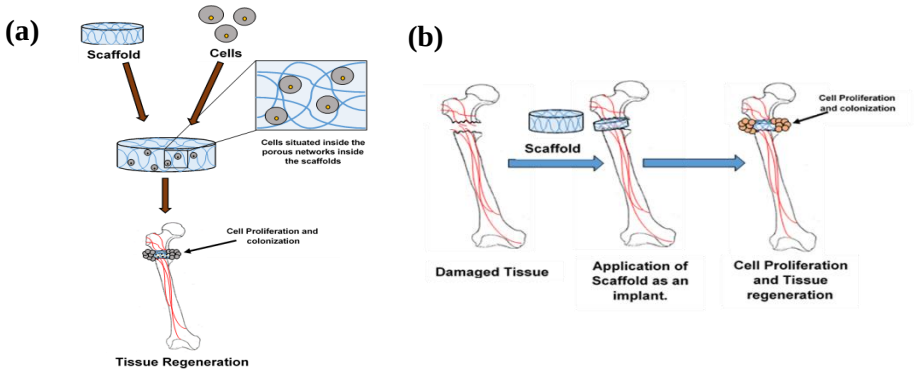


Figure 3. Tissue engineering construct with cells and scaffolds (a) and application of acellular scaffolds for BTR (b). (Self-representation)

b. Cellular TE construct that mimics extra cellular matrix:

Another significant approach of tissue engineering involves the utilization of an acellular biomaterial that essentially mimics the characteristics of extra cellular matrix of living tissue (Figure 4) [25]. In this case, the scaffold will be used as "implant material" which will stimulate the significant proliferation and colonization of cells and thereby facilitate tissue regeneration process [2, 25].

1.3. Biomaterial & Scaffolds in BTR

Biomaterials are the natural or synthetic material that has a notable therapeutic usage in the context of repair and restore/replace of biological tissue.

The principle objective of biomaterial includes the stimulation of tissue regeneration phenomenon through delivering a notable signal which are necessary for cell proliferation and growth and thereby facilitate the regeneration of healthy tissue.

1.3.1 Biomaterials in TE & Bone regeneration

Different types of biomaterial have been used in tissue engineering [2] are discussed below.

a. Natural Biomaterials

There are different natural biomaterials like protein based biomaterial (Silk, Collagen etc), poly saccharide based biomaterial (Chitosan, Alginate, Hyaluronic acid) used in BTR [26].

b. Artificial Biomaterials

The artificial biomaterials which are used in BTR involves Metals (Titanium, Gold), Ceramics (calcium phosphates and carbonates), Polymers (Poly lactic acid, poly glycolic acid etc.).

1.3.2 Classification of biomaterials on the basis of its generation

The biomaterials and its types can also be classified on the basis of the following: First generation, Second generation, Third Generation, Fourth generation of biomaterials.

1.3.3 Scaffolds for BTR

Scaffolds are the artificial structures that facilitate the three dimensional (3D) tissue formation.

The scaffolds materials might be used as "acellular systems" or "cell laden structures" for tissue engineering applications.

The following significant characteristics of the scaffold materials are *important* to be considered before its application [27]:

a. **Extra Cellular Matrix (ECM) mimicking material**. The scaffold material must mimic the natural extra cellular matrix and provide the necessary structural support to the cells.

b. **Geometry and architecture**. The internal geometry and structural architecture of scaffold material (like interconnected porous structures and pore sizes) ensures the cellular attachment for tissue engineering.

c. **Porosity**. It is an important factor for scaffold material. A significant porous scaffold material will ensure the cell attachment and facilitate significant vascularization inside the scaffold during regeneration.

d. **Mechanical property**. The stability of the scaffold material depends upon its appropriate mechanical property. The compressive strength of trabecular bone ranges from 0.22 MPa- 10.44 MPa and cortical bone ranges from 130-200 MPa.

e. **Biodegradability**. The gradual and controlled biodegradability is an important aspect of good tissue engineering scaffold material.

f. **Biocompatibility**. The term biocompatibility can be defined as "the ability of a material to perform with an appropriate host response in a specific application". The scaffold material must augment the cell attachment, proliferation and differentiation process through providing stimulatory signals to the surrounding cells and tissues.

1.3.4 Significant biological characteristics of the tissue engineering scaffold

The tissue engineering scaffold must be biocompatible and non-toxic. In addition, the cell attachment, colonization, proliferation and differentiation are the key biological requirement of the tissue engineering scaffold. On the other hand, the scaffold material should be accepted by host immune system. The immunologically inert tissue engineering materials has been recently utilized efficiently.

The most important characteristics that should be present in the tissue engineering scaffold is "*Bioactive*" property. In recent years, different bioactive compounds have been introduced in the composition of scaffold materials like bioactive calcium phosphates (hydroxyapatite, tri-calcium phosphate etc.) to enhance the efficiency of the scaffold material/implant [1, 2, 3]. The bioactive tissue engineering scaffold material will efficiently facilitate the cell migration/differentiation, successful integration of the implant within the host, and tissue formation.

1.4. Polymeric hydrogel scaffolds

1.4.1 Hydrogel scaffold

Hydrogels can be defined as a class of bio-inspired and bio-functionalized biomaterials composed of hydrophilic cross-linked polymeric three dimensional networks structure, which can absorb and retain high quantity of water and thus has the similarity with a living tissue.

A hydrogel contain following properties [28]:

- Hydrogel is made up of hydrophilic polymeric materials which are cross-linked either by covalent bonds or intra/intermolecular physical interactions.
- They can swell significantly and can retain notable amount of water without dissolving.
- Hydrogels due to its structural attributes has considered as biocompatible.

1.4.2 Polymeric hydrogels used in tissue engineering application for BTR

Different polymeric hydrogels were used in BTR applications. Polymeric hydrogels like alginate, collagen, chitosan, silk fibroin, PCL, PVA, PEG, PLGA, BC, gelatin-nanocellulose, hyaluronic acid, dextran based hydrogels were used in BTR.

1.4.3 Role of polymeric hydrogel scaffold in BTR

Hydrogel scaffold can efficiently mimic bone extra cellular matrix [3, 14]. In the context of bone tissue engineering, hydrogel as a scaffold material should contain the following characteristics (Figure.4) to promote bone cell colonization/proliferation, tissue growth and thereby facilitate regeneration:

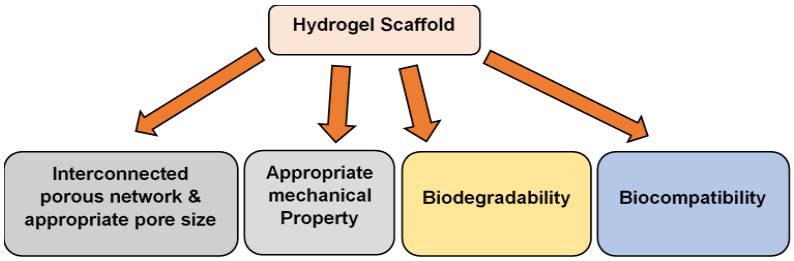


Figure 4. Property of hydrogel for bone tissue engineering

1.5 Bacterial cellulose (BC) and BTR

Cellulose is considered as significant and naturally abundant biopolymer composed of a linear homopolysaccharides developed by β-D-glucopyranose units linked through β -1,4 glycosidic bonds. Cellulose is produced by the green plants as well as some bacteria. Additionally, cellulose is also synthesized by certain algae and tunicates.

Bacterial cellulose (BC) is cellulose majorly is synthesized by bacteria of the genera, *Gluconobacter*. In 1886, Brown A.J. discovered the development of pellicle structure by bacteria which is similar to plant cellulose. BC is devoid of lignin and hemicellulose and thus differs from the plant cellulose. Additionally, BC has Iα and Iβ crystalline domains, where plant cellulose has only Iβ crystalline domain. The BC has different properties like water rendition capacity, biocompatibility etc.

1.5.1 Synthesis and production of BC

The BC production is done through fermentation process. The different components of a BC production are involves appropriate bacterial strain (*Gluconobacter* sp.), HS culture media, pH 4-7, 28-30ºC, Static/dynamic mode of production and post production treatment.

1.5.2 Mechanism of biosynthesis of BC and its use in BTR

The BC fibrillar matrix is composed of numerous three dimensional BC nano-fibers that ultimately form a hydrogel sheet like structure with high surface area. *Gluconobacter* is the most commonly used bacterium in BC production. The bacteria under appropriate carbon-nitrogen nutrient condition, and optimum temperature (28-30ºC) and pH (pH: 4-7) condition produce ribbon like cellulose I and thermodynamically stable cellulose-II polymers. The glucose units form the bacterial cellulose through cellular biosynthetic pathway. The bacteria first produce protofibrils (2-4 nm) of glucose chains which are then secreted by bacterial cell wall and produce microfibrils and then aggregated to form cellulose fiber ribbons (80 nm). BC has been extensively used in biomedical application. The advantage of BC over other polymers involves the fact that BC has significant controllable three dimensional fibrous network structure and notable biocompatibility. BC has a notable use as bone tissue engineering scaffold due to its remarkable similarity with bone extra cellular matrix and collagen.

1.6 Calcium and its importance in BTR

Different calcium based bioactive compounds like calcium phosphate and calcium carbonate [3, 29] have been utilized in bone tissue engineering scaffold material. Calcium based bioactive scaffolds will provide two types of stimulatory signals to the cells:

1.6.1 Extracellular [Ca^{2+}] based signaling

In osteoclast mediated normal bone resorption process, calcium ions (Ca^{2+}) are generally moves out from the bone matrix and this causes local increase of Ca^{2+} concentration (nearly 40 mM). Bioactive calcium agents like calcium phosphates and calcium carbonate equipped with tissue engineering scaffold can also lead to the development of elevated extracellular calcium concentration [30-32]. Different

macropores and micropores of tissue engineering scaffold facilitate the efficient release of calcium and phosphate for the local increase of concentration of this bioactive mineral. It has been reported that increase in local Ca^{2+} concentration can also stimulate notable intra cellular calcium signaling. Additionally, the high concentration of calcium subsequently initiate MAPK/ERK signaling pathway which ultimately causes the gene expression (like *bone morphogenetic protein-2* or *bmp-2* gene) for osteoblast proliferation and migration.

