Tomas Bata University in Zlín
Faculty of Technology

Doctoral Thesis

Biomineralized and Stimuli Responsive Hydrogel for Biomedical Applications

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Degree Course	2808V006 Technology of Macromolecular Substances
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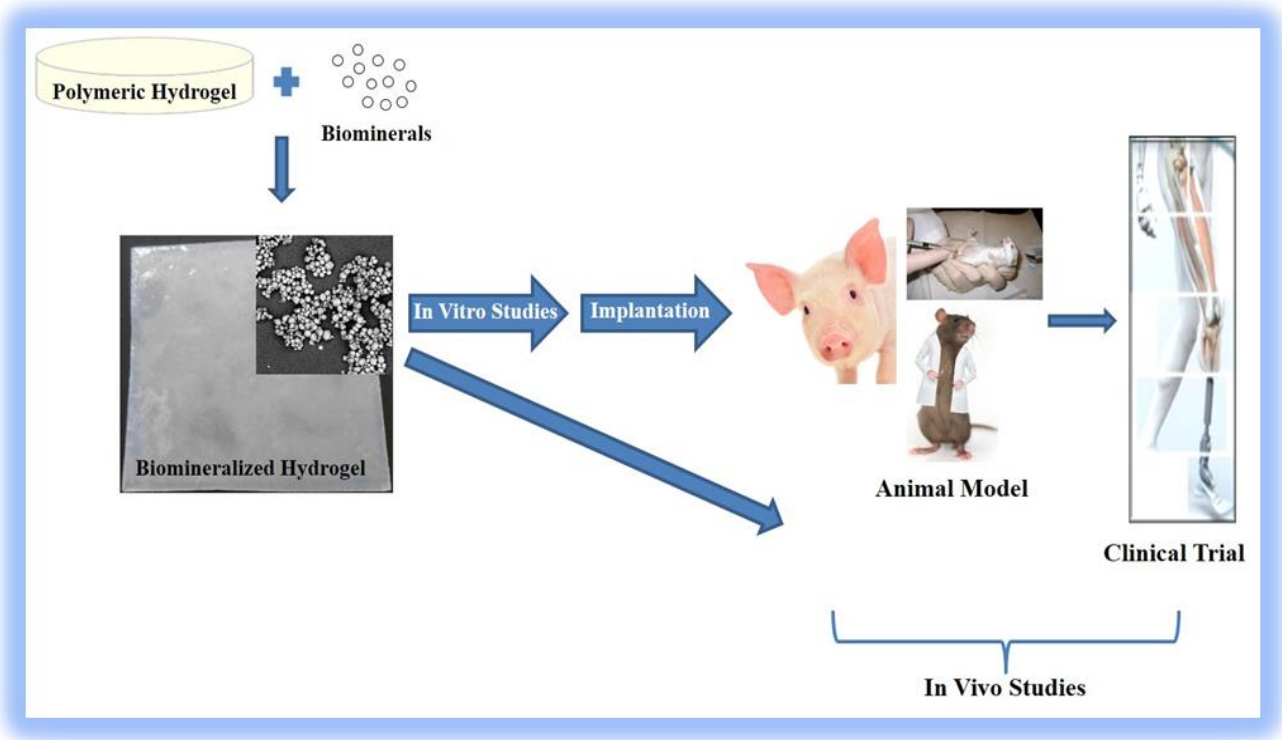
Zlin, December 2015

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them"

(*Sir William Henry Bragg)*



Proposed Future Application of Biomineralized Hydrogels

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ABSTRACT

In the last decade, tissue engineering and regenerative medicine actively focus on scaffolds which have a three dimensional structure for a better regeneration of tissue. Depending on the type of regeneration needed, the scaffolds can be prepared for both hard and soft tissues. Hard tissues are basically represented by bone composites containing organic matrix reinforced by inorganic minerals in the form of a hybrid structure. A relatively new concept in the development of scaffolds for the hard tissues is the formation of Biomimicry formed in the matrix through biomineralization. In these cases, the matrix is mostly represented by a biomaterial in which the crystal structure of minerals grows. From many available biomaterials hydrogels are preferred especially due to their ability to store large amount of liquid and create environment favourable for regeneration of living tissue.

The current doctoral thesis focuses on a research of establishing possible ways of biomimetic preparation of scaffolds through a mineralization process in the hydrogel matrix. Through a simple liquid diffusion technique, aqueous solutions of Na_2CO_3 and $CaCl_2$ were incorporated into the blend of PVP-CMC hydrogels, which was chosen to be an ideal matrix. A number of mineralized samples was proposed and prepared - differing in strength of concentration of the biomimetic process, formation of mineral crystal structure and different characteristics of the formed scaffold structure. These newly formed scaffold structures were named "**Biomineralized ($CaCO_3$) PVP-CMC hydrogel**."

Identifying the -CO_3 presence in the hydrogel structure, and thereby confirming the success of the biomineralization process was carried out by the FTIR method, which encountered the peaks at 1405cm^{-1} and 871cm^{-1}. XRD method then identified the calcite in the porous structure of PVP-CMC hydrogel. Morphological evaluation of the biomineralized structure through the SEM analysis proved that the micro-pores distribution within the hydrogel structure appeared in the range of 1-170 micrometres.

The level to which the pores were filled up by the calcite was evaluated by measurement of density of the samples in relation to non-filled system. Information about the composition of the scaffold system was added by the TGA measurement. Viscoelastic properties of the prepared systems were measured using parallel plate rheometer (ARES), where complex viscosity, storage and loss

moduli were evaluated. As per the expectations, the more intense the biomineralization process was, the lower the parameters determining the share of elasticity of the mineralized scaffold were and the higher the values determining the viscous properties. On top of that, mineralized scaffolds showed more difficulties to deformation in load in relation to non-filled hydrogels which can influence the ways of application of hard biomineralized scaffolds.

Further, the conditions influencing the regeneration effectivity of mineralized scaffolds were investigated. The scaffolds were evaluated from the points of view of swelling ability of biological solutions containing glucose, urea and physiological solutions. Experiments were carried out under physiologically relevant conditions. Evaluation was done based on relative increase of volume of the scaffold. The highest swelling ability was in the presence of urea solution followed by the physiological solution. Glucose solution had the least swelling ability. Eventually, the starting experiments evaluating cytotoxicity were carried out. Mouse embryonic fibroblasts were placed into the biomineralized scaffold for 24, 48 and 72 hours and also MG 63 Osteosarcoma cells were retained for 1 and 7 day period. However, viability with both the cells reached more than 80% which proves that the new type of biomineralized scaffold is of non-toxic nature.

Key words: mineralization, hydrogel PVP-CMC, $CaCO_3$, scaffold, viscosity, porosity, cytotoxicity.

SOUHRN

V posledních deseti letech se tkáňové inženýrství a regenerativní medicína velmi aktivně věnuje scaffoldům, které mají 3dimensionální strukturu pro lepší podporu obnovy tkáně. Scaffoldové struktury navíc mohou být připraveny podle potřeby regenerace jak pro tuhé, tak pro měkké tkáně. Tuhé tkáně v podstatě představují kostní kompozity, které mají organickou matrici vyztuženou anorganickými minerály ve formě hybridní struktury. Relativně novým konceptem ve vývoji scaffoldů pro tyto tuhé tkáně je tvorba tzv. biomimikrů, které se formují v matrici biomineralizací. Matrici v těchto případech většinou představuje biomateriál, ve kterém vyrůstá krystalická struktura minerálů. Z množství dostupných biomateriálů jsou upřednostňovány hydrogely, a to zejména pro schopnost absorbovat velké množství kapalin a vytvářet příznivé prostředí pro regeneraci živé tkáně.

Předkládaná doktorská práce se zaměřuje na výzkum možností biomimetické přípravy scaffoldů mineralizačním procesem v hydrogelové matrici. Za optimální matrici byla zvolena směs PVP-CMC hydrogelů, do které byly difusním procesem vpraveny vodní roztoky Na_2CO_3 a $CaCl_2$. Byla navržena a připravena řada mineralizovaných vzorků s rozdílnou koncentrací pro hodnocení biomimetického procesu, tvorby krystalické minerální struktury a vlastností vzniklého scaffoldu. Nově vzniklé struktury scaffoldů byly označeny jako "**Biomineralizovaný ($CaCO_3$) PVP-CMC hydrogel".**

Identifikace přítomnosti skupiny -CO_3 ve struktuře hydrogelu a tím potvrzení úspěšnosti procesu biomineralizace byla provedena metodou FTIR, která zjistila přítomnost peaků 1405cm^{-1} a 871cm^{-1}. Metodou XRD pak byla identifikována přítomnost kalcitu v porézní struktuře PVP-CMC hydrogelu. Morfologické hodnocení biomineralizované struktury bylo provedeno pomocí SEM, které ukázalo, že se distribuce mikropórů ve struktuře hydrogelu pohybuje v rozmezí od 1 do 170 mikrometrů.

Zaplněnost pórů kalcitem pak byla stanovena měřením hustoty vzorků v relaci k neplněnému systému. Informace o skladbě scaffoldového systému byly doplněny pomocí měření na TGA. Viskoelastické vlastnosti připravených systémů byly měřeny standardním postupem na rotačním reometru Ares. U vzorků byla posuzována komplexní viskozita a soufázový a ztrátový modul. S intenzivnějším biomineralizačním procesem se podle očekávání snižovaly parametry stanovující elastický podíl mineralizovaného scaffoldu, a zvyšovaly hodnoty určující viskozní

vlastnosti. Mineralizované scaffoldy navíc vykazovaly obtížnější deformovatelnost při zatížení v relaci k neplněným hydrogelům, což může mít v praxi vliv na způsob aplikace tuhých biomineralizovaných scaffoldů.

Dále byly studovány podmínky, které mohou ovlivňovat regenerační účinnost mineralizovaných scaffoldů. Scaffoldy byly posuzovány podle nasákavosti roztoků simulujících biologické tekutiny, které obsahovaly např. glukózu, močovinu či fyziologický roztok. Experimenty byly prováděny za fyziologicky relevantních podmínek. Hodnocení bylo prováděno podle poměrného narůstání objemu scaffoldu a bylo zjištěno, že nejvyšší schopnost nasávat má močovinový roztok, následovaný roztokem fyziologickým. Roztok obsahující glukózu pak prokázal nejnižší stupeň nasákavosti. Konečně pak byly provedeny počáteční experimenty hodnotící cytotoxicitu. Byly použity fibroblasty myších embryonálních tkání umístěné do biomineralizovaného scaffoldu po dobu 24, 48 a 72 hodin a buňky MG 63 Osteosarcoma po dobu 1 až 7 dnů. Životnost obou druhů buněk dosahovala více než 80% což prokazuje, že nový typ biomineralizovaného scaffoldu (PVP-CMC-$CaCO_3$) má netoxický character.

Klíčová slova: mineralizace, hydrogel PVP-CMC, $CaCO_3$, scaffold, viskozita, porozita, cytotoxicita.

ACKNOWLEDGEMENTS

The entire doctoral study would have been impossible without the support and guidance of a large number of individuals and organizations, especially Tomas Bata University in Zlin, Czech Republic for providing scholarship for International Students for pursuing Doctoral Study,which I have availed.

I had the unbelievably good fortune of working with Associate Professor Nabanita Saha (*supervisor of my doctoral study*) who provided me invaluable guidance from the beginning, constantly supported and encouraged me throughout the span of my doctoral studies. She nudges my research in the right path and nurtured me how to stand ourselves as a true professional in life. It has been an absolute honor for me to work under her guidance and learn many things about research.

I owe my sincere gratitude to my co-supervisor, Professor Petr Saha, Rector and Head of Polymer Centre, Tomas Bata University in Zlin for his kind advice, continuous motivation and providing opportunity to get additional financial support which has given a remarkable impact towards scientific research.

I am undoubtedly grateful to Professor Takeshi Kitano, Polymer Centre, Faculty of Technology, Tomas Bata University in Zlin for his help and support in learning Rheology. His total guidance in doing viscoelastic measurements and interpreting the results, have made me to acquire knowledge in the new area of science for the first time.

I would like to extend my big thanks to my colleagues/friends (Oyunchimeg Zandraa, Haojei Fei, Radek Vyroubal) for their help whenever required. Also, it's my privilege to thank all the friends and members of Polymer Centre (especially Dr. Petr Humpolicek, Michal Machovsky, Ludmila Zalesakova) for their technical assistance and support as and when needed.

I also got the opportunity to give tons of thanks to Dr. Tapas Chaudhuri, Head of Research and Development, Charotar University of Science and Technology, Changa, India, to boost me to peruse the doctoral study at Tomas Bata University after completing my Master study under his guidance and supervision.

I would also like to thank to Almighty for helping me to reach at this stage to understand the value of education and to complete my doctoral study. Finally and most importantly, a great deal of thanks and appreciation, goes to my parents, Mrs. Shashi Shah and Mr. Jaswant Shah, who have constantly supported me throughout my life. I also express my thanks to my other family members, relatives and friends in India for their love and encouraging me always to achieve my goal.

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SYMBOLS AND ACRONYMS

FTIR	Fourier transform infrared spectroscopy
SEM	Scanning electron microscopy
TGA	Thermogravimetric analysis
XRD	X-ray diffractomerter
PVP	Polyvinylpyrrolidone
PP	Polypropylene
PMMA	Poly (methylmethacrylate)
PTFE	Poly (tetrafluoroethylene)
PHB	poly-3- hydroxybutyrate
PBS	polybutylene succinate
PCL	Polycaprolactone
PA	Polyanhydrides
PVA	Polyvinyl alcohol
CMC	Carboxymethylcellulose
BIM	Biologically induced mineralization
BCM	Biologically controlled mineralization
MMM	Matrix mediated mineralization
Hap/HA	Hydroxyappatite
ACP	Amorphous calcium phosphate
OCP	Octa calcium phosphate
CaOx	Calcium oxalate
COM	Calcium oxalate monohydrate
COD	Calcium oxalate dihydrate
BTE	Bone tissue engineering
CaP	Calcium phosphate
PLGA	Poly(L-glycolic acid)
CC	Calcium carbonate
TCP/BCP	Bi/Tri-calcium phosphate
PNIPAAm	Poly(N-isopropylacrylamide)
PLLA	Poly (L-lactic acid)
ECM	Extra cellular matrix
PEG	Polyethyleneglycol
PDLLA	Poly-DL-lactide
PS	Polystyrene
DAT	Desaminotyrosine

HA-CPN	Hyaluronic acid-g-chitosan-g-poly (N-isopropylacrylamide)
SPHCs	Superporous hydrogel composites
DMEM	Dulbecco/Vogt modified eagle's minimal essential medium
PNIPAMAC	Poly (N-isopropylacrylamide-co-acrylic acid)
CS	Chitosan
FDA	Food and drug administration
CPC	Calcium phosphate cement
ACC	Amorphous calcium carbonate
GS	Glucose solution
US	Urea solution
PS	Physiological solution
G'	Storage modulus
G''	Loss modulus
η	Complex viscosity
Ws	Weight of swollen biomineralized scaffold
$\mathbf{W_d}$	Weight of dry biomineralized scaffold (initial)
$\mathbf{W_t}$	Weight of sample at the deswelling time, t,
$\mathbf{W_{t0}}$	Initial weight of the fully swollen sample
ρ	Apparent density of mater
m	Mass of hydrogel after incubating in water
d	Diameter (cm) of hydrogel after incubation
h	Height (cm) of hydrogel after incubation in water

LIST OF PUBLICATIONS (Published /Submitted)

Paper I

Preparation of $CaCO_3$ based Biomineralized PVP-CMC Hydrogels and their Viscoelastic Behaviour

Rushita Shah (50%), Nabanita Saha, Takeshi Kitano and Petr Saha
Journal of Applied Polymer Science, 2014, Vol. 131, No.10.
DOI: 10.1002/APP.40237, ISI **Impact Factor**: 1.6 (year 2014)
[Indexed in Web of Science and Scopus]

Paper II

Influence of Strain on Dynamic Viscoelastic Properties of Swelled (H_2O) and Biomineralized ($CaCO_3$) PVP-CMC hydrogels

Rushita Shah (50 %), Nabanita Saha, Takeshi Kitano and Petr Saha
Journal of Applied Rheology, 2015, Vol. 25, No.3, 33979
DOI: 10.3933/ApplRheol-25-33979, ISI **Impact Factor**: 1.078 (year 2014)
[Indexed in Web of Science and Scopus]

Paper III

Mineralized polymer composites as biogenic bone substitute material

Rushita Shah (25 %), Nabanita Saha, Takeshi Kitano and Petr Saha
AIP Conference Proceedings 1664, 070012, (2015)
DOI: 10.1063/1.4918447
[Indexed in Google scholar, will be available in Web of Science too.]

Paper IV

Stimuli responsive and biomineralized scaffold: an implant for bone-tissue engineering

Rushita Shah (40 %), Nabanita Saha, Ronald.N.Zuckermann and Petr Saha
SPE ANTEC 2015 Conf. Proc, Orlando, Florida, USA.
http://legacy.4spe.org/conferences/antec2015/titles.html

Paper V

Influence of Temperature, pH and Simulated Biological Solutions on Swelling and Structural Properties of Biomineralized ($CaCO_3$) PVP-CMC Hydrogel

Rushita Shah (50 %), Nabanita Saha and Petr Saha

Progress in Biomaterial-a springer open journal, 2015, Vol.4, No.2, p.123-136

Paper VI

Properties of biomineralized ($CaCO_3$) PVP-CMC hydrogel with reference to the cytotoxicity test using fibroblasts cells

Rushita Shah (40 %), Nabanita Saha, Zdenka Kucekova, Petr Humpolicek and Petr Saha

International Polymeric Materials and Polymeric Biomaterials (**Submitted in October 2015)**

1. INTRODUCTION

1.1 Emergence of Innovative Biomaterial

Biomaterial research is one of the important fields of interest among the modern medicine. The need for the development of techniques to facilitate the re-generation of failed or destroyed tissues/organs or any part of the body remains as a challenging factor in the field of biomaterials [1, 2]. The knowledge of incorporating any material (natural or synthetic) in medicine, modifying it and making it contact with the biological systems can be referred to as biomaterial [3]. Theoretically, any material (natural or man-made) can be considered as biomaterial till the conditions when it fulfills to be useful in any biomedical applications. Today's researchers are much focused in improving the methodology during the development of any biomaterial to have little risks in patients. The key factor in using any biomaterial is its biocompatibility, biofunctionality, biodegradability etc. [1, 2, 4].

Today's biomaterials are successfully utilized from the wound dressing to tendon and ligament repair of the human body. The choice of any biomaterial depends on its type of manufacturing conditions, surgeon's preference towards it, and reacted towards the immunological aspect of the body [1]. Biomaterial includes *metals*, *ceramics*, *composites* and *polymers*. Recently, there has been a resurgence of interest in utilizing the natural products as materials in medicines. Natural products obtained from plant or animal world can be referred to as natural biomaterial. These natural materials are emphasized more as they are said to have resemblance and familiar to the living body systems [1].

The use of natural materials can be traced back from thousands of years e.g. silk, which are produced to protect the cocoon of the silk moth, has great properties that include beauty, strength durability and antimicrobial. Some of the fascinating capabilities of natural materials include self-healing, self-replication, reconfiguration, chemical balance, and multifunctionality [4]. Other materials such as bone, collagens etc. are made in the body that is useful in making the materials. Hence, the fabrication of biologically derived material is safe, non-toxic and non-pollutant to the environment. Apart from these qualities, they are also biodegradable and recycled by nature. There are several studies going on for improving the prosthetics that includes hips, teeth, structural support to bones etc. [1].

In any of the biomedical applications, the criteria to select biomaterials are based on their material chemistry, molecular weight, solubility, shape and structure, hydrophilicity / hydrophobicity, lubricity, surface energy, water

absorption, degradation and erosion mechanism [5]. Polymeric based materials offer great advantages like biocompatibility, versatility of chemistry and the biological properties which are significant in the application of tissue engineering and organ substitution. Till now researchers have successfully attempted to grow skin and cartilage, bone and cartilage, liver, heart valves and arteries, bladder, pancreas, nerves, corneas and various other soft tissues [5].

1.2 Biomaterials in Medicine [6]

As per the European Society of Biomaterials, a biomaterial will come in contact with the biological system, in order to evaluate, treat or replace any functional organ or tissues inside the human body. So it's essential for the material scientist to find safe and effective material to be used in the body. To accomplish this, some important criteria to be set as under,

- The material should mimic the mechanical performance of the tissues to be replaced.
- Their biocompatibility which defines as "the ability of materials to respond to the host in a specific situation".
- Their inertness towards the body response.

1.3 Classification of Biomaterials

The role of biomaterials has been influenced considerably by advances in many areas of biotechnology and science [7]. The detailed classification of biomaterials and their uses are given as mentioned below:

[A] *Metallic Biomaterial*: The first metal alloy used was the "vanadium steel" as bone fracture plates. Sometimes, metallic elements are present in natural form in the human body, such as iron (Fe) in red blood cells, cobalt (Co) in the synthesis of vitamin B12 etc. [8]. Metals in form of iron (Fe), chromium (Cr), cobalt (Co), nickel (Ni), titanium (Ti), tantalum (Ta), niobium (Nb), molybdenum (Mo), and tungsten (W) were used to make the implants. The main concern dealing with the metallic based biomaterials is the biocompatibility issues. They can corrode easily in the in-vivo environment; hence it weakens the implant and can cause harmful effects on the tissues and organs.

[B] *Ceramic Biomaterial*: Ceramics are generally hard in nature. Its constitutes mainly in the areas of orthopedics and dentistry. However, ceramics as biomaterials has similarity to the physiological environment, due to its basic

constitution of ions (i.e. calcium, potassium, magnesium, sodium, etc.) which are found in physiological environment and others whose toxicity is very limited (zirconium and titanium). Ceramics used in fabricating implants can be classified as inert (e.g. Alumina, zirconia, silicone nitrides, and carbons), bioactive or surface reactive (e.g. glass ceramics and dense hydroxyapatites) and biodegradable or resorbable (calcium phosphates and calcium aluminates) [9].

[C] *Polymeric Biomaterial*: The main benefit of using the polymeric based biomaterial as compared to metals and ceramics is the ease of manufacturability, processability, reasonable cost and availability with desired mechanical and physical properties. The polymeric systems include acrylics, polyamides, polyesters, polyethylene, polysiloxanes, polyurethane, polyvinylpyrolidone (PVP), polypropylene (PP), poly (methylmethacrylate) (PMMA), poly (tetrafluoroethylene) (PTFE) etc. have their applications in dental materials, implants, dressings, extracorporeal devices, encapsulates, polymeric drug delivery systems, tissue engineered products, orthoses etc. [7].

[D] *Composite Biomaterial*: Composite material, usually refers to the material wherein two or more components are combined together with different chemical/physical properties. They are extensively used in dentistry and prosthesis designers are now incorporating these materials into other applications. When they combine, a new material formed has the characteristics different from individual components. They have peculiar properties and are stronger than single element which they are made. This composite biomaterial has several biomedical applications like in dental fillings, orthopedic implants, composite bone plates etc. [10].

[E] *Biodegradable Polymeric Biomaterial*: These types of materials can easily degrade in nature either by hydrolytic mechanisms or enzymes. They have two major advantages:

1) They do not elicit permanent chronic foreign-body reactions due to the fact that they are gradually absorbed by the human body.
2) Some of them have the capacity to regenerate tissues, so-called tissue engineering, through the interaction of their degradation with immunologic cells like macrophages.

The examples of the biodegradable polymers include: poly-3- hydroxybutyrate (PHB), polybutylene succinate (PBS), polycaprolactone (PCL), polyanhydrides (PA), polyvinyl alcohol (PVA), starch derivatives, derivative of cellulose such as cellulose acetate, carboxyl methyl cellulose (CMC), and nitrocellulose [7,10].

2. BIOMIMETIC and BIOMINERALIZATION

The evolution in the nature has introduced effective biological mechanisms. So, imitating these mechanisms has brought improvement in the life. Here, the subject introduced for copying, imitating and learning from biology coined *Bionics* by Jack Steele, of the US Air Force in 1960 at a meeting at Wright-Patterson Air Force Base in Dayton, Ohio and Otto H. coined the term *Biomimetics* in 1969. Biomimetics basically originated from ancient Greek "Bios" (life, nature) and Mimesis (imitation, copy) [11].

The exquisite designs of the organisms on earth have motivated many engineers and scientists. The development of airplane in 1903 by Wright Brothers, is itself a history in the research, as the entire qualities of its working was mimicked from God's handiwork (*i.e from flight of birds*). However, since the ancient times, biomimetics have been know till today in this modern age. It is well understood that nature is and will continuously be the guidance to the material scientists ever. Biomimetics is an important area of science that studies how nature designs, process and fabricate higher polymer composite structure, i.e. bones, teeth, shells, etc. as well as soft structures like cartilage, skin, etc. and implement this process to manufacture new materials with the distinctive properties. Thus, after understanding the fundamentals and simultaneously applying to construct new biomimetic material, its processing route can be determined [11, 12].

Among the higher vertebrate's i.e. human beings, body's muscular system are continuously formed and resorbed. But, somehow with increasing age, there is a gradual decrease noticed in the bone mass and its density. At such point, it becomes essential to repair the degenerated bone and again restore its biological function. Ideally, any replacement material, should mimic the functions of living tissues in its mechanical, chemical and biological aspect. Of course such concepts are easier to describe within paper then implementing in the real clinical aspects [13]. After all, this transfer of knowledge from nature to technology always lies in the hands of scientists and engineers.

"Biomineralization" is the field of biologically produced materials, such as shells, ivory, and teeth, and of the processes that contribute to the establishment of these hierarchically structured organic-inorganic composites or

"Biomineralization" refers to the operation by which organisms form minerals [14].

The development at the biochemistry has given number of exciting research areas in the form of biological, chemical, and earth sciences field of studies. Among all these wonderful topics the study of biomineral formation is perhaps the most fascinating. It is pointed that biomineralization lies in the interface between

life and earth as a new generation of scientists that brings cross-disciplinary training and new methods to solve most intimidating problems among the living beings.

The 1st book on biomineralization got published in 1924 in German by W. J. Schmidt. In this process mostly living organisms, produces such a chemical environment which helps in controlling the growth and nucleation of any mineral phase, thus ultimately providing hierarchical structural order providing higher physical properties. However, it had been noted by the earth scientist that minerals produced biologically contain the compositions that reflect the external environment in which animal lived [14, 15]. Some of the common examples of biomineralization in nature include teeth, bones, kidney stones, skeletons of algae, magnetotactic bacteria, etc. The ultimate hallmark with the biomineralization process results in organic-inorganic hybrid material with complex shapes, organizations, and high mechanical properties like high resistance and lightness [16].

Organic molecules present in the ionic solutions can have an impact on the morphological structure as well as crystal orientation, nucleation and growth. Also, phase transformation occurs with the activation of free energy transformations within the system. However, sometimes it is difficult to understand the exact way of controlling the biomineralization mechanisms which itself is challenged in the area of material science. However, according to Mann, [17] its necessary to study the detailed reactivity and bonding of such organized structure developed after biomineralization process.

Biomineralization on this earth have played an important role from the beginning of life, i.e. when water came into existence. The first perception coming into focus with this process co-incides with the appearance of “fossils” thus practically with the origin of life, originated the concept of biomineralization [17, 18]. On the whole, this process brings many biological systems together for e.g. involving cells, controlling the organic-inorganic structure which is formed by several combinations of ions, thus facilitating the unique expression in all kinds of species.

2.1 Basic Process of Biomineralization

Biomineralization process is divided into two fundamental groups on the basis of their degree of biological control [14].

Biologically Induced Mineralization (BIM):

- It's the precipitation of minerals by interaction between biological activity and the environment. In this process, the organism can usually alter certain parameters of its direct environment (pH, concentration of CO_2, etc.) and thereby favor the formation of particular minerals.
- However, the organism has no means of directly governing the type and habit (mineral structure) of the precipitated mineral.
- Minerals generated by processes of this are heterogeneous in nature (i.e. with irregular shape)

Biologically Controlled Mineralization (BCM) or Matrix Mediated Mineralization (MMM):

- In BCM the use of minerals of micro-organisms take place at the intracellular level. Here, the mineral crystals are formed and deposited within the organic matrices and vesicles for different purposes.
- BCM is defined by more distinct composition, size and shape of the intracellular formed crystals.
- Organisms have a significant degree on the control of crystallization of minerals.

As for e.g. consider the role of organic matrix in an inorganic nucleation process similar to the aspect that any enzyme is added in the solution. However, in both the cases, activation energy differs. Several factors are responsible for this, like lattice geometry, surface charge distribution, surface relaxation, need to be considered in organic-inorganic interface [19].

2.2 Biominerals Involved in Biomineralization

The term biominerals refers to the minerals produced by the organisms. Also, it can be said that the mineralized products are composite material formed by both the mineral and organic component [14]. Mineralized tissues available in nature are in several forms as shown in Figure 1. Table 1 illustrates a few types of biominerals already found in nature.

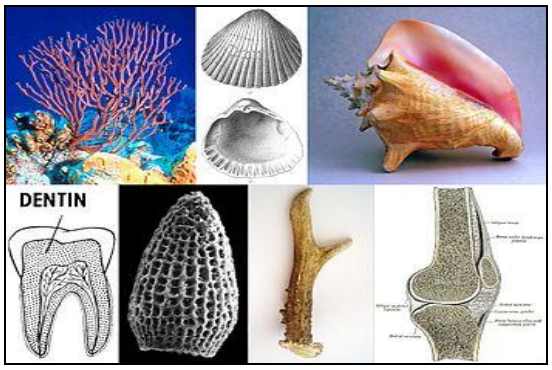


Figure 1: *Mineralized tissues present in nature: seasponge, seashells, conch, dentin, radiolarian, antler, bones etc. [20]*

Table 1: *Minerals produced by biologically induced and controlled mineralization process [14]*

Name	Formula
Carbonates	
Calcite	$CaCO_3$
Aragonite	$CaCO_3$
Vaterite	$CaCO_3$
Phosphates	
Octacalcium phosphate	$Ca_8H_2(PO_4)_6$
Brushite	$CaHPO_4{\cdot}2H_2O$
Francolite	$Ca_{10}(PO_4)6F_2$
Calcium Pyrophosphate	$Ca_2P_2O_7{\cdot}2H_2O$
Sulfates	
Gypsum	$CaSO_4{\cdot}2H_2O$
Barite	$BaSO_4$

Name	Formula
Oxides	
Magnetite	Fe_3O_4
Amorphous Ilmenite	$Fe+2TiO_3$
Amorphous Iron Oxide	Fe_2O_3
Manganese Oxide	Mn_3O_4
Sulfides	**Sulfides**
Pyrite	FeS_2
Hydrotroilite	$FeS{\cdot}nH_2O$
Sphalerite	ZnS
Wurtzite	ZnS
Fluorides	**Fluorides**
Fluorite	CaF_2
Hieratite	K_2SiF_6

2.3 Growth Mechanism of Biominerals

Biominerals in both vertebrates and invertebrates system are famous for their crystal morphologies and composite structures. Any Material scientist in this area tends to concentrate on how the biominerals are formed within the organic matrix [19, 21]. Also, the morphological control within the biomineralization process can be categorized in several ways like, the insoluble organic matrix which helps in growth and nucleation of the crystals, vesicular compartments that help to

control the ions during the process and several macromolecules like sulphated or phosphorylated glycoproteins which are included within the crystals thus controlling the crystal shape [22]. For the crystal growth and nucleation to be accomplished, biomineral needs a particular localized zone that maintains the supersaturation state during nucleation formation [15, 23]. The actual size of the site of mineral deposition tends to diffuse into the system or utilize the compartment. In pure solutions, inorganic mineral leads to the crystal growth from ions towards active sites like on the crystal surface and finally terminates after supersaturation stage is attained [24].

Also, phase transformation modifies the structure of the precursor phase such as amorphous or hydrated phase, thus decreases the thermodynamic stability. For e.g. the growth of HAp within the biological system takes place with the formation of several intermediates like initially, nucleation of ACP (amorphous calcium phosphate) followed by OCP (octa calcium phosphate) occurs thus finally forming HAp [24].

Nucleation process is also a major mechanism to be considered which deals with the formation of a new phase from the old phase, thus consequently increasing its free energy compared to the older one. However, solid surface or any foreign surface can cause higher influence on the nucleation process because interfacial free energy between solid surface and crystal is comparatively lower than crystal with the solution because of the stronger bonding between the solid surface and the crystal. Here, it can be considered that the bonding strength depends on the surface chemistry of the substrate. If the substrate atomic structure matches with the planes of nucleating phase, the lattice strain gets minimized and thus the substrate promotes greater bonding. This, ultimately, makes enthalpy with interfacial free energy smaller and simultaneously leading to the nucleation process on the proper plane [15, 24].

2.4 Models Displaying Process of Biomineralization

The formation of complex organic-inorganic complex material is a widespread biological phenomenon (biomineralization) which occurred in all organisms ranging from prokaryotes (e.g.,magnetite nanocrystals in certain bacteria) to eukaryotes in formation of bones and teeth in humans [25].

As a brief idea about presenting the process biomineralization, few examples are explained below.

a) Biomineralization in Prokaryotic organisms

Iron Biomineralization

Some bacterias have also unique characteristic properties of depositing iron minerals such as ferrihydrite and goethite, iron oxide, magnetite, Fe_3O_4 etc. Among them, Magnetotactic bacterias generally biomineralize either iron-oxide (particle range: 30-120 nm diameter) or iron-sulfide via biologically controlled process and the each crystals gives distinctive crystal morphology as shown in Figure 2 [25- 27]. Further, Magnetite is also found in the 'teeth' of chitin that increases the hardness of the teeth thus enabling the mollusk to scrape algae off rocks.

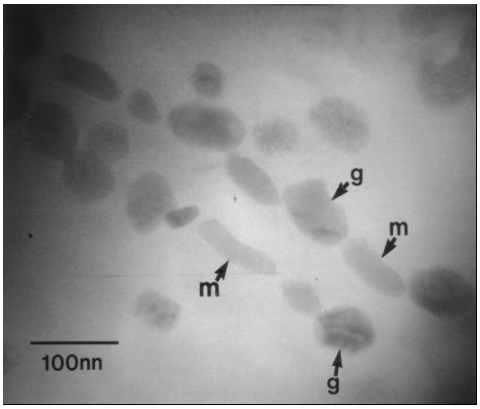


Figure 2: *Brightfield scanning-transmission electron micrograph of tooth-shaped magnetite in an uncultured magnetotactic bacterium [27]*

Sulphate Biomineralization

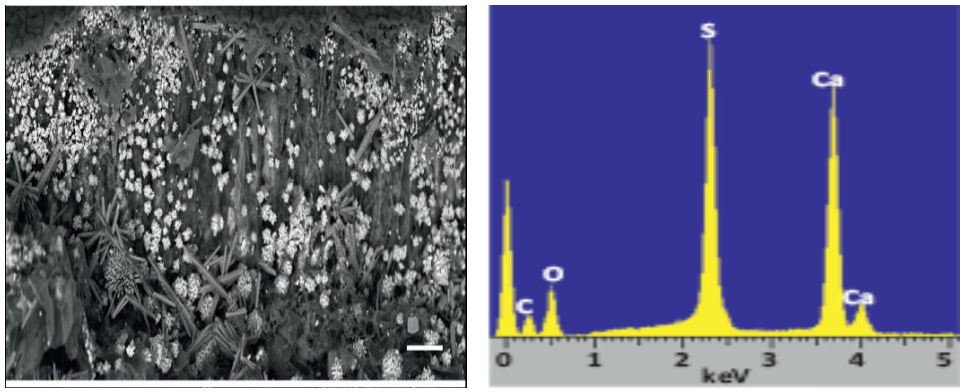
There is huge deposition of sulphur in the form of H_2S or SO_4 with the help of bacterias. As an example of using the process of mineralization, in the jellyfish Aurelia Aarita, the epidermal intracellular (vesicle) formation with statoliths of Gypsum, $CaSO_4 \cdot 2H_2O$ occurs within the jelly fish [25]. Also, there is silica deposition in the marine organisms, diatoms and sponges. However, 95% (dry weight) silica is found in the walls of Diatoms.