1.6.2 Mechanical stimulus and signaling

Calcium based compounds like calcium carbonate are considered as rigid filler in the filled-in polymer system. The presence of calcium based bioactive agents also enhances the mechanical property of the tissue engineering scaffold. Thus composite material containing polymeric matrix and rigid reinforcing material could also provide mechanobiological signals to the bone cells to proliferate along with biochemical cues [33-34]. The mechanobiological stimulus provided by extracellular matrix (ECM) and tissue engineering scaffold is at first recognized by a eukaryotic transmembrane cell-ECM receptor, *Integrin*. The bone cell cycle progression and bone cell proliferation are dependent on *Integrin-dependent cell signaling pathway* that also depends on the hard and soft matrix properties. However, actin cytoskeletal tension also plays a vital role in cell proliferation event.

1.7 Cell and cell biology in BTR

1.7.1. Bone cells

The maintenance of the animal bone structure includes periodical process of bone formation and bone resorption [17]. The bone majorly consists of 3 types of cells: Osteoblast (bone forming cells), Osteoclast (bone resorbing cells), and Osteocyte (mechano-sensing cells) and Mesenchymal stem cells.

1.7.2. Types of cells utilized in bone tissue engineering

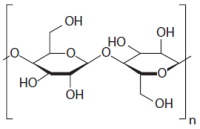
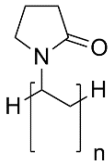
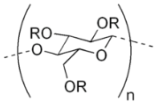
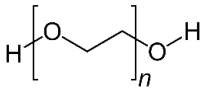
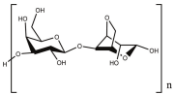
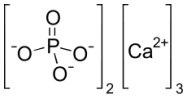
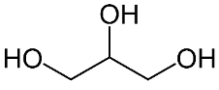
The selection of cell source in bone tissue engineering study is a significant criterion for successful application. Different cell lines like MC3T3-E1, COS-7, MG-63, SAOS-2, AD-SC have been used as *in vitro* model for bone tissue engineering [26].

2. EXPERIMENTATION

2.1 Materials for calcium reinforced BC based hydrogel preparation

2.1. a. The materials used in this study

Table 1. Materials used in the preparation of hydrogel scaffolds

Materials	Chemical formula	Description
Bacterial cellulose (BC)		*BC* is cellulose, which majorly synthesized by bacteria of the genera, *Gluconobacter*. BC is composed of a linear homopolysachharides developed by β-D-glucopyranose units linked through β -1, 4 glycosidic bonds. BC is devoid of lignin and hemicellulose and thus differs from the plant cellulose.
Polyvinylpyrrolidone (PVP)		*PVP* is a water-soluble polymer made from the monomer *N*-vinylpyrrolidone. It is a biocompatible polymer used in biomedical purposes like wound dressing.
Carboxy-methyl cellulose (CMC)	 R = H or CH_2CO_2H	*CMC* is a water-soluble polymer. It is a biocompatible polymer. It is used in cosmetic application and other biomedical uses.
Polyethylene glycol (PEG)		*PEG* is a hydrophilic polymer. It is also biocompatible polymer and reduces cell and tissue cytotoxicity
Agar		*Agar* is a mixture of two components: the linear polysaccharide agarose, and a heterogeneous mixture of smaller molecules called agaropectin. Agar is considered as a gelling agent.
β-tricalcium phosphate (β-TCP)		*β-TCP* is a calcium salt of phosphoric acid. It is considered as a biocompatible bio-ceramics.
Hydroxyapatite (HA)	$Ca_{10}(PO_4)_6(OH)_2$	*HA* is a naturally occurring mineral form of calcium apatite. It is an integral component of animal bone.
Glycerin		*Glycerin* or *Glycerol* is a simple hydrophilic polyol compound. It is used as a humectant in the preparation of hydrogel.

2.1. b. Polymers and their purpose of use for the preparation of the hydrogel scaffold

This study utilizes polymers like BC for biocompatibility and mechanical property; PVP for biocompatibility; CMC for biocompatibility and swelling property; PEG for cellular cytotoxicity lowering agent and Agar for gelling agent.

2.1. c. Sources of the utilized materials in this study

Table 2. Sources of the materials in this study

Materials		Description	Source
For ***CaP reinforced BC based hydrogel scaffolds***			
Polymers	*BC (Raw material)*	Synthesized polymer	*G. xylinus* CCM 3611^T bacteria
	PVP	PVP K30, mol wt: 40, 000	Fluka, Switzerland
	CMC	Sodium carboxymethyl cellulose	Sinopharm Chemical Reagent Co Ltd. (SCRC), China
	PEG	PEG 3000, mol wt. 2700-3300	Fluka, Switzerland
	Agar	Agar powder for gelling agent	Fluka, Switzerland
Other agents	*Glycerin*	Humidity retention agent	Lach-Ner Ltd., Neratovice, Czech Republic
Calcium Phosphates	*Hydroxyapatite*	CaP powder, mol. Wt.: 502.31 g/mol	Sigma Aldrich, St. Louis, MO, USA
	β-tri calcium phosphate	CaP powder, mol. Wt.: 310.18 g/mol	Fluka, Switzerland
For *in vitro* bio-mineralization of CaP reinforced BC based hydrogels to prepare ***calcium phosphate and calcium carbonate reinforced BC based hydrogel scaffolds***			
Sodium carbonate		Na_2CO_3; molecular weight 286.14 g/mol	Sigma Aldrich, St. Louis, MO, USA
Calcium chloride		$CaCl_2$; molecular weight 110.99 g/mol, 97.0%	Penta, Czech Republic

2.2 Methodology of calcium reinforced BC based hydrogel scaffold preparation

2.2.1 Synthesis of raw material i.e. Bacterial cellulose

The methodology of BC synthesis is described as follows:

BC (holding 99% H_2O) was synthesized in presence of basal synthetic Hestrin-Schramm (HS) nutritive medium (pH 7.0) using *Gluconacetobacter xylinus CCM 3611^T* (syn. *Acetobacter xylinum*) at 30°C for 15 days. 100 mL bacteriological culture bottles were inoculated with 5 mL of H.S. medium containing 96 × 10^8 cells/mL bacteria [bacteria counted at 550 nm wavelength]. The freshly prepared BC pellicle was treated with 0.5N NaOH solution and then heated at 80°C for 1 hour to remove the possible contaminations from the BC pellicle (Figure. 5). The newly produced BC pellicle was then treated with deionized water and the water was refreshed after 2h of interval until the pH reached a neutral value. Thereafter, a homogenous suspension of BC (particle size: ranging from *159 nm-351.03 nm*) from the obtained BC mat was prepared by grinding [10-12 mins] the BC mat in distilled water [3].

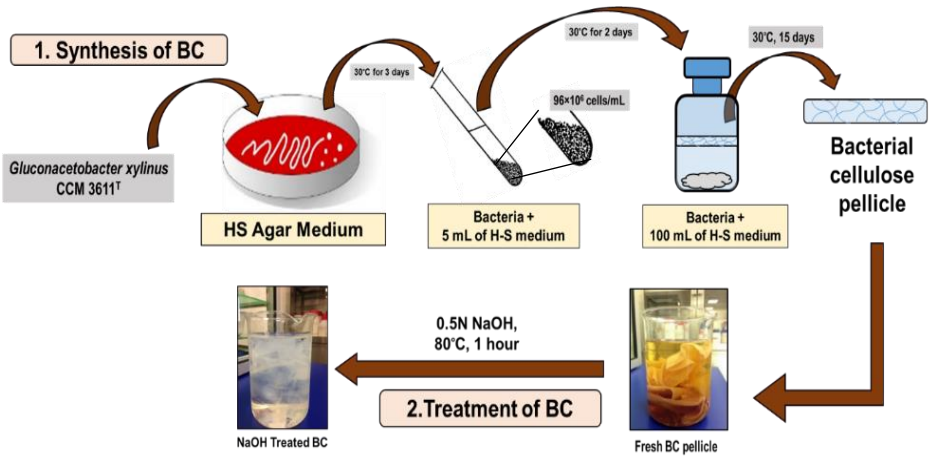


Figure 5. BC production in H.S. medium. (Self-representation)

2.2.2 Preparation of calcium reinforced BC based hydrogel scaffolds

The *calcium reinforced BC based hydrogel scaffolds* were developed in the form of *calcium phosphate reinforced BC based hydrogel scaffolds* and *calcium phosphate and calcium carbonate reinforced BC based hydrogel scaffolds*. The preparation of the hydrogel scaffolds is described below:

(a) *Preparation of calcium phosphate reinforced BC based hydrogel scaffolds*

Calcium reinforced filled BC based hydrogel scaffolds were prepared by applying the BC (holding 99% water) with CMC and PVP (Table 3). Polyethylene glycol (PEG) was also used to reduce the risk of tissue damage and other significant cytotoxic effects. Agar was used as gelling agent and glycerin was used as humectant. Additionally, calcium phosphates like β-TCP and HA were given to develop the inorganic *calcium phosphate filled BC based hydrogel scaffolds* (Figure. 6) .

The hydrogel scaffolds were developed following the solvent casting method, applying moist heat and pressure. 100 mL polymer solutions were prepared in 250mL sealed glass bottles under 15 lbs (107 KPa) pressure and 120°C temperature for 20 minutes. Two sets of polymer solutions were prepared; where one set was without inorganic calcium phosphate, and another set was with inorganic calcium phosphates (β-TCP/HA). 25 mL polymer solution from each sealed glass bottles was then poured into 75 mm diameter petri-dishes and then allowed to cool at room temperature (22-25°C). Finally, smooth, round shaped, off white color BC based scaffolds with and without calcium phosphate were achieved. The hydrogel scaffolds without calcium phosphate were termed as "*BC-PVP*" and "*BC-CMC*" and the calcium phosphate reinforced scaffolds were termed as "*BC-PVP-β-TCP/HA*" and "*BC-CMC-β-TCP/HA*".

Furthermore, on the basis of cell biological study, the "*BC-PVP-β-TCP/HA*" scaffold was selected and then different *calcium phosphate reinforced BC based hydrogel scaffolds* (BC-PVP-β-TCP/HA_10:90; BC-PVP-β-TCP/HA_20:80; BC-PVP-β-TCP/HA_40:60; BC-PVP-β-TCP/HA_50:50; BC-PVP-β-TCP/HA_60:40) (Diameter: 75 mm; Thickness: 5.9-6.2 mm) were prepared with different calcium phosphate i.e. β-TCP and HA ratios (Table 3 and 4).

Table 3. *Composition of calcium reinforced BC based hydrogel scaffold*.

Sample index		PVP (g)	CMC (g)	BC (g)	PEG (g)	Agar (g)	Glycerin (mL)	β-TCP/HA (g)	Water (mL)
Scaffolds without calcium	BC-PVP	0.5	0.0	0.5	1	2	1	0.0/0.0	95
	BC-CMC	0.0	0.5	0.5	1	2	1	0.0/0.0	95
Scaffolds with calcium phosphate	BC-PVP-β-TCP/HA	0.5	0.0	0.5	1	2	1	0.2/0.8	94
	BC-CMC- β-TCP/HA	0.0	0.5	0.5	1	2	1	0.2/0.8	94
Scaffolds with	BC-PVP-β-TCP/HA-$CaCO_3$	0.5	0.0	0.5	1	2	1	0.2/0.8	94

calcium phosphate and calcium carbonate	**BC-CMC- β-TCP/HA-$CaCO_3$**	0.0	0.5	0.5	1	2	1	0.2/0.8	94

Table 4. *Composition of calcium phosphate reinforced BC based hydrogel scaffold*

Calcium phosphate reinforced BC based hydrogel scaffolds	PVP (g)	BC (g)	PEG (g)	Agar (g)	Glycerin (mL)	β-TCP/HA (g)	Water (mL)
BC-PVP	0.5	0.5	1	2	1	0.0/0.0	95
BC-PVP β-TCP/HA _10:90	0.5	0.5	1	2	1	0.1/0.9	94
BC-PVP- β-TCP/HA_20:80	0.5	0.5	1	2	1	0.2/0.8	94
BC-PVP- β-TCP/HA_40:60	0.5	0.5	1	2	1	0.4/0.6	94
BC-PVP- β-TCP/HA_50:50	0.5	0.5	1	2	1	0.5/0.5	94
BC-PVP- β-TCP/HA_60:40	0.5	0.5	1	2	1	0.6/0.4	94

(b) Preparation of calcium phosphate and calcium carbonate reinforced BC based hydrogel scaffolds

The *calcium phosphate reinforced BC based hydrogel scaffolds* (only BC-PVP-β-TCP/HA_20:80 and "BC-CMC-β-TCP/HA_20:80) were further *in vitro* bio-mineralized for incorporation of additional $CaCO_3$ (Table. 3). The *in vitro* bio-mineralization process was performed following the simple liquid diffusion technique. Two different ionic solutions (i.e., Na_2CO_3 and $CaCl_2$) were used at fixed concentration ratios (5.25/100 mL and 7.35/100 mL) to prepare *calcium phosphate and $CaCO_3$ filled BC based hydrogel scaffolds*. In this study, the bio-mineralization of hydrogel matrix was carried out for 60 min by keeping the test samples in each ionic solution. The *calcium phosphate reinforced BC based hydrogel scaffolds* were first immersed in 100 mL solution of $CaCl_2$. H_2O for 30 min and then transferred into 100 mL of Na_2CO_3 solution and kept them for 30 min. In this procedure, *calcium phosphate and $CaCO_3$ filled BC based in vitro bio-mineralized hydrogel scaffolds* were achieved, which were finally termed as "*BC-PVP-β-TCP/HA-$CaCO_3$*" and "*BC-CMC- β-TCP/HA-$CaCO_3$*" respectively (Figure. 6).

2.3 Physical characterization

The physical characterization of *calcium reinforced BC based hydrogel scaffolds* were performed.

Most of the characterization study was performed with freeze dried or lyophilized samples. The calcium reinforced hydrogel scaffolds were freeze dried for 24 h, using Scanvac Cool Safe 110-4 PRO, Lynge; at -80°C temperature and 0–5 kPa pressure.

Thereafter, specific dimensions of the sample sections were selected for the characterization analysis. The methodologies of the physical properties characterization are the following:

2.3.1 Swelling study

The *swelling analysis* was performed through *gravimetric study* with physiological saline solution (Ringers solution; pH 7.40) at human physiological temperature (37°C± 1°C) and human fever temperature (39°C). Circular sections (20 mm diameter) of the hydrogel scaffolds were soaked for specific time intervals (5 min, 60 min, 120 min, 180 min, 240 min, 300 min, 360 min). The absorptivity of the hydrogel scaffolds is measured by the degree of swelling which is defined by the following equation: Degree of swelling (%) = $[(W_s-W_d)/W_d] \times 100$……(1)
Where, W_s and W_d are weight of the swollen and dried hydrogel scaffold sections.

The data for swelling study was presented as the mean ± standard error of the mean. Three repeats of experiments were conducted (n=3). Statistical differences between control and treated groups, as well as between the studied samples were assessed using one-way analysis of variance (ANOVA) followed by a suitable post-hoc test (Dunnett/Bonferonni) by using GraphPad Prism version 5.00 for Windows (San Diego, CA, USA) and MS Office 2010 (Redmond, WA, USA). The P value is expressed as $P<0.0001_{n=3}$ for the level of significance.

2.3.2 Porosity

Porosity is one of important factors of bone tissue engineering scaffold. The porosity can be defined as the percentage of void spaces inside a tissue engineering scaffold [18]. The *liquid displacement method* used for the determination of porosity. This method involves the immersion of freeze dried sample sections with specific

dimensions (10 mm^2) into absolute ethanol for 48 hours. The porosity of the sample is calculated by the following: P= (W2- W1)/ρV1…… (2)

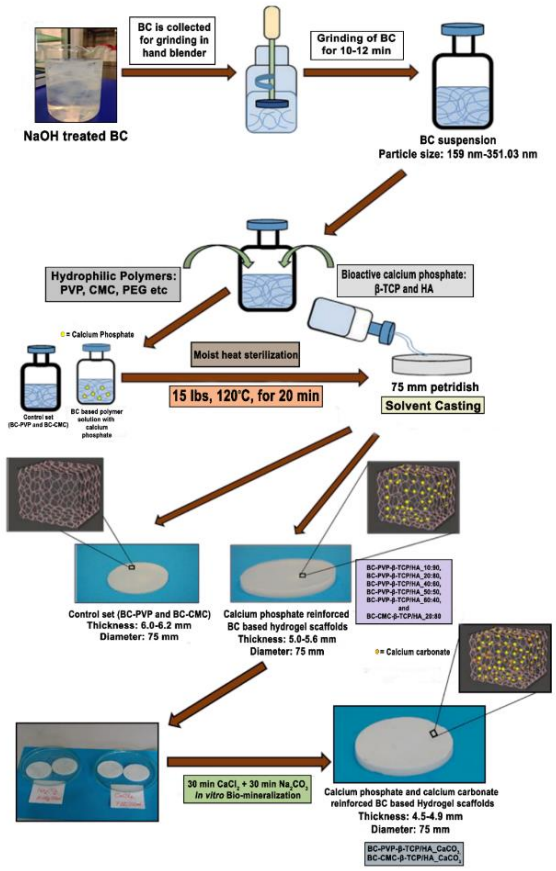


Figure 6. Preparation of calcium reinforced BC based hydrogel scaffolds. (Self-representation)

Where, Where, W1 and W2 are the weight of the hydrogel scaffolds before and after the immersion into absolute ethanol and V1 is the volume of the scaffolds before immersion into ethanol, q is the constant of the density of ethanol at room temperature.

The data for porosity study was presented as the mean ± standard error of the mean. Three repeats of experiments were conducted (n=3). Statistical differences between control and treated groups, as well as between the studied samples were assessed using one-way analysis of variance (ANOVA) followed by a suitable post-hoc test (Dunnett/Bonferonni) by using GraphPad Prism version 5.00 for Windows (San Diego, CA, USA) and MS Office 2010 (Redmond, WA, USA). The P value is expressed as $P<0.0001_{n=3}$ for the level of significance.

2.3.3 Biodegradation study

The biodegradation study was performed through the analysis of the following methods:

(a) Weight Loss profile

The hydrolytic degradation experiment was performed through *gravimetrically* by incubation of the freeze dried sample sections (20 mm diameter) in solution (Physiological Saline [i.e. Ringer] solution; pH: 7.4) maintaining at 37°C for 28 days (i.e. 4 weeks). The saline solution was changed weekly. The sample sections were removed at specific time (i.e. 7 days, 14 days, 21 days, 28 days) from the solution. They were then freeze dried after being washed by distilled water. The percentage of weight loss was evaluated according to the following equation:

$$\text{Weight loss (\%)} = [(W_0 - W_t)/W_0] \times 100 \quad \ldots\ldots(3)$$

Where, W_0 = weight at before degraded sample and W_t = weight of degraded samples at time t.

(b) Gel content analysis

Changes in gel content of the hydrolytically degraded calcium phosphate reinforced BC based hydrogel scaffolds samples (20 mm diameter) were analyzed to indicate the degradation phenomenon. The weights of the freeze dried hydrogel scaffolds were measured initially (W_0) and after a specific time (7, 14, 21, or 28 days) (W_t). The gel content of the scaffolds was measured through the following equation:

$$\text{Gel content} = W_t/W_0 \times 100 \quad \ldots\ldots(4)$$

(c) Gel Density Study

The density of the freeze dried *calcium phosphate reinforced BC based hydrogel scaffolds* samples (20 mm diameter) was studied at specific times (7, 14, 21, and 28 days) during the degradation study (in a physiological saline solution). The gel density (ρ) was determined by the following equation:

$$\rho = W/\pi \times (d/2)2 \times h \quad \ldots\ldots(5)$$

where; W = weight of the scaffold, $\pi = 3.14$, d = diameter of the samples, and h = thickness of the samples.

(d)pH Change and Calcium Release in the medium

The pH change of the medium (i.e., a physiological saline solution in which *calcium phosphate reinforced BC based hydrogel scaffolds* sections were immersed) was also analyzed with a pH meter (Lovobond pH meter, Amesbury, UK). Additionally, the percentage of calcium released by the *calcium phosphate reinforced BC based hydrogel scaffolds* at specific times (i.e., 7, 14, 21, and 28 days) during degradation was also studied because loss of calcium from the scaffolds might also indicate the loss of mechanical property. The percentage of calcium release from the scaffolds was studied by X-ray fluorescence (ARL Quant X, Spectrometer, Thermo Scientific, Waltham, MA, USA) in the presence of helium atmosphere.

The data for biodegradation study was presented as the mean ± standard error of the mean. Three repeats of experiments were conducted (n=3). Statistical differences between control and treated groups, as well as between the studied samples were assessed using one-way analysis of variance (ANOVA) followed by a suitable post-hoc test (Dunnett/Bonferonni) by using GraphPad Prism version 5.00 for Windows (San Diego, CA, USA) and MS Office 2010 (Redmond, WA, USA). The P value is expressed as $P<0.0001_{n=3}$ for the level of significance.