Biomineralization in Plant kingdom:

The most commonly biminerals found in plants are *calcium oxalate crystals, calcium carbonate, and silica.* Calcium is one of the essential plant nutrient with many fundamental functions in cellular metabolism [28]. In most plants, calcium required for cellular metabolism is maintained at 10^{-7} M or less. More often, plants accumulate calcium in excess of cytoplasmic requirements. The most abundant

minerals within plants is calcium oxalate crystals are the most common types of biominerals in plants; they account for 3-80% of plant dry weight and up to 90% of the total calcium of a plant [25].

Silica is 2nd abundant biomineral found in the earth's crust either in the form of silicon dioxides or silicates (Silica is 2nd abundant biomineral found in the earth's crust either in the form of silicon dioxides or silicates). Silica is also present in plants. As for example in Acacia Robeorum, shown in Figure 3 [28,29].



(a) **(b)**

Figure 3: *(a) Part of a cross section of a phyllode (Acacia robeorum) showing a large amount of amorphous and/or druse biominerals, (b) Spectra of amorphous and/or druse biominerals [29]*

Also, the dry mass of rice husks at harvest contains up to 20% silica within it (Figure 4) [30]. Further, the biomineralized silica found in the bundles of bamboo trees can be easily recognized by their elemental composition as well as their morphology.

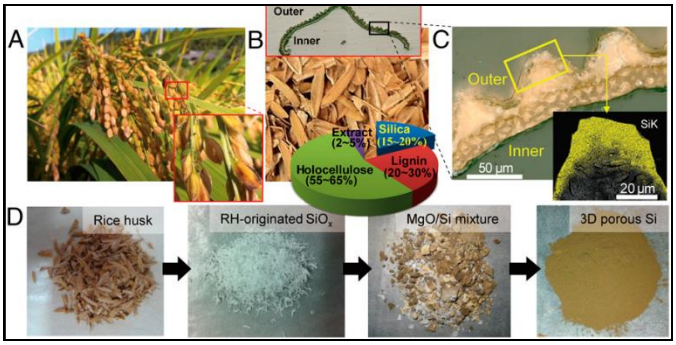


Figure 4: *Generation of 3D nanoporous Si from a rice plant [30]*

b) Carbonate biomineralization in prokaryotic and higher eukaryotic organisms

Among all the biominerals obtained till now, carbonates are among the widely distributed mineral [31]. In prokaryotes, for e.g. hierarically structured biominerals is found in mollusk shells wherein shells are organized into multilayered composites with different phases of calcium carbonate. Biominerals like calcium oxalate and phosphate are found as a pathological mineral in kidney stones in human beings. Usually, this kidney stone is generated from supersaturated calcium oxalate (CaOx) that crystallizes in renal tubules of the kidney. This gives two polymorphs, calcium oxalate monohydrate (COM) and dehydrate (COD) which crystallizes in renal tubules to produce two polymorphs, calcium oxalate monohydrate (COM) and dehydrate (COD). The kidney stone disease is correlated to COM, that forms layered polycrystalline aggregates through nucleation, growth, aggregation process and finally the crystal in the form of stones get aggregated and attached to epithelial cells [32, 33, 34].

In all the vertebrates, including humans, calcium phosphate based biominerals plays an important role. Especially teeth and bones are considered as store house of biominerals because of their distinctive forms, functions, and high degree of mineralization [25]. Bones comprises of extremely small nano-crystals of hydroxyapatite, which are embedded within an organic matrix. The mineralization of the bone is governed by hormones like vitamin D, parathyroid etc. as well as bone cells i.e. osteoblasts, osteoclasts, and osteocytes. However, the mineral ion Ca^{2+} and inorganic phosphate [Pi] are the key factors for controlling entire mineralization process of the bones [35].

Bone is one of the major mineralized tissues of the body basically organized into cancellous (trabecular bone or spongy bone) and cortical bone which has stones as a functional unit. The structure of cancellous bone is depicted in Figure 5. Bone forming cells (i.e., osteoblasts) and bone destructing cells (i.e., osteocytes) are present in osteons of cortical bone [35]. The inorganic minerals like calcium and phosphate are found in the extracellular matrix of collagen in the form of hydroxyapatite crystal ($Ca_{10}(PO_4)_6(OH)_2$). Mineralization process within the insoluble organic substrate, i.e., collagen occurs with the crystal nucleation of the hydroxyapatite. Till now there are reports suggesting that bone apatite is an intermediate between amorphous and crystalline calcium phosphate [36].

Recent research in the field of biomineralization is the utilization of calcium carbonate biomineral but somehow there exists different morphologies dealing with calcium carbonate and calcium phosphate. As described by Bonnuci, the amorphous calcium phosphate (ACP) and octacalcium phosphate OCP are known

to be precursors to HAp crystal formation within the collagen matrix, thus thereafter the crystals nucleate into crystalline phase [17,36,37].

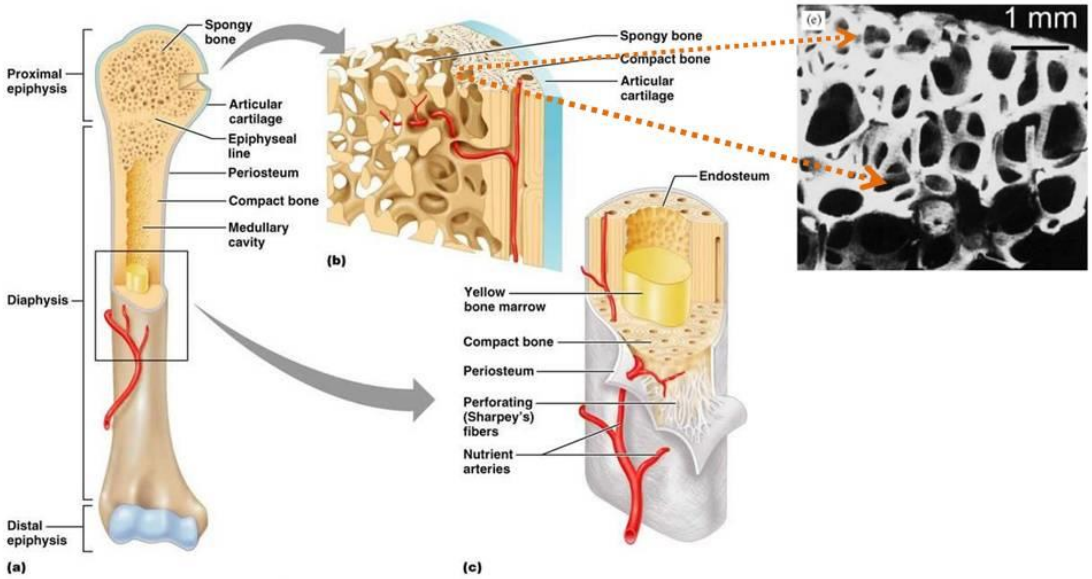
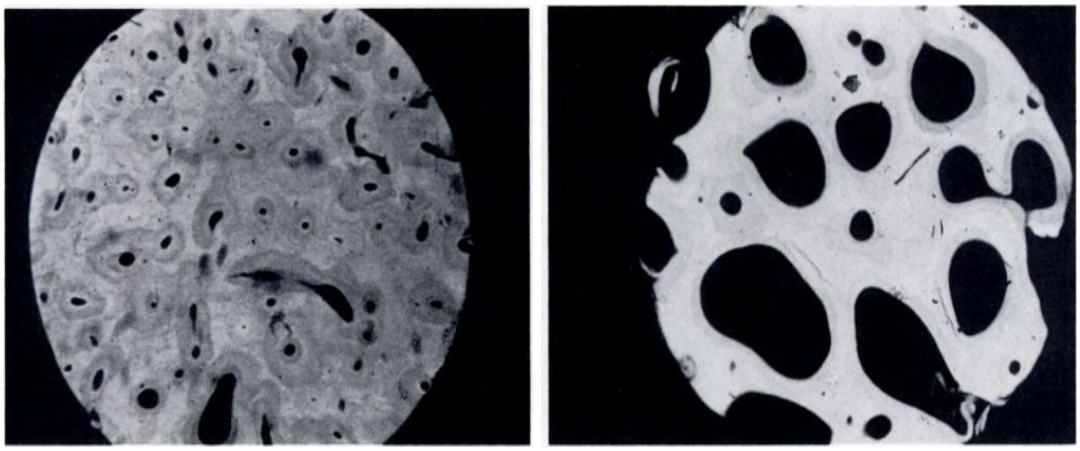


Figure 5: *Structure of cancellous bone [37]*

The study related with the physical and mechanical property dealing with the bone and simultaneously co-relating with any prepared biomaterial (in the form of scaffolds) is of great interest [38]. On the other hand, scaffold based biomaterial / biomineralized biomaterial, if possess more or less similar characteristics of normal bone, it can be applicable as bone substitute. However, the study of physical properties of bone had been kept into the theoretical frame of reference with the help of rheological science [38]. Bone is analyzed as fiber composite because of the presence of both collagen fibers as well as inorganic minerals thus forming a true composite [39]. It is obvious that the living bone systems are continuously underloaded due to either, travel; fixation; prolonged bed rest; or stress shielding from surgical implants, etc. thus, fraction of the bone mass is resorbed resulting in reduction of bone densities and thinning. This suggests that the skeletal system senses changes in sustained mechanical load patterns [40]. Also, there is an increase chance of osteoporosis type of disease due to the reduction in bone mass inside the body [41]. The microradiograph of normal bone and osteoporotic bone is shown in the Figure 6.



(a) **(b)**

Figure 6: Microradiograph of (a) normal bone (b) osteoporotic bone[41]

Further, the poroelasticity (theory explaining interaction of fluid and solid phase with porous medium) can also be determined wherein the deformations in hard tissues are smaller as compared to soft tissues and also the moduli with the hard tissues like bones are much stronger then soft ones under given shear load [40]. However, bone is considered under the viscoelastic material, i.e. the stress depends not only on the strain, but also on the time history of the strain that gives creep. For bones or any biomaterial mainly *elasticity*, *viscosity*, *plasticity* and *strength* is taken into account [38,42,43]. The normal bone can have modulus around 5 GPa and in the similar way the modulus and strength pertaining to cancellous bones and cortical bone are reported in the range of 0.04-1 GPa and 12-20 GPa respectively (modulus values), and 1.0-7.0 and 150 respectively (strength values) [39,44].

3. MATERIALS FOR TISSUE ENGINEERING

As per the definition, ‘‘It’s an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function”. Basically, it deals with the understanding of tissue formation and regeneration and aims to develop new tissues. Researchers are involved in combining the knowledge of physics, chemistry, engineering, materials science, biology and medicine in an integrated manner [45].

This exciting field of tissue engineering is challenged by several aspects which involves in-vitro culturing of human cells on extracellular matrix for e.g. Scaffolds, before being implanted into the human body, microstructures found in the native tissues, etc. Further, there is also challenge in developing functionally active material that will particular cell based interactions, facilitates cell and nutrients

penetrations, mechanical and degradation properties etc. [46]. Today's scientists are more oriented in developing mostly every tissue of the human body. Till now potential tissue engineered products that have already been implemented are cartilages, bones, muscles, heart valves, skin, etc. The most important material that serves an important role in tissue engineering techniques is the use of porous scaffolds that will act as three dimensional template for initial cell attachment and tissue formation for in vitro and in vivo [47]. Ideally, any scaffold prior to its utilization in any of the biomedical field should fulfill the needed requirements like high porous structure with an interconnected porous network for easy transfer of nutrients and some metabolic wastes, flexible enough to be prepared in various shapes and sizes, biodegradable in nature, suitable surface chemistry for the cells to grow and proliferate [48].

3.1 Rationale for Bone-Tissue Engineering (BTE)

There are many reasons to develop bone-tissue engineering alternatives because of the demand that large filler based materials are needed for reconstructing orthopedics defects and also orthopedic implants which are mechanically suitable to their biological environment [49]. In today's society, critical size defects in bone are common, which can occur either in battlefield injuries, accidents, falling and breaking a bone or with increasing age, etc. [50]. Sometimes bone cannot heal itself and has to be surgically repaired [50, 51]. Also, due to diseases like cancer or osteosarcoma, surgical resection needs to be performed often times [52]. In such cases, a large amount of bone is removed and replaced by implants/prosthetics. Thus, here the autografts and allografts serves as an important method for curing defects. But issues associated with infections or rejection with the implants are also seen as a drawback. Thinking about such cases, researchers are taking interest in tissue engineering approach to generate new bone tissues using temporary scaffolds which will degrade by itself after replacing with the native tissues [52]. After all, this process will be just take a single surgical step. The field of bone-tissue engineering (BTE) came into existence nearly three decades ago. BTE requires the collaborative efforts of scientists, engineers and surgeons to achieve the goal of creating bone grafts that enhance bone repair and regeneration. The classic model highlighting in the bone-tissue engineering is the need of a biocompatible scaffold that closely mimics the natural bone extracellular matrix niche, osteogenic cells to lay down the bone tissue matrix and sufficient vascularization to meet the growing tissue nutrient supply and clearance needs [49, 53].

The design of scaffolds (*schematic diagram*) with their criteria's is given below, in Figure 7. [54,55]

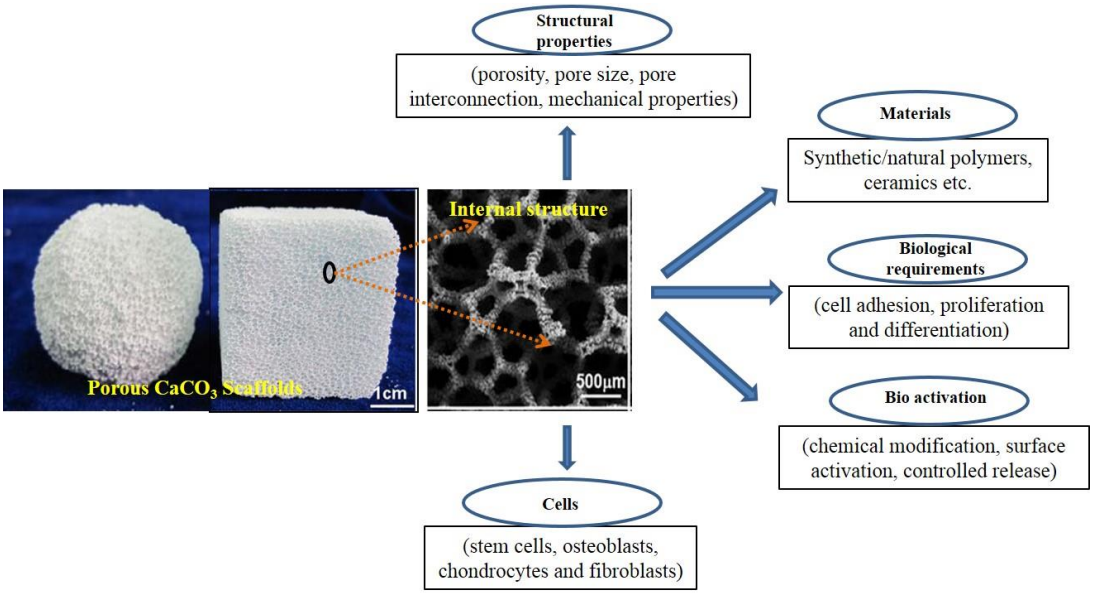


Figure 7: *Optimal design of scaffolds for bone tissue engineering [54]*

The criteria for scaffolds to be utilized in bone tissue engineering is given below, [54,56]

1) The material should be biocompatible in nature and enhance cell attachment, differentiation, and proliferation.
2) The composition of the material should lead to the controlled degradation ability to enhance the formation of new tissues.
3) The material should have adequate mechanical strength to withstand the load inside the body and remain non-immunogenic and non-corrosive.
4) The porosity with the material should be greater than 90% and diameters between 300-500 µm for cell penetration, tissue ingrowth and vascularisation, nutrient delivery and should possess rough inner surfaces (which facilitate cell attachment).
5) The material should possess the capacity for vascularization, encourage osteoconduction within the host bone and also brings about a strong bond between the scaffold and host bone [57].
6) The synthesis of the material and fabrication of the scaffold should be suitable for commercialization.

3.2 Principles and Materials Used in Bone-Tissue Engineering (BTE)

The basic anatomic structures involved in bone regeneration are multicellular units formed by osteoblasts cells (derived from mesenchymal stem cells or bone marrow stromal stem cells and are bone forming cells) and osteoclast cells (derive from hematopoietic progenitors) which are bone resorbing cells. Usually in the bone forming process, osteoblasts are required for osteoclast differentiation process. For the bone regeneration, in an early phase, osteoblasts precursor and growth factors that are obtained from graft material are included on the sites of augmentation and this is followed by osteoconduction. Osteoconduction is a function of a bone graft that provides a tri-dimensional scaffold for the growth of host capillaries and osteoprogenitor cells. Thereafter, the osteoblast precursors differentiate into mature osteoblasts under the influence of osteoinductors and synthesize new bone during the first weeks [53, 58, 59].

As for example: natural and synthetic ceramics (hydroxyapatite (HA) and various calcium phosphate (CaP) compositions, and their composites i.e. HA/ poly (lactic-co-glycolic acid) (PLGA).

Osteoinductivity has already been demonstrated with CaP-based biomaterials in the form of sintered ceramics, cements, coatings, alumina ceramics, bioglass, polymer/ceramic composites etc. [60].

There are some hybrid materials obtained by combination of 2 or more composite materials with enhanced functionalities and properties [53,61-63] which are classified in the form of *co-polymers*, *polymer-polymer blends* and *polymer-ceramic composites* as stated below in Table 2.

Table 2: *Hybrid biomaterials*

Classes of Hybrid Biomaterials		
Co-polymers	**Polymer-polymer blends**	**Polymer-ceramic composites**
Co-polymers are defined as being derived from two or more monomeric species. Examples: PLGA-PCL, PLGA copolymerized with PLL, and PLA- co-polymerized PCL.	Polymer blends involve a mixture of two polymers. Intermolecular H-bonding, Van der Wall's interactions are used to prepare such blends. *Examples:* PLGA blends with polyphosphazenes.	Composite material with inorganic hydroxyapatite crystals (HA) and organic gel matrices are used in BTE. *Examples:* Composites of HA and various polymers, like poly (lactic acid), PLGA, gelatin, chitosan, collagen have been prepared.

Another important type of biomaterial is utilized in BTE is hydrogel that possess biocompatibility and desirable properties and have long been used in this field of BTE. It has the capacity to mimic the extracellular matrix topography and delivering required bioactive agents that promote tissue regeneration. Hydrogel as scaffolds to be used in the BTE has several desirable qualities like: bone growth enhancement, should have correct mechanical and physical properties for requisite applications, no harmful effects occur on the surrounding tissue due to processing of sterilization, without loss of properties, and easily available to surgeon on short notice etc. [64].

Mainly, bone regeneration in hydrogel based scaffolds in vivo needs the penetration of bone cells in the matrix. However, it has been verified that higher porosity of scaffolds enhances the osteogenesis process. Here, the Table 3 gives few examples of some composite scaffolds depicting their porosities and accordingly their reported application [65].

Table 3: *Composite scaffolds and their porosities in BTE [65]*

Composite	Fabrication technique	Pore size (mm)	Application
Collagen/ hydroxyapatite	Freeze-drying	30–100	Rabbit periosteal cells in vitro
Titanium/calcium phosphate	a) Sintering b) Soaking	50–200	Femoral defects in rabbits, Human osteoblasts in vitro, Canine bone-in growth chamber
Poly(L-lactide-co-D,L-lactide)/ b-tricalcium phosphate	Salt-leaching	125–150	Cranial defects in rabbits
Poly(lactide-co-glycolide)/collagen/apatite	Salt-leaching	355–425	Femoral defects in rabbits

Further, few models depicting bone graft substitutes already available at commercial basis in the market and their successful utilization are shown in the Table 4 [59,60].

Table 4: *Bone graft substitutes commercially available [66,67]*

Company and its Commercially available product	Composition	Commercially available forms	FDA status
Biomet Osteobiologics *ProOsteon® 500R*	Coralline-derived HA/CC composite	Granular or block	Bone Void Filler
Exactech *OpteMxTM*	HA/TCP biphasic combination	Granules, sticks, rounded wedges	Bone Void Filler
(IsoTis OrthoBiologics) ***Integra*** *MozaikTM*	80% highly purified, b-TCP/20% highly	Strip and putty	Extremities, Pelvis, Spine Bone Void

	purified .Type-1 collagen		Filler
Medtronic Spinal & Biologics *MasterGraft® Granules*	Biphasic calcium phosphate (15% HA / 85% b-TCP)	Granules	Bone Void Filler
Medtronic Spinal & Biologics *MasterGraft® Matrix*	Biphasic calcium phosphate and collagen	Compression resistant block	Bone Void Filler: Must be used with autogenous bone marrow

4. INTRODUCTION OF HYDROGEL AS A SCAFFOLD BIOMATERIAL

4.1 General Characteristics of Hydrogels

Hydrogels are 3D materials with the ability to absorb large amount of water, flexible in nature, biodegradability, biocompatibility etc. [68-77], and their swollen state is maintained either by physical or chemical cross-linking. It is derived from natural or synthetic materials to augment or replace any tissues or organs of living tissues. Further, biomaterials also have to accomplish some specific requirements, such as non-toxic, desired functionality, sterilizability and biocompatibility [68]. They have recorded their potential applications in the field of biomedical and pharmaceuticals like area of tissue engineering as scaffolds for cell therapeutics, wound healing, cartilage/bone regeneration, drug release, etc. However, the 1st application of the use of hydrogel was in 1960, when Wichterle and Lim introduced the use of hydrophilic networks of cross-linked poly (2-hydroxyethyl methacrylate) (pHEMA) as soft contact lens material [78]. The presence of soft-tissue, higher permeability and efficiency of membrane to release any entrapped molecules in a controlled way made hydrogels to be explored in different biomedical fields [79]. The absence of cross-linking points made the hydrophilic linear polymer chains dissolve in water because of the compatibility aspect between polymer chains and water. However in presence of such cross-linking points, solubility is counter-balanced by the retractive force of elasticity, induced

by cross-linking points of the network. When this force becomes equal, swelling is said to reach an equilibrium point. Generally the presence of specific groups such as –COOH, –OH, $–CONH_2$, –CONH– and $–SO_3H$ makes the hydrogel absorb water within it [80-82].

The presence of physical and chemical cross-linking within hydrogel maintains its three dimensional integrity throughout. Some peculiar features of both physical and chemical cross-linking structure within hydrogel is given below [83].

Chemical Cross-linked hydrogels:

This hydrogels consists of linear polymer chains which are covalently bonded with each other via cross-linking agents, forming the 3D network structure inside them. The network formed cannot be reshaped and/or resized since the polymer is no longer soluble in solvents and heating to melt-process can only degrade the polymer once cross-linking takes place. Sometimes the cross-linking agents used turn out to be toxic in nature.

Physical Cross-linked hydrogels:

In the case of physical cross-linked hydrogels, physical junction domains are associated with chain entanglements, hydrophobic interaction, hydrogen bonding, crystalinity and / or ionic complication. Reversible crosslinking points allow solvent casting and/or thermal processing and make ease of preparation and bulk formation. However, it is observed the weak mechanical properties in the swollen state [37].

4.2 Classification of Hydrogels

Depending on the preparation method, ionic charges, the rate of degradation, nature of swelling and cross-linking, etc., hydrogels can be classified as shown in Table 5 [84-86].

Table 5: *Hydrogel classification [84-86]*

Classification of hydrogels based on different approaches	
Origin	Natural , Synthetic and Semi-synthetic hydrogel
Durability	Durable or Biodegradable hydrogel
Polymeric composition	Homopolymeric, Co-polymeric and Multi-polymeric Interpenetrating network based hydrogels.
Configuration	Amorphous, Semi-crystalline and Crystalline hydrogels.
Network electric charge	Nonionic (neutral), Ionic (including anionic or cationic). Amphoteric electrolyte (ampholytic) containing both acidic and basic groups. Zwitterionic (polybetaines) containing both anionic and cationic groups in each structural repeating unit.
Stimuli responsive	Chemical stimuli and Physical stimuli response based hydrogels

4.3 Hydrogels as Smart Biomaterials:

a) Stimuli responsive hydrogels

Stimuli responsive hydrogels can undergo large changes in their swelling behavior, network changes, and mechanical strength in response to any environmental changes. Stimuli responsive hydrogels can be considered as *intelligent* or *smart* hydrogels. Mainly stimuli responsive hydrogels are categorized in two ways as shown in Figure.8.

1) physical responsive hydrogels and 2) chemical responsive hydrogels [83].

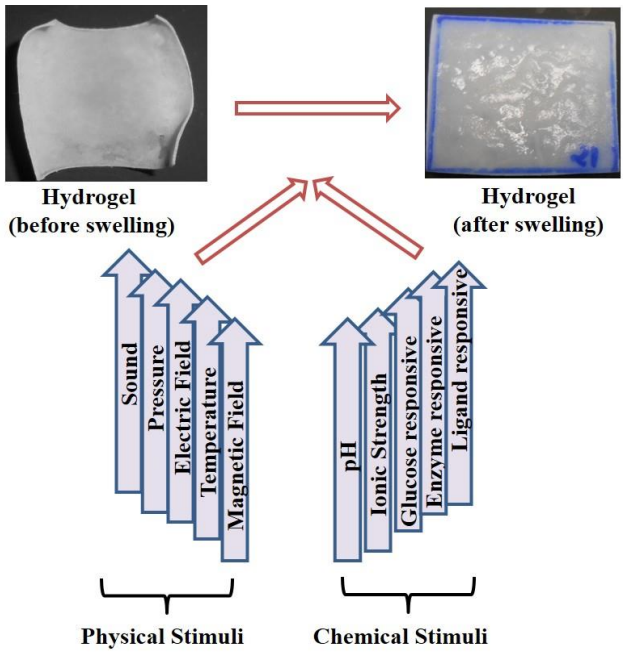


Figure 8: *Different stimuli acting on the hydrogels*

b) Bio-responsive based hydrogels: These hydrogels apart from having all the general properties of the hydrogels, they also persists unique sensitivity towards any biological target such as nutrient, growth factor, receptor, antibody, enzyme etc [87]. Their application is as novel drug delivery system wherein both degradable and responsive to stimuli is required. Examples: PLLA with Dextran and PNIPAAm based hydrogel, PEG based hydrogels.

c) Injectable based hydrogels: These type of hydrogels have huge water content ability to homogeneously encapsulate cells and efficient mass transfer, and have similar structure to extracellular matrix (ECM), which has moldability (they can adapt to the defect shape), possibility of in vivo delivery in a minimally invasive way (resulting in a faster recovery, smaller scar size and less pain for patients). These hydrogels are applicable in the biomedical and pharmaceutical fields, such as vehicles for drug/gene/cell delivery, and tissue engineering such as: heparin, alginate, chitosan, agarose based hydrogels (natural polymers). PVA, PEG/PEO, PEG based hydrogels (synthetic polymers) [88-90].

d) Biomineralized hydrogels: Biominerlized hydrogels are gel like matrices forming organic-inorganic hybrid composite material when comes in contact with any biomineral through the process of biominerlization. These kinds of hydrogels

are generally applicable in bone-tissue engineering or as dental implants. As for example, PVA hydrogel with calcium and magnesium phosphate formation, cellulose hydrogels, mineralization in cellulose gel membranes etc. [91].

4.4 Methodology to Fabricate Hydrogel based Scaffold Biomaterials

The fabrication of any scaffolds to be used in tissue-engineering depends on the surface properties of the materials and its purpose of effectiveness. It is observed that most of the techniques used moist heat or pressure to the polymer or dissolving in any organic solvent to give definite shape to it. However, it becomes mandatory to select appropriate technique to develop any scaffold material on the basis of its application. Here, a few techniques are shown in Table 6 with their application in the biomedical field [5].

Table 6: *Scaffolds fabrication technique in biomedical applications.*

Method	**Polymers**	**Unique factors**	**Application**
Solvent casting/salt leaching method	Absorbable polymer (PLLA, PLGA, collagen, HA/nylon etc.)	Biodegradable controlled porous scaffolds	Bone and cartilage tissue engineering
Ice particle leaching method	PLLA & PLGA	Control of pore structure and production of thicker scaffolds	Porous 3D scaffolds for bone tissue engineering
Gas foaming/salt leaching method	PLLA, PLGA & PDLLA	Controlled porosity and pore structure sponge	Drug delivery and tissue engineering
Solvent evaporation technique	PLGA, PLAGA	High-density cell culture, due to the extended surface area	Bone repair
Particle aggregated scaffold	Chitosan, HAP	High mechanical stability	Bone, cartilage, or osteochondral tissue engineering
Freeze drying method	PLGA, PLLA, PGA, PLGA/PPF, Collagen, and Chitosan	3D porous sponge structure, durable and flexible	Tissue engineering scaffolds
Thermally induced phase separation	PEG, PLLA	Highly porous scaffold for cellular transplantation	Complicated shapes for tissue engineering applications
Ceramic scaffold fabrication			
Sponge replication method	PU sponge, PVA, TCP, BCP or calcium sulfate	Interconnected porous ceramic scaffolds	Bone tissue engineering
	Coating on: metals,		

Simple calcium phosphate coating method	glasses, inorganic ceramics and organic polymers (PLGA, PS, PP, silicone, and PTFE), collagens, fibres of silk, and hairs	Improve biocompatibility or enhance the bioreactivity	Orthopedic application
Fibrous scaffold fabrication			
Nanofiber electrospinning process	PGA, PLA, PLGA, PCL copolymers, collagen, elastin, and so forth	High surface area, biomechanical, and biocompatibility	Drug delivery, wound healing, soft tissue synthetic skin, and scaffolds for tissue engineering

5. IMPORTANCE OF GEL LIKE MATRICES IN BIOMINERALIZATION

Historically, the gel like matrices has been has been preferred in order to control the purity, morphology, and optical quality of the crystals developed in the later stage. A challenge in the field of biomineralization is to select the appropriate model for its phenomenon. Matrix mediated mineralization is focused wherein several extracellular matrices can be used for e.g. three-dimensional macromolecular assembly of proteins; polysaccharides based hydrogels, and/or glycoproteins, etc. [92]. These matrixes being porous, fibrous or hydrated in nature can provide better framework for the organic-inorganic structural phenomenon. Several studies were carried out utilizing matrix-mediated mineralization using several natural sources like bacterias, algaes etc. to prepare composite biomaterial. Till today, different hydrogel has been prepared for example alginate hydrogel, agarose, poly (N-isopropylacrylamide-co-vinyl phosphonic acid), bacterial cellulose, and Polyvniyl alcohol as matrices and biomineralization process were successfully performed [92].

5.1 Significance of calcium derivatives in BM with respect to clinical trials

Calcium derivatives have shown potential applications for bone and teeth related dis-orders because of their property of being biocompatible in nature with natural bones/teeth, and also biodegradable in nature. The development of CPC (calcium phosphate cement) was started three decades ago with the formation of

$CaPO_4$ paste for dental restoration and repair [93]. Combes et.al reported the first $CaCO_3$ cement that constitutes biphasic material i.e. ACC (doped with Mg or Strontium) and crystalline vaterite [93].

The filling of dental caries, generating newly formed bone-tissues, etc. have been successfully accomplished using several calcium derivatives.

Many reports exist wherein the powders such as hydroxyapatite (HAp) and β-tricalcium phosphate (β-TCP) and cemented a-tricalcium phosphate (α-TCP) have been used for bone-filling materials in clinical use [94]. These days a trend is towards the utilization of biomaterials with soft interface, flexible structures and self-standing characteristics are strongly desired [94].

One of the most important derivatives of calcium that has long history in its application is $CaCO_3$ [95]. It's widely used in paint, plastics, inks, foods, pharmaceuticals etc. [93, 96]. Moreover, it is low cost, safe, biocompatible, osteoinductive , possess s lower rate of degradation, etc. The importance of $CaCO_3$ has attracted researchers and apart from this it is believed that $CaCO_3$ can be perfect for its utilization as biomedical cement resorption and replacement by bone tissue [97]. Naturally occurring biomineralization of $CaCO_3$ is shown in the Figure 9.

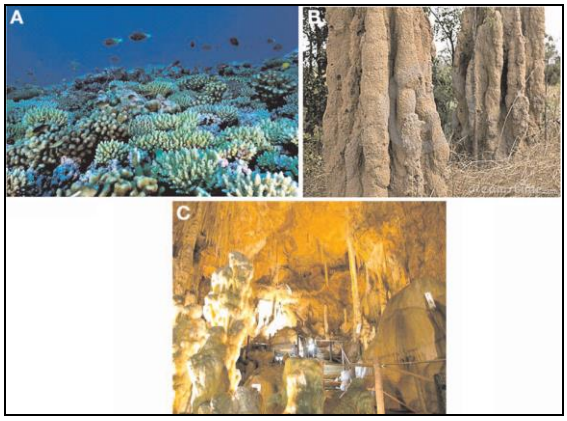


Figure 9: *Biomineralization of calcium carbonates in natural structures (A) Corals (B) Anthills (C) Limestonecaves [31]*

Calcium carbonate can exist with any of its three polymorphs i.e. calcite, vaterite and aragonite [74]. Among them calcite is in trigonal crystalline form present in nature and its thermodynamically most stable. Its utilization as bonding with the bones without any apatites have already been reported. Vaterite constitute the least stability, but once comes in contact with water, starts attaining the stable form. It has hexagonal system. Aragonite forms orthorhombic structure and being biocompatible in nature, it has drawn the interest for further research. Reports exists for the use of aragonite in the drug delivery as well as for bone repair and

regeneration because of being more denser in nature compared to other polymorphs [98].

5.2 Growth and Progress in Mineral based Biomaterial Preparation (2005-2015)

Nature has also presented us beautiful structures in the form of bones, teeth and shells which cannot be regained once lost. Mayans in 2000BC were using tooth –shaped shells (made of $CaCO_3$) for dental implants.So development of such structures using organic-inorganic hybrid synthesis is focused. In the last 10 years, material scientists have achieved a lot of progress in the field of biomineralization. Several polymer based matrices used in carrying out the biomineralization process in the span of these 10 years are briefly described below.

Hydrogel composite based on natural polymer matrices:

Year 2006-2008: Collagen-hydroxyapatite composites, fibrin glue with calcium phosphate granules, fibrin gel with $CaPO_4$ mineralization formed by gel diffusion system, formation of HAp in agarose gels via electrophoresis and alternate soaking approaches etc. were developed [99,100].
Year 2009-2011: gel composite (GC) comprised of calcium alginate hydrogel and nano hydroxyapatite/collagen (nHAC), alginate with HAp scaffolds, formation of octacalcium phosphate within alginate hydrogels, calcium carbonate mineralization within alginate hydrogels, composite formed of desaminotyrosine (DAT) functionalized gelatin (Gel–DAT) with HAp etc. [101,102].
Year 2012-2014: Amorphous calcium phosphate (ACP) and hydroxyapatite (HAp) mineralization in peptide hydrogels, calcium carbonate crystallization in agarose, gelatin and sodium alginate hydrogel, apatitic nanoparticles included into matrigel, agarose hydrogel for biomimetic mineralization to generate enamel like tissues, bacterial cellulose with gelatin and HAp, xanthan gum hydrogel and HAp formation, apatite mineralization behavior on polyglutamic acid hydrogels, incorporation of biphasic calcium phosphate microparticles into hyaluronic acid-g-chitosan-g-poly (N-isopropylacrylamide) (HA-CPN) hydrogel, calcium carbonate crystal growth in the silk fibroin hydrogel and also in silk fibroin/sodium alginate nanofiber hydrogels by the vapor diffusion method, carboxymethyl cellulose—hydroxyapatite hybrid hydrogel, keratin based hydrogel incorporated with HAp composite, alginate-calcium phosphate gels etc. [103-112].
Year 2015: Incorporation of nanosilicates within collagen based hydrogels [113], Inclusion of $CaCO_3$ within 2D templates of peptoid nanosheets [114].