2.4 Morphological characterization

Morphological characterization of *calcium reinforced BC based hydrogel scaffolds* was performed through *scanning electron microscope (SEM) analysis*. Small sections from different regions of the freeze dried scaffold were selected and analyzed through SEM by:

(1) *"Phenome Pro"* (Phenome World, The Netherlands) table SEM, operating in the secondary electron imaging mode at an accelerating voltage of 5–20 kV. The image magnification was 100X – 10kX.

(2) *"NOVA nanoSEM"* (FEI, USA), operating in the secondary electron imaging mode at an accelerating voltage of 5–20 kV. The image magnification was 100X–10kX.

On the other hand, the pore size is an important factor for bone tissue engineering scaffold. The pore size distributions were measured through SEM image analysis by ImageJ/Fiji software (NIH, USA).

The data for pore size distribution was analyzed and presented as the mean ± standard error of the mean. Statistical differences between control and treated groups, as well as between the studied samples were assessed using one-way analysis of variance (ANOVA) followed by a suitable post-hoc test (Dunnett/Bonferonni) by using GraphPad Prism version 5.00 for Windows (San Diego, CA, USA) and MS Office 2010 (Redmond, WA, USA). The P value is expressed as $P<0.0001$ and $P<0.01$ for the level of significance.

2.5 Chemical characterization

The *chemical characterization* of *calcium reinforced BC based hydrogel scaffolds* was performed by:

(1) Fourier Transform Infrared Spectroscopy (FTIR)

The freeze dried samples of *calcium reinforced BC based hydrogel scaffolds* were used for *FTIR* analysis and performed at room temperature (20–22°C). A small amount of sample section with flat surface was selected from each freeze dried hydrogel scaffolds. Three repeats of experiments were conducted. FTIR was performed at wave number 600–4000 cm^{-1} with a uniform resolution of 2 cm^{-1}. The ATR-FTIR analysis was conducted by using NICOLET 320 FTIR spectrophotometer with the "Omnic" software package.

(2) Energy Dispersive X-ray Fluorescence (EDX) Study

The presence of calcium (Ca), phosphate (P) in the *calcium phosphate reinforced BC based hydrogel scaffolds* were determined by *scanning electron microscope enabled energy dispersive X-ray fluorescence* (SEM-EDX) at an accelerated voltage of 15 kV. For this study, sections from freeze dried samples were selected.
The SEM-EDX study was also performed in triplicate.

2.6 Thermal characterization

The thermal behavior of *calcium reinforced BC based hydrogel scaffolds* was analyzed by the following methods:

(1) Thermo-gravimetric analysis (TGA)

TGA of *calcium reinforced BC based hydrogel scaffolds* was performed by the TA Q500 apparatus (TA Instruments, USA). The analysis was performed with 7-8 mg amount of freeze dried sample with platinum pan, at the constant heating rate of

10°C/min between the temperature ranges from 25–700°C under nitrogen atmosphere (50 mL/min).
The TGA was performed in triplicate (n = 3).

(2) Differential scanning calorimetry (DSC)

DSC of freeze dried samples of *calcium phosphate reinforced BC based hydrogel scaffolds* was performed to determine the thermal characteristics of the hydrogel scaffolds. 5–8 mg of the sample was taken. The DSC was measured with a differential scanning calorimeter (Mettler Toledo, Columbus, Ohio, USA) at a temperature range of -50 to 500°C in the presence of the nitrogen gas flow rate at 50 mL/min.
The DSC study was performed in triplicate (n = 3).

2.7 Mechanical Property analysis

The mechanical characterization of *calcium reinforced BC based hydrogel scaffolds* was performed through the following methods:

(1) Viscoelastic behavior analysis

The viscoelastic property of the *calcium reinforced BC based hydrogel scaffolds* samples was studied by using a modular compact rheometer testing device (Anton Paar, MCR 502, Austria) and "Rheoplus" software package for data analysis. Dynamic frequency sweep analysis was conducted in the linear viscoelastic region (LVR) at 1% strain amplitude at room temperature (28°C), and human physiological temperature (37°C). The viscoelastic behavior was analyzed with both "before dry sample" sections (i.e. samples before freeze dried) and "swelled samples" sections (i.e. sample sections after swelling in physiological saline solution) (20 mm diameter) in the oscillation mode with the angular frequency range from 0.1 to 100 rad s^{-1} and in some fixed angular frequencies (0.39 rad s^{-1}, 3.9 rad s^{-1}, 39 rad s^{-1}) at 37°C. In addition, viscoelastic behavior of swelled *calcium phosphate reinforced BC based hydrogel scaffolds* were studied at human fever temperature (39°C) from 0.1 to 100 rad s^{-1}. The tan δ value was also determined. The influence of angular frequency on storage (G') and loss (G'') modulus and complex viscosity (η*) was calculated by the following equation:

$$\eta^* = [(G'/\omega)^2 + (G''/\omega)^2]^{1/2} \quad \text{.....(6)}$$

The viscoelastic analysis was performed in triplicate (n = 3). The data were presented as mean± standard error of the mean.

(2) Compression test

The compressive strength of *calcium reinforced BC based hydrogel scaffold*s was determined by using Testometric M350-5CT, England, UK at room temperature (20°C) with a load cell having a full scale 300 kgf. Sections (20 mm diameter) from freeze dried hydrogel samples were taken for the study. The study was done with a compression rate of 1 $mm.min^{-1}$.

The compression study was performed in triplicate (n = 3). The data were presented as mean± standard deviation of the mean.

2.8 Biological Property analysis

Biological characterizations of the bone tissue engineering scaffold were performed on the basis of antibacterial study and *in vitro* analysis.

2.8.1 *Antimicrobial study*

The antibacterial activity of all *calcium reinforced BC based hydrogel scaffolds* were studied by following the *agar disc diffusion* method. The bacteria selected for this study are *Staphylococcus aureus* (*S. aureus CCM 4516; Gram + ve bacteria*) and *Escherichia coli* (*E. coli CCM 4517; Gram – ve bacteria*). 8 mm circular specimens were selected from the all *calcium reinforced BC based hydrogel scaffolds*. Specimens were sterilized by treating with 1 mL of 96% ethanol for 40 min. Then the specimens were dried at 30–32°C and again UV sterilized for 30 minutes. 100 mL of bacterial suspension was used for this study. The analysis was performed using sterile nutrient agar (2%) medium. Finally, testing plates were then incubated in a temperature controlled incubator at 37°C for 24 h. The antibacterial property was analyzed by observing the zone of inhibition. The antibacterial property analysis was performed in triplicate (n = 3).

2.8.2 *Cell biological study*

The cell biological analysis of *calcium reinforced BC based hydrogel scaffolds* was performed through the following studies:

2.8.2. a Biocompatibility study

Assessment of bio-compatibility of a tissue engineering scaffold material is a complex procedure. It involves different safety and pre-clinical evaluation. The biological assessment of medical devices is regulating by internationally recognized standards i.e. International Organization for Standardization (ISO) standard ISO-10993-5. Cell cytotoxicity and viability is the most important consideration of biological assessment of the scaffold.

i. Cell culture

Cells (Human fibroblast, Lep-3; Mouse bone explant cells, BEC; human osteosarcoma cells, Saos-2; Human mesenchymal stem cells, MSC) were used as model systems in our study. Cells were obtained from the Cell culture collection of the Institute of Experimental Morphology, Pathology and Anthropology with Museum—Bulgarian Academy of Sciences (IEMPAM-BAS). The cultures were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The cultures were kept in a humidified incubator (Thermo Scientific, HEPA Class 100, Waltham, MA, USA) at 37°C under 5% CO_2 in air. For routine passages, the cells were detached using a mixture of 0.05% trypsin and 0.02% EDTA. The cell cultures were passaged 2–3 times per week (1:2 to 1:3 split). The experiments were performed during the exponential phase of cell growth. During investigations performed the BEC cells were on their 44–50th passages.

ii. Sample preparation

Circular sections (6 mm in diameter) of the six freeze dried *calcium reinforced BC based hydrogel scaffolds* were taken for this study. Sections were placed in the 48-well cell culture plate, treated with 50 µL of 96% ethanol for 40 min after which the ethanol was removed and dried at 30–32°C to complete dryness. Then, the materials were sterilized under the exposure of UV radiation for 80–90 min.

Indirect and direct experiments were performed for evaluating the influence of the materials on cell viability and proliferation.

iii. Indirect experiments

For cell viability/proliferation study, the cells (i.e., Lep-3, BEC, Saos-2, MSC) were seeded in 96-well flat-bottomed microplates at a specific concentration of (1×10^4 cells/well for Lep-3 and BEC; 7.5×10^3 cells/well for Saos-2) in fresh DMEM medium with 10% FBS. At the 24^{th} h, the culture medium from each well was removed and changed with 100 µL DMEM containing hydrogel scaffolds extract (sample extracts, obtained after 1-, 3-, 5-and 7-day incubation periods).

The percent of viable cells was determined using MTT (*3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)* test. The cells were incubated for 3 h with MTT solution (0.3 mg MTT in 10 mL DMEM) at 37°C under 5% CO_2 condition. The formed blue MTT formazan was extracted with a mixture of absolute ethanol and DMSO (1:1, v/v). The quantitative analysis was performed by absorbance measurements in an automated microplate reader (Tecan, Sunrise™, Grödig, Austria) at 540/620 nm.

iv. Direct experiment

Each material was placed in bottom of a 48 well cell culture plate on the drop (20 µL) of FBS for 30 min at 30–32°C in order to stick the sample to the surface of the well. Thereafter, 1 mL of DMEM (containing 10% FBS and antibiotics) was given to the wells (containing sterilized sample sections as well as empty wells that serve as controls).

The cells (5×10^4 cells/well, Lep-3, BEC, Saos-2) were seeded directly on the material sample placed on the bottom of a 48-well cell culture plate and incubated for 1, 3, 5, and 7 days in CO_2 incubator at 37°C. The number and viability of the cells were determined before seeding using an automated cell counter and the trypan blue dye exclusion technique (zero time). The cell viability was found to be >95% in all experiments performed. At the start of the experiment the cell numbers were equal in all wells/ between all different samples, and they were cultured in equal conditions. Cells were grown in wells without materials, which served as controls. The effect of the materials on cell viability and proliferation was studied by MTT test. MTT concentration corresponded to the volume of the plate. Like those discussed in

indirect experiment, the cells here were also incubated in MTT solution at 37°C under a 5% CO_2 condition. The quantitative analysis was performed following the extraction of blue colored formazan.