Hydrogel composite based on synthetic polymer matrices:

Year 2006-2008: Formation of HAp in PVA/PVP hydrogel, poly(lactide ethylene oxide-fumarate) (PLEOF)/apatite composite, Incorporation of calcium carbonate in polyacrylamide gels using double diffusion technique, hydroxyapatite reinforced polyvinyl alcohol hydrogel (HA/PVA-H) composite etc. were reported [115,116].
Year 2009-2011: Development of poly(2-hydroxyethyl methacrylate) (pHEMA)-hydroxyapatite (HA) composite, inclusion of nanosized hydroxyapatite particles within cyclic acetal hydrogels etc, pHEMA–hydroxyapatite (HA) superporous hydrogel composites (SPHCs), nanocomposite hydrogels made from poly(ethylene glycol) (PEG) and hydroxyapatite nanoparticles (nHAp) etc. [117,118].
*Year 2012-2014:*Thermoresponsive microgels, poly(N-isopropylacrylamide) (PNIPAM) and poly- (N-isopropylacrylamide-co-acrylic acid) (PNIPAMAC) were used as templates for the mineralization of amorphous calcium carbonate (ACC), oligo(poly(ethylene glycol) fumarate) hydrogel with HAp formation, poly(ethylene oxide phosphonamidate) hydrogels template for mineralization of calcium carbonate and hydroxyapatite, PVA based hydrogel with calcium and magnesium phosphate mineralization, poly(p-phenylene-sulfoterephthalamide) and $CaCO_3$ formation by in situ mineralization [119-126].
*Year 2015:*Graphene was incorporated within PVA hydrogel as matrix, introduction of calcium carbonate affine groups to the hydrogel matrix by copolymerizing acrylic acid and [2-(methacryloyloxy)ethyl] trimethylammonium chloride [127-129].

Hydrogel composite with calcium derivatives for BTE

HAp incorporated within starch/N-vinylpyrrolidone (starch/NVP), $CaCO_3$ hydrogel with gelatin and cynnamaldehyde as bone substitute, Polylactide coated TCP and BCP for bone regeration in female White Rabbits, insoluble chitin and chitosan with soluble polymers like poly- (acrylic acid) (PAA), poly (aspartic acid), and poly (glutamic acid) coated with thin films of $CaCO_3$, PCL with calcium carboante nano-fibers for guided bone tissue regeneration, collagen- $CaCO_3$ and collagen -HAp microspheres serve promising potential in bone regeration and reconstruction medicines, $CaCO_3$ in agarose, chitosan, electrospun fibers for hard stable tissue scaffods material, $CaPO_4$ based ceramic scaffolds used for osteoconduction and osteoinduction in bone formation, pHEMA/PCL with carbonated substituted HAp/HA for bone tissue engineering, $CaCO_3$ crystal formation within hydroxypropylmethyl cellulose hydrogel template is also reported, Coral $CaCO_3$ scaffold with the thin layer of hydroxyapatite composite

have proved to be ideal filler for bone defect and serve as a biodegradable scaffold for bone – regeneration after clinical trials when used for bone augmentation in the 13 year-old female patient with osteochondroma (benign tumor of the bones) [130-139].

As a model examples herein few clinical applications of calcium derivatives utilized in dental or in bone regeneration are discussed.

Calcium Phosphate cements and Chitosan derivatives, tricalcium phosphate/CS, alginate/HAp and CS/HAp supported faster osteoblast differentiation and growth with osteogenic effects (Figure 10) [140].

Zhao et al. prepared a 3D lattice of HAp/CS/gelatin (a composite structure similar to that of the human bone) by in situ precipitation and proposed to be used for bone tissue engineering, thus inducing favorable adhesion, growth and osteogenic differentiation of cultured human mesenchymal cells [141].

Further, the bone regeneration in rabbits was successfully accomplished using polylactide-coated ß-tricalcium phosphate (TCP) / biophasic calcium phosphate (BCP). This calcium phosphate served as a safe and important bone graft substitute [142].

Also in one case, the bioengineered teeth generated from three-dimensionally arranged dental epithelial and mesenchymal cells, some growth factors and additives in collagen gels by in vitro. Thus, the bioengineered tooth obtained shows dispersion of blood vessels and nerve fibers when transplanted into mouse maxilla, thus resulted in successful tooth replacement as shown in Figure 11 [143].

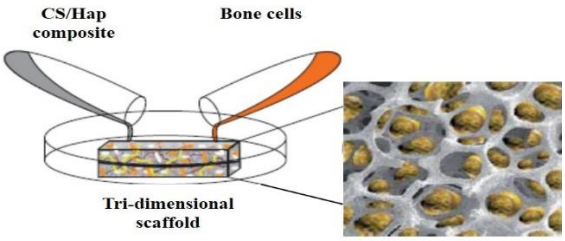


Figure 10: *Osteoblast Colonization within tri-dimensional scaffold [141]*

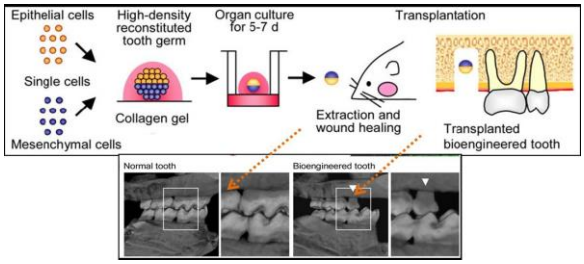


Figure 11: *Schematic representation of the transplantation technology used for the generation of reconstituted tooth germ and MicroCT images of the occlusion of normal as well as bioengineered teeth [143]*

6. MOTIVATION IN THE RESEARCH

- The physical cross-linked hydrogels being mechanically weak, it's essential for some additives to make it physically stronger. As, mineralization is much in demand by the material scientists, addition of biogenic minerals in form of Calcite/Phosphate/ HA etc. This makes the material stronger and improves its mechanical properties.

- Several methodologies were already established for the preparation of biomineralized hydrogel. These techniques include thermally induced phase separation, solvent casting/particle leaching, solid free form, microsphere sintering, and scaffold coating. For getting dense scaffolds to be used in bone-tissue regeneration, it is required to add any form of biomineral ($CaPO_4$ or $CaCO_3$) or bioactive ceramic like hydroxyapatite (HAP), $Ca_{10}(PO_4)_5(OH)_2$, into the biopolymer matrix. Already there are reports of HAp/ $CaPO_4$ /$CaCO_3$ utilized in the organic phase of hydrogel following the biomimetic mineralization process.

- Considering all these above mentioned aspects and to accomplish the target, the approach of this doctoral thesis was motivated to study on calcite filled biomineralized hydrogel scaffold, where mineralization of $CaCO_3$ will be done using PVP-CMC hydrogel matrix (already established the preparation technique of PVP-CMC hydrogel by the research team of Tomas Bata University in Zlin) and reported [62-65]. Simple liquid diffusion technique was selected to accomplish the formation of biomineralized hydrogel using biomineral solutions (in lower and higher concentration) of $CaCl_2$ and Na_2CO_3. Also, no report is there till now wherein PVP-CMC hydrogel is used as matrix for biomineralization (except the work of TBU in Zlin) [144-148].

7. AIMS OF DOCTORAL STUDIES

The target to be achieved throughout the doctoral study is to develop a user friendly and biocompatible biomaterial in the form of '**biomineralized hydrogel scaffold'** which could be applicable in the bone-tissue regeneration, bone tissue engineering. The main tasks to be fulfilled in the doctoral thesis are as follows:

- **Preparation of biomineralized hydrogel using biomimetic mineralization technique.** This include the use of 'PVP-CMC Hydrogel' as a matrix for biomineralization and ionic solutions (calcium chloride and sodium carbonate) to form biominerals i.e. calcium carbonate.

- **Characterizations of the novel biomineralized hydrogels:** in the form of physical, structural (morphological, physical-chemical), rheological, mechanical property, absorptivity, porosity, swelling-deswelling, thermal analysis, cytotoxicity, biocompatibility etc.

- **Study on stimuli responsive behaviour of novel biomineralized materials** in different pH, temperature and various physiological fluids.

- **To evaluate the biocompatibility of novel biomineralized materials:** with respect to in vitro **cytotoxicity assay** (using mouse embryonic fibroblast cell and MG 63 human osteosarcoma cells) considering its application in biomedical purposes (especially as bone substitute material/ dental implant)

8. SYNOPSIS

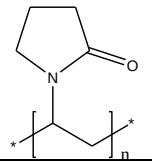
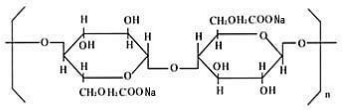
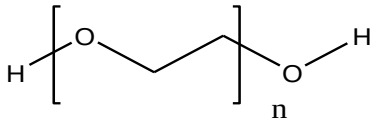
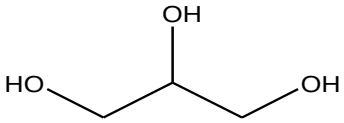
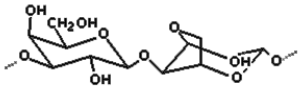
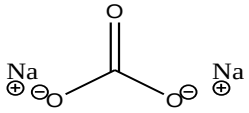
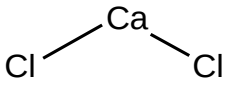
The principal aim of this work is to develop novel biomaterial in the form of biomineralized hydrogel with the vision to utilize the material in the biomedical field (especially in bone-tissue engineering). This novel biomaterial can serve as a promising future in the scientific area of tissue engineering. Thus, this thesis is stated in the form of synopsis of the work accomplished in achieving the aim of developing a novel biomaterial and accordingly, the details with the work has been reported in the form of **six** research articles. Out of the six articles, *four* (3 published and 1 submitted) are published in ISI Journals and *two* are published in (peer reviewed) international conference proceedings, available in Web of Science /Google Scholar.

The present doctoral thesis emphasized particularly on four main goals/aims as already described in section 8.

1. **Preparation of biomineralized hydrogel using biomimetic mineralization technique**

The main challenge lying in the field of biomineralization is the selection of an appropriate in-vitro model. Matrix mediated mineralization is usually preferred because matrix being three dimensional porous and hydrated in nature provides better framework for any of the inorganic crystals of biominerals in the form of $CaCO_3$, $CaPO_4$, HAp etc., to grow and develop some distinctive biomaterial with appropriate structural and mechanical properties. Different methodologies have been implemented for the development of such biomaterial as described in detail in section 4.4, Table 6. Inspired by the fascinating features of biomimetic and biomineralization process, the strategy to develop mineralized hydrogel was designed. The detail with the materials utilized to construct such novel type of biomineralized hydrogel is depicted in Table 7. In the preliminary step, the matrix of PVP-CMC hydrogel, which is already reported by the researchers of Tomas Bata University in Zlin was prepared following solution casting technique and using physical cross-linking agent (i.e. moist heat and pressure) to achieve soft, white PVP-CMC hydrogel (*Paper 1*). These fresh samples are usually kept for 48 – 72 hrs at room temperature (22-25 ^{o}C) to achieve dry state of hydrogel. During the preparation of PVP-CMC hydrogel, different volume of polymer solution were also taken to obtain hydrogel with varying thickness (*Paper V).*

Table 7: *Description of raw materials*

Name of compounds to prepare Biomineralized hydrogel	**Chemical Structure of the compounds**	**Product ordered from Company**
PVP (Polyvinyl pyrrolidone)		PVP K30 (PVP: molecular weight 40,000), from Fluka, Switzerland,
CMC (carboxymethyl cellulose)		CMC from Sinopharm Chemical Reagent Co-Ltd (SCRC), China
PEG (Poly-ethylene glycol)		Polyethylene glycol 3000 (PEG: average molecular weight 2700-3300), from Fluka, Switzerland,
Glycerol		Glycerin from Lachema, Czech Republic
Agar		Agar from Fluka, Switzerland
Sodium Carbonate		$CaCl_2$: molecular weight 110.99g/mol, 97.0%), Penta, Czech Republic
Calcium Chloride		Na_2CO_3: molecular weight 105 g/mol) from Sigma Aldrich, Czech Republic

To prepare the novel biomaterial, (i.e. biomineralized hydrogel) liquid diffusion technique was implemented and two different ionic solutions were used i.e., Na_2CO_3 and $CaCl_2$ *(Paper I)*. The hydrogel thereafter obtained is designated as "biomineralized-($CaCO_3$) PVP-CMC hydrogel". This material can also be termed as calcite-filled hydrogel or mineralized polymeric complex (MPC). Further, it is important to remark here that different concentration of ionic solutions were selected and the range falls from higher to lower concentration (i.e., **Na_2CO_3:**10.5-0.007 g/100ml and **$CaCl_2$:**14.7-0.007 g/100ml). Thus, nine kinds of biomineralized hydrogel samples were prepared *(Paper VI)*. All the biomineralized hydrogels

possess ivory in color, slight flexible and rubbery in nature. The schematic diagram explaining the phenomenon of mineralization process within the polymeric matrix and fabrication of biomineralized hydrogels, is shown clearly in the Figure 12. The physical appearance of the prepared biominerlized hydrogel (in terms of change in weight and thickness) has been thoroughly described in *paper V*.

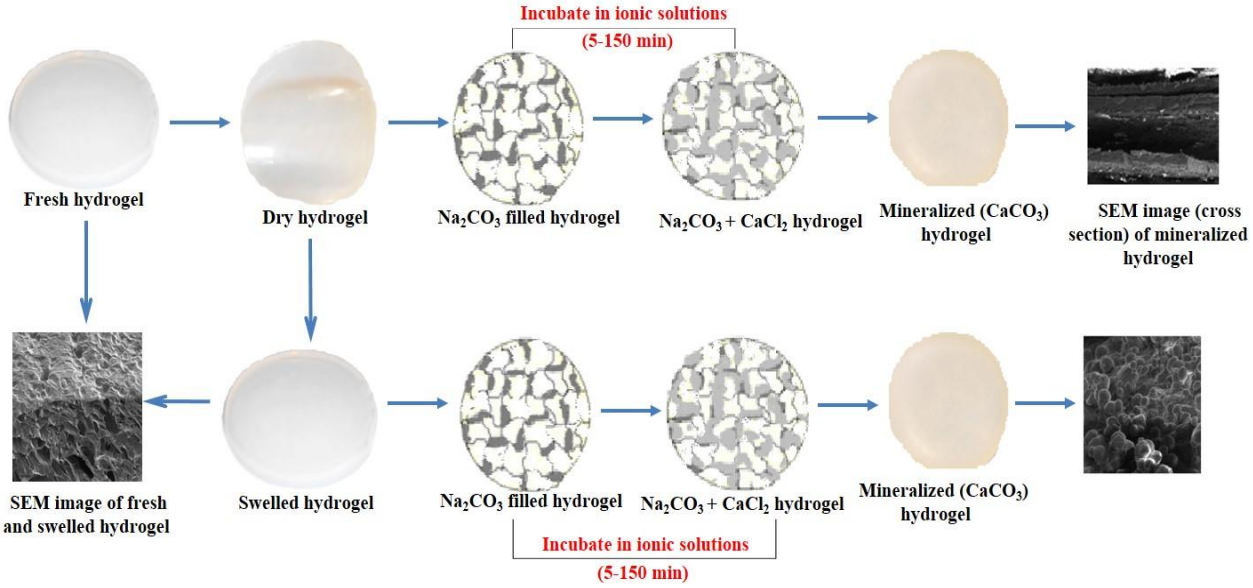


***Figure 12**: Schematic approach for the biomineralization of PVP-CMC based hydrogel*
[Journal of Applied Polymer Science, 2014, Vol. 131, No.10]

2. Characterizations of the novel biomaterial i.e. biomineralized hydrogel:

To design and fabrication of any biomaterial in the form of hydrogel scaffolds/matrix, the main hurdle corresponding to its engineering is, its characteristics and thus more efforts is needed to be focused regarding this aspect. In drawing upon diversified applications dealing with the use of polymers, several desired properties like: *good adhesion*, *flexibility*, *smoothness*, *rigidity*, *crack resistance*, etc. are taken into consideration. Thus, for the characterization and subsequently understanding the polymeric system, there exist several ways for accomplishment, i.e. via spectroscopy, microscopy, rheology, thermal analysis, surface chemistry, chromatography, physical testing and so on.

The characterization techniques were selected to accomplish the aim of this doctoral study, is given below.

Physical-chemical properties of biomineralized hydrogel

FTIR and **XRD**

To determine the structural analysis of prepared novel biomineralized hydrogel (having different amount of bio-mineral i.e. $CaCO_3$) attenuated total reflectance ATR-FTIR was used with NICOLET 320 FTIR Spectrophotometer with "Omnic" software package. However the internal phase characterization of the calcite formed within PVP-CMC hydrogels were obtained using X-ray diffractometer X´Pert PRO (PANalytical, The Netherlands) with CuK a radiation of α= 0.1540598 nm.

Figure 13a shows the FTIR of the prepared novel bio-material, wherein the strong absorption bonds related to CO_3^{2-} appear at 1405 cm^{-1} and sharp peaks are noticed at 871 cm^{-1} which indicates the incorporation of $CaCO_3$ into PVP-CMC hydrogel matrix (*Paper I and VI*). On the other hand, XRD also confirmed the internalization of the calcite formation within the PVP-CMC hydrogel by showing the pattern of exhibited peaks at 2θ = 23.10(012), 29.46(104), 36.03(110), 39.48(113), 43.24 (202), 47.62(018), 48.61 (116) (Figure 13b). *(Paper VI)*

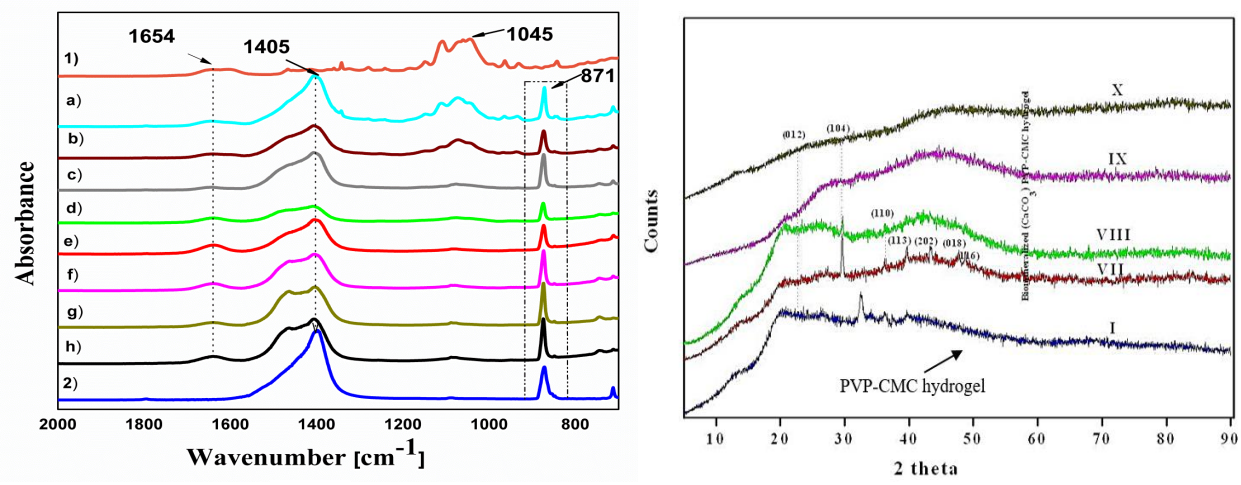


Figure 13: *FTIR (a) and XRD (b) spectra of biomineralized ($CaCO_3$) PVP-CMC hydrogel*

[Source: Journal of Applied Polymer Science, 2014, Vol. 131, No.10 (Paper I), and International Polymeric Materials and Polymeric Biomaterials (Paper VI, Submitted in October 2015)]

Morphological analysis of biomineralized hydrogel

SEM

The surface and internal morphologies of the prepared biomineralized-($CaCO_3$) PVP-CMC hydrogels samples were determined by scanning electron microscopy which was carried out on VEGA II LMU (TESCAN) operating in the high-vacuum/secondary electron imaging mode at an accelerating voltage of 5-20 kv and magnification was 100 x-10 kx as shown in Figure 14 (*Paper I*). Based on the results depicted in paper I, the further details with the structural changes taking place inside the PVP-CMC hydrogel matrix after altering the concentration of ionic solutions were investigated and the manuscript dealing with this study has been submitted (*Paper VI*). Moving further, the details concerning about the macro-porous structure obtained from the cross-sections of the test samples were established using Image J software. (*Paper VI, submitted*)

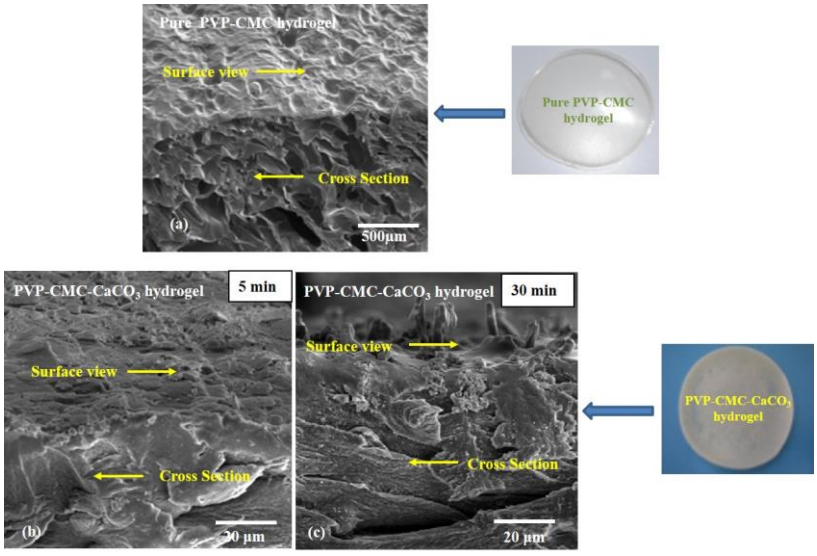
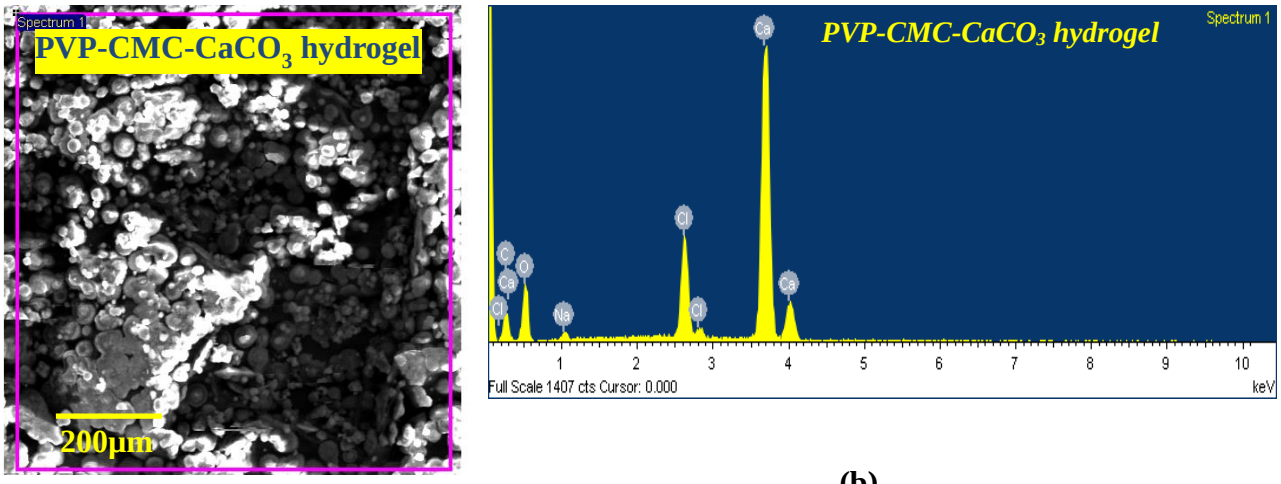


***Figure 14**: SEM Micrographs for Pure PVP-CMC and biomineralized ($CaCO_3$) PVP-CMC hydrogel* [Source: Journal of Applied Polymer Science, 2014, Vol. 131, No.10 (Paper I)]

Further, as a primary information the elemental analysis of the prepared biomineralized ($CaCO_3$) PVP-CMC hydrogel has been done with EDS. Figure 15 shows the selected area over the SEM image and correspondingly its EDS analysis has been determined. The presence of $CaCO_3$ found on the prepared novel hydrogel biomaterial is proven from the percentage atomic number as depicted in Table 8.



(a) **(b)**

Figure.15: *EDS analysis. (a) SEM micrograph of biomineralized ($CaCO_3$) PVP-CMC hydrogel (fabrication time 90 min) for EDS analysis (b) EDS elemental composition*

Table 8**:** *Elemental composition of $CaCO_3$ present in biomineralized ($CaCO_3$) PVP-CMC hydrogel*

Element	**Spectrum on crystals**
	Atomic (%)
Calcium	10.19
Carbon	31.13
Oxygen	54.38

Apparent bulk density of biomineralized hydrogel

The apparent density of biomineralized ($CaCO_3$) PVP-CMC hydrogel samples (test sample size: 25 mm x 25 mm diameter) were examined by soaking the test samples in distilled water up to the saturation time at room temperature (25-26 ˚C). The apparent density was determined using the equation as below,

$$\rho = 4m/\pi d\text{^}2h$$

ρ =apparent density of material (g/cm^3), m=mass (gm) of hydrogel after incubating in water till the saturation time (i.e. 90 mins), d= diameter (cm) of hydrogel after incubation, h=height (cm) of hydrogel after incubation in water

There exists a connection of porosity with apparent density of polymeric based hydrogels, so taking this into aspect apparent/bulk density was also examined with the biomineralized test samples. It was remarked that with decrease in biomineral concentration within the hydrogel, apparent density decreases but porosity gradually rises. (*Paper VI, submitted*)

Thermal analysis of biomineralized hydrogel

TGA

The thermal stability of the biomineralized PVP-CMC hydrogel was performed using TA Q500 apparatus (TA Instruments, US), with the constant heating rate of 10ºC/min from room temperature i.e. 25 ºC upto 700 ºC under Nitrogen atmosphere considering the fact that the weight loss for the inorganic compounds likes $BaSO_4$/$CaCO_3$ initiates after 600 ºC of temperature as shown in Figure 16. The weight loss for the individual compounds i.e. polymers PVP and CMC, as well as inorganic additive $CaCO_3$ were noted with respect to increase in temperature. However, degradation temperature for CMC is above 250 ºC that of PVP is 350 ºC and above 600 ºC calcite decomposition starts as reported in paper VI. (*submitted*)

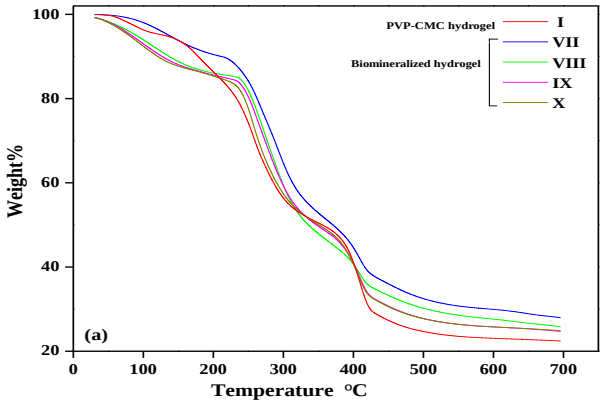


Figure 16: *Thermal Analysis of biomineralized ($CaCO_3$) PVP-CMC hydrogel*
[Source:International Polymeric Materials and Polymeric Biomaterials (Paper VI, Submitted in October 2015)]

Rheological property of biomineralized hydrogel

Another characterization technique that has good agreement showing the structure network of the polymeric biomaterial is Rheology. When dealing with polymeric structures like hydrogels, its flow property depends on its polymer constituents. The dynamic viscoelastic properties of swelled and biomineralized PVP-CMC hydrogels were found using, a parallel plate rheometer (ARES; Rheometrics Scientific, USA) testing machine with an "RSI Orchestrator" software package. The dynamic viscoelastic properties in the form of storage modulus G', loss modulus G'', complex viscosity η* and tan δ for both water swelled and mineralized hydrogels at defined time interval (5-150 mins) as well as particular angular frequency (ω´= 0.39, 3.9, 39 rad/s) has been broadly studied. Paper I (already published) reports about the rheological properties (storage modulus, loss modulus, complex viscosity and Tan delta) of biomineralized ($CaCO_3$) PVP-CMC hydrogel at 1% strain against time and angular frequency. It was found that all the hydrogels (fresh, swelled in water and $CaCO_3$ filled) exhibited stable and linear viscoelastic curve maintaining the typical characteristics of cross-linked gels or any solid material.

Paper II, deals with modifying the results compared to paper I and focusing only on the viscoelastic behavior of the prepared biomaterial in terms of both 1% and 10% strain. The relationship between G' and G'', η* and Tan delta of biomineralized hydrogel with respect to angular frequency region (0.1 – 1 rad/s) and time interval of 10 mins (minimum time to fabricate mineralization hydrogel) and 90 mins (supersaturation time of mineralization to fabricate mineralization hydrogel) was documented (Figure 17). Also, G' and G''and η* in response to time interval from 5-150 mins at the fixed angular frequencies (ω = 0.39, 3.9, 39 rad/s) in both 1% strain and 10 % was found as revealed in Figure 18. Lastly, the strain sweep measurements were also reported at lower and higher strain.

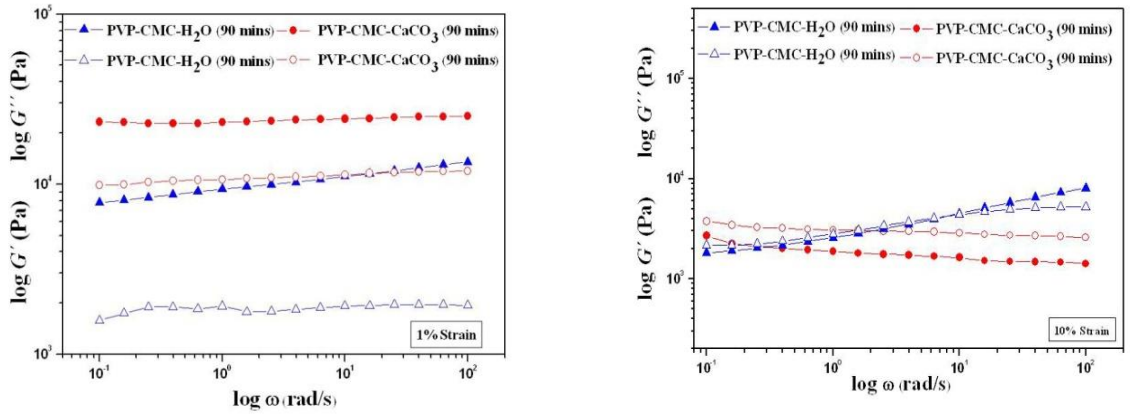
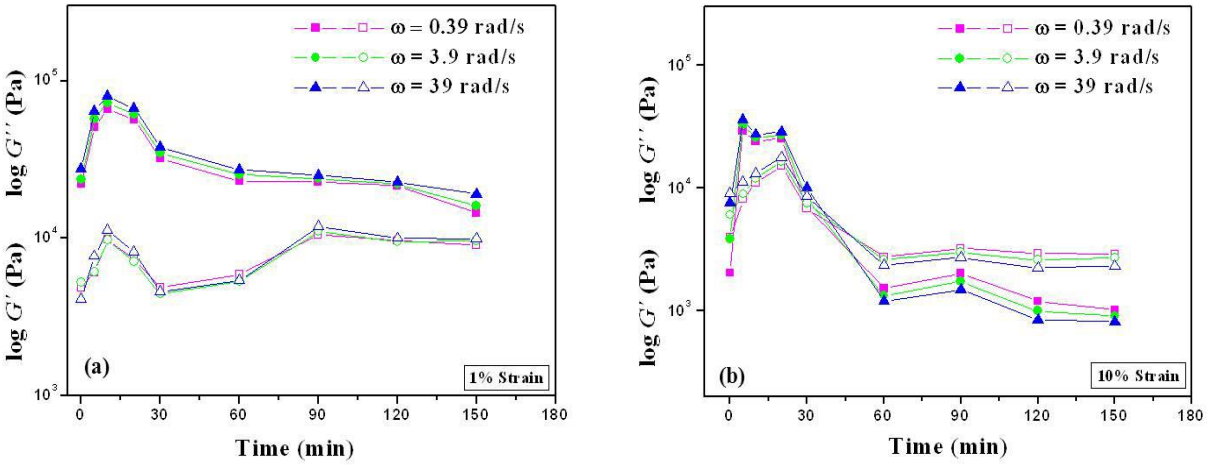


Figure 17: *Dynamic viscoelasticity (storage (filled symbol) and loss moduli (open symbol)) of PVP-CMC-H_2O and PVP-CMC-$CaCO_3$ as a function of angular frequency under 1 % and 10% strain.* [Source:Journal of Applied Rheology, 2015, Vol. 25, No.3, 33979 (Paper I)]



(a) **(b)**

Figure 18: *Storage (filled symbol) and loss moduli (open symbol) vs. $CaCO_3$ absorption time plots of PVP-CMC hydrogel as a parameter of angular frequency (0.39, 3.9 and 39 rad/s) under different strains (a) 1% strain and (b) 10% strain* [Source Journal of Applied Rheology, 2015, Vol. 25, No.3, 33979 (Paper II)]

Altogether by compiling the results through the frequency and strain sweep measurements accomplished at 1% and 10 % strain for both swelled and biomineralized hydrogel, it was found that elastic property (G') is more predominant than viscous property (G"), however, both properties (moduli) changed significantly at higher strain over wide range of angular frequency region,

and the nature of biomineralized hydrogel turned from elastic to viscous. Moreover, it was noticed that calcite filled mineralized scaffolds (PVP-CMC-$CaCO_3$) always has an increased complex shear modulus compared to unmineralized hydrogel scaffold (PVP-CMC-H_2O) as shown in Figure 18. Unmineralized scaffold deformed easier than the mineralized scaffold under a given shear load (i.e. 1% and 10 % strain). This observation is matching with report of the rheological measurement of biomineralized ovalbumin scaffold for bone tissue engineering [44]

On the whole, this study sounds to be significant to identify the effect of changing strain (from lower 1% to higher 10% strain) on the prepared novel biomaterial to recommend its future application.

Mechanical property of biomineralized hydrogel

Young modulus and tensile strengths at break of biomineralized ($CaCO_3$) PVP-CMC hydrogels were determined in a tensile testing machine (Model number M350-5 CT with Testometric type – DBBMTCL-10 kgF) with the cross-head speed of 20 mm/min. Here, in our case the modulus obtained ranged between 868-980 MPa, (Figure 19) which is fitted within the modulus values of cancellous bones (i.e. 0.04 -1.0 GPa or 40 -1000 MPa) (*Paper VI, submitted*)

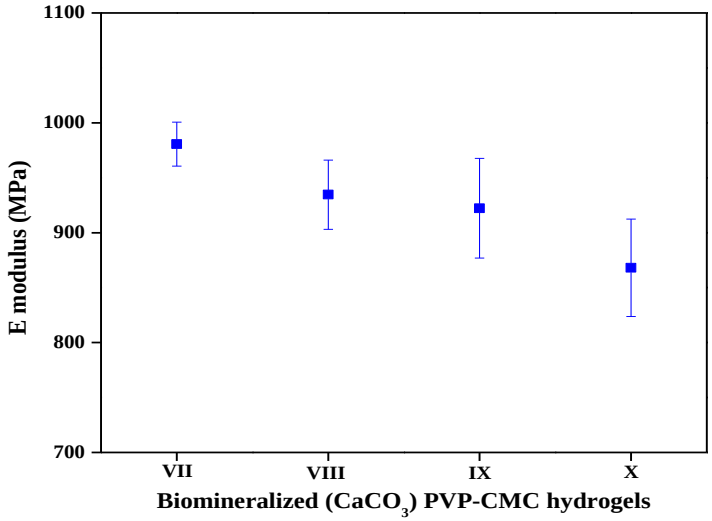


Figure 19: *Elastic Modulus/Young Modulus of biomineralized ($CaCO_3$)PVP-CMC hydrogel* [Source: International Polymeric Materials and Polymeric Biomaterials (Paper VI, Submitted in October 2015)]

Absorptivity study of PVP-CMC hydrogel with respect to water and ionic solutions

Evaluation of the absorptivity by the matrix of PVP-CMC with respect to water and ionic minerals is already reported (*Paper I and II*). The PVP-CMC matrix showed higher and faster water uptake capacity compared to ionic solutions due to polymeric interaction within the matrix. There always exists an intermolecular non-covalent interaction in form of either columbic repulsion, hydrogen bonding etc. within polymeric material which leads to the rise in the absorption rate but the presence of ionic solution which ultimately represents the uptake potential of calcite, absorptivity slows down gradually. Finally, the supersaturation time for the uptake of ionic solutions was noticed around 90 mins (Figure 20). After this, the calcite deposition continued, but only over the surface of the hydrogel matrix. (*Paper I and II)*

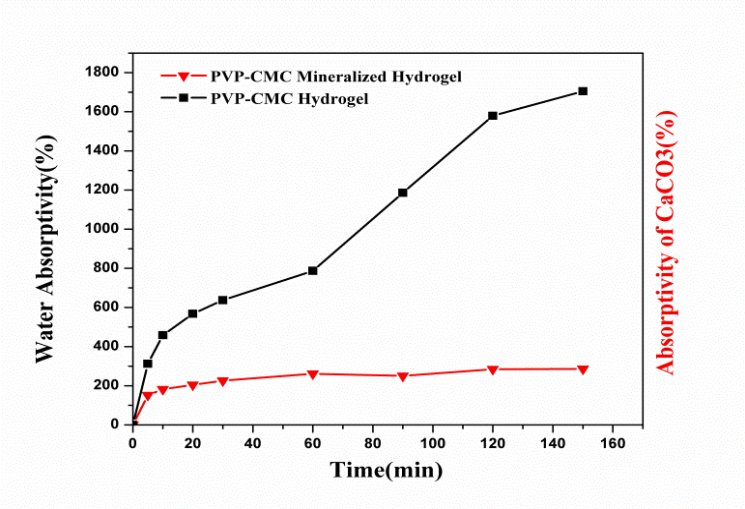


Figure 20: *Absorption behavior of PVP–CMC hydrogel in presence of water and ionic solutions* [Source: Journal of Applied Polymer Science, 2014, Vol. 131, No.10 (Paper I)]

3. Study on stimuli responsive behaviour of novel biomineralized materials

Stimuli responsive nature is the fundamental property dealing with the polymers. Materials responding to any external stimuli can be either chemical or physical based stimuli like change in temperature, pH, salt solution, etc. This type of materials therefore termed as “Smart Material” [149].

Swelling is one of the exciting feature and important criteria to be taken into consideration for any biomaterial before its application [149]. This swelling

behavior gets readily affected when comes in contact with external solutions like ions, salts, etc. Also, exposure of polymer based hydrogels in water or saline solution can provide useful information on the structure of the network of hydrogel and also the cross-linking density [150].

The swelling study was performed using dry sample (diameter: 25 mm x 25 mm and thickness: 0.1 - 0.4 mm) of biomineralized ($CaCO_3$) PVP-CMC hydrogel. Gravimetric technique has been used to evaluate the swelling performance of biomineralized PVP-CMC hydrogel. The swelling percentage (%) was calculated from the below mentioned equation wherein W_s = weight of swollen biomineralized scaffold in water/ionic solution/simulatged biologiocal fluids and W_d = weight of dry biomineralized scaffold (initial)

$$\text{Swelling \%} = (W_s\text{-}W_d/W_d) \times 100\ldots\ldots \text{[1]}$$

Further, the deswelling or water retention (%) was obtained through the following equation, [11] wherein, W_t = weight of sample at the deswelling time, t, and W_{t0} = initial weight of the fully swollen sample.

$$\text{De-Swelling \%} = (W_t)/ (W_{t0}) \times 100\ldots\ldots \text{[2]}$$

For fulfilling the required goal, firstly focus was given to observe the change during the swelling behavior of biomineralized hydrogels in physiological solution. This solution is considered to be similar to human blood plasma and conditions were kept favorable as human body temperature (< 35 - >40 ^oC) and pH of body fluid 7.3-7.5. It has been observed that irrespective of thickness the biomaterial (PVP-CMC-$CaCO_3$) is shown a higher rate of swelling within 30 min and then increased more or less constant rate as shown in Figure 21. (*Paper III*)

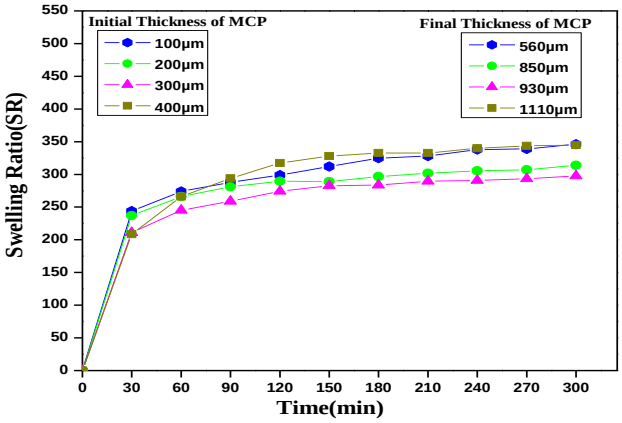


Figure 21: *Swelling behaviour of biomineralized hydrogels in physiological solution*
[Source:AIP Conference Proceedings 1664, 070012, (2015), DOI: 10.1063/1.4918447 (Paper III)]

Now, the confirmation regarding the prepared biomaterial being stimuli responsive, was perceived when deswelling and reswelling phenomenon were observed simultaneously after performing the swelling behavior of the prepared novel material in the physiological solution and the details, particularly dealing with swelling-deswelling-reswelling and again deswelling process is reported in ANTEC-2015 proceeding (*Paper IV*). The apparent changes seen in thickness during the entire process of swelling-deswelling is shown here in Table 9.

***Table 9**: Effect of swelling-reswelling-deswelling on thickness of the biomineralized scaffolds*

Thickness* of Biomineralized Scaffolds				
Time	**Swelling** (mm)	**Deswelling** (mm)	**Reswelling** (mm)	**Deswelling** (mm)
0	0.40	1.09	0.63	1.18
30	0.83	0.95	0.84	1.09
60	0.93	0.85	0.99	0.94
90	0.97	0.79	1.05	0.91
120	1.03	0.72	1.10	0.85
150	1.09	0.98	1.13	0.81
180	1.09	0.63	1.18	0.80

*Average data of three samples

[Source: http://legacy.4spe.org/conferences/antec2015/titles.html, SPE ANTEC 2015, conference proceeding, (Paper IV)]

Further, the detailed features highlighting only the stimuli responsive behavior of the prepared novel biomaterial (with varying sample thickness: 0.1-0.4 mm, size: 25 mm x 25 mm) was conducted in different aqueous pH (4 - 9), temperature (10 - 40 ^{o}C) as shown in Figure 22 (a and b) and simulated biological solutions (SBS: glucose solution (GS), urea solution (US), physiological solution (PS), experimental condition: temperature: 37 ^{o}C, pH = 7.5) (Figure 23). Based on the observation and intensive study with this topic, the obtained results are reported into the Journal of "Progress in Biomaterial" (*Paper V)*.

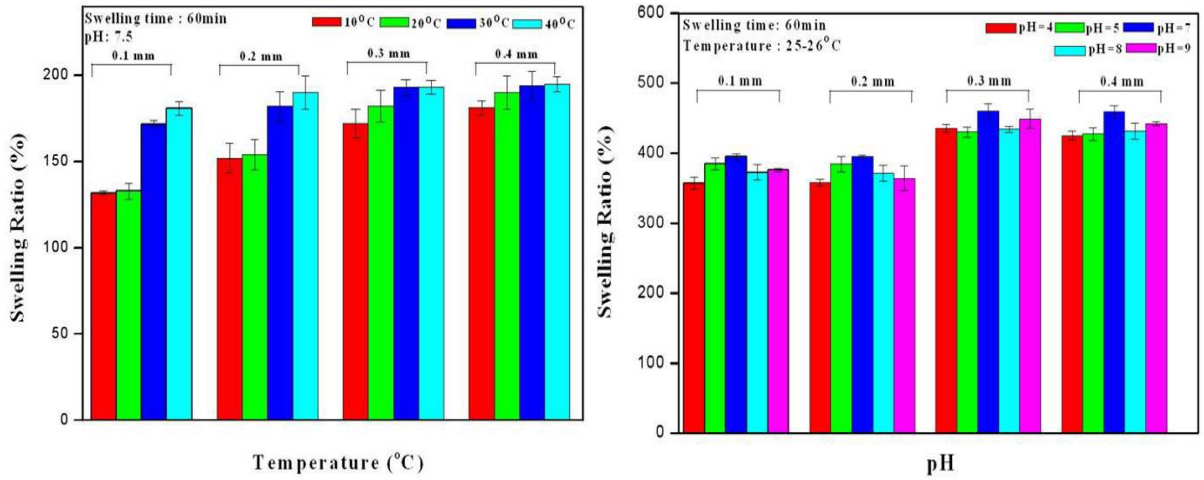


Figure 22: *Effect of temperature (a) and pH (b) on swelling of biomineralized ($CaCO_3$) PVP-CMC hydrogel* [Source:Progress in Biomaterial-a springer open journal, 2015, Vol.4, No.2, p.123-136 **(**Paper V)]

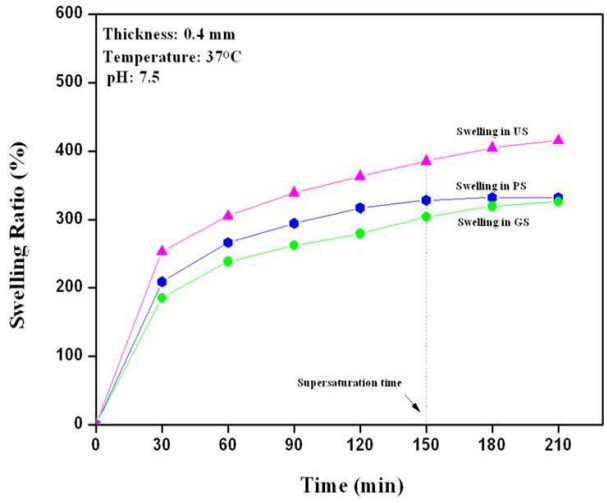


Figure 23: *Swelling behaviour and super saturation time of biomineralized ($CaCO_3$) PVP-CMC hydrogel in simulated biological solutions.*[Source:Progress in Biomaterial-a springer open journal, 2015, Vol.4, No.2, p.123-136 **(**Paper V)]

Overall, the results obtained through the entire study showed that there was no deformation in the test samples. Only minor changes were noticed in their internal structure when analyzed through SEM, (*Paper IV and V*). Also, one more important observation need to mention here is that the novel biomaterial (PVP-CMC-$CaCO_3$) does not lose any $CaCO_3$ particle when performed stimuli responsive experiment under any sort of environmental conditions. These strongly

emphasize that $CaCO_3$ particles are strongly bonded with the polymer matrix, i.e., PVP-CMC hydrogel.

4. To evaluate the biocompatibility of novel biomineralized materials : with respect to in vitro cytotoxicity assay

Any sort of biomaterial prepared require a number of prerequisite before its application. The main purpose of executing biocompatibility testing is to determine the toxicity of any prepared biomaterial intended to be useful without causing any harm to human beings. This study includes cytotoxicity, hemocompatibility, irritation / intracutaneous reactivity and sensitization tests [151]. Several properties possessed by materiáls can affect biocompaibility such as material‘s chemical composition, its mechanical properties, leachates (for e.g. toxic substance) releasing out from the materiál that can cause the reduction/cell death etc. [152,153]. For any biomateriál prior to judge it as biocompatible, firstly it has to undergo several clinical trials extending from in-vitro (i.e living cells) to in-vivo (i.e. model animals) experiments [154]. The use of International Standard ISO-10993, "Biological Evaluation of Medical Devices Part 1: Evaluation and Testing" describes about the biocompability test for the materials that can have application in human beings.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (colorimetric assay) is popular approach to determine the toxicity of any novel biomaterial [155, 156]. The principle governing this MTT Assay is as under,

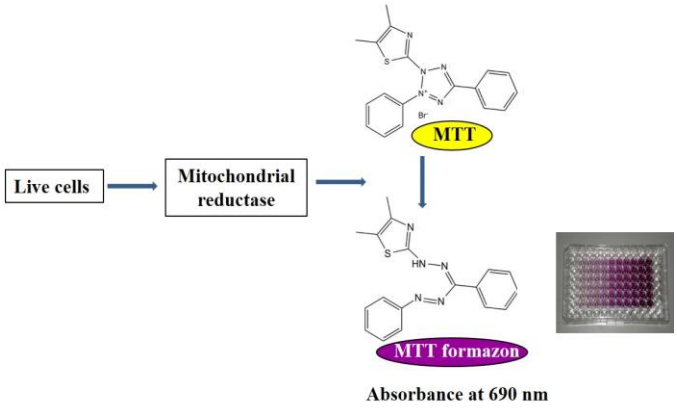


Figure 24: *principle of MTT Assay [148,149]*

The final goal related to this study is more devoted towards the biocompatibility of the material. The cytotoxicity test of biomineralized hydrogels were accomplished using mouse embroynic fibrobalsts in 2 ways, 1) by conducting cytotoxicity of hydrogel extracts and 2) by cytotoxicity in direct contact. Before extract preparation, all the test samples were disinfected under UV-radiation source (258 nm) emitted from low pressure Hg lamp (UV-C Long Life 30 W/G30TB, Phillips, The Netherlands).

Thereafter, the extract of all samples was prepared according to ISO standard 10993-12. The process of extraction was performed in chemically inert closed containers using aseptic techniques at 37 ± 1 °C under stirring for 24 ± 1 hrs. After extraction step (i.e. on 2^{nd} day), the parent extracts (100 %) were then diluted in culture medium (DMEM) to obtain a series of dilutions with concentrations of 75, 50, 25, 10, and 1 % which has been used further for cell viability test. All extracts were used within 24 hrs. Then on 3rd day MTT Assay was carried out and the absorbance parameter was measeured at 570 nm by an Infinite M200 PRO (Tecan, Switzerland). The cell viability was noted after every 24 hrs and continued till 72 hrs.

For cytotoxocity study in direct contact test samples (size: 15mm diameter) were chosen. Cells in concentration $1x10^5$ cells/ml were taken in the petri dishes and the test samples were put into the middle of the dish. The cell growth and morphology were observed after every 24, 48 and 72 hrs and micrograph were taken using inverted phase contrast microscope IX 80 (Olympus). (*Paper VI-submitted*)

In the case of mouse embroynic fibrobalsts cell, the test samples prepared with higher ionic concentration solution, reached high levels of cytotoxicity, so here, focus was given to investigate the properties of biomineralized ($CaCO_3$) PVP-CMC hydrogel samples prepared with the lower ionic concentration (i.e. samples indexed as VII-X). The results showed, the cell viability above 80% in all the extracts concentrations of hydrogels for all time of exposition, resulting the fact that all the samples (VII-X) were without any cytotoxic effect. The manuscript prepared with the details of cytotoxicty study is already submitted (*Paper VI*).

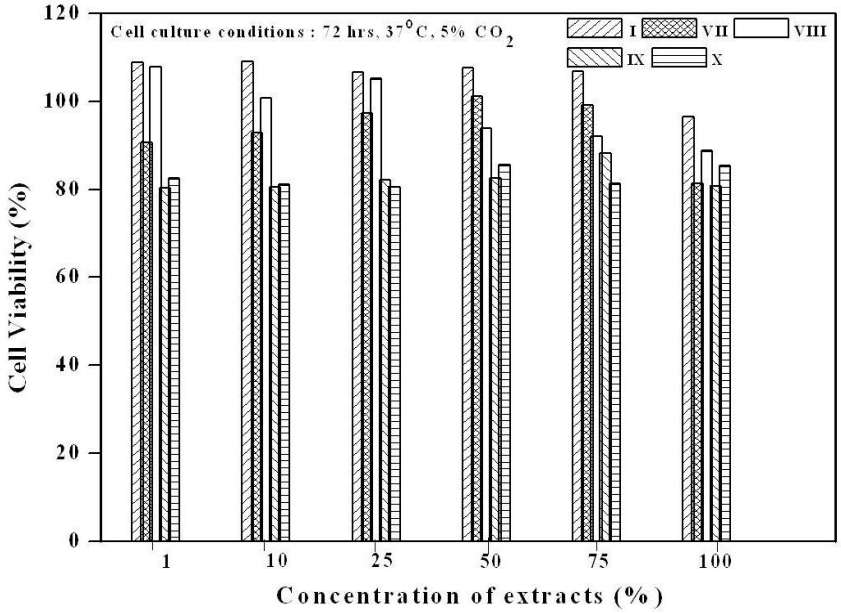


Figure 25*: Cell viability of fibroblasts in presence of biomineralized ($CaCO_3$) PVP-CMC hydrogel extracts for 72 hrs.* [Source: International Polymeric Materials and Polymeric Biomaterials (Paper VI, Submitted in October 2015)]

Also, the cytotoxicity test of prepared biomineralized hydrogels was carried out in presence of MG 63 human osteosarcoma cells. Here, test samples (II-VII) prepared with higher ionic concentration solution were used which shows positive response. MTT assay was performed by culturing the cells in presence of hydrogel leachant/extract (leachant prepared in sterile media for 7 days at 37 °C). The results of the MTT (i.e. the level of toxicity with MG 63 human osteosarcoma cells) were determined after 1 day and 7 day incubation period. Further, the attachment of MG 63 cells on to the PVP-CMC hydrogel surface was checked by staining the cells with Live/Dead staining. For this the cultures were maintained in a humidified incubator at 37 °C, 5% CO_2 for 7 days. After completion of 7 days, cells were washed with sterile PBS (pH 7.4) and incubated in staining solution and fianlly, the stained cells were visualized under fluorescence microscope. In order to assess the osteostimulatory capability of the prepared novel biomaterial, alkaline phosphatase activity (ALP) was measured using alkaline phosphatase assay kit. In ALP activity, conversion of colorless p-nitrophenyl phosphate into colored p-nitrophenol takes place, that is quantified spectrophotometrically at 405 nm using a multiplate reader and simultaniously, the total DNA content is also quantified. (*Manuscript under preparation in co-operation with Indian Institute of Technology-Guwahati*)

9. CLOSING REMARK AND CONTRIBUTION TO SCIECNE AND TECHNOLLOGY

As per the goals set to accomplish during the entire period of doctoral studies, following conclusions were drawn:

- Biomineralization in today's scientific era is considered as hot topic with the trend of continuous progress. Inspired by biomimetic and biomineralization process, the thesis entitled as "Biomineralized and stimuli responsive hydrogel for biomedical applications" represents the overview of the development of novel calcite filled hydrogel and its detail features examined through several characterization techniques to understand its possible future applications.
- For conducting biomimetic biomineralization, primarily, PVP-CMC hydrogel using solvent casting technique was prepared (work already invented by the TBU researchers). This PVP-CMC hydrogel being transparent, flexible, and hydrophilic in nature, also truly serves as an extracellular matrix for carrying out the mineralization process. Further, to achieve novel biomaterial in terms of biomineralized hydrogel, assimilation of $CaCO_3$ was successfully attained through simple liquid diffusion technique within PVP-CMC hydrogel matrix.
- $CaCO_3$ within the PVP-CMC hydrogel matrix showed an excellent absorptivity, high stability, good organic and inorganic interaction, thus facilitating high mechanical property in terms of modulus and tensile strength and predicting its future application as scaffolds for bone regeneration.
- The stimuli responsive investigation of biomineralized PVP-CMC hydrogel was conducted which showed an active response (i.e. change in swelling ratio) in all types of stimulus (i.e. Temperature, pH and simulated biological solutions), thus confirming the prepared material as "smart biomaterial". Also, there was no deformation of the matrix or loss of $CaCO_3$ noticed, which confirms the strong bonding of calcite within the 3D polymeric structure.
- The broad and extensive study performed related to the viscoelastic behavior of the novel biomaterial showed that the unmineralized scaffold deformed

easier than the mineralized scaffold under a given shear load (i.e. at 1% and 10 % strain). However, the material is stable, uniform and maintain the gel characteristics throughout the strain.

- Lastly, the biocompatibility study in terms of cytotoxicity was performed using mouse embryonic fibroblast cells and more than 80% cell viability was seen thus reflecting total non-toxic nature of the prepared novel biomaterial.

Through the entire work accomplished, finally a regenerated and functionalized hydrogel membrane was obtained which can be biogenic and bioactive in nature. It is supposed to have potential application in the field of bone tissue engineering as bone repair/ regeneration, tissue replacement, dental implant, etc. Above all, it can also serve as a needful biomaterial for the welfare of man-kind.

10. FUTURE PLAN

The ultimate goal of the doctoral studies was to develop scaffold for biomedical application especially in bone tissue engineering, so the next immediate goal will be to address the above challenge and improve the scaffold accordingly.

- For accomplishing this, firstly a detailed study exhibiting the mechanical strength of the prepared novel biomaterial in correspondence to that of native bone (both to enable load-bearing and stimulate osteogenesis) will be performed.
- Attention will be given to achieve uniformly biomineralized (calcite filled) hydrogel scaffold. Further, degradation study (in vitro) of biomineralized (calcite filled) hydrogel scaffold will be carried out.
- In vitro study will also be performed with suitable cell lines for e.g. human bone marrow-derived mesenchymal stem cell, MG 63 human osteosarcoma cells , osteoblast cell line-MC3T3-E1,osteoblast cell line (SaOS-2) etc.
- However, with some more detailed studies and challenges listed above for fulfilment with the prepared novel biomaterial; **the final goal of our research is seeking its application in the biomedical field,** i.e. bone-tissue regeneration/bone substitute/dental implant *(especially preferred to be utilized as cancellous-spongy bone)*.

BIBLIOGRAPHY

[1] IGE.O.O.; UMORU, E.L.; A.SUNDAY. Natural Products: A Minefield of Biomaterials , *ISRN Materials Science.* 2012, doi:10.5402/2012/983062.
[2] Handbook of Materials for Medical Devices (#06974G) ASM International. Chapter 1 Overview of biomaterials and their use in medical devices, 2003. http://asmiinternational.org/bookstore.
[3] COBURN, C.J.; PANDIT, A. "Chapter 13: Development of naturally-derived biomaterials and optimization of their biomechanical properties. *Topics in Tissue Engineering*, 2007, Vol. 3, p. 1–23.
[4] CAO, Y.; WANG, B. "Biodegradation of silk biomaterials." *International Journal of Molecular Sciences*, 2009, Vol. 10, No. 4, p. 1514–1524.
[5] DHANDAYUTHAPANI, B.; YOSHIDA, Y.; MAEKAWA, T.; KUMAR, D.S. Polymeric Scaffolds in Tissue Engineering Application: A Review. *International Journal of Polymer Science*, 2011, Vol. 2011, doi:10.1155/2011/290602.
[6] GENTLEMAN, E.; BALL, M.D.; STEVENS, M.M. ©*Encyclopedia of Life Support Systems (EOLSS)*, Medical sciences, Vol-II. Biomaterials. ISBN: 978-1-84826-284-3 (e-Book).
[7] PARIDA, P.; BEHERA, A.; MISHRA, S.C. Classification of Biomaterials used in Medicine. *International Journal of Advances in Applied Sciences*, 2012, Vol.1, No.3, p. 31-35.
[8] INCT-BIOFABRIS. BIOMATERIALS. [Viewed 2015-10-17] Available from: http://biofabris.com.br/en/biomateriais/
[9] REGI, M.V. Ceramics for medical applications. *Journal of the Chemical Society*, Dalton Transactions, 2001, p.97-108.
[10] J.R. DAVIS, © 2003 ASM International. HANDBOOK OF MATERIALS FOR MEDICAL DEVICES, Ohio, 2003, Chapter one. Biomaterials. ISBN: 0-87170-790-X. Also. Also available in PDF from: http://uotechnology.edu.iq/appsciences/filesPDF/material/lectures/4c/8-Biochmical1.pdf
[11] COHEN, Y.B. Biomimetics: mimicking and inspired-by biology, *Procedings of the SPIE Smart Structures Conference*, San Diego, CA, 2005, SPIE, Vol. 5759-02.
[12] MANN, S. Oxford Chemistry Masters: 5, Biomineralization: principles and concept in Bioinorganic Material Chemistry, oxford university press, New York, 2001, ISBN-13: 978-0198508823.
[13] Ashcraft, A. New design innovations from biomimetics, *Creation*, 2010, Vol.32, No.3, p.21-23.

[14] MA, M.G, SUN, R.C. Advances in Biomimetics, Prof. Marko Cavrak (Ed.), 2011, Chapter two. Biomineralization and Biomimetic Synthesis of Biomineral and Nanomaterials. ISBN: 978-953-307-191-6, InTech, DOI: 10.5772/13838. Also available from: http://www.intechopen.com/books/advances-in-biomimetics/biomineralization-and-biomimetic-synthesis-of-biomineral-and-nanomaterials
[15] WEINER, S.; DOVE, P.M. An Overview of Biomineralization Processes and the Problem of the Vital Effect. *Reviews in Mineralogy and Geochemistry*, 2003, Vol. 54, p. 1-29.
[16] BENCHERIF, S.A. et.al. Injectable preformed scaffolds with shape-memory properties. *Proceedings of National Academic of Sciences*, 2012, Vol. 109, p.19590-19595.
[17] H. A. LOWENSTAM, S. Weiner, On Biomineralization, Oxford University Press, New York, 1989, p. ix, 324.
[18] WEINER, S. Organization of extracellularly mineralized tissues: a comparative study of biological crystal growth, *CRC Critical Reviews in Biochemistry*, 1986, Vol.20, No.4, p.365-408.
[19] MANN, S. Biological Inorganic Chemistry, Ivano Bertini, Harry B.Gray, Edward I. Stiefel, Joan.S.Valentine (Ed) , California, USA, 2007,ISBN 10: 1-891389-43-2, ISBN 13: 978-1-891389-43-6.
[20] Mineralized tissues. [Viewed 2015-10-17] Available from: https://en.wikipedia.org/wiki/Mineralized_tissues
[21] CUIF, J.P et al. Layered Growth and Crystallization in Calcareous Biominerals: Impact of Structural and Chemical Evidence on Two Major Concepts in Invertebrate Biomineralization Studies. *Minerals*, 2012, Vol.2, p. 11-39.
[22] WEINER, S.; ADDADI, L. Acidic macromolecules of mineralized tissues - the controllers of crystal-formation. *Trends in Biochemical Sciences*, 1991, Vol. 16, 252-256.
[23] Gilbert, P.U.P.A.; Abrecht,M.; Frazer, B.H. The Organic-Mineral Interface in Biominerals. *Reviews in Mineralogy & Geochemistry, 2005, Vol. 59, p. 157-185.*
[24] YOREO, J.J.DE.; VEKILOV, P.G. Principles of Crystal Nucleation and Growth. *Reviews in Mineralogy and Geochemistry*, 2003, Vol. 54, No. 1, p. 57-93, DOI: 10.2113/0540057.
[25]JANREN, A.H.; Skinner, H.C.W. Biomineralization, Treatise on Geochemistry, *Heinrich D. Holland and Karl K. Turekian* (Ed), Copyright © 2003 Elsevier Ltd, Vol.8, p. 117–184, ISBN: 0-08-044343-5.
[26] BLAKEMORE, R. P.; BLAKEMORE N. A. In Iron Biominerals (eds. R. B. Frankel and R. P. Blakemore, 1990, Chapter: Magnetotactic magnetogens. Plenum, New York, p. 51–67.

[27] BAZYLINSKI, D. A.; FRANKEL, R.B. Biologically Controlled Mineralization in Prokaryotes, *Reviews in Mineralogy & Geochemistry – Reviews in Mineralogy and Geochemistry*, 2003, Vol. 54, No. 1, p. 217-247, DOI: 10.2113/0540217.
[28] HE, H.; VENEKLAAS, E.J.; KUO, J.; LAMBERS, H. Physiological and ecological significance of biomineralization in plants. *Trends in Plant Science*, 2014, Vol. 19, No. 3, 166-174.
[29] SPANGENBERG, D. B. In Mechanisms of Biominerization in the Invertebrates and Plants, N. J. Watabe and K. M. Wilbur (Ed), 1976, Intracellular statolith synthesis Auralia Aurita, University of South Carolina Press, Columbia, SC, p. 231–418.
[30] JUNGA, D.S. et.al. Recycling rice husks for high-capacity lithium battery anodes, *Proceedings of National Academic of Science*, 2013, Vol. 110, No.30, p.12229–12234.
[31] DHAMI, N.K.; REDDY, M.S.; MUKHERJEE, A. Biomineralization of calcium carbonates and their engineered applications: *A Review, Frontiers in Microbiology Terrestrial*, 2013, Vol. 4, No. 314, p.1-13.
[32] RIMER, J. D., WESSON, J. A. WARD, M. D. Pathological Biomineralization of Calcium Oxalate Kidney Stones. *Food, Pharmaceutical & Bioengineering Division*, AICHE proceedings, 2008.
[33] WESSON, J.A.; WARD, M.D. Pathological Biomineralization of Kidney Stones. Medical Mineralogy and Geochemistry, *Elements*, 2007, Vol. 3, No.6, p.415-421.
[34] GOWER, L.B. PIPL, Biomimetics Laboratory, Department of Materials Science and Engineering, University of Florida. Also Available from: http://gower.mse.ufl.edu/research.html.
[35]Singh, A.P. Bone Mineralization Process, Also Available from: http://boneandspine.com/bone-mineralization-process/
[36]E. BONUCCI, Calcification in Biological Systems. *C R C Press LLC*, Boca Raton, 1992, p. 43, ISBN: 0-8493-5735-7.
[37] Cancellous Bone Tissue, Copyright © 2013 Health, Medicine and Anatomy Reference Pictures. Also Available from: http://healthfavo.com/cancellous-bone-tissue.html,
[38] SEDLIN, E.D. A Rheologic Model for Cortical Bone: A Study of the Physical Properties of Human Femoral Samples. *Acta Orthopaedica Scandinavica*, 1965, Vol. 36, Sup 83, p.1-77.
[39] BUREAU, M.N.; LEGOUX, J.G.; DENAULT, J. Implantable biomimetic prosthetic bone. Patent No: WO2006074550 A1, 2006.

[40] COWIN, S.C. Bone poroelasticity. *Journal of Biomechanics*, 1999, Vol.32, p. 217-238.
[41] DICKENSON, R.P.; HUTTON, W.C.; STOTT, J.R.R. The Mechanical Properties of Bone in Osteoporosis. *The Journal of Bone and Joint Surgery*,1981, Vol. 63-B, No. 2, p.233-238.
[42] LAKES, R. Biomechanics II: Bone viscoelasticity, bone creep, bone relaxation. Also, Available from:
http://silver.neep.wisc.edu/~lakes/Biom2.html
[43] JOHNSON, M.; KATZ, J.L. Some new developments in the rheology of bone. *Biorheology.Supplement*, 1984, Vol. 1, p.169-174.
[44] SHEETS, K.T. Mechanical and Cellular Response to Biomineralization of Ovalbumin Scaffolds for Bone Tissue Engineering. Virginia: Virginia Polytechnic Institute and State University, 2010. Doctoral Thesis. Also available in PDF from: http://scholar.lib.vt.edu/theses/available/etd-05132010
235423/unrestricted/Sheets_KT_T_2010.pdf
[45] SALGADO, A.J.; COUTINHO, O.P.; REIS, R.L. Bone Tissue Engineering: State of the Art and Future Trends. *Macromolecule. Bioscience*, 2004, Vol. 4, p.743–765.
[46] OZKAN, S.; KALYON, D.M.; YU, X. Functionally graded b-TCP/PCL nanocomposite scaffolds: In vitro evaluation with human fetal osteoblast cells for bone tissue engineering. *Journal of Biomedical Materials Research Part A*,2009, Vol. 92A, No. 3, p.1007-1018.
[47] SHI, J.; XING, M.M.Q.; Zhong, W. Development of Hydrogels and Biomimetic Regulators as Tissue Engineering Scaffolds. *Membranes*, 2012, Vol.2, p.70-90, DOI:10.3390/membranes2010070.
[48] HUTMACHER, D.W. Scaffold design and fabrication technologies for engineering tissues — state of the art and future perspectives. *Journal of Biomaterial Science. Polymer Edition*, 2001, Vol. 12, No. 1, p. 107–124.
[49] BURG, K.J.L.; PORTE, S.; KELLA, J.F. Biomaterial developments for bone tissue engineering. *Biomaterials*, 2000, Vol.21, p.2347-2359.
[50] COVEY, D.C.; et al. Orthopaedic war injuries: from combat casualty care to definitive treatment: a current review of clinical advances, basic science, and research opportunities. *Instructional Course Lecture*, 2008, Vol. 57, p.65-86.
[51] PAPE, H.C. Bone defects and nonunions—what role does vascularity play in filling the gap?. *Injury*, 2010, Vol.41, No. 6, p. 553–554.
[52] HEARE, T.; HENSLEY, M.A.; DELL'ORFANO, S. Bone tumors: osteosarcoma and Ewing's sarcoma. *Current Opinion in Pediatrics*, 2009, Vol.21, p.365-72.