The data were presented as mean±standard error of the mean. Statistical differences between control and treated groups were assessed using one-way analysis of variance (ANOVA) followed by suitable post-hoc test by using GraphPad Prism version 5.00 (San Diego, CA, USA) for Windows and MS Office 2010 (Redmond,WA, USA).

2.8.2.b DNA damage analysis

The presence of single and double stranded DNA damages within the cells induced by the effect of *calcium reinforced BC based hydrogel scaffolds* was assessed by single cell gel electrophoresis (Comet assay) at alkaline pH. Cells (Lep-3) were seeded in 6-well flat-bottomed microplates at a concentration of 3×10^5 cells/well. At the 24^{th} hour the culture medium was removed and changed with sample extract media (3-days modified media) and control media was also prepared. The cells were mixed with 1.4% of low-gelling agarose (Sigma Type II) and immediately spread onto microscopic slides pre-coated with 0.5% normal agarose. Then the cells were lysed for 1 h in a lysis solution (1 M NaCl, 50 mM EDTA pH 8, 30 mM NaOH, 0.1% N-lauroylsarcozine; pH 10). After 1-h incubation in the denaturing solution (30 mM NaOH, 10 mM EDTA; pH 12.6) for DNA unwinding, the slides were electrophoresed for 20 min at 0.46 V/cm in the same denaturing buffer. At the end of the electrophoresis, the slides were subsequently dehydrated for 5 min in 75% and in 96% of ethanol. Comets were observed under Leitz epifluorescent microscope (Orthoplan, VARIO ORTHOMAT 2) using a 450–490 nm band-pass filter following staining of microgels with the fluorescent dye SYBR green I (Molecular Probes, Eugene, OR, USA). 1000 randomly chosen objects per each probe and treatment were taken for quantification. Two repetitions of the experiment were done and standard deviations were quantified. In all cases they were very small.

2.8.2.c Apoptosis/necrosis analysis

The ability of the materials to induce cells death was evaluated by APOPTOSIS detection kit, (ANNEXINV—GFP-Certified Apoptosis/Necrosis detection kit, Enzo Life Sciences). Cells were spinned down at 400 g for 5 min at room temperature and carefully re-suspended in 1 mL cold 1×PBS (2.68mM KCl, 1.47mM KH_2PO_4, 1.37mM NaCl, 8mM Na_2HPO_4), pH 7. Spinning down follows at the same conditions

and the pellet was re-suspended in 510 µL Dual Detection Reagent (500µL 1×binding buffer, 5µL Apoptosis Detection reagent/Annexin V-Enzo Gold; 5µL Necrosis Detection Reagent). Samples were incubated at room temperature for 10 min at dark and were analyzed via cytometry using 488 nm laser at FL 2 and FL 3 channels for apoptosis and necrosis detection respectively. Results were quantified with FlowJo software. Two repetitions of the experiment were done. Data quantitation included estimation of the percentage of cells undergoing apoptosis and necrosis and the ratio between these percentages in comparison to the control cells is given in PDU (procedure data units).

2.8.2. d Bone marker analysis

The activity of alkaline phosphatase (ALP) was assessed in the 5 days modified cell culture medium with cells (Saos-2 and MSC cells). The cells were seeded in 6-well flat-bottomed microplates at a concentration of 2×10^4 for Saos-2 cells/well to 4×10^4 for MSC cells/well. Sample volume was adjusted with 80 µL/well with assay buffer. Alkaline phosphatase (ALP) activity was evaluated by standard colorimetric kit as recommended by the manufacturer protocol. The data presented here as mean± error of the mean.

2.8.2.e Cell-biomaterial interaction study

The samples prepared as described in biocompatibility analysis. The cells (7×10^5 cells/well, Lep-3) were seeded directly on sample materials in a 48-well cell culture plate and left in an incubator (Thermo Scientific) at 37 °C, 5% CO_2. After 7 days' incubation period, the culture medium was removed and the sample sections were washed with 4% glutaraldehyde for 1h followed by washing with double distilled water. The samples were then subjected to dry by filter system CORNING 431097 (0.22 µm) under low pressure for 2 h and were left for 2 days at 30–32 °C for complete drying. Finally, samples were prepared for SEM analysis by a standard procedure and observed under scanning electron microscope (JEOL JSM-5510, Tokyo, Japan) at an accelerating voltage of 10 kV.

3. MOTIVATION OF THE DOCTORAL STUDY

Different pathological and traumatic events lead to significant bone fracture situation [1]. On the other hand, aging and bone related diseases also increase the probability of bone fracture. Research indicated that about 75 million individuals have been troubled by osteoporosis in Europe, USA and Japan [11]. Study has shown that bone related diseases like osteoporosis causes two million bone fractures every year and this number will increase to three million by 2025.

Treatment methods including bone-autografting and allografting have been used for so long. However, various limitations also exist for the successful utilization of these methods [1]. The autograph based approach involves the potential surgical risks like infection and inflammation, bleeding, hypersensitivity, and damage of donor tissue are also prominent [1]. Thus, a novel approach is important to solve this fracture related problem. Different biomaterial and scaffolds from metal to ceramic have been successfully used in tissue engineering application. However, they have also limitations.

Cellulose is considered as significant and naturally abundant biopolymer. Bacterial cellulose has been extensively used in biomedical application. The advantage of BC over other polymers involves the fact that BC has significant controllable three dimensional fibrous network structures and notable biocompatibility. BC has a significant use as bone tissue engineering scaffold due to its remarkable similarity with bone extra cellular matrix and collagen. The BC based 3D hydrogel thus can be also utilized in BTR application.

Bone cells composed of different receptors and ion channels which can successfully receive extracellular calcium that is also responsible for the growth of the cells [1, 2]. Studies demonstrated that incorporation of bioactive compounds within a bone tissue engineering scaffold has a significant role in increase of *osteoconductive* and *osteoinductive* property of the scaffold [2]. This doctoral study focuses the development of *calcium reinforced BC based hydrogel scaffolds*, which could provide "osteoconduction" through its efficient scaffold structure and also "osteoinduction" through providing osteogenic signal to the bone cells.

4. AIM AND BRIEF SUMMARY OF THE THESIS

The major ***aim*** of this doctoral study is the *preparation and characterization of calcium reinforced BC based hydrogel scaffold for bone tissue regeneration application.* Hence, this doctoral thesis work is directed on the following aspects (as mentioned below).

- **Preparation of *calcium reinforced BC based hydrogel scaffolds***
  - *Preparation of raw material, i.e. bacterial cellulose*
    BC, one of the key raw material is synthesized for the preparation of hydrogel scaffolds.
  - *Preparation of calcium reinforced polymeric hydrogel scaffolds*
    - *Calcium phosphate reinforced BC based hydrogel scaffolds*
    - *Calcium phosphate and calcium carbonate reinforced BC based hydrogel scaffolds.*
- **Evaluation of properties of the *calcium reinforced BC based polymeric hydrogel scaffolds*** on the basis of:
  - *Structural property*
    - Morphology (Surface/Cross section)
    - Physio-chemical property
    - Swelling
    - Porosity
    - Biodegradation
    - Thermal analysis
    - Mechanical property analysis
  - *Functional property*
    - Biological property analysis
      - *Antibacterial property analysis*
      - *Cell biological property analysis*
- **Selection of a suitable *composition* for polymeric hydrogel scaffold** on the basis of *its structural* and *functional* (biological activity) *properties* to recommend for its application for bone tissue regeneration.

The ***brief summary*** of this doctoral work which were also submitted/accepted, published and in preparation in the form of 7 articles are explained below:

Article I entitled "***Inorganic calcium filled bacterial cellulose based hydrogel scaffold: novel biomaterial for bone tissue regeneration***" emphasized the structure, physio-chemical characterization of *calcium reinforced BC based hydrogel scaffolds* (i.e. *calcium phosphate reinforced BC based hydrogel scaffolds* and *calcium phosphate and calcium carbonate reinforced BC based hydrogel scaffolds*). At first, the raw material, BC was synthesized in presence of basal synthetic Hestrin-Schramm (HS) nutritive medium (pH 7.0) using *Gluconacetobacter xylinus CCM 3611^T* (syn. *Acetobacter xylinum*), incubated at 30°C for 15 days. Then the BC was treated in 0.5N NaOH solution and heated at 80°C for 2 hours for decontamination. Thereafter, a homogenous suspension of BC (particle size range: 159 nm -351.03 nm) from the obtained BC mat was prepared by grinding (10-12 mins) the BC mat in distilled water. The grinded BC suspension (50 mL BC suspension ≅ 0.5 g of BC) was then used to prepare BC based hydrogel. Additionally, synthetic polymers like PVP and/or CMC (used for biocompatibility), PEG (used for reducing the cytotoxic effect), Agar (used as a gelling agent), and Glycerin (used as a humectant) were also utilized to prepare BC-PVP and BC-CMC hydrogel matrix. Furthermore, *calcium reinforced BC based hydrogel scaffolds* were then prepared in the form of *calcium phosphate reinforced BC based hydrogel scaffolds* & *calcium phosphate and calcium carbonate reinforced BC based hydrogel scaffolds* (i.e. *in vitro* bio-mineralized scaffolds). Earlier research by other researchers reported that the ratio of β-TCP: HA= 20:80 was found significant for osteogenic growth of bone cells. Thus, β-TCP: HA= 20:80 was initially selected for this study. *Calcium phosphate reinforced BC based hydrogel scaffolds (BC-PVP-β-TCP/HA_20:80 and BC-CMC-β-TCP/HA_20:80)* were prepared by incorporating calcium phosphate [in the form of β-tri-calcium phosphate (β-TCP) and hydroxyapatite (HA) in the ratio of 20:80] within the BC-PVP and BC-CMC hydrogel matrix. Thereafter, the *calcium phosphate reinforced BC based scaffolds* were *in vitro* bio-mineralized for the preparation of *calcium phosphate and calcium carbonate reinforced BC based hydrogel scaffolds (BC-PVP-β-TCP/HA-$CaCO_3$ and BC-CMC-β-TCP/HA-$CaCO_3$)*. BC-PVP and BC-CMC were kept as control set. Thereafter, the hydrogel scaffolds were characterized for BTR application. FTIR and TG analysis indicated the presence of BC and calcium phosphate and calcium carbonate within the hydrogel scaffolds. SEM study established the porous structures (50-200 μm) within the scaffolds. Swelling study

indicated significant swelling ability of *calcium phosphate reinforced BC based hydrogel scaffolds*. Compressive strength (0.24-0.60 MPa) of the *calcium reinforced BC based hydrogel scaffolds* were found similar like human trabecular bone. On the other hand, antibacterial study indicated that *calcium phosphate & calcium carbonate reinforced BC based hydrogel scaffold*, especially *BC-CMC-β-TCP/HA-$CaCO_3$* hydrogel scaffold has a faint antibacterial property only for *S. aureus*. Furthermore, notable human fibroblast (Lep-3) cell viability was also found. The results indicated that *calcium reinforced BC based hydrogel scaffolds* have the necessary characteristics to be utilized in bone regeneration application. These above mentioned observations and results were ***reported and published*** in "*International Journal of Polymeric Materials and Polymeric Biomaterials*" (**2019**), 68:1-3, 134-144. DOI: https://doi.org/10.1080/00914037.2018.1525733 (Web of Science Indexed, Q2 [Polymer Science], J_{imp}: 2.263)