[53] AMINI, A.R.; LAURENCIN, C.T.; NUKAVARAPU, S.P. Bone Tissue Engineering: Recent Advances and Challenges, *Critical Reviews in Biomedical Engineering*, 2012, Vol.40, No.5, p. 363–408.
[54] CHEN, Q.; ROETHER, J.A.; BOCCACCINI, A.R. Topics in Tissue Engineering, N Ashammakhi, R Reis, & F Chiellini (Eds), 2008, Chapter six. Tissue Engineering Scaffolds from Bioactive Glass and Composite Materials. Vol. 4.
[55] SUBIA, B.; KUNDU, J.; KUNDU, S.C. Tissue Engineering, Daniel Eberli (Ed), 2010. Chapter 6. Biomaterial scaffold fabrication techniques for potential tissue engineering applications. ISBN 978-953-307-079-7, DOI: 10.5772/8581
[56] BOSE, S.; ROY, M and BANDYOPADHYAY, A. Recent advances in bone tissue engineering scaffolds. *Trends in Biotechnology*, 2012, Vol.30, No. 10, p.546–554. doi:10.1016/j.tibtech.2012.07.005.
[57] MAROLT, D.; KNEZEVIC, M.; NOVAKOVIC,G.V. Bone tissue engineering with human stem cells. *Stem Cell Research & Therapy*, 2010, Vol.1:10.
[58] SHEIKH, Z.; SIMA, C.; GLOGAUER, M. Bone Replacement Materials and Techniques Used for Achieving Vertical Alveolar Bone Augmentation, *Materials*, 2015, Vol. 8, p. 2953-2993. doi:10.3390/ma8062953
[59] URIST, M.R. Bone transplants and implants. *In Fundamental and Clinical Bone Physiology*. 1980, p. 331–368.
[60] BARBIERI, D.; RENARD, A.J.S.; DE BRUIJN, J.D.; YUAN, H. Heterotopic bone formation by nano-apatite containing poly (d,llactide) composites. *European cells and materials,* 2010, Vol.19, p.252-261.
[61] ULERY, B.D.; NAIR,L.S.; LAURENCIN, C.T. Biomedical Applications of Biodegradable Polymers. *Journal of Polymer Science Part B: Polymer Physics,* 2011,Vol.49, No. 12, p.832–864.
[62] BAILLARGEON, A.L.; MEQUANINT, K. Biodegradable Polyphosphazene Biomaterials for Tissue Engineering and Delivery of Therapeutics. *BioMed Research International*, 2014, Vol. 2014, Article ID 761373.
http://dx.doi.org/10.1155/2014/761373
[63] WEIA, G.; MA, P.X. Structure and properties of nano hydroxyapatite /polymer composite scaffolds for bone tissue engineering. *Biomaterials*, 2004, Vol.25, p.4749–4757.
[64] PARK, J.B. The use of hydrogels in bone-tissue engineering, *Medicina Oral Patologia Oral Cirugia Bucal*. 2011, Vol.16, No.1, p.115-118.
[65] KARAGEORGIOU, V.; KAPLAN, D. Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials*, 2005, Vol.26, p.5474–5491.
[66] Summary of typical bone-graft substitutes that are commercially available – 2010. Also available from:

http://www.aatb.org/aatb/files/ccLibraryFiles/Filename/000000000323/BoneGraftSubstituteTable2010.pdf
[67] GREENWALD, A.S. et al. Bone Graft substitutes. Facts, fictions and Applications. *Journal of bone and joint surgery*, 2001, Vol.83, No. 2, p. 98-103.
[68] ROSIAK, J.M.; YOSHII, F. Hydrogels and their medical applications. *Nuclear Instruments and Methods in Physics Research B*, 1999, Vol.151, p.56-64.
[69] ROY, N.; SAHA, N.; KITANO, T.; SAHA, P. Novel Hydrogels of PVP–CMC and Their Swelling Effect on Viscoelastic Properties. *Journal of Applied Polymer Science,* 2010, Vol. 117, p.1703-1710.
[70] SAHA, N.; SAARAI, A.; ROY, N.; KITANO,T.; SAHA,P. Polymeric Biomaterial Based Hydrogels for Biomedical Applications Journal of Biomateial and Nanobiotechnology, 2011, Vol. 2, p.85-90.
[71] SAHA, P.; SAHA, N.; ROY, N. "Hydrogel Wound Covering" Patent no: 302405, Czech Republic.
[72] ROY, N.; SAHA, N.; KITANO, T.; SAHA, P. Biodegradation of PVP–CMC hydrogel film: A useful food packaging material. *Carbohydrate Polymers,* 2012, Vol. 89, p.346-353.
[73] KUANG, M.; WANG, D.; GAO, M.; HARTMANN, J.; MOHWALD, H. A Bio-inspired Route to Fabricate Submicrometer-Sized Particles with Unusual Shapes - Mineralization of Calcium Carbonate within Hydrogel Spheres. *Chemistry of Mater*ial, 2005, Vol. 17, p.656-660.
[74] ZHUWEI, D.; et al. Biomimetic Nucleation and Morphology Control of $CaCO_3$ in PAAm Hydrogels Synthesized from Lyotropic Liquid Crystalline Templates. *Chinese Journal of Chemistry*, 2009, Vol.27, p. 2237-2241.
[75] BENCHERIF, S.A. et al. Injectable preformed scaffolds with shape-memory properties. *Proceedings of the National Academy of Sciences,* 2012, Vol. 109, p.19590-19595.
[76] VALLÉE, F. et al. Synthesis and rheological properties of hydrogels based on amphiphilic alginate-amide derivatives, *Carbohydrate Research*, 2009. Vol.344, p.223–228.
[77] AMINI, A.A.; NAIR, L.S. Injectable hydrogels for bone and cartilage repair. *Biomedical Material*, 2012, Vol. 7, No.2, doi:10.1088/1748-6041/7/2/024105
[78]VLIERBERGHE, S.V.; DUBRUEL, P.; SCHACHT, E. Biopolymer-Based Hydrogels As Scaffolds for Tissue Engineering Applications: A Review. *Biomacromolecules, 2011, Vol*. 12, p.1387–1408.
[79] SLAUGHTER, B.V. et.al. Hydrogels in Regenerative Medicine. *Advanced Material,* 2009, Vol. 21, p. 3307–3329.

[80] PEPPASA, N.A.P.; LEOBANDUNGA, B.W.; ICHIKAWA, H. Hydrogels in pharmaceutical formulations. *European Journal of Pharmaceutics and Biopharmaceutics*, 2000, Vol.50, p.27-46.
[81] PEPPAS, N.A.; HILT, J.Z.; KHADEMHOSSEINI, A.; LANGER, R. Hydrogels in Biology and Medicine: From Molecular Principles to Bionanotechnology. *Advanced Material,* 2006. Vol. 18, p.1345–1360.
[82] PARK, J.H.; BAE, Y.H. Hydrogels based on poly(ethylene oxide) and poly(tetramethylene oxide) or poly(dimethyl siloxane): synthesis, characterization, in vitro protein adsorption and platelet adhesion. *Biomaterials,2002,Vol*. 23, p. 1797–1808.
[83] PATEL, A.; MEQUANINT, K. Biomedical Engineering - Frontiers and Challenges.Prof. Reza Fazel. (Ed), 2011, Chapter: Hydrogel Biomaterials. *InTech,* DOI: 10.5772/24856.
[84] AHMED, E.N. Hydrogel: Preparation, characterization, and applications: A review, *Journal of Advanced Research*, 2015, Vol. 6, p.105–121.
[85] El-SHERBINY, I.M.; YACOUB, M.H. Hydrogel scaffolds for tissue engineering: Progress and challenges, Global Cardiology Science and Practice, 2013, Vol.2013, No. 3, p.316-342. http://dx.doi.org/10.5339/gcsp.2013.38.
[86] EBARAM, M. et al. Smart Biomaterials, NIMS Monographs, © National Institute for Materials Science, Japan, 2014, DOI: 10.1007/978-4-431-54400-5_2.
[87] ULIJN, R.V. et al. Bioresponsive Hydrogels. *Materials Today*, 2007, Vol. 10, No.4, p.40-48.
[88] TAN, H.; MARRA, K.G. Injectable, Biodegradable Hydrogels for Tissue Engineering Applications. *Materials,* 2010, Vol. 3, p.1746-1767, DOI:10.3390/ma3031746.
[89] LI, Y.; RODRIGUES, J.; TOMA´ S, H. Injectable and biodegradable hydrogels: gelation, biodegradation and biomedical applications. *Chemical Society Reviews,* 2012, Vol. 41, p.2193–2221.
[90] LEE, A.L.Z.; NG, V.W.L.; GAO,S.; HEDRICK, J.L .; YANG, Y.Y. Injectable Biodegradable Hydrogels from Vitamin D Functionalized Polycarbonates for the Delivery of Avastin with Enhanced Therapeutic Efficiency against Metastatic Colorectal Cancer. *Biomacromolecules,* 2015, Vol. 16, p.465−475.
[91] RAUCH, M.W. etal. Mineralization of Calcium Carbonates in Cellulose Gel Membranes. *European Journal of Inorganic Chemistry*, 2012, Vol.2012, No.32, p. 5192–5198.
[92] SMITH, E.A.; LI, H.; KEENE, E.C.; SHE, Z.W.; ESTROFF, L.A. Crystal Growth of Calcium Carbonate in Hydrogels as a Model of Biomineralization. *Advanced Functional Material,* 2012, Vol.22, p. 2891–2914.

[93] TAS, A. C. Developments in Porous, Biological and Geopolymer Ceramics: Ceramic Engineering and Science Proceedings, M. Brito, E. Case, W. M. Kriven, J. Salem and D. Zhu,(Eds), 2007. Use of Vaterite and Calcite in Forming Calcium Phosphate Cement Scaffolds, John Wiley & Sons, Inc., Hoboken, NJ, USA. Vol.28, No. 9, DOI: 10.1002/9780470339749.ch14.
[94] NONOYAMA, T. et.al. Calcium phosphate biomineralization in peptide hydrogels for injectable bone-filling materials. *Soft Matter*, 2012, Vol. 8, p.11531–11536.
[95] YUFEI, MA.; FENG, Q.; BOURRAT, X. A novel growth process of calcium carbonate crystals in silk fibroin hydrogel system. *Materials science and Engineering C*, 2013, Vol.33, p.2413-2420.
[96] Natural calcite powder. Available from: http://naturalcalcitepowder.com/
[97] DIZAJ, S.M et al. Calcium Carbonate Nanoparticles; Potential in Bone and Tooth Disorders. Pharmaceutical Sciences, 2015, Vol. 20, p.175-182.
[98] ISLAM, K.N. et.al Facile Synthesis of Calcium Carbonate Nanoparticles from Cockle Shells. *Journal of Nanomaterials Volume*, 2012, p. 1-5, Article ID 534010, DOI:10.1155/2012/534010.
[99] WATANABE, J.; AKASHI, M. Novel Biomineralization for Hydrogels: Electrophoresis Approach Accelerates Hydroxyapatite Formation in Hydrogels. *Biomacromolecules*, 2006, Vol.7, p.3008-3011.
[100] WATANABE, J.; AKASHI, M. Anisotropic hydroxyapatite formation inside agarose gels by integration of electrophoretic and alternate soaking approaches. *Journal of Biomaterial Science and Polymer Edition*, 2008, Vol. 19, No. 12, p. 1625–1635.
[101] SHIRAISHI, N. et al. Preparation and characterization of porous alginate scaffolds containing various amounts of octacalcium phosphate (OCP) crystals. *Journal of Material Science: Material Medicines*, 2010, Vol. 21, p.907–914.
[102] NEFFE, A.T.; LOEBUS,A.; ZAUPA, A.; STOETZEL , C.; MÜLLER ,F.A.; LENDLEIN. Gelatin functionalization with tyrosine derived moieties to increase the interaction with hydroxyapatite fillers. *Acta Biomaterialia*, 2011, Vol.7, p.1693–1701.
[103] SMITH, E.A.; LI ,H.; KEENE , E.C.; SEH , Z.W.; ESTROFF, L.A. Crystal Growth of Calcium Carbonate in Hydrogels as a Model of Biomineralization. *Advanced Functional Material*, 2012, Vol. 22, p.2891–2914.
[104] CAO, Y.; MEI, M.L.; LI, Q.L.; LO, E.C.M.; CHU, C.H. Agarose Hydrogel Biomimetic Mineralization Model for the Regeneration of Enamel Prismlike Tissue *Tissue. Applied Material Interface,* 2014, Vol.6, p.410−420.

[105] CHENA, J.P.; TSAIA, M,J.; LIAO, H.T. Incorporation of biphasic calcium phosphate microparticles in injectable thermoresponsive hydrogel modulates bone cell proliferation and differentiation. *Colloids and Surfaces B: Biointerfaces*, 2013, Vol. 110, p.120– 129.
[106] JING, W.; et.al. Laser Patterning of Bacterial Cellulose Hydrogel and its Modification With Gelatin and Hydroxyapatite for Bone Tissue Engineering, *Soft Materials*, 2013, Vol. 11, p. 173–180.
[107] MING, J.; ZUO, B. Crystal growth of calcium carbonate in silk fibroin/sodium alginatehydrogel. *Journal of Crystal Growth*, 2014, Vol. 386, p.154–161.
[108] PASQUI D,; TORRICELLI P,; DE CAGNA M,; FINI M,; BARBUCCI R.Carboxymethyl cellulose—hydroxyapatite hybrid hydrogel as a composite material for bone tissue engineering applications. *Journal of Biomedical Material Research Part A*, 2014, Vol. 102A, p.1568–1579.
[109]WANG X. et al. Effect of Bioglass on Growth and Biomineralization of SaOS-2 Cells in Hydrogel after 3D Cell. *BioprintingPLoS* One, 2014, Vol.9, No. 11, e112497. DOI:10.1371/journal.pone.0112497.
[110] ALVES, C.D. et.al. Gelation and biocompatibility of injectable alginate–calcium phosphate gels for bone regeneration. *Journal of Biomedical Material Research Part A*, 2014, Vol. 102A, p.808–817.
[111] NAKATA, R.; TACHIBANA, A.; TANABE, T. Preparation of keratin hydrogel/hydroxyapatite composite and its evaluation as a controlled drug release carrier. *Materials Science and Engineering*, 2014, Vol.41, p.59–64.
[112] ROEDER, L.; RECKENTHÄLER, M.; BELKOURA, L.; ROITSCH,S.; STREY, R.; SCHMIDT, A.M. Covalent Ferrohydrogels Based on Elongated Particulate Cross-Linkers. *Macromolecules*, 2014, Vol.47, p.7200−7207.
[113] XAVIER, J.R. et al. Bioactive Nanoengineered Hydrogels for Bone Tissue Engineering: A Growth-Factor-Free Approach. *ACS Nano*, 2015, Vol.9, No.3, p. 3109–3118.
[114] JUN, J.M.V.; ALTOE, M.P.V.; ALONI, S.; ZUCKERMANN, R.N Peptoid nanosheets as soluble, two-dimensional templates for calcium carbonate mineralization. *Chemical Communication*, 2015, Vol.51, 10218.
[115] WATSUBO, T.; SUMARU, K.; KANAMORI, T.; SHINBO, T.; YAMAGUCHI, T. Construction of a New Artificial Biomineralization System. *Biomacromolecules*, 2006, Vol. 7, p.95-100.
[116] HELBIG, U. Growth of calcium carbonate in polyacrylamide hydrogel: Investigation of the influence of polymer content. *Journal of Crystal Growth*, 2008, Vol.310, p.2863–2870.

[117] PHADKE,A.; ZHANG,C.; HWANG,Y.S.; VECCHIO,K.; VARGHESE, S. Templated Mineralization of Synthetic Hydrogels for Bone-Like Composite Materials: Role of Matrix Hydrophobicity. *Biomacromolecules,* 2010, Vol.11, p.2060–2068.
[118] ÇETIN, D.; KAHRAMAN, A.S.; GÜMÜSDERELIO˘GLU, M. Novel Scaffolds Based on Poly (2-hydroxyethyl methacrylate) Superporous Hydrogels for Bone Tissue Engineering. *Journal of Biomaterials Science,* 2011, Vol. 22, p.1157–1178.
[119] XIA, Y.; GU,Y.; ZHOU,X.; XU ,H.; ZHAO,X.; YASEEN, M.; LU, J.R. Controllable Stabilization of Poly(N-isopropylacrylamide)-Based Microgel Films through Biomimetic Mineralization of Calcium Carbonate.*Biomacromolecules*, 2012, Vol. 13, p. 2299−2308.
[120] FENG-JU,Z.; XIN-GUO, Y.; YU, Z.; KUN-KUN, G.; MING-JUN, W.; WEN-FENG, W. Crystallization of calcium carbonate in hydrogels in presence of meso-tetrakis (4-hydroxylphenyl) porphyrin. *Journal of Central South University*, 2012, Vol.19, p.1802−1807.
[121] NEJADNIK,M.R.; MIKOS, A.G.; JANSEN, J.A.; LEEUWENBURGH, S.C.G. Facilitating the mineralization of oligo(poly(ethylene glycol) fumarate) hydrogel by incorporation of hydroxyapatite nanoparticles. *Journal of biomedical materials research A,* 2012, Vol. 100A, No.5, p.1316-1323.
[122] FEDOROVICH, N.E.et.al. The osteoinductive potential of printable, cell-laden hydrogel-ceramic composites. *Journal of biomedical materials research*, 2012, Vol.100a, No. 9, p.2412-2420.
[123] ZHANG, L.; Nontoxic poly(ethylene oxide phosphonamidate) hydrogels as templates for biomimetic mineralization of calcium carbonate and hydroxyapatite architectures. *Journal of Material Science,* 2013, Vol.48, p.288–298.
[124] MIYAZAKI, T.; MUKAI, J.; ISHIDA, E.; OHTSUKI, C. Apatite mineralization behavior on polyglutamic acid hydrogels in aqueous condition: Effects of molecular weight. *Bio-Medical Materials and Engineering*, 2013,Vol.23, p.339–347.
[125] NISHIO,T.; TANAKA,Y.; NAKA, K. Preparation of Composites of Liquid-Crystalline Matrix of Poly(p-phenylene-sulfoterephthalamide) and $CaCO_3$ by In Situ Mineralization. *Journal of Applied polymer Science,* 2015, DOI: 10.1002/app.41455.
[126] DOUGLAS, T.E.L. et al. Composites of polyvinyl alcohol (PVA) hydrogel and calcium and magnesium phosphate formed by enzymatic functionalization. *Materials Letters*, 2014, Vol. 137, p.62–67.
[127] GUARINO V, GALIZIA M, ALVAREZ-PEREZ M, MENSITIERI G, AMBROSIO L. Improving surface and transport properties of macroporous

hydrogels for bone regeneration. *Journal of Biomedical Material Research Part A*, 2015, Vol. 103A, p.1095–1105.
[128] TOUMIA, Y.;et al. Soft Confinement of Graphene in Hydrogel Matrixes. *Journal of Physical Chemistry, Part B,* 2015, Vol.119, p.2051−2061.
[129] RAUNER, N.; BUENGER, L.; SCHULLER ,S.; TILLER, J.C. Post-Polymerization of Urease-Induced Calcified Polymer Hydrogels. *Macromolecular Rapid Communication, 2015,* Vol.36, p. 224−230.
[130] FU, k. et al. Characterization of a biodegradable coralline hydroxyapatite/calcium carbonate composite and its clinical implementation. *Biomedical Materials*, 2013, Vol.8, No. 6, 065007 p. 1-11 doi:10.1088/1748-6041/8/6/065007.
[131] JIN, Z.; JUN, L.Y.; XIANG, C.G. Synthesis of $CaCO_3$ crystals using hydroxypropylmethyl cellulose hydrogel as template. *Chinese Science Bulletin*, 2007, Vol. 52, No. 13, p.1796-1801.
[132] BASSETT, D.C.; MARELLI, B.; NAZHAT , S.N.; BARRALET, J.E. Stabilization of Amorphous Calcium Carbonate with Nanofibrillar Biopolymers. *Advance Functional Material*, 2012, Vol. 22, p.3460–3469.
[133] JUHASZ, J.A.; BEST, S.M.; BONFIELD, W. Preparation of novel bioactive nano-calcium phosphate—hydrogel composites. *Science and Technology of Advanced Materials*, 2010, Vol. 11 014103 (7pp) doi:10.1088/1468-6996/11/1/014103.
[134] WANG, J.; YAN, H.; CHEN, T.; WANG, Y.; LI, H.; Zhi, W.; Feng, B.; Weng, J.; Zhu,M. Porous nanoapatite scaffolds synthesized using an approach of interfacial mineralization reaction and their bioactivity. *Journal of Biomedical Material Research Part B*, 2014, Vol.102B, p.1749–1761.
[135] DEWI, A.H.; ANA, I.D.; JANSEN, J. Calcium Carbonate Hydrogel Construct with Cynnamaldehyde Incorporated to Control Inflammation during Surgical Procedure, *Journal of Biomedical Materials Research Part A* 'Accepted Article', DOI: 10.1002/jbm.a.35571.
[136] ARAKAKI, A et al. Biomineralization-inspired synthesis of functional organic/inorganic hybrid materials: organic molecular control of self-organization of hybrids. *Organic Biomolecular Chemistry*, 2015, Vol.13, p. 974–989.
[137] SCHMIDLIN, P.R.; NICHOLLS, F.; KRUSE ,A.; ZWAHLEN, R.A.; WEBER, F.E. Evaluation of moldable, in situ hardening calcium phosphate bone graft substitutes. *Clinical Oral Implants Research 00,* 2011, p.1–9 DOI: 10.1111/j.1600-0501.2011.02315.x
[138] WEI, O. Novel synthesis strategy for composite hydrogel of collagen /hydroxyapatite microsphere originating from conversion of $CaCO_3$ templates. *Nanotechnology,* 2015, Vol.26, p.1-8.

[139] RAAFAT, A.I. et al. Characterization and Bioactivity Evaluation of (Starch/N-vinylpyrrolidone)—Hydroxyapatite Nanocomposite Hydrogels for Bone Tissue Regeneration. *Journal of Applied Polymer Science*, 2013, DOI: 10.1002/APP.38113

[140] PENICHE, C.; et.al. Chitosan / hydroxyapatite-based composites. *Biotecnología Aplicada*, 2010, Vol. 27, p. 202-210.

[141] ZHAO,F.; GRAYSON,W.L.; Ma, T.; BUNNELL,B.; LU, W.W. Effects of hydroxyapatite in 3-D chitosan-gelatin polymer network on human mesenchymal stem cell construct development. *Biomaterials*, 2006, Vol.27, No.9, p.1859-67.

[142] SCHMIDLIN, P.R.; NICHOLLS, F.; KRUSE, A.; ZWAHLEN, R.A.; WEBER, F.E. Evaluation of moldable, in situ hardening calcium phosphate bone graft substitutes. *Clinical Oral Implant Research 00*, 2011, p.1–9, DOI: 10.1111/j.1600-0501.2011.02315.x

[143] IKEDA, E. et al. Fully functional bioengineered tooth replacement as an organ replacement therapy. *Proceedings of National Academic of Science*, 2009, Vol. 106, No. 32, p.13475–13480, DOI: 10.1073/pnas.0902944106.

[144] SHAH, R.; SAHA, N.; KITANO, T.; SAHA, P. Preparation of $CaCO_3$-based Biomineralized Polyvinylpyrrolidone–Carboxymethylcellulose Hydrogels and their Viscoelastic Behavior. *Journal of Applied Polymer Science*, 2014, Vol.131, No. 10, DOI: 10.1002/app.40237

[145] SHAH, R.; SAHA, N.; KITANO, T.; SAHA, P. Mineralized polymer composites as biogenic bone substitute material. Polymer Processing Society, PPS 30, 2014, AIP Conference Proceedings, 1664, 070012 (2015); http://dx.doi.org/10.1063/1.4918447.

[146] SHAH, R.; SAHA, N.; KITANO, T.; SAHA, P. Influence of Strain on Dynamic Viscoelastic Properties of Swelled (H_2O) and Biomineralized ($CaCO_3$) PVP-CMC hydrogel. *Journal of Applied Rheology*, 2015, Vol. 25, No.3, 33979 (10 pages)

[147] SHAH, R.; SAHA, N.; ZUCKERMANN, R.N.; SAHA, P. Stimuli responsive and biomineralized scaffold: an implant for bone-tissue engineering. SPE ANTEC, 2015, Conference Proceedings (online available)

[148] SHAH, R.; SAHA, N.; SAHA, P. Influence of Temperature, pH and Simulated Biological Solutions on Swelling and Structural Properties of Biomineralized (CaCO3) PVP-CMC Hydrogel. Progress in Biomaterial-a Springer Open Journal, 2015, Vol.4, No.2, p.123-136.

[149] SADEGHI, M.; HOSSEINZADEH, H. Synthesis and super-swelling behavior of a novel low salt-sensitive protein-based superabsorbent hydrogel: collagen-g-poly(AMPS). *Turkish Journal of Chemistry*, 2010, Vol. 34, p.739 – 752.
[150] NINDIYASARI, F, et.al. Effect of Hydrogel Matrices on Calcite Crystal Growth Morphology, Aggregate Formation, and Co-Orientation in Biomimetic Experiments and Biomineralization Environments. *Crystal Growth and design*, 2015. DOI: 10.1021/cg5018483.
[151]Biocompatibility Testing. Pacific Biolabs. Available from: http://www.pacificbiolabs.com/testing_biocompatibility.asp
[152] LALUPPA, J.A.; McAdams, T.A.; PAPOUTSAKIS, E.T.; MILLER, W.M. Culture materials affect ex vivo expansion of hematopoietic progenitor cells. *Journal of Biomedical Material Research*, 1997, Vol. 36, No.3, p.347–359.
[153] WATAHA, J.C. Predicting clinical biological responses to dental materials. *Dental Materials*, 2012,Vol. 28, No.1, p.23–40.
[154] ZHANG, M. et.al. Properties and biocompatibility of chitosan films modified by blending with PEG. *Biomaterials*., 2002, Vol.23 No.13, p.2641–2648.
[155] Cell death assays for drug discovery Part 3. 2011, Editor, Molecular and Cell Biology. Available from: http://www.aleralabs.com/Latest/cell-death-assays-for-drug-discovery-part-3.html
[156] MTT Assay. Available from: https://en.wikipedia.org/wiki/MTT_assay

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Publications:

Thesis

- **Shah Rushita**: *Studies of biodegradation of caprolactam using soil bacterial isolates*. Master Thesis. 2011. Charotar University of Science and Technology (CHARUSAT) Changa, India. (*M.Sc. thesis*).

Journal (*International with Impact Factor*)

1. **SHAH, R.;** SAHA, N.; KITANO, T.; SAHA, P. Preparation of $CaCO_3$-based Biomineralized Polyvinylpyrrolidone–Carboxymethylcellulose Hydrogels and their

Viscoelastic Behavior, *Journal of Applied Polymer Science*. 2014, DOI: 10.1002/APP.40237. [Indexed in *Web of Science and Scopus*]

2. **SHAH, R.;** SAHA, N.; KITANO, T.; SAHA, P. Influence of Strain on Dynamic Viscoelastic Properties of Swelled (H_2O) and Biomineralized ($CaCO_3$) PVP-CMC hydrogel. *Journal of Applied Rheology*, 2015, Vol. 25, No.3 (2015), 33979 (10 pages). [Indexed in *Web of Science and Scopus*]

3. **SHAH, R.;** KRONEKOVA, Z.; ZAHORANOVA, A.; ROLLER, L.; SAHA, N.; SAHA, P.; KRONEK, J. In vitro study of partially hydrolyzed poly (2-ethyl-2-oxazolines) as materials for biomedical applications. *Journal of Materials Science: Materials in Medicine.* 2015, Vol. 26, No. 4:5485. DOI: 10.1007/s10856-015-5485-4. [Indexed in *Web of Science and Scopus*]

4. **SHAH, R.;** SAHA, N.; SAHA, P. Influence of Temperature, pH and Simulated Biological Solutions on Swelling and Structural Properties of Biomineralized ($CaCO_3$) PVP-CMC Hydrogel, Progress in Biomaterial-a springer open journal, 2015, Vol.4, No.2, p.123-136

5. **SHAH, R.;** SAHA, N.; KUCEKOVA, Z.; HUMPOLICEK, P.; SAHA, P. Properties of biomineralized ($CaCO_3$) PVP-CMC hydrogel with reference to the cytotoxicity test using fibroblasts cells. *International Journal of polymeric material and biopolymeric materials. (submitted in october 2015)*

Book Chapters / Journal

1. SAHA, N.; **SHAH, R**.; VYROUBAL, R.; KITANO, T.; SAHA, P. Morphology, Absorptivity and Viscoelastic Properties of Mineralized PVP-CMC Hydrogel, Novel Trends in Rheology V Book Series: AIP Conference Proceedings 1526 (2013) 292 – 300. ISSN: 0094-243X, ISBN: 978-073541151-7. [Indexed in *Web of Science and Scopus*]

2. SAHA, N.; VYROUBAL, R.; **SHAH, R**.; KITANO, T.; SAHA, P. Effect of Strain on Viscoelastic Behavior of Fresh, Swelled and Mineralized PVP-CMC Hydrogel, Novel Trends in Rheology V Book Series: AIP Conference Proceedings 1526 (2013) 301-309, ISSN: 0094-243X, ISBN: 978-073541151-7. [Indexed in *Web of Science and Scopus*]

3. **SHAH, R**.; SAHA, N.; KITANO, T.; SAHA, P. Mineralized polymer composites as biogenic bone substitute material. Polymer Processing Society, PPS 30, June 8-12 2014, Ohio, Cleveland, USA. AIP Conference Proceedings, 1664, 070012 (2015); http://dx.doi.org/10.1063/1.4918447. [Indexed in Google scholar, will be available in Web of Science as well]

4. **SHAH, R**.; VYROUBAL, R.; FEI, H.; SAHA, N.; KITANO, T.; SAHA, P. Preparation of bacterial cellulose based hydrogels and their viscoelastic behavior. Novel Trends in Rheology VI, 28-29 July, 2015, Zlín, Czech Republic. AIP Conference Proceedings 1662, (2015) 040007, ISSN:0094-243X,ISBN: 978-073541306-1

Conference contributions (*National/International*)

1. **SHAH, R**.; PATEL, E.; SHAH, R. Comparison of nanostructured ZnO films prepared by Solution Based Techniques. State Level Seminar on Condensed Matter Physics- with Nano-science Flavours organized by Department of Physics, Sardar Patel University, Feb 04, 2011.Anand, Gujarat, India
National Conference On Recent Trends In Theoretical And Experinmental Physics, at VP & RPTP Science College, Vallabh Vidhyanagar, 18-19 Feb,2011, Anand,Gujarat, India.

2. **SHAH, R.;** IYER, B.; SAHA, N. Studies on Biodegradation of caprolactam by soil bacterial isolate RC-2. Journal of Current opinion of Biotechnology, European Biotechnology Congress, 16-18 May, 2013, Bratislava, Slovakia. *(conference attended,oral presentation)*[Abstract Indexed in *Web of Science*]

3. VYROUBAL, R.; SAHA, N.; VESELA, D.; **SHAH, R.;** SAHA, P. Biomimetic nucleation and growth of $CaCO_3$ in bacterial cellulose produced by Gluconacetobacter xylinus (Acetobacter xylinus). Journal of Current opinion of Biotechnology, European Biotechnology Congress, 16-18 May, 2013, Bratislava, Slovakia. *(conference attended, poster presentation)*[Abstract Indexed in *Web of Science*]

4. **SHAH, R.;** KRONEK.J.; KRONEKOVA, Z.; UCNOVA, L.; LACIK,I.; SAHA, N. , SAHA, P. Partial Hydrolysis of Poly(2-ethyl-2-oxazolines) to Poly(2-oxazoline-co-ethylene imines): Synthesis, Cytotoxicity and Complexing Efficiency with the Perspective for Biomedical Applications**.** European Polysaccharides Network Of Excellence (EPNOE) 2013 International Polysaccharide conference, (Poster presentation) 21-24th October, 2013, Nice, France. (*conference attended, poster presentation*) [Abstract available in the Book of Abstracts]

5. SAHA, N.; VYROUBAL, R.; **SHAH, R**.; KITANO, T.; SAHA, P. *Effect on crystal structure of biomimetic $CaCO_3$ mineralization in biomaterial based extracellular matrix.* 12th International Symposium on Biomineralization, Freiberg, Germany, 27-30 August, 2013, Abstract available in Conference Proceedings (Published online)

6. **SHAH, R.;** KRONEK.J.; KRONEKOVA, Z.; UCNOVA, L.; LACIK,I.; SAHA, N. , SAHA, P. Partialy Hydrolyzed Poly (2-ethyl-2-oxazolines): Synthesis, Cytotoxicity and Efficiency. Plastko 2014, 8-9 April 2014, Zlin, Czech Republic. Conference proceedings, PLASTKO, 2014: ISBN 978-80-7454-335-7, available in CD. *(conference attended,oral presentation)*

7. KRONEK, J.; KRONEKOVA, Z.; **SHAH**, R.; ZAHORANOVA, A.; PAULOVICOVA, E.; PAULOVICOVA, L.; MIKULEC, M.; PETRENCIKOVA, N. The effect of structure and functionality on biotolerability of polymers based on 2-oxazolines, 8th Czech-Slovak Conference,

Polymery, 6-9th October 2014, Zamek Trest, Czech Republic. Abstract available in Conference Proceedings (Published online)

8. SAHA, N.; ALTOE**,** V.; **SHAH, R**.; BABAYAN, V.; ALONI, S.; ZUCKERMANN, R.N.; SAHA P. PVP-CMC Hydrogel as a Matrix for Biomineralization of Fe3O4 micro-nano Particles. 1st International Conference nanoFIS2014 – Functional Integrated nanoSystems, Graz, Austria, 3-5th December 2014. [Abstract available in Conference Documentation]

9. **SHAH, R.;** VYROUBAL, R.; FEI, H.; ZANDRAA, O.; SAHA, N.; KITANO, T.; SAHA, P. Bacterial Cellulose based nanobiocomposites for biomedical applications. Second Symposium on Advances in Sustainable Polymers **(**ASP-15), CoE-SUSPOL, Department of Chemical engineering, IIT Guwahati, India, 21-22nd January, 2015. [Extended Abstract available Conference Proceedings]

10. **SHAH, R.;** SAHA, N.; ZUCKERMANN, R.N.; SAHA, P. Stimuli responsive and biomineralized scaffold: an implant for bone-tissue engineering, SPE ANTEC, 23-25th March, 2015, Florida, USA, [Conference Proceedings available online].

11. SAHA, N.; **SHAH, R.;** SAHA, P. Calcium carbonate filled hydrogel as scaffold for bone tissue engineering., workshop/conference COST Action MP 1301, Bulgaria Academy of Science, 13-14 October, Sofia, Bulgaria. (*oral presentation*)

Project Accomplished

1. Project IGA/FT/2013/011,"Development of Innovative Extracellular Polymer Matrix for Biomedical Applications", Internal Grant Agency of Tomas Bata University in Zlin.
(1. 3. 2013 – 30. 11. 2013): **Member**
2. Project IGA/FT/2014/015, "Cellulose based composites for biomedical applications", Internal Grant Agency of Tomas Bata University in Zlin.(1.3.2014-30.11.2014): **Applicant**
3**.** Project IGA/CPS/2015/008, "Bacterial Cellulose based hydrogel electrolyte: A novel supercapacitor material", Internal Grant Agency of Tomas Bata University in Zlin.(1.3.2015-30.11.2015): **Member**
4. KONTAKT-II LH14050 (Czech-American Cooperation Project: 17140050302/2104): **Member**

Patents / Utility Model

1 SAHA, N., **SHAH, R.,** SAHA, T.;SAHA, P. Hydrogel-based biomaterial sensitive to magnetic stimuli, Utility Model, Univerzita Tomáše Bati ve Zlíně, Zlín, CZ, Reg.no. 27466, 2014.

Paper I

Preparation of $CaCO_3$ based Biomineralized PVP-CMC Hydrogels and their Viscoelastic Behaviour

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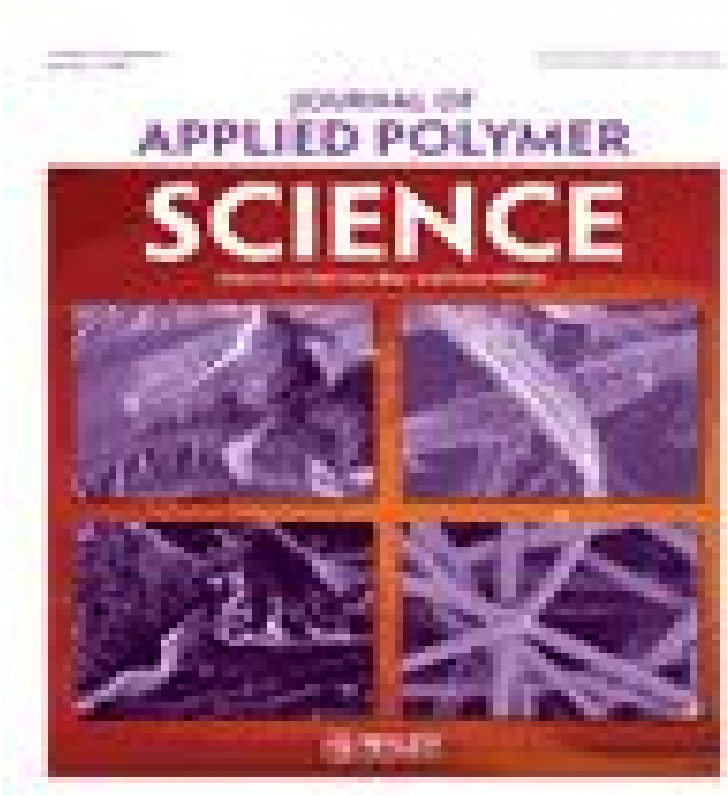
Journal of Applied Polymer Science, 2014, vol. 131 (10)
DOI: 10.1002/APP.40237
[Available in Web of Science and Scopus]



Journal of Applied Polymer Science

Preparation of $CaCO_3$-based Biomineralized Polyvinylpyrrolidone–Carboxymethylcellulose Hydrogels and their Viscoelastic Behavior

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ABSTRACT: In the blend of natural and synthetic polymer-based biomaterial of polyvinylpyrrolidone (PVP) and carboxymethylcellulose (CMC), fabrication of $CaCO_3$ was successfully accomplished using simple liquid diffusion technique. The present study emphasizes the biomimetic mineralization in PVP–CMC hydrogel, and furthermore, several properties of this regenerated and functionalized hydrogel membranes were investigated. The physical properties were studied and confirmed the presence of $CaCO_3$ mineral in hydrogel by Fourier transform infrared spectroscopy and Scanning electron microscopy. Moreover, the absorptivity of water and mineral by PVP–CMC hydrogel was studied to determine its absorption capacity. Further, the viscoelastic properties (storage modulus, loss modulus, and complex viscosity) of mineralized and swelled samples (time: 5–150 min) were measured against angular frequency. It is interesting to know the increase of elastic nature of mineralized hydrogel filled with $CaCO_3$ maintaining the correlation between elastic property and viscous one of pure hydrogel. All these properties of biomineralized hydrogel suggest its application in biomedical field, like bone treatment, bone tissue regeneration, dental plaque and tissue replacement, etc. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40237.