Article II entitled "***Rheological Performance of Bacterial Cellulose based non-mineralized and mineralized hydrogel scaffolds***" focused the analysis of rheological/viscoelastic property of *calcium reinforced BC based hydrogel scaffolds*. Here non-mineralized hydrogel scaffolds referred to *calcium phosphate reinforced BC based hydrogel scaffolds (BC-PVP-β-TCP/HA_20:80 and BC-CMC-β-TCP/HA_20:80)* and mineralized hydrogel scaffolds referred to *calcium phosphate and calcium carbonate reinforced in vitro bio-mineralized BC based hydrogel scaffolds (BC-PVP-β-TCP/HA-$CaCO_3$ and BC-CMC-β-TCP/HA-$CaCO_3$)*. BC-PVP and BC-CMC were kept as control set. The viscoelastic property analysis indicated the mechanical strength of the hydrogel scaffolds in room temperature (28°C). It is observed that all the *calcium reinforced BC based hydrogel scaffolds (BC-PVP-β-TCP/HA_20:80 and BC-CMC-β-TCP/HA_20:80, BC-PVP-β-TCP/HA-$CaCO_3$ and BC-CMC-β-TCP/HA-$CaCO_3$)* were significantly viscoelastic in nature. They maintained high elastic property (expressed in terms of storage modulus [G']) than viscous property (expressed in terms of loss modulus [G"]). These above-mentioned results were ***reported and published*** in "*AIP Conference Proceedings related to Novel Trends in Rheology VII"* (**2017**), 1843, 050008-1–050008-7. DOI: https://doi.org/10.1063/1.4983000 (Web of Science Indexed; Book Chapter, ISBN: 978-0-7354-1513-3).

Article III entitled "***Biocompatibility and Biological Efficiency of Inorganic Calcium Filled Bacterial Cellulose Based Hydrogel Scaffolds for Bone Bioengineering***" focused the study of biocompatibility and cell biological efficiency analysis of *calcium reinforced BC based hydrogel scaffolds* (ie. *calcium phosphate reinforce BC based hydrogel scaffold [BC-PVP-β-TCP/HA_20:80 and BC-CMC-β-TCP/HA_20:80] and calcium phosphate and calcium carbonate reinforced BC based hydrogel scaffolds [BC-PVP-β-TCP/HA-$CaCO_3$ and BC-CMC-β-TCP/HA-$CaCO_3$])*. BC-PVP and BC-CMC were also kept as control set. Earlier, the *Article I* and *Article II* indicated that *calcium reinforced BC based hydrogel scaffolds* has the necessary physiochemical and mechanical property to induce the cell growth for bone tissue regeneration. To analyse the effect of hydrogel scaffolds on the cellular level, the biocompatibility study was performed with human diploid fibroblast, Lep-3 (from 3 month's old human embryo) cell and mouse bone explant cells (BEC; from 2-3 months old ICR mice). The cell proliferation was found notable for 3-7 days of incubation with *calcium reinforced BC based hydrogel scaffolds*. In addition, the fibroblast (Lep-3) apoptosis was found insignificant for *BC-PVP-β-TCP/HA_20:80* hydrogel scaffold. Additionally, cell-biomaterial interaction study through SEM analysis demonstrated the notable growth of Lep-3 cell on to the surface of *BC-PVP-β-TCP/HA_20:80* hydrogel scaffold. Thus, according to the research observation, the "***BC-PVP-β-TCP/HA****"* hydrogel scaffold was found a better combination among the *scaffold*s that can be further analysed for bone tissue regeneration application. The above-mentioned observations were ***reported and published*** in "*International Journal of Molecular Sciences*" (**2018**), 19 (12):3980. DOI: 10.3390/ijms19123980 (Web of Science Indexed, Q2 [Multidisciplinary, Chemistry, Biochemistry & Molecular Biology], J_{imp}: 4.183)

Article IV entitled "***Swelling and rheological study of calcium phosphate filled bacterial cellulose based hydrogel scaffold***" involved the analysis of swelling and thereby study of viscoelastic property of the swelled *calcium reinforced BC based hydrogel scaffolds*. Earlier, *Article III* demonstrated that the "***BC-PVP-β-TCP/HA****"* hydrogel scaffold has the necessary physiochemical and cell biological characteristics to be utilized bone regeneration application. Thus, in this work, keeping the *BC-PVP-β-TCP/HA_20:80* as a reference sample, different "*BC-PVP-β-TCP/HA"* scaffolds were developed and analysed on the basis of swelling and viscoelasticity. The β-TCP and HA were incorporated in the ratio of 10:90, 20:80, 40:60, 50:50, 60:40 into the scaffold matrix to achieve the *calcium phosphate*

reinforced BC based hydrogel scaffolds and were termed as, "BC-PVP-β-TCP/HA_10:90" (for β-TCP: HA= 10:90), BC-PVP-β-TCP/HA_20:80" (for β-TCP: HA= 20:80), BC-PVP-β-TCP/HA_40:60" (for β-TCP: HA= 40:60), BC-PVP-β-TCP/HA_50:50" (for β-TCP: HA= 50:50), BC-PVP-β-TCP/HA_60:40" (for β-TCP: HA= 60:40). *BC-PVP-β-TCP/HA_20:80* was kept as reference sample. BC-PVP scaffold was also used as a control. Analyses of swelling and viscoelastic property is important in respect to the utilization of the *calcium phosphate reinforced BC based hydrogel scaffolds* in bone tissue regeneration application. Thus, *the developed calcium phosphate reinforced hydrogel scaffolds (i.e. BC-PVP-β-TCP/HA_10:90, BC-PVP-β-TCP/HA_20:80, BC-PVP-β-TCP/HA_40:60, BC-PVP-β-TCP/HA_50:50, BC-PVP-β-TCP/HA_60:40)* were first analyzed on the basis of swelling at physiological temperature (37^{o}C) in physiological saline solution (Ringer solution; pH. 7.4), then the viscoelastic property of the scaffolds was analyzed both at room temperature (28^{o}C) and physiological temperature (37^{o}C) regime. Furthermore, the morphological characterization of the hydrogel scaffolds at "before swelling" and "after swelling" was also analyzed. All the BC based hydrogel scaffolds have shown notable viscoelastic property both at 28^{o}C and 37^{o}C. However, among the all *calcium phosphate reinforced BC based hydrogel scaffolds*, the *BC-PVP-β-TCP/HA_20:80* and *BC-PVP-β-TCP/HA_50:50* hydrogel scaffolds were found significant in the context of swelling and rheological property. The above-mentioned results were ***reported and published*** in "*Journal of Applied Polymer Sciences*" (**2020**), 137, 48522. DOI: 10.1002/app.48522. [Web of Science Indexed, Q2 [Polymer Science], J_{imp}: 2.188).

Article V entitled "***Viscoelastic behavior of Calcium Phosphate Packed Bacterial Cellulose -Polyvinylpyrrolidone based Hydrogel Scaffolds at Human Fever Temperature***" emphasized the analysis of swelling and viscoelastic property of *calcium phosphate reinforced BC based hydrogel scaffolds* at altered human physiological temperature i.e. human fever temperature (39^{o}C). Earlier, *Article IV* demonstrated and reported that all *calcium phosphate reinforced BC based hydrogel scaffolds* showed significant swelling and rheological behavior at both room temperature (28^{o}C) and human physiological temperature (37^{o}C). Especially, *BC-PVP-β-TCP/HA_20:80*, *BC-PVP-β-TCP/HA_50:50* and also *BC-PVP-β-TCP/HA_40:60* hydrogel scaffolds were showing notable swelling and rheological property among all *calcium phosphate reinforced BC based hydrogel scaffolds*. Thus, this work focused the analysis of swelling and viscoelastic property at human fever

temperature (39°C) with *BC-PVP-β-TCP/HA_20:80*, *BC-PVP-β-TCP/HA_40:60* and *BC-PVP-β-TCP/HA_50:50* hydrogel scaffolds. It has been observed that all the three *calcium phosphate reinforced BC based hydrogel scaffolds* were showing significant swelling ability and viscoelastic behavior at human fever temperature (39°C). The above-mentioned result was ***reported and accepted for publication*** in "*AIP conference proceedings related to PPS Europe-Africa 2019 Regional Conference*" (**2020**) (Will be indexed in Web of Science, Book Chapter).