KEYWORDS: biomimetic; swelling; functionalization of polymers; $CaCO_3$; biomineralized hydrogel

Received 18 July 2013; accepted 30 November 2013
DOI: 10.1002/app.40237

INTRODUCTION

Over the past decade, a number of applications of hydrogels including the polymeric ones have arisen because of their advantageous and potential properties, such as high water holding capacity, equilibrium swelling degree, flexibility because of their three-dimensional cross-linked structures, biodegradability and good biocompatibility.[1–8] Many natural and synthetic hydrogels are already reported as having potential biological applications and considered as promising material for tissue engineering, as scaffolds, bone-tissue, and dental transplants.[4–9] Further, inspired by natural biogenic minerals, many researchers have attempted to mimic natural biomineralization through organic–inorganic hybridization in order to develop a new material for medical applications.[10,11] Biomineralization occurs in specific micro-environment and is finely tuned by cells and specialized biomacromolecules that offer great advantage for specific cell–material interaction. Biominerals like teeth, bones, and shells have beautiful and functional structure that cannot be achieved artificially but by the osteogenic process, they introduced the process of biomineralization and have drawn the attention to the next generation inorganic synthesis methodology[12] which makes it possible to prepare biomineralized polymeric material. Through mesocrystal theory, it is possible to understand the mechanism of biomineralization processes found in nature.[7,8,10]

A challenge in the field of biomineralization is identifying the appropriate *in vitro* model for its phenomenon. There exist several methods of forming organic–inorganic hybrid materials, which include thermally phase-induced separation, solvent casting / particle leaching, microsphere sintering, scaffold coating, etc.[13] With respect to scaffolds forming, as for materials, mineralization is generally required to incorporate the bioactive mineral like as calcium carbonate or calcium phosphate into a polymer matrix. It is reported that only powders of hydroxyapatite and β-trichlorophosphate have been used for bone-filling materials in clinical use.[12] These materials are highly bioactive and biodegradable, but they were mechanically unstable and flow out of surrounding tissue after long-term use. So it will be beneficial to utilize biomaterials having soft interface, strong and flexible structure.[12] Nature produces a wide variety of

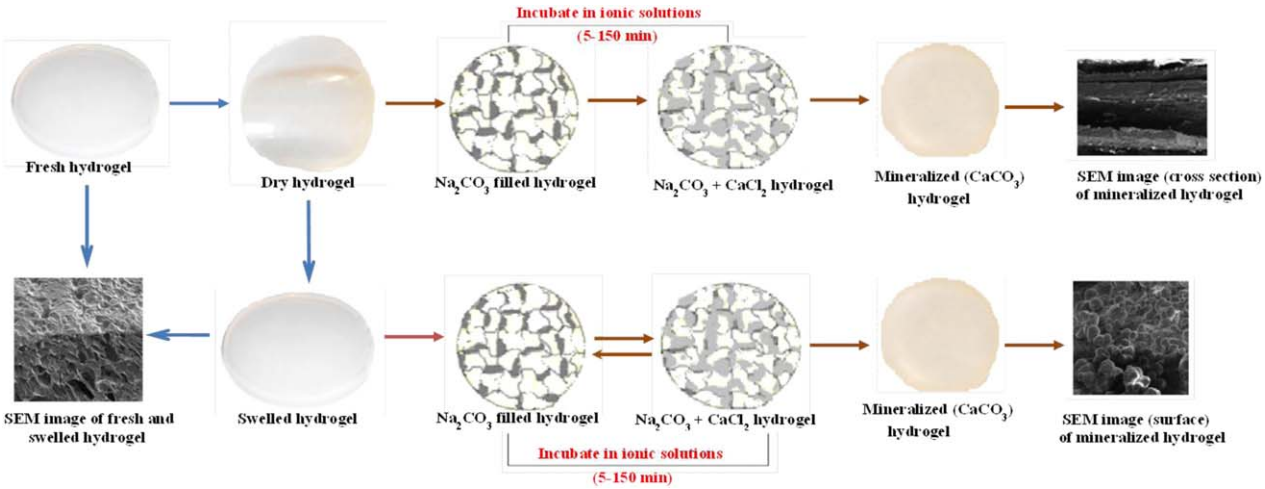

Figure 1. Schematic approach for the biomineralization of PVP–CMC-based hydrogel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

attractive and highly functionalized mineralized tissues using simple inorganic salts. The well-known fascinating natural biomineral is calcium carbonate ($CaCO_3$). This mineral exists in three different polymorphs of vaterite, aragonite, and calcite. Among them aragonite and calcite are mostly found as the polymorphs in natural system. All these polymorphs transform into most stable form, calcite, in presence of water.[9,14–18]

Several studies were carried out with template mediated mineralization using biological sources such as bacteria, algae, viruses, proteins, and biopolymers to obtain organic–inorganic composite hybrids. The matrix mediated mineralization process depends on the behavior and characteristic properties of extracellular matrix, which can be proteins, polysaccharides, or glycoproteins.[19] These matrices are often three-dimensional, fibrous, porous, and hydrated networks that can provide structural framework inorganic minerals to grow and so organic–inorganic hybrid composite materials or crystals can develop the materials with unique mechanical or structural properties. The growth of crystals in gels has emerged as a platform for biomineralization process. Various gel matrices have been used as materials motivating the researchers to study in depth the interaction of gel like matrices in association with mineralization by biological organisms.[16] Till today, many matrices, for example alginate hydrogel,[20] agarose,[21] Poly(N-isopropylacrylamide-Co-Vinyl phosphonic acid),[22] bacterial cellulose,[23] and Polyvniyl alcohol,[24] have been used for carrying out mineralization process. Tomas Bata University in Zlin researchers have already developed a polysaccharide-based hydrogel termed "polyvinylpyrrolidone (PVP) and carboxymethylcellulose (CMC) hydrogel" applying a simple physical cross-linking agent (moist heat and pressure) without using any chemical cross-linking agents.[3–6] Thus, in this study it was decided to use a new biomaterial, PVP–CMC hydrogel, as an extracellular matrix for the preparation of $CaCO_3$-based biomineralized scaffold. This calcite-based biomaterial will be useful for specific biomedical applications like bone tissue regeneration, enhancement of bone healing process, drug delivery, etc.

Moreover, among other interesting properties of hydrogels it is essential to know about viscoelastic behavior of biomineralized hydrogel. The viscoelastic properties depend on several intrinsic polymeric properties, nature of cross-linking, water content, as well as concentration of $CaCO_3$ present in biomineralized hydrogel.[7,8]

In this article, we describe the method of preparation of mineralized PVP–CMC hydrogel and its characterization in terms of physical appearance, physico-chemical structure, equilibrium swelling capacity in presence of water and equilibrium $CaCO_3$ uptake capacity from ionic solutions, and their dynamic viscoelastic properties (storage modulus, loss modulus, and complex viscosity) of mineralized and swelled samples were measured against angular frequency under low strain amplitude.

EXPERIMENTAL

Mineralized PVP–CMC hydrogel was prepared by simple liquid diffusion based technique[7,8,10] and it possesses several applications in the biomedical field. Then, characterization of the samples following standard method was done.

Materials

PVP K30 (PVP: molecular weight 40,000), polyethylene glycol 3000 (PEG: average molecular weight 2700–3300) and agar were supplied by Fluka, Switzerland; carboxymethyl cellulose (CMC) was purchased from Sinopharm Chemical Reagent (SCRC), China; glycerin was obtained from Lachema, Czech Republic; calcium chloride ($CaCl_2$: molecular weight 110.99 g/mol, 97.0%), Penta, Czech Republic; sodium carbonate-10-hydrate (Na_2CO_3: molecular weight 286.14 g/mol) was obtained from Sigma Aldrich.

Preparation of Biomineralized Hydrogel

The biomineralized hydrogel was prepared using ionic solution of calcium chloride ($CaCl_2$) and sodium carbonate (Na_2CO_3) by the following simple liquid diffusion technique as represented in Figure 1. The hydrophilic 'PVP–CMC hydrogel' was prepared following the solvent casting method applying only physical (moist heat and pressure) cross-linking agent.[3–6] The obtained fresh hydrogel was soft, smooth, off-white, and round (diameter 80 mm and thickness 6 mm). These fresh samples

were kept for 48–72 hrs at room temperature (22–25°C) for air drying as well as for freeze drying. The dry PVP–CMC hydrogel (diameter: 75 mm, thickness: 0.8–1.00 mm) was used for the preparation of biomineralized as well as water swelled PVP–CMC hydrogels. In the process of biomineralized hydrogel preparation, the dry PVP–CMC hydrogel was used as a matrix where $CaCl_2.2H_2O$ (14.7%, 1*M*, pH = 8.4) solution and Na_2CO_3 (10.5%, 1*M*, pH= 7.4) solution were used (the concentration and pH of $CaCl_2$ and Na_2CO_3 solution was maintained as mentioned in Ref.[10]) as the source of Ca^{+2} and CO_3^{-2}, respectively. The dry hydrogel film first immersed in the 50 mL solution of $CaCl_2.2H_2O$ and then transferred into 50mL Na_2CO_3 solution. In this way, mineralization of $CaCO_3$ in hydrogel matrix was carried out from 5 to 150 min keeping the test samples in each ionic solution for certain period and transferred them after equal incubation time interval. Finally, rubbery, round (diameter: 80 mm, thickness: 1–2 mm) and ivory hydrogels were obtained and designated as "mineralized hydrogel".

Simultaneously, the dry PVP- CMC hydrogel was incubated in demineralized water at room temperature for the same duration. After a certain period (i.e. after 150 min), the water-soaked hydrogel became as good as a fresh hydrogel but the thickness of the water-soaked hydrogel sample varied with incubation period. The water-soaked hydrogel was termed as "swelled hydrogel".

Scanning Electron Microscopy

The morphology of samples was determined by scanning electron microscopy (SEM). The SEM observation was carried out on VEGA II LMU (TESCAN) operating in the high-vacuum/secondary electron imaging mode at an accelerating voltage of 5–20 kV. The samples were sputter coated with a thin layer of palladium/gold alloy to improve the surface conductivity and tilted 30° for better observation. The images were taken at magnification of 100×–10k×.

Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) spectra of the specimens (Fresh PVP–CMC hydrogel and mineralized hydrogel) were obtained at wavenumber of 2000–600 cm^{-1} at room temperature with uniform resolution of 2 cm^{-1}. For this, attenuated total reflectance ATR-FTIR was used with NICOLET 320 FTIR Spectrophotometer with "Omnic" software package.

Study of Swelling Behavior

The degree of swelling of PVP–CMC hydrogel was investigated in the presence of water and ionic solutions ($Na_2CO_3 + CaCl_2$) at room temperature (22–25°C) and swelling or mineralization time from 5 to 150 min.[3,25,26] The degree of swelling can be described by absorptivity of minerals and formation of $CaCO_3$ in PVP–CMC hydrogel. The samples were immersed in mineral solutions until 150 min and in every specified time interval, the weight of the swelled and mineralized samples was recorded.

The swelling depends on absorptivity of water and formation of biogenic mineral within the hydrogel which is defined by eq. (1), wherein W_s and W_d are weights of the swollen and the dry PVP–CMC hydrogels, respectively. The PVP–CMC hydrogel is swollen either because of absorption of H_2O and/or because of diffusion of ionic salts within the matrix which form $CaCO_3$ crystal structure within the matrix as well as surface of the hydrogel:

$$\text{Absorptivity}\% = [((Ws - Wd) \div Wd)] \times 100 \quad (1)$$

Measurement of Dynamic Viscoelastic Properties

The viscoelastic properties of hydrogels were examined by using a parallel plate rheometer testing device (ARES, Rheometrics Scientific, USA) and "TA Orchestrator" software for data evaluation. Dynamic frequency sweep tests were carried out at the temperature of 28°C to obtain the storage (G'- elastic part) and loss (G''- viscous part) moduli, and complex viscosity (η^*). The geometry of measuring parallel plate was 25 mm in diameter, with a gap between plates depending on sample thickness (approximately 1–1.7 mm for mineralized, 1.8–6 mm for swelled hydrogel, and 6 mm for a fresh one). Measurement was conducted in oscillation mode with the frequency range from 0.1 to 100 rad s^{-1} at 1% strain amplitude, which is considered as small strain amplitude to maintain the measurements within the linear viscoelastic region for homogeneous polymer substances. Influence of angular frequency and swelling time and mineralization time (time interval: 5–150 min) on G', G'', and η^*, which is calculated by the following equation of water swelled and mineralized hydrogel was discussed:

$$\eta* = \sqrt{((G' \div \omega)^2 + (G'' \div \omega)^2)} \quad (2)$$

RESULTS AND DISCUSSION

SEM Micrographs of Hydrogels

Figure 2 represents the SEM micrographs of pure PVP–CMC and biogenic mineralized (calcite) PVP–CMC hydrogels, which indicate both their surfaces and their cross-section structures. Figure 2(a) shows both structures of pure PVP–CMC hydrogel and gives the information of three-dimensional cross-linking network of hydrogel and the development of pores. In Figure 2(b–e), the surface and cross section of the hydrogels mineralized at different times are shown, and it is obvious that the granular shaped calcite crystals obtained by simple liquid diffusion technique are regenerated and accumulated continuously into the PVP–CMC hydrogel matrix. During the mineralization process the pores get filled up with $CaCO_3$. The fact that during the mineralization process the impregnated hydrogel membranes are incubated to ionic solutions of (Na_2CO_3+$CaCl_2$) is shown in Figure 1. $CaCO_3$ accumulation is increased with respect to incubation time in ionic solution. Here, the Ca^{+2} ions are subjected to diffusion in the membrane and they act as nucleation sites for the carbonate mineralization. When the pores of PVP–CMC hydrogels are filled with $CaCO_3$, the nucleation of Ca^{+2} ions continuous on the surface of the hydrogel which is clearly visible at Figures 2(c,d,e).

FTIR Analysis of Hydrogels

The FTIR spectra of pure $CaCO_3$, calcite-based PVP–CMC hydrogel and nonmineralized PVP–CMC hydrogels are shown in Figure 3. From the spectrum some important characteristics can be described as follows: 1654 cm^{-1} for C=O vibration, peak in the region between 1000 and 1200 cm^{-1} representing the presence of CMC of PVP–CMC hydrogel which corresponds

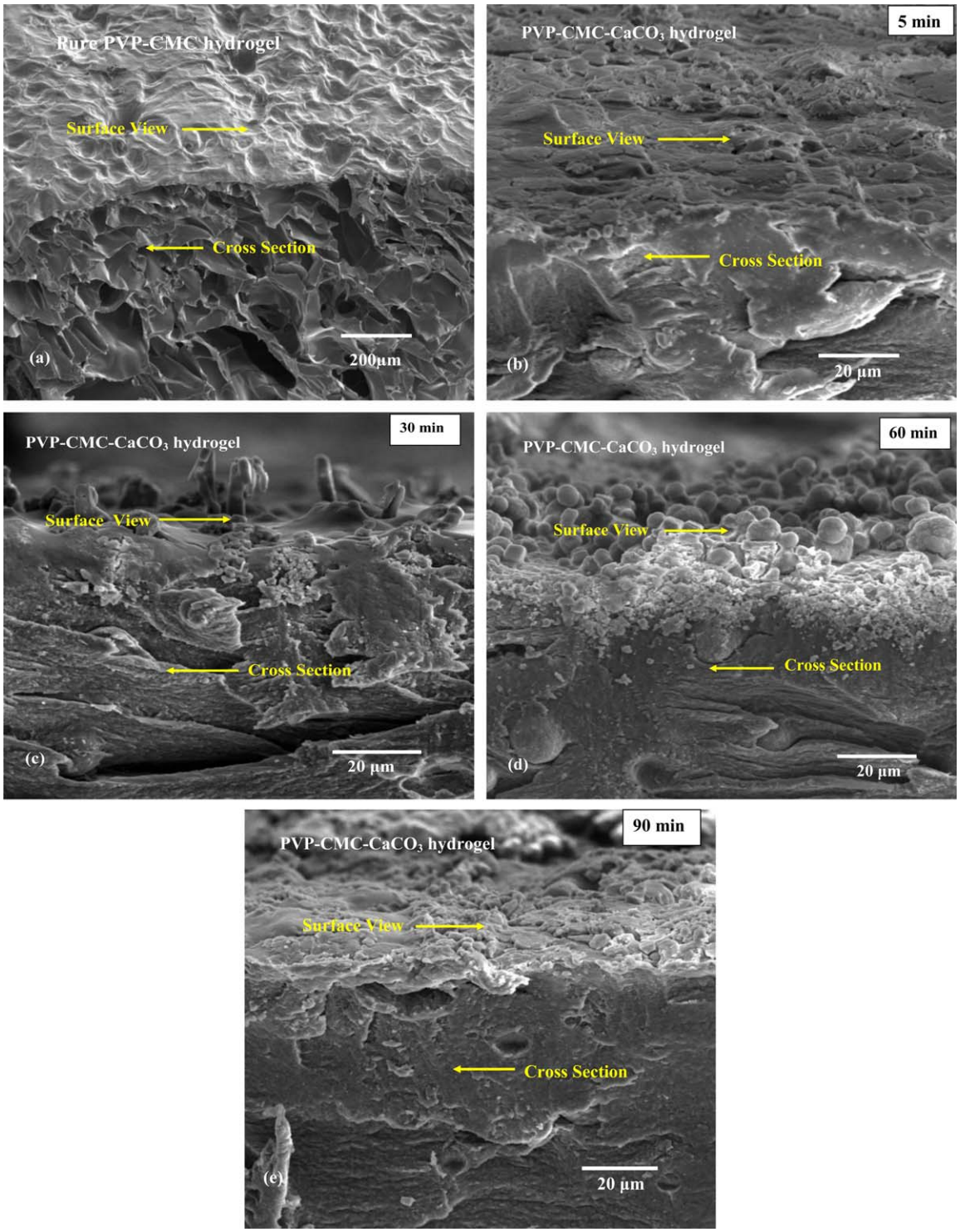

Figure 2. SEM micrographs of PVP–CMC hydrogels: (a) pure PVP- CMC; (b) biogenic mineralized (calcite) PVP–CMC hydrogels (duration 5 min); (c) biogenic mineralized (calcite) PVP–CMC hydrogels (duration 30 min); (d) biogenic mineralized (calcite) PVP–CMC hydrogels (duration 60 min); and (e) biogenic mineralized (calcite) PVP–CMC hydrogels (duration 90 min). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

to the -C-O, C-CH stretching and CO and C-O-C bending of the glycoside ring of CMC[3,27]. Here, it is clearly visible from the spectrum that CMC related peak is present in nonmineralized hydrogel but during the process of mineralization and with the increase in time, the intensity of CMC at particular wavenumber almost disappears, which confirms that there is deposition of calcium carbonate taking place within the hydrogel. Further, when considering the mineralized PVP–CMC hydrogel (a–h)

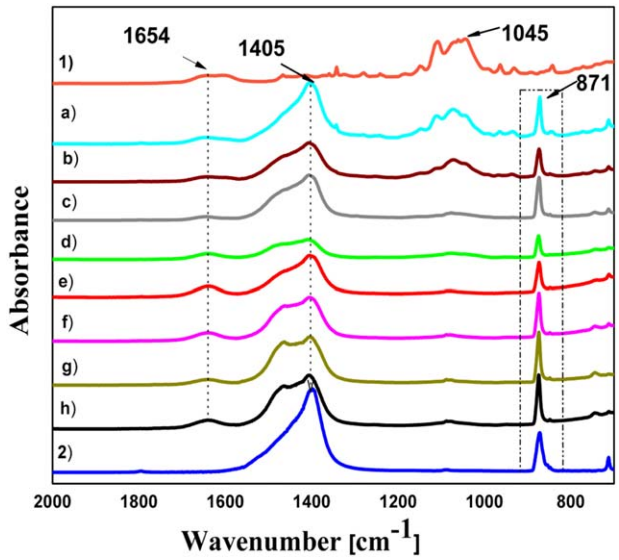

Figure 3. FTIR spectra of (1) PVP–CMC hydrogel, (2) pure $CaCO_3$ and biomineralized ($CaCO_3$) PVP–CMC hydrogel in the order time of mineralization, i.e. 5, 10, 20, 30, 60, 90, 120, and 150 mins. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and pure $CaCO_3$ strong absorption bonds related to CO_3^{2-} appear at 1405 cm^{-1} and sharp peaks are noticed at 871 cm^{-1}, which indicates the incorporation of $CaCO_3$ into PVP–CMC hydrogel.[7,8]

Absorptivity of H_2O and $CaCO_3$ within Hydrogels

The absorptivity of PVP–CMC hydrogel was studied for 150 min in the presence of water and mineral solutions (i.e. Na_2CO_3 and $CaCl_2$) to observe the H_2O uptake capacity as well as capacity to accumulate the biogenic mineral in the form of $CaCO_3$ (as shown in Figure 5). It can be seen from the figure that the swelling or water uptake capacity of PVP–CMC hydrogel is faster and higher in quantity compared to ionic solutions that finally represent the uptake capacity of calcite (i.e. $CaCO_3$). Hydrogels possess high water retention capacity.[3] There is an increase in polymeric interaction of fresh hydrogel related with the increase in time that leads to high sorption rate, and it finally leads to the water or minerals uptake potential of the hydrogel. The mineralized / calcite-based hydrogel is prepared using dry or water-swelled hydrogel film in ionic solutions wherein Ca^{+2} ions on the top layer act as nucleation sites and are slowly subjected to diffusion inside the membrane.[10] This leads to impregnation of calcite crystals in the cross-linking network and of the pores inside the hydrogel structure. However, the absorption rate of hydrogel would be expected from the increase in surface area with the increasing porosity of the hydrogels.[25] From Figure 4, it is clear that in the case of mineralized hydrogel, the rate of accumulation of $CaCO_3$ within PVP–CMC hydrogel increases with time but there is not much difference observed in thickness of the mineralized hydrogel after 90 min though investigation continued until 150 min. Thus, we can consider that for biomineralization of PVP–CMC hydrogel (thickness 6 mm) with $CaCO_3$, 90 min is the optimum condition to reach the equilibrium state for diffusion of ionic salts within the matrix to fill the pores of the hydrogel. After 90

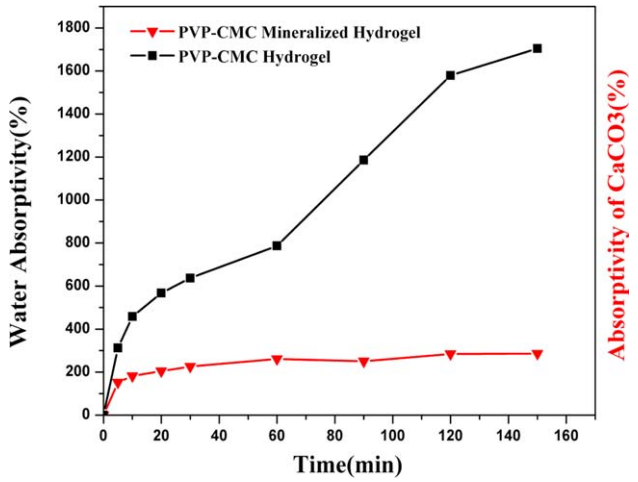

Figure 4. Absorption behavior of PVP–CMC hydrogel in presence of water and ionic solutions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

min, the biomimetic mineralization process continued but the $CaCO_3$ crystal formation occurs on the surface of hydrogel which is ascertained by Figures 2(c,d,e).

Viscoelastic Properties of Hydrogels

The viscoelastic properties of PVP–CMC hydrogel of fresh (before dry sample), water swollen (swelled in water for 5 to 150 min) and mineralized (mineralized in ionic salt solution for 5–150 min) hydrogel samples were measured at room temperature. Figure 5 reveals the typical dynamic viscoelastic properties of 90 min swelled and mineralized (filled with $CaCO_3$) PVP–CMC hydrogels as a function of angular frequency (ω). In the figure, the storage modulus (G') and loss modulus (G'') of these hydrogels are compared with those of fresh PVP–CMC hydrogel, because a gel (elastic solid) property can be defined based

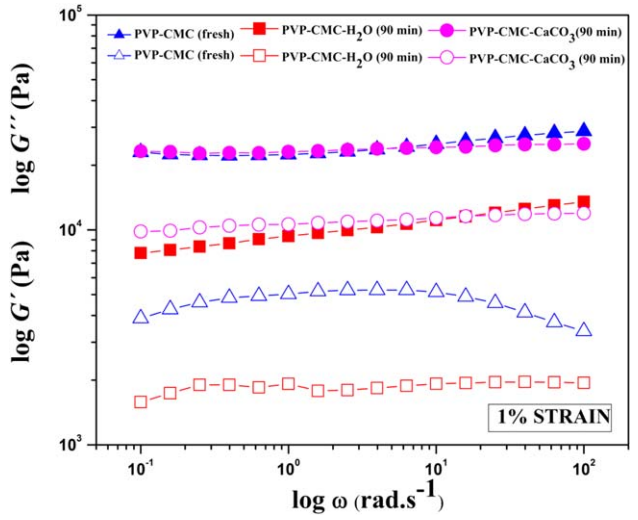

Figure 5. Storage modulus (G',filled symbol) and loss modulus (G'',open symbol) as function of angular frequency (ω) at 28°C and 1% strain for fresh (before drying), swelled in water (90 min) and mineralized with $CaCO_3$ (90 min) PVP–CMC hydrogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

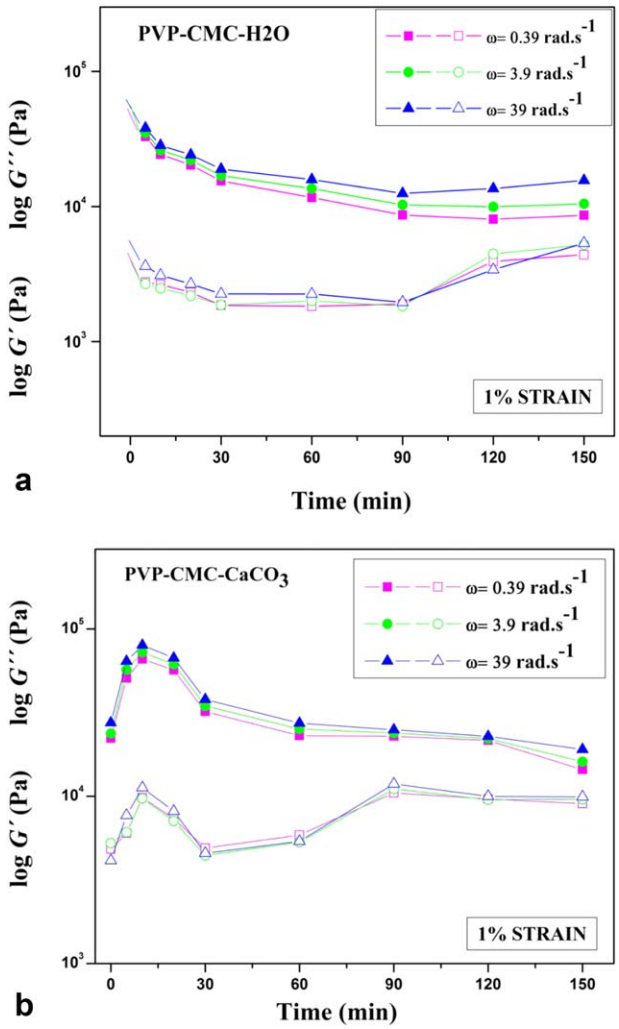

Figure 6. (a) Effect of swelling time (0–150 min) on storage modulus (G', filled symbol) and loss modulus (G'', open symbol) as a parameter of angular frequency(ω) of 0.39, 3.9, and 39 rad s^{-1} for water swelled PVP–CMC hydrogel. (b) Effect of swelling time (0–150 min) on storage modulus (G', filled symbol) and loss modulus (G'', open symbol) as a parameter of angular frequency(ω) of 0.39, 3.9, and 39 rad s^{-1} for mineralized ($CaCO_3$) PVP–CMC hydrogel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

on the concept that the storage modulus (G') is dominant in the gel phase whereas the loss modulus (G'') is dominant in the sol phase.[28,29] As can be seen in Figure 5, in case of fresh, swelled, and mineralized hydrogels the values of G' are higher than G'' across the wide range of angular frequency (ω: 0.1–100 rad s^{-11}), and dependence of these moduli on ω is not high, which is characteristic for cross-linked hydrogels. It is also noticeable that the difference in values of G' and G'' in mineralized hydrogel is not much compared to viscoelastic nature of fresh and swelled hydrogel although both moduli of mineralized hydrogel are higher than those of swelled hydrogel, which seems to confirm that the presence of $CaCO_3$ within the PVP–CMC hydrogel enhances the cross-linkage number within the hydrogel matrix and then induces the increase of viscous nature. The elastic behavior of hydrogel shows its influences compared to the viscous nature. Moreover all the hydrogels exhibited the linear curve which is also confirmed by typical characteristics for cross-linked gels or solid materials.[7,8,28,29] The results presented in Figure 5, also proved that there is not much difference in elastic properties of fresh and 90 min mineralized hydrogels but the values of elastic properties is decreased in the case of swelled hydrogel because of uptake of water (as shown in Figure 4) within the hydrogel matrix.

Relationships between G' and G'' and swelling or mineralizing time as a parameter of angular frequency (ω: 0.39, 3.9, and 39 rad s^{-1}) are shown for swelled hydrogels and mineralized ones in Figures 6(a,b), respectively. It can be seen from the figures that even though the swelled hydrogel decreases its elastic property (G') with time monotonously because of the incorporation of water into the pores of three-dimensional cross-linked PVP–CMC hydrogel [Figure 6(a)], the viscous property (G'') of this hydrogel seems to be changed with swelling time as G' except the materials after long time swelling.

The mineralized/calcite-filled hydrogel [Figure 6(b)] show very different behavior form to that of the swelled hydrogel at shorter mineralization time, that is, after both moduli reach the maximum values and then the values decrease gradually after short time. Here, it is essential to mention that irrespective of changes in angular frequencies at 1% strain the G' and G'' values of swelled and mineralized PVP–CMC hydrogel showed the same trend of viscoelastic nature [Figures 6(a,b)]. Though, the swelled hydrogel has been examined at different time intervals, the 90-min swelled samples exhibited mostly the same value of storage modulus (G') at different angular frequencies ($\omega = 0.39$, 3.9, and 39 rad s^{-1}). Similarly, the 90 min mineralized hydrogel also exhibited the same values at different angular frequencies ($\omega = 0.39$, 3.9, and 39 rad s^{-1}) regardless of G' and G''. Therefore, it can be predicted that 90 min is the optimum duration for performing the biomineralization process following the liquid diffusion technique to prepare calcite-filled PVP–CMC hydrogel at room temperature when thickness of fresh hydrogel is 6 mm.

The effect of water uptake time and mineralization duration on complex viscosity (η^*) as a parameter of angular frequency (ω) are shown for water swelled PVP–CMC hydrogel as well as for $CaCO_3$-filled PVP–CMC hydrogel in Figures 7(a,b), respectively. As can be seen from Figure 7(a), the PVP–CMC hydrogel showed linear complex viscosity (η^*) irrespective of different angular frequencies ($\omega = 0.39$, 3.9, and 39 rad s^{-1}) and becoming soft when swelled from dry PVP–CMC hydrogel. Moreover, no sharp changes occurred within the internal cross-linking structure of PVP–CMC hydrogel. On the other hand, it has been noticed that there is no physical difference observed within the $CaCO_3$-filled mineralized hydrogel, either the liquid diffusion mineralization process is initiated using dry or freshly prepared hydrogel. Thus, the effect of complex viscosity (η^*) on mineralized PVP–CMC hydrogel has been compared with fresh hydrogel. It can be seen from Figure 7(b) that regardless of angular frequencies ($\omega = 0.39$, 3.9, and 39rad s^{-1}) and at the beginning, approximately up to 10 min the values of complex viscosity (η^*) are increased because of uptake of $CaCO_3$ within

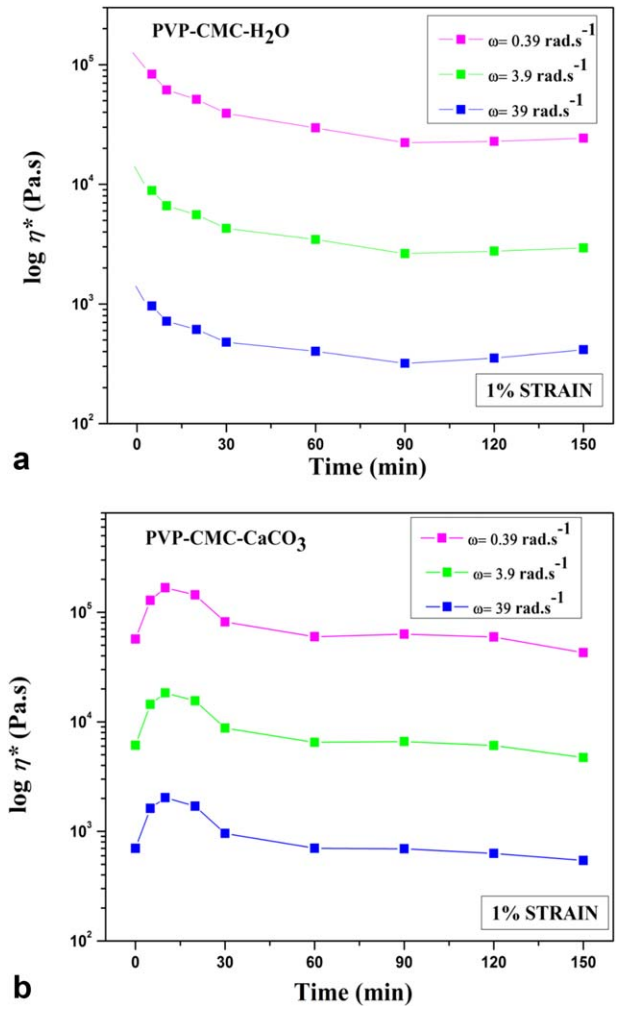

Figure 7. (a) Effect of time on complex viscocity (η^*) as a parameter of angular frequency(ω) for water swelled PVP–CMC hydrogel. (b) Effect of time on complex viscosity (η^*) for mineralized PVP–CMC hydrogel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the three-dimensional cross-linked structure of PVP–CMC hydrogel [see Figure 2(a)] and when the pore of PVP–CMC hydrogels are saturated with $CaCO_3$, the values of complex viscosity(η^*) start to decrease from 30 min mineralization time, thereafter it shows a steady linear value of complex viscosity (η^*) for the case of mineralized PVP–CMC hydrogel. The effect of time on complex viscosity (η^*) is also attributed to the result of $CaCO_3$ absorption behavior of PVP–CMC hydrogel (as shown in Figure 4).

The linear viscoelastic properties of frequency sweep experiment for minimum duration (5 min) and maximum duration (150 min) swelled in water and mineralized PVP–CMC hydrogels are depicted in Figures 8(a,b), respectively, where storage modulus (G', filled symbol) and loss modulus (G'', open symbol) are shown as a function of angular frequency (ω: 0.1–100 rad s^{-1}) at 28°C with 1% strain. These figures show more or less similar trends of viscoelastic behavior for both hydrogels that indicate the stable internal cross-linking structure of PVP–CMC hydrogel matrix.