Article VI entitled "***Calcium Phosphate Incorporated Bacterial Cellulose-Polyvinylpyrrolidone Based Hydrogel Scaffold: Structural Property and Cell Viability Study for Bone Regeneration Application***" emphasized the physical property analysis like void fraction, porosity, study of biodegradability and primary cell viability property. Earlier, *Article IV* and *Article V* were reported the analysis of the important implant properties like swelling and viscoelastic behavior of *calcium phosphate reinforced BC based hydrogel scaffolds*. It has been observed at human physiological temperature regime (37°C), *BC-PVP-β-TCP/HA_20:80*, and *BC-PVP-β-TCP/HA_50:50* hydrogel scaffolds have indicated notable swelling and viscoelastic behavior. Furthermore, at human fever temperature (39°C), BC-PVP-β-TCP/HA_20:80, BC-PVP-β-TCP/HA_40:60 and BC-PVP-β-TCP/HA_50:50 hydrogel scaffolds also have demonstrated notable swelling and rheological property. Hence, in this work, the physical property and primary cell viability behavior study were performed with *BC-PVP-β-TCP/HA_20:80*, *BC-PVP-β-TCP/HA_40:60* and *BC-PVP-β-TCP/HA_50:50* hydrogel scaffolds. BC-PVP was kept as control set. The porosity and void fraction of *BC-PVP-β-TCP/HA_50:50* hydrogel scaffold was found higher than other two calcium reinforced BC based hydrogel scaffolds. Additionally, *BC-PVP-β-TCP/HA_50:50* hydrogel scaffold has the highest degree degradation than *BC-PVP-β-TCP/HA_20:80* and *BC-PVP-β-TCP/HA_40:60* hydrogel scaffolds. On the other hand, compressive strengths of all the *calcium reinforced hydrogel scaffolds* were found comparable with the human cancellous bone. Furthermore, Saos-2 cell viability was found significant for all the *calcium phosphate reinforced BC based hydrogels*. Thus, in this work, this was suggested that the *BC-PVP-β-TCP/HA_20:80*, *BC-PVP-β-TCP/HA_40:60* and *BC-PVP-β-TCP/HA_50:50* hydrogel scaffolds can be further evaluated on the basis of detailed *in vitro* efficiency in the context of bone tissue regeneration application. The above-mentioned observations were ***reported and published*** in "*Polymers*" (**2019**), 11, 1821. DOI:10.3390/polym11111821 (Web of Science Indexed, Q1 [Polymer Science], J_{imp}: 3.164).

Article VII entitled "***In vitro efficiency of calcium phosphate incorporated bacterial cellulose based hydrogel scaffold for bone regeneration***" emphasizes the *in vitro* efficiency of *calcium reinforced BC based hydrogel scaffolds*. Earlier in *Article VI*, according to the physical, biodegradable, mechanical and primary cell viability property of three *calcium phosphate reinforced BC based hydrogel scaffolds*: *BC-PVP-β-TCP/HA_20:80*, *BC-PVP-β-TCP/HA_40:60* and *BC-PVP-β-TCP/HA_50:50* were suggested for the further analysis in the context of biological applicability for bone regeneration application. Thus, this work focuses in-depth analysis of *in vitro* cell biological efficiency of the *BC-PVP-β-TCP/HA_20:80, BC-PVP-β-TCP/HA_40:60* and *BC-PVP-β-TCP/HA_50:50 hydrogel scaffolds*. BC-PVP scaffold was kept as control set. The *in vitro* efficiency was studied on the basis of comparative analysis of the biocompatibility (through MTS analysis) with human osteosarcoma cell line, Saos-2 and human adipose derived mesenchymal stem cells (MSC). Additionally, ki-67 protein expression (marker for cell proliferation) analysis was performed. Furthermore, bone marker study (Alkaline phosphatase expression) was also analyzed. Finally, cell-biomaterial interaction was analyzed by SEM study. Albeit, notable biocompatibility was observed with all the hydrogel scaffolds however, a significant MSC biocompatibility was found for *BC-PVP- β-TCP/HA_20:80* and *BC-PVP- β-TCP/HA_50:50*. Both Saos-2 and MSC cells also expressed ki-67 protein for cell proliferation. Additionally, it was observed that both the cell line expressed notable alkaline phosphatase (bone marker) in presence of the *BC-PVP- β-TCP/HA_20:80* and *BC-PVP- β-TCP/HA_50:50* scaffolds extracts. Finally, the SEM study indicated a significant growth of Saos-2 cells on to the surface of the *calcium phosphate reinforced BC based hydrogel scaffolds: BC-PVP- β-TCP/HA_20:80* and *BC-PVP- β-TCP/HA_50:50*. Thus, these results indicated the future possibility of *BC-PVP- β-TCP/HA_20:80* and *BC-PVP- β-TCP/HA_50:50* hydrogel scaffolds to be utilized in bone tissue engineering application. These scaffolds were recommended for further analysis on the basis of *in vivo* evaluation for soft bone tissue engineering. The ***above-mentioned results are being prepared and will be submitted*** in a Web of Science indexed impact factor journal.

5. CONCLUDING REMARKS

5.1 Conclusion

This doctoral work focuses the ***development and characterization of a novel calcium reinforced BC based hydrogel scaffold suitable for BTR application***. The hydrogel scaffolds were developed with natural polymer, BC. Additionally, other synthetic polymers: PVP, CMC, PEG etc. were also utilized. *Calcium reinforced BC based hydrogel scaffolds* were prepared in the form of *calcium phosphate reinforced BC based hydrogel scaffolds (BC-PVP-β-TCP/HA and BC-CMC- β-TCP/HA)* and *calcium phosphate and calcium carbonate reinforced BC based hydrogel scaffolds (BC-PVP-β-TCP/HA_$CaCO_3$ and BC-CMC- β-TCP/HA_$CaCO_3$)*. The *calcium phosphate reinforced BC based hydrogel scaffolds* were *in vitro* bio-mineralized to develop *calcium phosphate and calcium carbonate reinforced BC based hydrogel scaffolds*. They were then characterized on the basis of structural and functional properties.

The **structural property** of calcium reinforced BC based hydrogel scaffolds was analyzed. Study indicated that the swelling ability and porosity of *BC-PVP-β-TCP/HA_20:80 and BC-CMC-β-TCP/HA_20:80* were significant and higher than *BC-PVP-β-TCP/HA_$CaCO_3$ and BC-PVP-β-TCP/HA_$CaCO_3$*. Moreover, among *calcium phosphate reinforced hydrogel scaffolds*, the swelling property was found notable for *BC-PVP-β-TCP/HA_20:80, and BC-PVP-β-TCP/HA_50:50* scaffolds. On the other hand, the FTIR and TGA studies indicated the presence of all of the necessary ingredients. The biodegradation of the *calcium phosphate reinforced hydrogel scaffolds* was also analyzed. Albeit, all the hydrogels were found hydrolytically degradable; however, *BC-PVP-β-TCP/HA_50:50* scaffold has shown notable hydrolytic degradation. On the other hand, all the hydrogel scaffolds were found to be viscoelastic in nature at room temperature (28^{o}C); however, the viscoelastic property of *BC-PVP-β-TCP/HA_20:80* and *BC-PVP-β-TCP/HA_50:50* scaffolds was notable at human physiological temperature (37°C) and human fever temperature (39°C). Additionally, the mechanical strength (i.e. compressive strength) of all *calcium reinforced BC based hydrogel scaffolds* were found similar with the human cancellous bone.

On the other hand, the **functional properties** of the *calcium reinforced BC based hydrogel scaffolds* were analyzed majorly on the basis of cell biological efficiency analysis. Antibacterial property of the hydrogel scaffolds was also analyzed with *Staphylococcus aureus* (*S. aureus* CCM [4516]) [gram +*ve* bacteria] and *Escherichia coli* (*E. coli* CCM [4517]) (Gram – *ve* bacteria). No significant antibacterial property was found. However, a faint antibacterial efficiency was only for *BC-CMC-β-TCP/HA_$CaCO_3$* with *S. aureus*. The *cell biological efficiency* was analyzed with *bone specific cells* with different cell lines like human fibroblast (Lep-3) cells, mouse bone explant cell line (BEC), human osteosarcoma cell line (Saos-2) and human mesenchymal stem cells (MSC). Albeit, all the *calcium phosphate reinforced BC based hydrogel scaffolds* and *calcium phosphate and calcium carbonate BC based hydrogel scaffolds* shown notable biocompatibility with human fibroblast (Lep-3) and bone explant cells (BEC); however, it was also observed that biocompatibility was significant for "*BC-PVP-β-TCP/HA_20:80*" hydrogel scaffold. Furthermore, the cellular apoptosis was also notably less for the above-mentioned scaffold. Thus, the "*BC-PVP-β-TCP/HA*" hydrogel scaffold was selected for further analysis and different "*BC-PVP-β-TCP/HA*" hydrogel scaffolds were prepared by applying different β-TCP and HA concentration ratios (w/w): *BC-PVP-β-TCP/HA_10:90, BC-PVP-β-TCP/HA_20:80, BC-PVP-β-TCP/HA_40:60, BC-PVP-β-TCP/HA_50:50, BC-PVP-β-TCP/HA_60:40*. On the basis of structural, physiochemical, mechanical/viscoelastic properties of the above-mentioned scaffolds, the *in vitro* efficiency was analyzed with *BC-PVP-β-TCP/HA_20:80, BC-PVP-β-TCP/HA_40:60, BC-PVP-β-TCP/HA_50:50* hydrogel scaffolds. Finally, the cell viability and biocompatibility of human osteoblast like cell line Saos-2 and human MSC confirmed the efficiency of the *BC-PVP-β-TCP/HA_20:80, BC-PVP-β-TCP/HA_50:50* hydrogel scaffolds. Additionally, cell biomaterial interaction and bone marker expression were also found significant for the *BC-PVP-β-TCP/HA_20:80, BC-PVP-β-TCP/HA_50:50* hydrogel scaffolds.

Hence, on the basis of above observations, the *calcium phosphate reinforced BC based hydrogel scaffolds*: ***BC-PVP-β-TCP/HA_20:80 and BC-PVP-β-TCP/HA_50:50*** were further recommended for the *in vivo* analysis and application in BTR.

5.2 Contribution of the doctoral work to society

This work focuses the preparation of hydrogel scaffold for BTR with osteoconductive and osteoinductive property. The hydrogel scaffolds were prepared with biopolymer, *BC*. Additionally, the bioactivity of the BC based hydrogel scaffold has been enhanced by reinforcing the scaffold with the incorporation of calcium mineral (calcium phosphate and/or calcium carbonate).

This study will elaborate the use of natural polymer based *calcium reinforced polymeric hydrogel scaffold* in bone tissue engineering application and can provide opportunity to the next generation scientific researchers to develop further modified and extremely specific polymeric hydrogel scaffolds intended for bone tissue regeneration application.