Tan δ of swelled (5 and 150 min) and mineralized (5 and 150 min) hydrogel samples as a function of angular frequency (ω) are depicted in Figure 9(a). It can be seen from the figure that Tan δ of the samples of 150 min swelled or mineralized hydrogel are higher than that of 5 min swelled or mineralized ones over whole range of angular frequency, and at initial frequency range the Tan δ remained steady and then gradually decreased when the PVP–CMC hydrogel samples are swelled or mineralized up to 150 min, whereas, for the case of 5 min swelled or mineralized hydrogel the Tan δ is gradually decreased and then increased slightly. From these results it can be said that the change from elastic to viscous nature of both swelled and mineralized hydrogels may be influenced strongly by the swelling or mineralization time. In order to clarify the dependence of

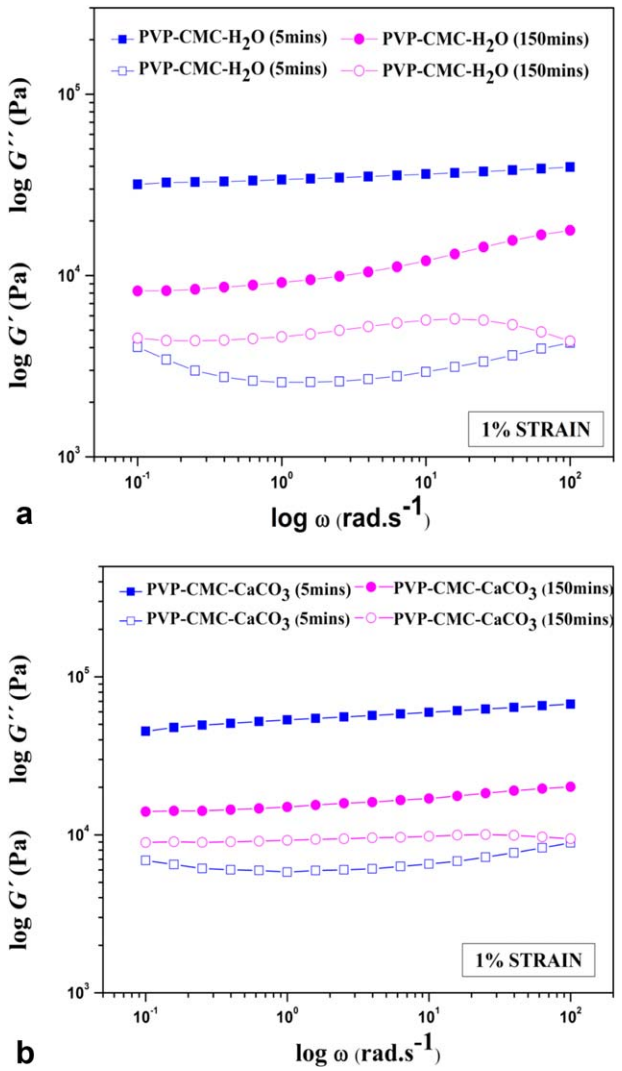

Figure 8. (a) Comparison of storage modulus (G',filled symbol) and loss modulus (G'', open symbol) between water swelled PVP–CMC hydrogel of 5 min swelling time and that of 150 min one as a function of angular frequency (ω).(b) Comparison of storage modulus (G', filled symbol) and loss modulus (G'', open symbol) between mineralized ($CaCO_3$) PVP–CMC hydrogel of 5 min mineralizing time and that of 150 min one as a function of angular frequency (ω). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

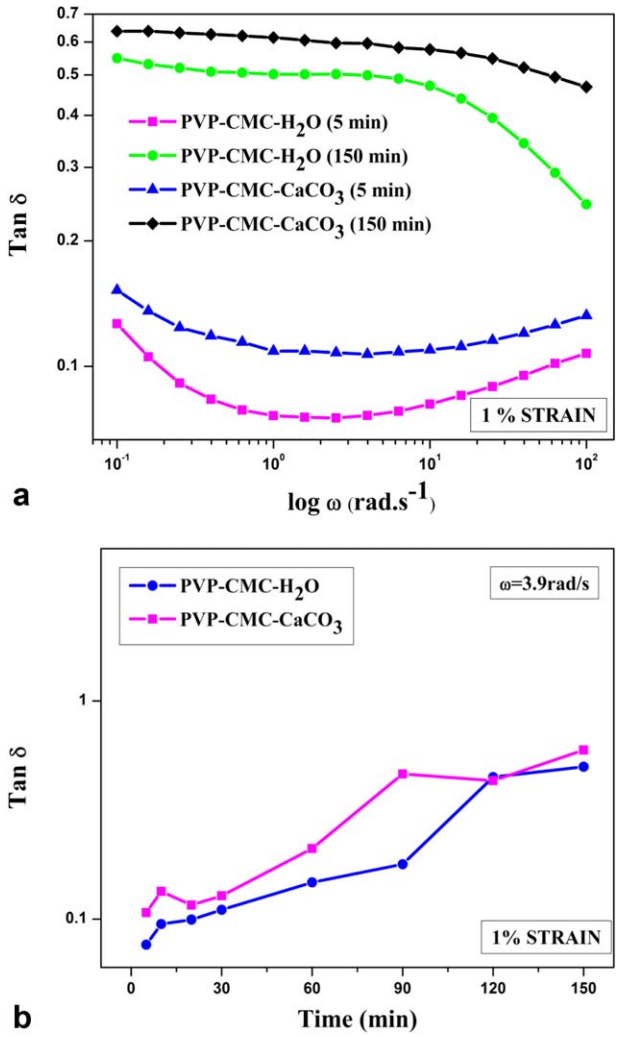

Figure 9. (a) Tan δ as a function of angular frequency(ω) for swelled (5 and 150 min) and mineralized hydrogels (5 and 150 min). (b) Tan δ as a function of swelling or mineralization time for swelled and mineralized hydrogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

viscoelastic nature on the time, the relationship between Tan δ and time of swelled and mineralized hydrogel at the angular frequency of 3.9 rad/s is shown in Figure 9(b). It is obvious from the figure that Tan δ of both hydrogels increases with the increase of swelling or mineralization time. It does not increase smoothly, which confirms that both hydrogels change from elastic nature to viscous one with the increase of time.

CONCLUSIONS

In this work, PVP–CMC hydrogel has been used as an extracellular matrix for biomimetic biomineralization study where the surfaces as well as pores were filled with $CaCO_3$. Finally a regenerated and functionalized hydrogel membrane, which would be biogenic and bioactive for medical applications like bone tissue repairing, bone regeneration, tissue replacement, dental plaque, etc., has been developed. Concerning the advantage of using the PVP–CMC hydrogel film, primarily, this hydrogel was invented at our laboratory, possible to be prepared in any size, shape, and thickness, and no chemical cross-linking agent was used. It is transparent, flexible, and hydrophilic in nature and moreover, it is possible to use it for biomineralization in fresh, dry, and also in swelled form. Thus, when liquid diffusion technique was implemented for the deposition of $CaCO_3$ within the PVP–CMC hydrogel matrix which showed an excellent absorptivity, good organic and inorganic interaction and finally a calcite-based new biomaterial termed as PVP–CMC-$CaCO_3$ or calcite-based PVP–CMC hydrogel was formed. This new material showed very stable and uniform viscoelastic properties maintaining its gel properties.

ACKNOWLEDGMENTS

The authors are thankful for the support of Research and Development for Innovation Operational Program co-funded by the European Regional Development Fund (ERDF) and national budget of the Czech Republic within the framework of the Centre of Polymer Systems project (reg. number: CZ.1.05/2.1.00/03.0111) and the support of the "Education for Competitiveness" Operational Program co-funded by the European Social Fund (ESF) and the national budget of the Czech Republic, within the "Advanced Theoretical and Experimental Studies of Polymer Systems" project (reg. number: CZ.1.07/2.3.00/20.0104).

REFERENCES

1. Kuang, M.; Wang, D.; Gao, M.; Hartmann, J.; Mohwald, H. *Chem. Mater.* **2005**, *17*, 656.
2. Zhuwei, D.; Cuixiang, L.; Haoran. L.; Dingjie, L. *Chin. J. Chem.* **2009**, *27*, 2237.
3. Roy, N.; Saha, N.; Kitano, T.; Saha, P. *J. Appl. Polym. Sci.* **2010**, *117*, 1703.
4. Saha, N.; Saarai, A.; Roy, N.; Kitano, T.; Saha, P. *J. Biomater. Nanobiotechnol.* **2011**, *2*, 85.
5. Saha, P.; Saha, N.; Roy, N. "*Hydrogel Wound Covering*" *Czech Pat. no: 302405, Czech Republic.*
6. Roy, N.; Saha, N.; Kitano, T.; Saha, P, *Carbohydr. Polym. 89*, **2012**, 346.
7. Saha, N.; Shah, R.; Vyroubal, R.; Kitano, T.; Saha, P. Novel Trends in Rheology V Book Series: AIP Conference Proceedings 2013; 1526, 292. ISSN: 0094-243X, ISBN: 978-073541151-7.
8. Saha, N.; Vyroubal, R.; Shah, R.; Kitano, T.; Saha, P. *Novel Trends in Rheology V Book Series: AIP Conference Proceedings* **2013**; *1526*, 301, ISSN: 0094-243X, ISBN: 978-073541151-7.
9. Olderøy, M.; Xie, M.; Andreassen, P. J.; Strand, L. B.; Zhang, Z.; Sikorski, P. *J. Mater. Sci. Mater. Med.* **2012**, *23*, 1619.
10. Rauch, M. W.; Dressler, M.; Scheel, H.; Opdenbosch, D. V.; Zollfrank, C. *Eur. J. Inorg. Chem.* **2012**, 5192.
11. Yang, X.; Akhtar, S.; Rubino, S.; Leifer, K.; Hilborn, J.; Ossipov, D. *Chem. Mater.* **2012**, *24*, 1690.
12. Nonoyama, T.; Ogasawara,H.; Tanaka,M.; Higuchi, M.; Kinoshita, T. *Soft Matter.* **2012**, *8*, 11531.

13. Huang, J.; Liu, G.; Song, C.; Saiz, E.; Tomsia, P. A. *Chem. Mater.* **2012**, *24*, 1331.
14. Aizenberg, J. *Adv. Mater.* **2004**, *16*, 1295.
15. Carrillo, N. A.; Quitral, V. P.; Pedram, Y. M.; Arias, L. *J. Eur. Polym. J.* **2010**, *46*, 1184.
16. Ni, M.; Ratner, D. B. *Surf. Interface Anal.* **2008**, *40*, 1356.
17. Ma, Y.; Feng, Q. *J. Solid State Chem.* **2011**, *184*, 1008.
18. Xia, Y.; Gu, Y.; Zhou, X.; Xu, H.; Zhao, X.; Yaseen, M.; Lu, R. *J. Biomacromolecules* **2012**, *13*, 2299.
19. Smith, E. A.; Li, H.; Keene, E. C.; Seh, Z. W.; Estroff, L. A. *Adv. Funct. Mater.* **2012**, *22*, 2891.
20. Zhang, F. J.; Yang, X. G.; Zhuang, Y.; Guo, K. K.; Wang, M. J.; Wei, W. F. *J. Cent. South Univ.* **2012**, *19*, 1802.
21. Li, H.; Estroff, A. L. *CrystEngComm*, **2007**, *9*, 1153.
22. Kim, Y. S.; Lee, C. S. *J. Appl. Polym. Sci.* **2009**, *113*, 3460.
23. Guzun, S. A.; Stroescu, M.; Jinga, S.; Jipa, I.; Dobre, T.; Dobre, L. *Ultrason. Sonochem.* **2012**, *19*, 909.
24. Sinha, A.; Agrawal, S.; Nayar.; Das, K. S.; Ramachandrarao, P. *J. Mater. Synth. Process.* **2002**, *10*, 149.
25. Mahdavinia, R. G.; Mousavi, B. S.; Karimi, F.; Marandi, B. G.; Garabaghi, H.; Shahabvand, S. *eXPRESS Polym. Lett.* **2009**, *5*, 279.
26. Junji, W.; Mitsuru, A. *Biomacromolecules* **2006**, *7*, 3008.
27. Pasqui, D.; Torricelli, P.; Cagna, M. D.; Fini, M.; Barbucci, R. *J. Biomed. Mater. Res. A* **2013**,00A,*00*,1.
28. Zhang, J. T.; Bhat, R.; Jandt, K. D. *Acta Biomater.* **2009**, *5*, 488.
29. Fathi, M.; Entezami, A. K.; Pashaei-Asl, R. *J. Polym. Res.* **2013**, *20*, 125, 10.1007/s10965-013-0125-5.

Paper II

Influence of Strain on Dynamic Viscoelastic Properties of Swelled (H_2O) and Biomineralized ($CaCO_3$) PVP-CMC hydrogels

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Journal of Applied Rheology, 2015, 25, 33979
DOI: 10.3933/ApplRheol-25-33979

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Influence of Strain on Dynamic Viscoelastic Properties of Swelled (H_2O) and Biomineralized ($CaCO_3$) PVP-CMC Hydrogels

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Received: 5.7.2014, Final version: 10.12.2014

Abstract:
This paper reports the rheological behavior of swelled and mineralized hydrogel prepared using polyvinylpyrrolidone (PVP) and carboxymethylcellulose (CMC) hydrogel as base polymer. Herein, the bio-mineral calcium carbonate ($CaCO_3$) was incorporated into the hydrogel using simple liquid diffusion method. The morphology of the swelled and mineralized hydrogel was analyzed through scanning electron microscopy. Further, the normalized time of absorptivity was identified from the time dependent absorptivity behavior of calcite and water filled PVP-CMC hydrogel. The effect of the biomineral ($CaCO_3$) and water on the dynamic viscoelastic properties, after penetrating inside the hydrogel matrix has been evaluated. The frequency sweep at 1 and 10 % strain and also strain sweep measurement were performed to determine the frequency and strain dependent viscoelastic moduli *G'* and *G"* of both swelled and mineralized hydrogel. At higher strain the both moduli showed significant change over wide range of angular frequency region and the nature of mineralized polymer composites (MPC) turned from elastic to viscous. Based on the observed basic properties, MPC (calcite based polymer composites) can be recommended for the treatment of adyanamic bone disorder and water swelled hydrogel can be acclaimed as a scaffold for burned wound dressing.

Key words:
PVP-CMC hydrogel, swelled and mineralized hydrogel, viscoelastic properties

1 INTRODUCTION

Hydrogels being hydrophilic polymeric materials are intensively in demand as their remarkable properties like huge water absorption capacity retaining their structural integrity, flexibility, biodegradability, biocompatibility etc. have made it possible to implement as soft material in many biomedical fields like scaffolds for tissue engineering, therapeutic drug delivery, as wound dressings and other medical device fabrication [1–12]. Hydrogels can be prepared from diversed materials that include synthetic polymers along and natural based polymers. These days' biopolymers are much in demand as they provide three dimensional environment and morphology close to extracellular matrices of native tissues [13–15].

Polymeric based hydrogels can be distinguished as either physical or chemical systems. Chemical gels can be explained as 3D-molecular network in which adjacent polymer chains are cross-linked covalently [16]. On other hand, physical gels have polymer chains linked together by secondary molecular forces like hydrogen bonding, van der Waals force, and covalent bond [16]. The polymeric biomaterial prepared using the blended form of natural and synthetic material is given much attention by the material scientists. Polyvinyl pyrollidone (PVP) being synthetic polymer is widely used in the biomedical applications because of its biocompatibility and nontoxic in nature. As such PVP alone has not much swelling properties but when mixed with any natural polymers or polysaccharides its properties are improved [1]. Polysaccharides like alginic acid/ alginate, cellulose/carboxymethyl cellulose (CMC), chitin and chitosan, based hydrogels being abundant in nature, non-toxic and biodegradable so are in focus [1, 15, 17, 18].

Biomimetic materials preparation is of great inspiration to the material scientist who is involved in understanding the mechanisms and principles behind several mineralization phenomenon [19]. Moreover, biomineralization offers another way to enhance specific cell-

material interactions [20]. However, the chemical and mechanical properties of the microenvironment affect the cellular response to the synthetic biomaterials. So, the main challenge in this field is to construct biomaterials to which cell responds towards tissue-specific phenotype [21]. These biomaterials have the capacity to augment mammalian teeth's and bones in form of implants and thus introduce the concept of biomimicking [19]. To achieve this, polymeric matrix should be prepared with biominerals, also their mechanical properties are to be focused. Several mineralization strategies are reported to prepare organic-inorganic hybrid materials like vapor diffusion technique, double diffusion, soaking method, solvent casting, particle leaching, scaffold coating [22]. Hence the use of inorganic synthesis methodology is in demand [19, 23]. The mechanism of this mineralization process in any of the organic-inorganic hybrid material can be explained by mesocrystal theory [5–7, 24]. A variety of templates are used for carrying out biomineralization process and several cations, anions and gases can diffuse through this type of matrices. Among them, the polymer scaffolds can provide compartmentalized crystallization environment and provides the facility for the anions or cations to get enter through the matrices similar to the native process of biomineralization.

Inorganic fillers like talc, glass fibers or biominerals in form of calcium carbonate or calcium phosphate, when added to the polymers matrices strengthen the properties of the particular matrix [25]. Among the biominerals used uptill now calcium carbonate has soft and strong interface. Apart from this, it overcomes the drawback of flowing out from the material. Further, there exist different polymorphs of calcium carbonate among which calcite has more stability [20, 26–30]. As these fillers interfere with the macromolecular structure of polymer matrix, the mechanical properties get altered of the newly developed complex organic-inorganic hybrid material [25]. It becomes essential to monitor the mechanical properties of such composite materials prepared by incorporating biominerals which are highly bioactive and biodegradable in nature to optimize stimulation conditions for facilitating its use in the tissue engineering or bone replacement. Bones being hard and composite material mostly comprised of biominerals in form of calcium phosphate and calcium hydroxyl-apatite. However, bone continuously remodels as per the movement and stress caused in the body of all vertebrates. Recent advances in the treatment of bone related disorders leads to the utilization of biodegradable polymers. These polymers can lead to the uptake of several biominerals in form of calcite; calcium phosphate etc and can fill up the bone defects or adyanamic bone disorder or bone cavities.

Due to wide range of applications of these types of mineralized materials, it is essential to know viscoelastic properties of material. Rheometry is one of the strongest tools to examine the microstructural changes taking place inside the materials without disrupting its surface or the whole material itself. The viscoelastic material functions in form of dynamic shear moduli, G' and G'', and tan δ can be determined [14–16, 18, 25, 31–33]. In this paper, we describe about the viscoelastic properties of mineralized PVP-CMC hydrogel and compare it with the water swelled PVP-CMC hydrogel. Apart from this, also the effect of strain on the newly formed matrix is discussed briefly.

2 EXPERIMENTAL

2.1 MATERIALS AND SWELLED AND MINERALIZED HYDROGEL PREPARATION

Water (H_2O) swelled and biomineralized ($CaCO_3$) PVP-CMC hydrogel were used in this study. Simple liquid diffusion technique [24] was implemented to prepare the water swelled and calcite incorporated PVP-CMC hydrogel and the following ingredients: PVP (0.2 %), CMC (0.8 %), PEG (1 %), Agar (2 %), and Glycerin (1 %) were used for the preparation of PVP-CMC hydrogel. PVP K30 (molecular weight 40,000), polyethylene glycol 3000 (average molecular weight 2700–3300) and agar were supplied by Fluka/Switzerland. Carboxymethyl cellulose (CMC) was purchased from Sinopharm Chemical Reagent Co-Ltd (SCRC)/China. Glycerin was obtained from Lachema/Czech Republic, calcium chloride (molecular weight 110.99 g/mol, 97.0 %) from Penta/Czech Republic, and sodium carbonate (molecular weight 105g/mol) was obtained from Sigma Aldrich.

The fresh PVP-CMC hydrogel was prepared using solvent casting technique applying controlled pressure, moisture and heat (i.e. 15 lb, 120 °C, and 15 min) in polymer solution. Finally, round, soft and off-white colored hydrogel was obtained with diameter of 80 mm and thickness of 6 mm. [1–4] To obtain mineralized hydrogel firstly, the fresh PVP-CMC hydrogel were kept under drying for around 48–72 hrs. The dry hydrogel (thickness: 0.8–1.00 mm) is soaked in the solution of minerals containing $CaCl_2$ (14.7 %) and Na_2CO_3 (10.5 %) as source of Ca^{+2} and CO_3^{2-} alternatively and was run till 150 mins. Finally the rubery, round and ivory colored hydrogel with thickenss of 1–2 mm and diameter of 80 mm were obtained and designated as mineralized hydrogel (PVP-CMC-$CaCO_3$) [5–7, 24]. On other hand, water swelled PVP-CMC hydrogel (PVP-CMC-H_2O) was obtained by soaking the dry PVP-CMC hydrogel at the same incubation period following the same liquid diffusion method.

2.2 MEASUREMENTS

Swelling behavior or absorptivity behavior

The swelling study [34, 35] of PVP-CMC hydrogel was carried out in water as well as in mineral solutions of $CaCl_2$ and Na_3CO_3. This swelling and the mineralization rate was in a specific time interval from 5 mins to 150 minutes to reach equilibrium mineralization state. The degree of swelling corresponds to the water as well as mineral absorptivity by the material, which is defined by Equation 1 where W_s and W_d are weights of swollen gel and dried gel, respectively.

$$Absorptivity(\%) = \left[(W_s - W_d) \div W_d\right] \times 100 \quad (1)$$

Morphology observation

Scanning Electron Microscopy was used to investigate the surface and interior morphologies of the freeze dried samples (before dry, water swelled and mineralized hydrogels). The scanning electron microscopy (VEGA II LMU (TESCAN)) was operated in the high vacuum/secondary electron imaging mode at an accelerating voltage of 5 – 20 kV). Before the images were taken, all the samples were freeze dried under – 81 °C for 72 hours and then lyophilized for 24 hours. Thereafter, the samples were sputter coated with a thin layer of palladium/gold alloy to improve the surface conductivity and tilted 300 for better observation. The images were taken at magnification of 100 – 10000 times.

Rheological properties

To evaluate the dynamic viscoelastic properties of swelled and mineralized PVP-CMC hydrogels, a parallel plate rheometer (ARES; Rheometrics Scientific, USA) testing machine with an "RSI Orchestrator" software package was used. A 25 mm diameter parallel plate with a gap of 2 – 3 mm was used, and dynamic oscillatory flow measurements were carried out under 1 % strain to maintain the measurements within the linear viscoelastic region (LVER) as the basic data and also under higher strain of 10 % for comparison. The storage G' and loss moduli G'', complex viscosity η^*, and tan δ were obtained as a function of a wide range of angular frequencies (0.1 – 100 rad/s). Further, the strain sweep tests were conducted in the range of 0.1 – 100 % strain at fixed angular frequency (0.35, 3.5, and 35 rad/s). All the measurements were carried out at 28 °C. The complex modulus $|G^*|$, complex viscosity $|\eta^*|$ and tan δ which gives the measure of the ratio of the energy loss to the energy stored indicating the entire viscoelastic nature of the sample are calculated by the following equations below, where ω is the angular frequency.

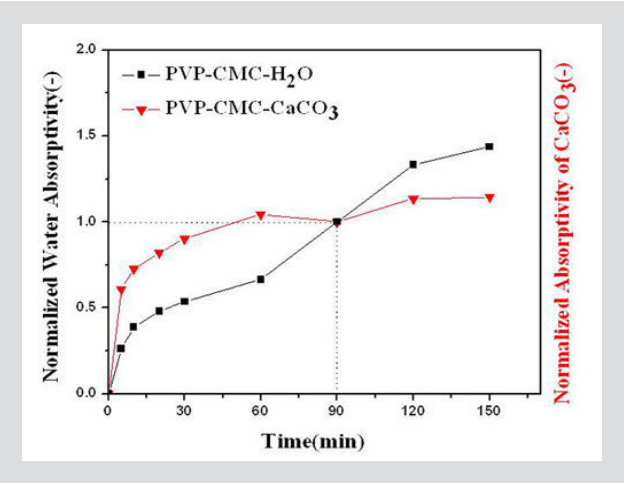

Figure 1: Time dependent absorptivity behavior of PVP-CMC-H_2O and PVP-CMC-$CaCO_3$ hydrogels. Both absorptivity of water and of $CaCO_3$ are normalized by the each value at the time of 90 minutes.

$$|G^*| = \sqrt{(G')^2 + (G'')^2} \quad (2)$$

$$|\eta^*| = (G^*/\omega) \quad (3)$$

$$Tan\delta = G''/G' \quad (4)$$

3 RESULTS AND DISCUSSION

This section will discuss about mainly the dynamic viscoelastic nature of calcium filled biomaterial which is preparing as an implant for bone tissue engineering including morphology of water swelled and biomineralized hydrogel matrix, absorption behavior of water, absorption of calcium and carbonate ions for the formation of $CaCO_3$.

3.1 ABSORPTIVITY BEHAVIOR OF HYDROGELS

The absorptivity behavior of PVP-CMC hydrogel with water and calcite filled was studied. The absorptivity behavior is always governed by hydrophobicity of the chain and the cross linking density. As there exists intermolecular non-covalent interaction, such as columbic repulsion, hydrogen-bonding and polar forces, this causes a very high sorption rate in the polymeric hydrogel [1]. If the calcite filled hydrogel is compared with the water swelled hydrogel then the uptake capacity is more in water swelled hydrogel due to high rate of polymeric interactions inside the matrix. The deposition of calcite mineral in the hydrogel matrix can be well understood by mesocrystal theory [24]. Initially the calcium ions get deposited on the top and then slowly penetrate inside the matrix structure of hydrogel. As long as the matrix gets filled up with the mineral solution thereafter the deposition will just be on the top of the hydrogel. It can be seen from Figure 1 which shows the relation between the normalized water absorptivity and absorptivity of

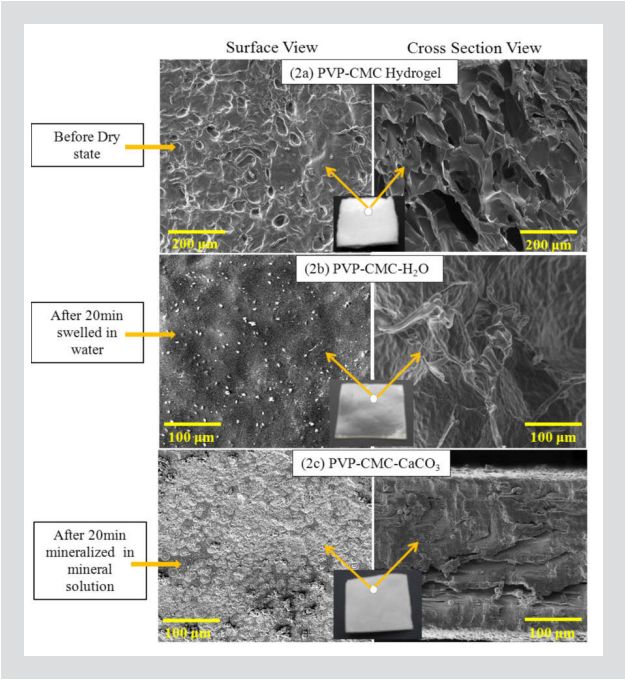

Figure 2: SEM Micrographs of a) PVP-CMC Hydrogel, b) PVP-CMC-H_2O, and c) PVP-CMC-$CaCO_3$.

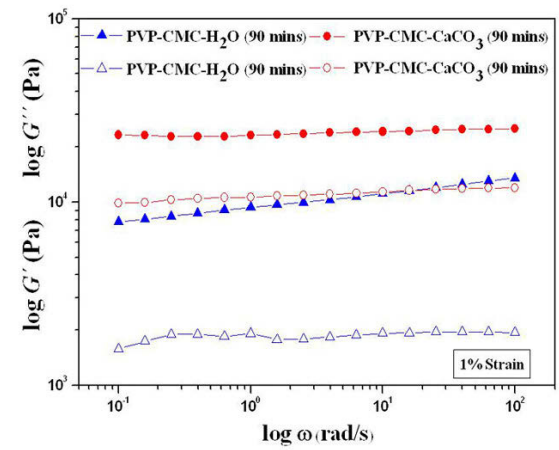

Figure 3: Dynamic viscoelasticity (storage (filled symbol) and loss moduli (open symbol) of PVP-CMC-H_2O and PVP-CMC-$CaCO_3$ at 90 minutes as a function of angular frequency under 1 % strain.

$CaCO_3$ and time that till 60 to 90 minutes there was the increase of the uptake of the mineral solution within the hydrogel but after that there was not much increase. This clearly states about the supersaturating state for the deposition of $CaCO_3$ within the hydrogel after 90 min absorption in presence of calcium chloride and sodium carbonate mineral solutions.

3.2 SEM MICROGRAPHS OF HYDROGELS

The surface and cross section morphology of before dry, water absorbed and mineralized (calcite filled) hydrogel is shown in Figure 2. PVP-CMC hydrogel which has been used as a matrix for biomimetic mineralization. Figure 2a exhibits the surface as well as internal morphology of PVP-CMC hydrogel (before drying). The 20 minutes water (H_2O) absorbed and mineral ($CaCO_3$) absorbed PVP-CMC hydrogels are depicted in Figure 2b and 2c, respectively. It can be seen from Figure 2b that after absorption, the observed pores of PVP-CMC hydrogel Figure 2a gets filled up with water and swelled slowly if the dry PVP-CMC hydrogel matrix placed in water. The observed white dots on the surface of hydrogel as shown in Figure 2b may be the scar of small water molecules. But, when the dry PVP-CMC hydrogel undergoes for mineralization following the liquid diffusion technique, the same pores filled up with $CaCO_3$ as depicted in Figure 2c. The saturation point regarding deposition and nucleation of calcium ions and absorption of water by dry PVP-CMC hydrogel is shown in Figure 1 and also reported earlier [7]. The surface image of PVP-CMC-$CaCO_3$ hydrogel is changing according to deposition of $CaCO_3$ with the increase of mineralization time however the surface image of water swelled hydrogel remained more or less same.

3.3 RHEOLOGICAL PROPERTIES OF HYDROGELS

Rheology is the sensitive method to determine the material characterization as the flow property can predict the polymer molecular weight and also the hydrogel network structure. The rheological behavior of any hydrogels depends on the polymer concentration. However, the blend of PVP with CMC increases the strength of hydrogel. Also, the addition of PEG usually increases the elasticity of the hydrogel and glycerin improves the flexibility of dry film of hydrogel. Thus, to evaluate the elastic properties of PVP-CMC hydrogel in the form of PVPCMC-H_2O and PVP-CMC-$CaCO_3$ the dynamic viscoelastic properties has been examined to understand its application on biomedical point of view.

The dynamic viscoelastic properties in the form of storage modulus G', loss modulus G'', complex viscosity η^*, and tan δ for both water swelled and mineralized hydrogels at defined time interval of 10 – 90 mins, were measured mainly at lower (1 %) and higher (10 %) strain at room temperature. It is observed through the SEM photographs that calcium is getting deposited on the top layer as nucleation sites and slowly penetrates inside the matrix of hydrogel as shown in Figure 2c. Till 90 mins there was an increase in the uptake of the calcite ions and smoothly filled the pores of hydrogel matrix but after that there was not much increase perceived in weight of the PVP-CMC hydrogel matrix as shown in Figure 1. On the other hand, it was clearly observed that after 90 min, calcium ions were just depositing on the surface of hydrogel and not penetrating inside. In case of water swelled hydrogel due to increase in polymeric interactions the uptake of water also rises (Figure 1). From all this observation it clearly indicates that 90 minutes is the saturation period and thereafter the stability is attained. From the above reason, we selected 90 and 10 minutes absorbed biomaterials as saturated and unsaturated materials, respectively for

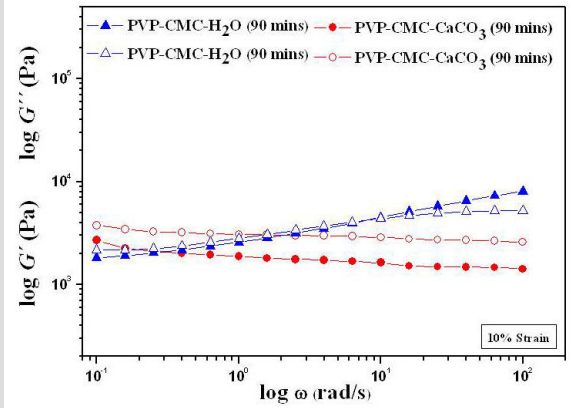

Figure 4: Dynamic viscoelasticity (storage (filled symbol) and loss moduli (open symbol)) of PVP-CMC-H_2O and PVP-CMC-$CaCO_3$ as a function of angular frequency under 10 % strain.

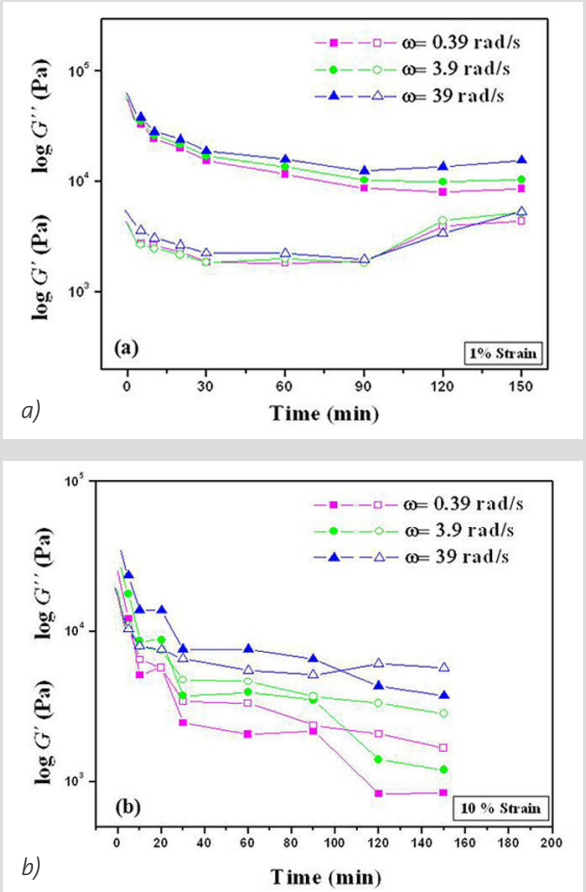

Figure 5: Storage (filled symbol) and loss moduli (open symbol) versus water (H_2O) absorption time plots of PVP-CMC hydrogel as a parameter of angular frequency (0.39, 3.9, and 39 rad/s) under different strains: a) 1 % strain and b) 10 % strain.

the evaluation of dynamic viscoelastic properties of PVP-CMC hydrogels, especially the $CaCO_3$ mineralized ones (i.e. PVP-CMC-$CaCO_3$).