LIST OF TABLES

LIST OF FIGURES

SYMBOLS AND ABBREVIATIONS

BC	Bacterial Cellulose
BTR	Bone Tissue Regeneration
PVP	Polyvinylpyrrolidone
β-TCP	β tricalcium phosphate
HA	Hydroxyapatite

LIST OF PUBLICATIONS

This doctoral work of Mr. Probal Basu, entitled "***Study on Calcium Reinforced Polymeric Hydrogel Scaffolds for Bone Tissue Regeneration*****"** involves the following articles:

Article I

Basu P., Saha N & Saha P. (**2019**) "Inorganic calcium filled bacterial cellulose based hydrogel scaffold: novel biomaterial for bone tissue regeneration", ***International Journal of Polymeric Material and Polymeric Biomaterial*** (Web of Science indexed, Q2 [Polymer Science], **J_{imp}: 2.263**), 68:1-3, 134-144. DOI: https://doi.org/10.1080/00914037.2018.1525733

Article II

Basu P., Saha N, Bandyopadhyay S, Saha P. (**2017**) "Rheological Performance of Bacterial Cellulose based non-mineralized and mineralized hydrogel scaffolds", ***AIP Conference Proceedings*** **of Novel Trends in Rheology VII** (Web of Science Indexed; Book Chapter) 1843, 050008-1–050008-7. DOI: https://doi.org/10.1063/1.4983000. ISBN: 978-0-7354-1513-3

Article III

Basu P, Saha N, Alexandrova R, Andonova-Lilova B, Georgieva M, Miloshev G, Saha P. (**2018**) "Biocompatibility and Biological Efficiency of Inorganic Calcium Filled Bacterial Cellulose Based Hydrogel Scaffolds for Bone Bioengineering", ***International Journal of Molecular Sciences*** (Web of Science Indexed, Q2 [Multidisciplinary, Chemistry, Biochemistry & Molecular Biology], **J_{imp}: 4.183**) ***19*** (12): 3980. DOI: 10.3390/ijms19123980

Article IV

Basu P, Saha N & Saha P. (**2020**) "Swelling and rheological study of calcium phosphate filled bacterial cellulose based hydrogel scaffold", ***Journal of Applied Polymer Science*** [Web of Science Indexed, Q2 [Polymer Science], **J_{imp}: 2.188**), 137, 48522. DOI: 10.1002/app.48522

Article V

Basu P., Saha N., Saha P. (**2020**) **"**Viscoelastic behaviour of Calcium Phosphate Packed Bacterial Cellulose -Polyvinylpyrrolidone based Hydrogel Scaffolds at Human Fever Temperature", ***AIP conference proceedings*** **of PPS Europe-Africa 2019 Regional Conference** (will be in Web of Science Indexed, Book Chapter), ***In Press.***

Article VI

Basu P., Saha N, Saha P. (**2019**) "Calcium Phosphate Incorporated Bacterial Cellulose-Polyvinylpyrrolidone Based Hydrogel Scaffold: Structural Property and Cell Viability Study for Bone Regeneration Application", ***Polymers*** [Web of Science Indexed, Q1 [Polymer Science], **J_{imp}: 3.164**), 11(11), 1821. DOI:10.3390/polym11111821

Article VII

Basu P., Saha N., Alexandrova R., Andonova-Lilova, B., Saha, P. "*In vitro* efficiency of calcium phosphate incorporated bacterial cellulose based hydrogel scaffold for bone regeneration", ***Manuscript is in preparation and will be submitted*** *to an Impact Factor Journal*.

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Curriculum Vitae

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Education:	*2016 – to date* Tomas Bata University in Zlin, Center of Polymer Systems (Faculty of Technology), Ph.D. studies in Chemistry and Materials Technology, Specialization: *Technology of Macromolecular Compounds* *2010 – 2012* Presidency College, University of Calcutta, Kolkata, West Bengal, India. Master of Science degree in Zoology. *2007 – 2010* Asutosh College, University of Calcutta, Kolkata, West Bengal, India, Bachelor of Science in Zoology.

Exposure of Other Laboratory and Training School Attended in Abroad:

1. **Short Term Scientific Mission (STSM) visit, supported by** European Cooperation of Science and Technology (COST) NEWGEN MP1301 (European Union), 15th June-15th July, 2017 for working at Institute of Experimental Morphology, Pathology and Anthropology with Museum-Bulgarian Academy of Sciences (IEMPAM-BAS), Sofia, Bulgaria.

2. **Training School "Non Living Materials meet living Biology"**, 9th - 12th May, 2017 at Patras, Greece. Organized by European Ceramic Society (ECerS) and COST MP1301 "NEWGEN".

Projects:

- *IGA/CPS/2017/003* Preparation and characterization of a Bacterial cellulose (BC) based hydrogel with enhanced mechanical and bio-adhesive property. (**Co-investigator**)
- *IGA/CPS/2018/008* Bacterial Cellulose based Bioactive and Functional Biomaterials: Preparation and Characterization **(Principal Investigator**)
- *IGA/CPS/2019/003* Calcium phosphate filled bacterial cellulose based hydrogel scaffolds. (**Principal Investigator**)

List of publications

1. **Basu P.**, Saha N., Saha P. (**2020**) Viscoelastic behavior of Calcium Phosphate Packed Bacterial Cellulose -Polyvinylpyrrolidone based Hydrogel Scaffolds at Human Fever Temperature, *AIP conference proceedings* related to *Europe-Africa PPS2019 conference*, November 18-21, Pretoria, South Africa, ***In Press.***

2. **Basu P**, Saha N & Saha P. (**2020**) Swelling and rheological study of calcium phosphate filled bacterial cellulose-based hydrogel scaffold, *J. Appl. Polym. Sci*, 137, 48522.

3. **Basu P,** Saha N, Saha P. **(2019)** Calcium Phosphate Incorporated Bacterial Cellulose-Polyvinylpyrrolidone Based Hydrogel Scaffold: Structural Property and Cell Viability Study for Bone Regeneration Application, *Polymers*, 11(11), 1821.

4. **Basu P**, Saha N & Saha P. (**2019**) Inorganic calcium filled bacterial cellulose based hydrogel scaffold: novel biomaterial for bone tissue regeneration, *Int J Polym Mater*, 68:1-3, 134-144.

5. **Basu P**, Saha N, Alexandrova R, Andonova-Lilova B, Georgieva M, Miloshev G, Saha P. (**2018**) Biocompatibility and Biological Efficiency of Inorganic Calcium Filled Bacterial Cellulose Based Hydrogel Scaffolds for Bone Bioengineering, *Int. J. Mol. Sci*. ***19***(12): 3980.

6. **Basu P**, Saha N, Bandyopadhyay S, Saha P. (**2017**) Rheological Performance of Bacterial Cellulose based non-mineralized and mineralized hydrogel scaffolds, *AIP Conf. Proc*. **1843**, 050008-1–050008-7.DOI: 10.1063/1.4983000

7. Bhattacharya S and **Basu P**. (**2016**) The Southern House Mosquito, *Culex quinquefasciatus*: profile of a smart vector. *J. Entomol. Zool. Stud.* 2016; **4**(2): 73-81.

- # Conferences/seminar/workshop Attended

1. **Basu P,** Saha N, Saha P (**2019**) Biomechanical property of calcium phosphate filled bacterial cellulose based hydrogel scaffolds, 25th Congress of the European Society of Biomechanics (ESB-2019), July 7th- 10th, 2019, Vienna, Austria**.** Abst Pap. P. 562 (ISBN: 978-3-903024-96-0).
2. **Basu P**, Saha N, Saha P (**2019**) Calcium filled bacterial cellulose based composite hydrogel for bone tissue engineering: Optical microscopy and bone marker analysis, 35th International Conference of the Polymer Processing Society (PPS-35), May 26-30, 2019, Çeşme-İzmir, Turkey.
3. **Basu P**, Saha N, and Saha P (**2018**) Inorganic Calcium filled Bacterial Cellulose based Scaffold for Bone Regeneration, 4th International Conference on Biomedical Polymers & Polymeric Biomaterials, 15th July – 18th July 2018, Krakow, Poland, Abst Pap. P. 05 (ISBN: 978-83-65955-10-4).
4. **Basu P**, Saha N, and Saha P (**2017**) Calcium Phosphate loaded bioadhesive biopolymer based hydrogel scaffold: a novel biomaterial for bone tissue engineering, Proceedings of Eight Workshop on "Experimental Models and Methods in Biomedical Research", 14-16th June-2017, IEMPAM-BAS, Sofia, Bulgaria. ISSN: 1314-9091.
5. **Basu P**, Saha N, and Saha P. (**2017**) Rheological Performance of Bacterial Cellulose based non-mineralized and mineralized hydrogel scaffolds, Novel Trends in Rheology VII, 26th- 27th July-2017, Tomas Bata University in Zlin, Czech Republic.
6. **Basu P**, Saha N, Saha P (**2017**) Calcium phosphate filled bacterial cellulose based hydrogel scaffolds for dental and orthopaedic application, European Cooperation of Science and Technology (COST) NEWGEN MP1301 (European Union) Workshop and WG Meeting (Cluj-Napoca, Romania) on Biomaterials for Dental and Orthopaedic applications, Abst. Pap. P. 10.

7. **Basu P**, Saha N, Saha P (**2016**) Functional Significance of Three Dimensional Hydrogel Scaffolds in Bone Tissue Engineering – A Review, European Cooperation of Science and Technology (COST) NEWGEN MP1301 (European Union), Workshop and WG Meeting (Zlin, Czech Republic) on Hydrogel/ Biomineralized Biomaterial for Bone Tissue Regeneration, Abst. Pap. P. 22 (ISBN: 978-80-7454-623-5).

List of Awards/Scholarship

1. **ITC Conference Grant (CA-15216-1480)** for participating *25th Congress of the European Society of Biomechanics (ESB-2019)*, July 7th-10th, **2019**, Vienna, Austria
2. Awarded **Third Position** in Power-point presentation and English language contest in "*Show off/ Zeig Dich*" competition, 18th April, **2018** at Faculty of Humanities, Tomas Bata University in Zlin, Czech Republic.
3. **Short Term Scientific Mission (STSM) Fellowship,** *COST Action NEWGEN MP1301*, 15th June-15th July, **2017** for working at IEMPAM-BAS, Sofia, Bulgaria.
4. Awarded **First Position** in Poster Presentation Contest at Training School "*Non Living Materials meet living Biology*", 9th –12th May, **2017** at Patras, Greece. Organized by European Ceramic Society (ECerS) and COST Action NEWGEN MP1301.

Professional Experience

1. **Research Assistant, August, 2012 - August, 2016**: Virus research and vector borne diseases research at Graduate Department of Zoology, Asutosh College (University of Calcutta) Kolkata, India.

2. **Guest Faculty, from September, 2013 - March, 2015:** Department of Zoology, Asutosh College (University of Calcutta), Kolkata-700026, West Bengal, India.

Study on Calcium Reinforced Polymeric Hydrogel Scaffolds for Bone Tissue Regeneration

Studie polymerních hydrogelových scaffoldů plněných vápníkem pro regeneraci kostní tkáně

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