In Figure 3, *G'* and *G"* as a function of angular frequency ω at 1 % strain are compared for both 90 minutes water swelled and mineralized PVP-CMC hydrogels. It is generally considered as storage modulus being dominant in gel phase whereas loss modulus in sol phase. As seen from the figure the storage modulus *G'* of water swelled and mineralized hydrogels are higher as compared to the loss modulus *G"* over the whole range of angular frequency (0.1–100 rad/s). The dependence of these moduli on frequency is not so high, which is general characteristics of the cross linked hydrogels [5–7, 36]. Also, it can be seen from the figure that the values of *G'* and *G"* of mineralized hydrogel are higher than both the moduli of water swelled hydrogel sample. This result may be due to the strong effect of $CaCO_3$ in the matrix for improving the both moduli. It is generally considered that the elastic property of hydrogels decreases slowly with the filling up of water molecules. Overall the trend of all the moduli sustain the plateau behavior at same level throughout whole frequency range confirms that the material is strictly/rigid cross-linked gel or solidlike material. *G'* and *G"* versus frequency curves at 10 % strain for the same materials in Figure 3 are shown in Figure 4. It can be seen from the comparison of both figures that, the behavior of both curves changes drastically with the increase of strain from 1 to 10 %. The values of *G'* and *G"* highly decrease in case of mineralized hydrogel as compared to swelled hydrogel and *G'* become lower than *G"* over the whole frequency range. Moreover, at lower frequency region from 0.1 to 1 rad/s both moduli of mineralized hydrogel gradually decrease with the increase of angular frequency yet they maintain the steady linear curve which confirms the firm adhesion of $CaCO_3$ biomineral with the matrix of PVP-CMC hydrogel. In the case of water swelled hydrogel, the values of *G'* exhibits the slightly lower values than *G"* in the lower frequency region. This indicates that the physical bonding and the entanglements in polymers are broken at lower angular frequency region (0.1–1 rad/s). Further, the increase of *G'* is also observed after certain value of angular frequency (10 rad/s). Here, the values of *G'* and *G"* are merged at some point then *G"* gradually decreases whereas *G'* increases. This shows that the polymer is flexible throughout.

The relationship between *G'* and *G"* of water swelled PVP-CMC hydrogel with respect to time interval from 5–150 minutes at fixed angular frequencies (0.39, 3.9, and 39 rad/s) is shown at 1 and 10 % strain in Figures 5a and 5b, respectively. The value of *G'* at 1 % strain gradually decreases with increase in time due to filling up of pores in hydrogel which is its peculiar arrangement, however *G'* is always higher compared to *G"* even at different angular frequencies. Further, there is the change observed in the viscous property of hydrogel. With the increase of swelling time, there is a sudden rise of *G"* and then gradually attains the stability (Figure 5a). It can be seen from the figure that even at different angular frequency the trend of *G'* and *G"* remains the same. With the increasing of strain from 1 to 10 %, there is drastic change observed in the behavior of *G'*

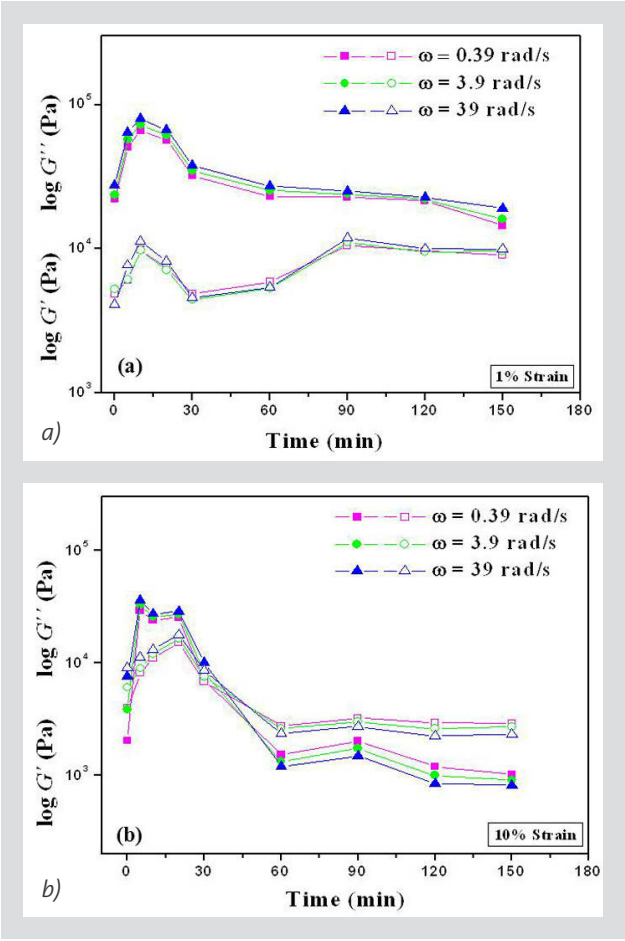

Figure 6: Storage (filled symbol) and loss moduli (open symbol) versus $CaCO_3$ mineralization time plots of PVP-CMC hydrogel as a parameter of angular frequency (0.39, 3.9, and 39 rad/s) under different strains: a) 1 % strain and b) 10 % strain.

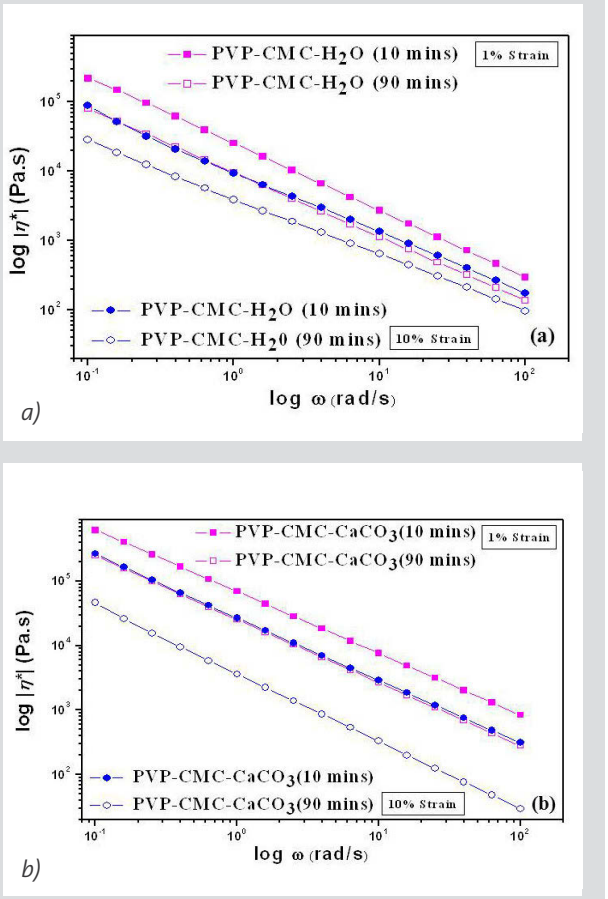

Figure 7: Angular frequency dependent complex viscosity under 1 and 10 % strain for 10 and 90 minutes mineralized or water swelled PVC-CMC hydrogels: a) PVC-CMC-H_2O hydrogel and b) PVC-CMC-$CaCO_3$ hydrogel.

and *G"* (Figure 5b). Both *G'* and *G"* values starts decreasing on applying higher strain (i.e. 10 % strain). However, it is observed that the decrease of *G'* is higher as compared to *G"* with the respective frequencies, especially at frequencies of 0.39 and 3.9 rad/s. The elastic properties of hydrogel gets loosen with the increase of time and the viscous nature of the gel at short absorption time is almost comparable with elastic nature. So, it is predicted that as higher strain is applied on hydrogel based biomaterial, the interactions of cross linking and bonding inside the polymer get weaken and separated and then the change of elastic to viscous nature of the gel is achieved. The relationship between *G'* and *G"* of mineralized PVP-CMC hydrogel with respect to time interval from 5–150 minutes at the fixed angular frequencies (0.39, 3.9, and 39 rad/s) is shown at 1 and 10 % strain in Figures 6a and 6b, respectively. In case of mineralized PVP-CMC hydrogel, at 1 % strain (Figure 6a), both *G'* and *G"* increases in shorter mineralization time and reach maximum values but thereafter gradually the values starts decreasing. At certain time interval of 90 minutes, the values of *G'* and *G"* are almost the same at different angular frequencies (0.39, 3.9, and 39 rad/s) for mineralized hydrogel. In comparison with Figure 5a for water swelled hydrogel, the trend in viscoelastic nature of both types of hydrogels is similar under 1 % strain at various angular frequencies. It can be concluded that 90 min is the optimum duration for bio mineralization process. On increase in the strain from 1 to 10 % in Figure 6b, the trend of *G'* and *G"* against time curves are the same. This clearly states that there exists the strong bonding of calcium ions with the polymeric chains of the PVP-CMC hydrogel.

Complex viscosity means the viscous property of the materials which is calculated from dynamic viscoelastic values and is considered as an important physical value for the materials such as gels in which it is difficult to measure the viscosity directly. It is known that complex viscosity coincides well with shear viscosity under low shear rate or low angular frequency for normal polymeric materials (liquids) in the linear flow condition. Complex viscosity η^* under 1 and 10 % strain versus angular frequency ω plots in double-logarithmic coordinates are shown in Figures 7a and 7b for 10 and 90 min water swelled PVC-CMC hydrogels and mineralized ones, respectively. From Figure 7a it is noticeable that the values of complex viscosity at both 1 and 10 % strain decreases linearly with the increase of frequency and also decreases monotonously with the increase of swelled time and strain. However this trend remains

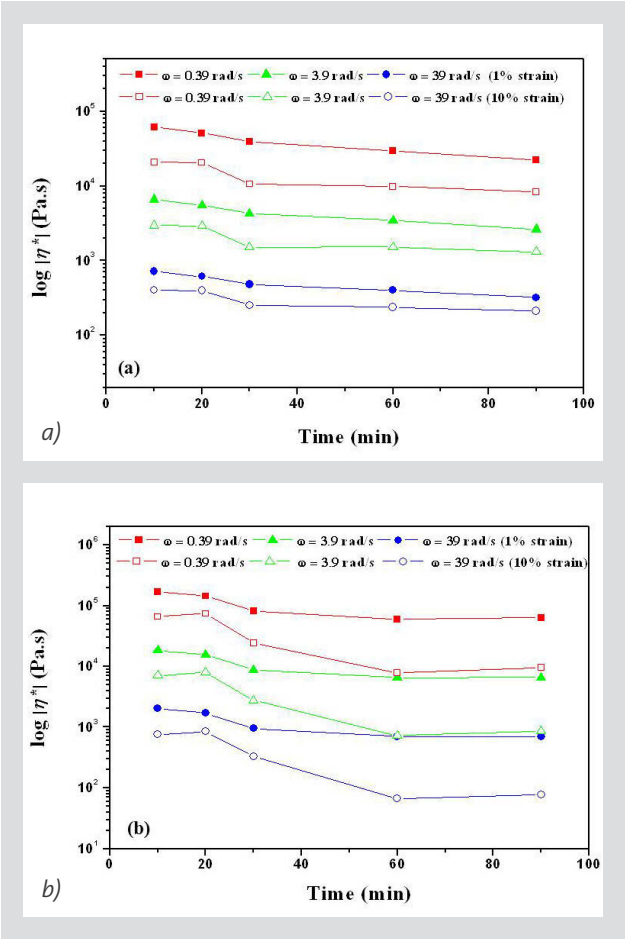

Figure 8: Time dependent complex viscosity η as a parameter of angular frequency under 1 and 10 % strain for mineralized or water swelled hydrogel: a) PVC-CMC-H_2O hydrogel and b) PVC-CMC-$CaCO_3$ hydrogel.*

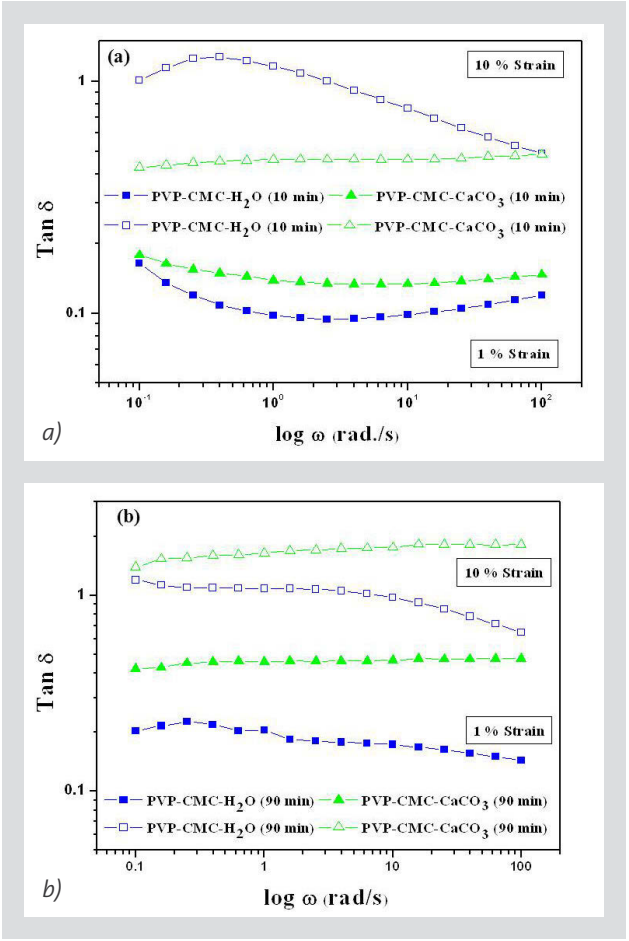

Figure 9: Loss angle tan δ as a function of angular frequency under 1 % (filled symbol) and 10 % strain (open symbol) for H_2O absorption and $CaCO_3$ mineralization PVP-CMC hydrogels with different times: a) 10 minutes and b) 90 minutes.

the same throughout as the true nature of polymeric based hydrogels. Th complex viscosity η^* of mineralized hydrogel as shown in Figure 7b exhibits more or less similar behavior to those of water swelled hydrogel in Figure 7a under both low and high strain values for both short and long absorption time sample.

Figures 8a and 8b shows the time dependence curves of complex viscosity η^* at the particular angular frequencies of 0.39, 3.9, and 39 rad/s for both mineralized and water swelled hydrogels, respectively. It is found clearly from these figures that η^* of both hydrogels simply decrease with the increase of angular frequency and strain. And also from both figures it can be decided that the values of viscosity parameter decreases from 10 mins up to around 40 min , but thereafter η^* of the samples get stable. This indicates that there is not much change in the structure of hydrogel after swelled in water and filling up with calcium. Loss angle tan δ defined as the ratio of loss modulus *G"* and storage modulus *G'* shows the correlation between viscous and elastic properties of the materials under oscillatory flow. The tan δ of water swelled (10 minutes) and mineralized (10 minutes) hydrogels as a function of angular frequency at 1 and 10 % strain is shown in Figure 9a. It is clear from the figure that tan δ of 10 min samples at 1 % strain for both swelled or mineralized hydrogel are lower as compared to 10 % strain, i.e. the increase of strain increases tan δ. At lower angular frequency region, the values of tan δ are almost steady and then gradually decrease and then start increasing with frequency at 1 % strain. As strain increases tan δ is initially stable but then gradually decreases. The tan δ values of both hydrogels at 1 % strain is low over the whole range of angular frequency, which means that elastic property is more predominant than viscous property. With the increase of strain, tan δ increases for both hydrogels, which means that *G"* approaches *G'* with the increase of strain (as shown in strain sweep curves later). In Figure 9b, tan δ with respect to angular frequency at 90 minutes of swelled and mineralized hydrogels is shown. Here, at 1 % strain the linear nature is observed in both water swelled and calcite filled hydrogel. However, in case of water filled hydrogel at certain high angular frequency the decrease in the values of Tan δ is noticed. Further on increasing the strain up to 10 % the values of tan δ in both the types of hydrogels is found to become higher. Angular frequency dependence of 90 minutes absorption hydrogels is considered to be lower than that of 10 minutes absorption ones.

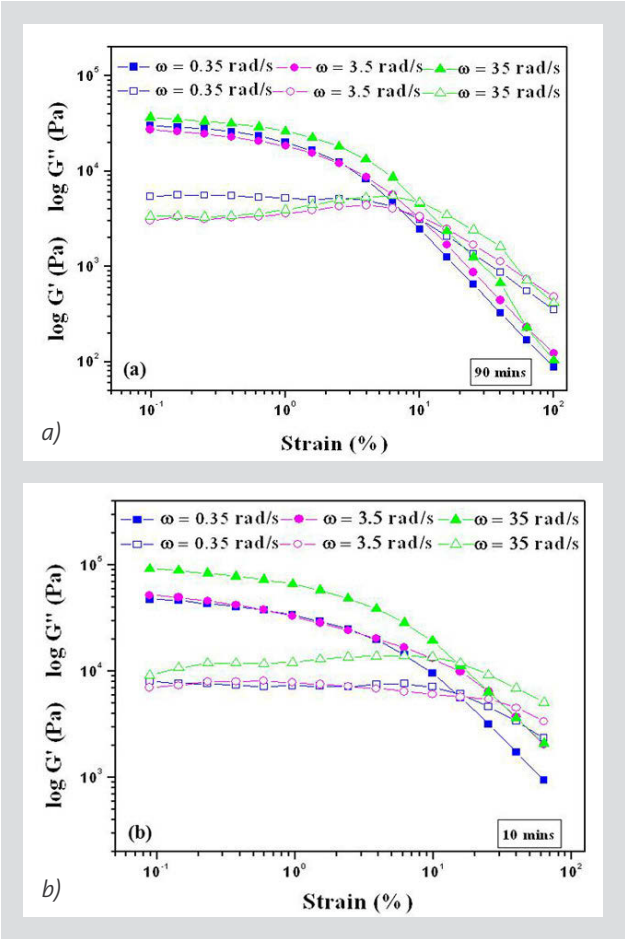

Figure 10: Storage modulus G' (filled symbol) versus strain and loss modulus G" (open symbol) versus strain plots as a parameter of angular frequency for PVP-CMC-$CaCO_3$ hydrogels with different times: a) 10 minutes and b) 90 minutes.

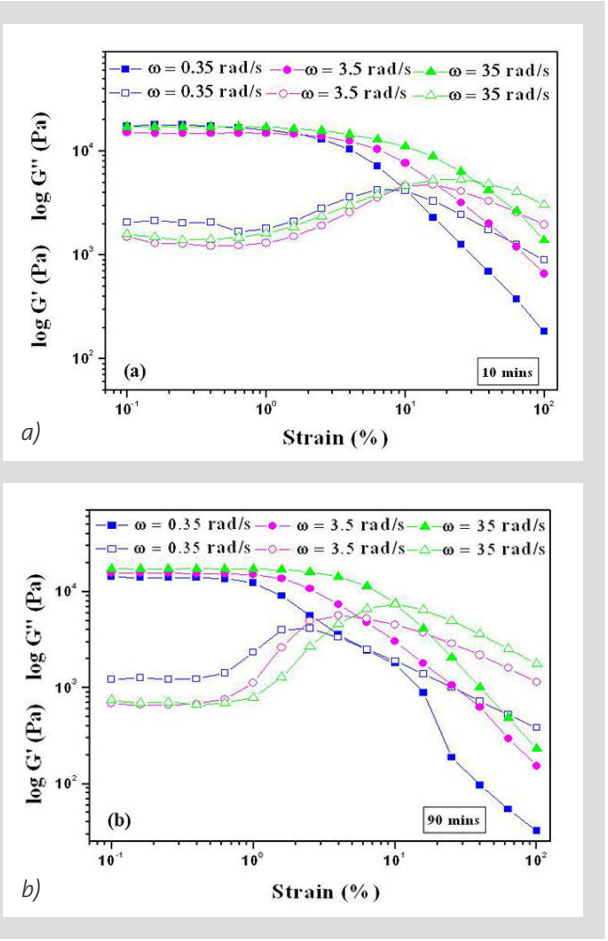

Figure 11: Storage modulus G' (filled symbol) and loss modulus G" (open symbol) versus strain plots as a parameter of angular frequency for PVP-CMC-H_2O hydrogels with different times. a) 10 minutes and b) 90 minutes.

The results of strain sweep measurements for both hydrogels are shown in Figures 10 and 11. In this experiment, strain was changed from 0.1 to 100 % under constant angular frequency. Figure 10a shows the behavior of *G'* and *G"* with respect to strain at the particular angular frequencies of 0.35, 3.5 and 35 rad/s for $CaCO_3$-PVP-CMC hydrogels with 10 min absorption time. Initially the trend is same for both the moduli with steady linear curve indicating the strong adhesion of calcium ions with the polymeric chain but slowly the decline of the slope of the curve is observed in both moduli with the increase of strain in the low and middle range of strain, and with further increase of strain, both moduli decrease sharply. With increase in angular frequency from 0.35 to 35 rad/s, there is the increase of *G'* and also *G"* exhibits the same trend as *G'*. Similarly, in case of *G"* the increase of the values with increasing frequency is noticed. As shown in the figure, both moduli approach each other with the increase of strain and with further increase of strain *G"* becomes higher than *G'*. This behavior seems to mean that the gel losses its stiffness (elastic property) with the increase of strain and the point is achieved wherein elastic and viscous nature coincides. This can predict that the material is slowly getting transformed into the viscous property predominant ones. Figure 10b shows the behavior of *G'* and *G"* with 90 mins of absorption time. Here, also initially with increasing the strain from 0.1 to 100 % the steady curve is obtained in both *G'* and *G"* but after applying certain strain suddenly fall down is noticed in both moduli. Further, also with the increase of angular frequency both moduli do not increase monotonously as in case of the sample with 10 mins of absorption time shown in Figure 10a. Storage modulus values increase only at $\omega = 35$ rad/s whereas *G"* decreases with the increase of the frequency from 0.35 to 35 rad/s. Overall in the dependence of both *G'* and *G"* of this hydrogel on strain, it is noticed the similar trend to the behavior of the melts of polymers and their blends.

Figure 11a shows the *G'* and *G"* versus strain curves of 10 minutes water absorbed hydrogels at various angular frequencies (0.35, 3.5, and 35 rad/s). In comparison with the results of 10 min mineralized hydrogels in Figure 10a, we are able to observe the remarkable change of *G"* curves which show the increase of the value with the increase of strain and the decrease with further increase of strain, showing the maximum point at the critical value of strain, and this critical strain increases with the increase of angular frequency. Although the reason of this behavior is not clear at present, it seems

to be relating with the gradually fracture of the internal structure of water absorbed hydrogel under higher strain region, and as the result, viscous nature of the gel increase more quickly in comparison with that of mineralized gel shown in Figure 10a in which we do not observe clearly this behavior. In Figure 11b for 90 minutes water absorbed hydrogels, the trend of the curves is similar to 10 mins curves although there is the change of the absolute values of *G'*, *G"*, and critical strain.

4 CONCLUSION

The liquid diffusion technique was used to achieve the PVP-CMC-H_2O (water swollen hydrogel scaffold) and PVP-CMC-$CaCO_3$ (biomineralized polymer composites (MPC)). The morphological image confirms the existence of porous structure within the PVPCMC hydrogel matrix as well as uptake of water by dry PVP-CMC hydrogel and the deposition of $CaCO_3$ within the hydrogel matrix. Both PVP-CMC-H_2O and PVP-CMC-$CaCO_3$ are flexible in nature. Water swelled PVP-CMC biomaterial maintained its elastic and viscous behavior depending upon its water uptake. In the case of PVP-CMC-$CaCO_3$, the mineralized polymer composites, 90 min is the optimum duration for biomineralization process. The frequency sweep at 1 and 10 % strain and also strain sweep measurement were performed to determine the frequency and strain dependent viscoelastic moduli (*G'* and *G"*) of both swelled and mineralized hydrogel. At low strain, elastic property expressed by *G'* was more predominant than the viscous properties expressed by *G"*, however both moduli changed significantly at higher strain over wide range of angular frequency region. Further, the nature of mineralized polymer composites (MPC) turned from elastic to viscous. The calcite filled biomaterial investigated here can be utilized in biomedical applications like adyanamic bone disorder and water swelled hydrogel can be acclaimed as a scaffold for burned wound dressing.

ACKNOWLEDGEMENTS

The authors are thankful for the support of Operational Programme Research and Development for Innovation co-funded by the European Regional Development Fund (ERDF) and national budget of Czech Republic within the framework of the Centre of Polymer Systems project (CZ.1.05/2.1.00/03.0111) and the support of the Operational Programme "Education for Competitiveness" co-funded by the European Social Fund (ESF) and the national budget of the Czech Republic, within the "Advanced Theoretical and Experimental Studies of Polymer Systems" project (CZ.1.07/2.3.00/20.0104). This work was conducted within the framework of COST Action MP1301 "New Generation Biomimetic and Customized Implants for Bone Engineering".

REFERENCES

[1] Roy N, Saha N, Kitano T, Saha P: Novel hydrogels of PVP–CMC and their swelling effect on viscoelastic properties, J. Appl. Polym. Sci. 117 (2010) 1703 – 1710.
[2] Saha N, Saarai A, Roy N, Kitano T, Saha P: Polymeric biomaterial based hydrogels for biomedical applications, J. Biomater. Nanobiotechnol. 2 (2011) 85 – 90.
[3] Saha P, Saha N, Roy N: Hydrogel Wound Covering, Patent No: 302405, Czech Republic
[4] Roy N, Saha N, Kitano T, Saha P: Biodegradation of PVP–CMC hydrogel film: A useful food packaging material, Carbohydrate Polymers 89 (2012) 346 – 353.
[5] Saha N, Shah R, Vyroubal R, Kitano T, Saha P: Morphology, absorptivity and viscoelastic properties of mineralized PVP-CMC Hydrogel, in Novel Trends in Rheology V, AIP Conference Proceedings 1526 (2013) 292 – 300.
[6] Saha N, Vyroubal R, Shah R, Kitano T, Saha P: Effect of strain on viscoelastic behavior of fresh, swelled and mineralized PVP-CMC hydrogel, in Novel Trends in Rheology V, AIP Conference Proceedings 1526 (2013) 301 – 309.
[7] Shah R, Saha N, Kitano T, Saha P: Preparation of $CaCO_3$-based biomineralized polyvinyl-pyrrolidone-carboxymethylcellulose hydrogels and their viscoelastic behavior, J. Appl. Polym. Sci. 131 (2014) 40237.
[8] mKuang M, Wang D, Gao M, Hartmann J, Mohwald H: A bio-inspired route to fabricate submicrometer-sized particles with unusual shapes – Mineralization of calcium carbonate within hydrogel spheres, Chem. Mater. 17 (2005) 656 – 660.
[9] Zhuwei D, Cuixiang L, Haoran L, Dingjie L: Biomimetic nucleation and morphology control of $CaCO_3$ in PAAm hydrogels synthesized from lyotropic liquid crystalline templates, Chine. J. Chem. 27 (2009) 2237 – 2241.
[10] Bencherif SA, Sands RW, Bhatta D, Arany P, Verbeke CS, Edwards DA, Mooney DJ: Injectable preformed scaffolds with shape-memory properties, PNAS 109 (2012) 19590 – 19595.
[11] Vallée F, Müller C, Durand A, Schimchowitsch S, Dellacherie E, Kelche C, Cassel JC, Leonard M: Synthesis and rheological properties of hydrogels based on amphiphilic alginateamide derivatives, Carbohydrate Research 344 (2009) 223 – 228.
[12] Amini AA, Nair LS. Injectable hydrogels for bone and cartilage repair, Biomed. Mater. 7 (2012) 024105.
[13] Killion JA, Kehoe S, Geever LM, Devine DM, Sheehan E, Boyd D, Higginbotham CL: Hydrogel/bioactive glass composites for bone regeneration applications: Synthesis and characterisation, Mater. Sci. Eng. C 33 (2013) 4203 – 4212.
[14] Jahromi SH, Grover LM, Paxton JZ, Smith AM: Degradation of polysaccharide hydrogels seeded with bone marrow stromal cells, J. Mech. Behavior Biomed Mater. 4 (2011) 1157 – 1166.

[15] Xu J, Liu Z, Erhan SZ: Viscoelastic Properties of a biological hydrogel produced from soybean oil, J. Am. Oil Chem. Soc. 85 (2008) 285 – 290.
[16] Jones DS, Lawlor MS, Woolfson AD: Rheological and mucoadhesive characterization of polymeric systems composed of poly(methylvinylether-co-maleicanhydride) and poly(vinylpyrrolidone), designed as platforms for topical drug delivery, J. Pharma. Sci. 92 (2003) 995 – 1007.
[17] Rao RR, Jiao A, Kohn DH, Stegemann JP: Exogenous mineralization of cell-seeded and unseeded collagen–chitosan hydrogels using modified culture medium, Acta Biomaterialia 8(2012) 1560 – 1565.
[18] Ahearne M, Yang Y, El Haj AJ, Then KY, Liu KK: Characterizing the viscoelastic properties of thin hydrogel-based constructs for tissue engineering applications, J. R. Soc. Interface 2 (2005) 455 – 463.
[19] Munro NH, MacGrath KM: Hydrogels as biomimetic mineralisation scaffolds, Bioins. Biomim. Nanobiomat. 1 (2011) 26 – 37.
[20] Nonoyama T, Ogasawara H, Tanaka M, Higuchi M, Kinoshita T: Calcium phosphate biomineralization in peptide hydrogels for injectable bone-filling materials. Soft Matter 8(2012) 11531 – 11536.
[21] Olderøy M, Xie M, Andreassen JP, Strand BL, Zhang Z, Sikorski P: Viscoelastic properties of mineralized alginate hydrogel beads, J. Mater. Sci.: Mater. Med. 2 (2012) 1619 – 1627.
[22] Yang X, Akhtar S, Rubino S, Leifer K, Hilborn J, Ossipov D: Direct "Click" Synthesis of hybrid bisphosphonate–Hyaluronic acid hydrogel in aqueous solution for biomineralization, Chem. Mater. 24 (2012) 1690–1697.
[23] Huang J, Liu G, Song C, Saiz E, Tomsia PA: Role of molecular chemistry of degradable pHEMA hydrogels in three-dimensional biomimetic mineralization, Chem. Mater. 24 (2012) 1331–1337.
[24] Rauch MW, Dressler M, Scheel H, Opdenbosch DV, Zollfrank C: Mineralization of calcium carbonates in cellulose gel membranes, Eur. J. Inorg. Chem. (2012) 5192 – 5198.
[25] Tucker JD, Lear PL, Atkinson GS, Lee S, Lee SJ: Use of polymeric compatibilizers in polypropylene/calcium carbonate Composites, Korean J. Chem. Eng. 17 (2000) 506 – 509.
[26] Aizenberg J: Crystallization in pattern: A bioinspired approach, Adv. Mater. 16 (2004) 1295 – 1302.
[27] Carrillo NA, Quitral VP, Pedram YM, Arias L: Crystal growth of $CaCO_3$ induced by monomethylitaconate grafted polymethylsiloxane, Euro. Polym. J. 46 (2010) 1184 – 1193.
[28] Ni M, Ratner DB: Differentiating calcium carbonate polymorphs by surface analysis techniques – An XPS and TOF-SIMS study, Surf. Interface Anal. 40 (2008) 1356 – 1361.
[29] Ma Y, Feng Q: Alginate hydrogel-mediated crystallization of calcium carbonate, J. Solid State Chem. 184 (2011) 1008 – 1015.
[30] Xia Y, Gu Y, Zhou X, Xu H, Zhao X, Yaseen M, Lu RJ: Controllable stabilization of Poly(N-isopropylacrylamide)-based microgel films through biomimetic mineralization of calcium carbonate, Biomacromolecules 13 (2012) 2299–2308.
[31] Moura MJ, Figueiredo MM, Gil MH: Rheological study of genipin cross-linked chitosan hydrogels, Biomacromolecules 8 (2007) 3823 – 3829.
[32] Xiao L, Ould Eleya MM, Gunasekaran S: Rheology and swelling behavior of whey protein based hydrogels, Proceedings of the 3rd International Symposiumon Food Rheology and Structure (ISFRS 2003) 169 – 173.
[33] Orbach R, Harpaz IM, Abramovich LA, Mossou E, Mitchell EP, Forsyth VT, Gazit E, Seliktar D: The rheological and structural properties of fmoc-peptide-based hydrogels: The effect of aromatic molecular architecture on self-assembly and physical characteristics, Langmuir 28 (2012) 2015–2022.
[34] Mahdavinia RG, Mousavi BS, Karimi F, Marandi BG, Garabaghi H, Shahabvand S: Synthesis of porous poly-(acrylamide) hydrogels using calcium carbonate and its application for slow release of potassium nitrate, eXPRESS Polymer Letters 5 (2009) 279 – 285.
[35] Junji W, Mitsuru A: Novel biomineralization for Hydrogels: Electrophoresis approach accelerates hydroxyapatite formation in hydrogels, Biomacromolecules 7 (2006) 3008 – 3011.
[36] Fathi M, Entezami AK, Pashaei-Asl R: Swelling/deswelling, thermal, and rheological behaviour of PVA-g-NIPAAm nanohydrogels prepared by a facile free-radical polymerization method, J. Polym. Res. 20 (2013) 125.

Paper III

Mineralized polymer composites as biogenic bone substitute material

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AIP Conference Proceedings 1664, 070012, (2015)
DOI: 10.1063/1.4918447
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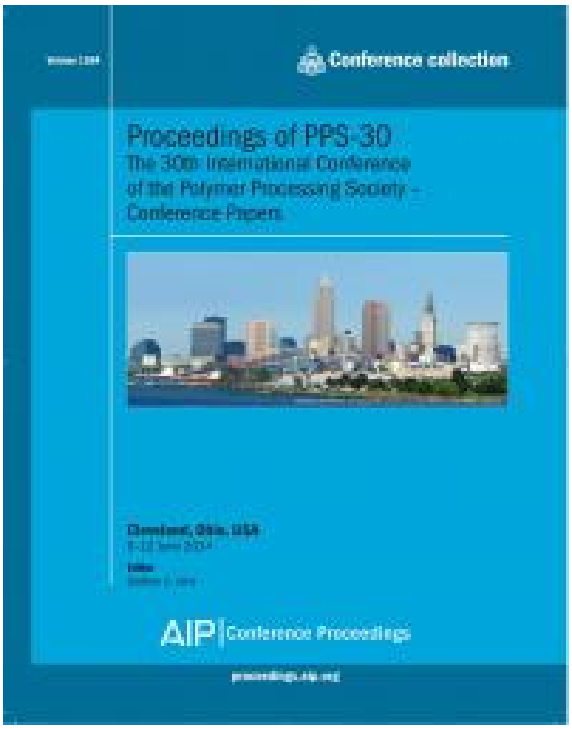

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Citation: AIP Conference Proceedings **1664**, 070012 (2015); doi: 10.1063/1.4918447
View online: http://dx.doi.org/10.1063/1.4918447
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Mineralized Polymer Composites as Biogenic Bone Substitute Material

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Abstract: Mineralized polymer composites (MPC) are recognized as potential fillers of bone defects. Though bioceramics exhibits quite a good bone-bonding and vascularization, it is considered to be too stiff and brittle for using alone. Thus, the use of polymer scaffold instead of bioceramics has several advantages including combining the osteoconductivity and bone-bonding potential of the inorganic phase with the porosity and interconnectivity of the three-dimensional construction. Aiming the advantages of ceramic-polymer composite scaffolds, the calcium carbonate ($CaCO_3$) based biomineralized scaffold was prepared, where the PVP-CMC hydrogel was used as an extracellular matrix. This paper is reported about the morphology, swelling trend (in physiological solution) and viscoelastic behavior of (90 min mineralized) MPC. The dry MPC are off-white, coarse in texture, comparatively less flexible than the original PVP-CMC based hydrogel film, and the deposition of granular structures on the surface of the hydrogel film confirms about the development of biomineralized scaffold/polymer composites. Irrespective of thickness, the dry MPC shows higher values of swelling ratio within 30 min, which varies between 200-250 approximately. The dynamic viscoelastic nature of freshly prepared MPC was investigated applying 1% and 10% strain. At higher strain the viscoelastic moduli (G' and G") show significant change, and the nature of MPC turns from elastic to viscous. Based on the observed basic properties, the MPC (calcite based polymer composites) can be recommended for the treatment of adyanamic bone disorder.

Key words: Adyanamic bone disorder, biomineralization, calcium carbonate, PVP-CMC hydrogel, polymer composite scaffold.
PACS: 81.07 Pr, 81.05 Rm, 87.85 jf, 83.85Cg.

INTRODUCTION

Biomimetic materials inspired in biology are of interest in these days for material scientist in several biomedical fields. The properties of hydrogels as unique biomaterials came in focus since 20th century with the development of soft contact lenses [1]. In the swollen state, they are soft and rubbery, thus resembling a living tissue [2]. Ongoing investigations on natural and synthetic hydrogels have established their potential use in several biomedical applications such as drug delivery systems, soft contact lenses, implants, bone-tissue regeneration, dental plaques etc. [3-5]. However, natural based polymeric materials, polysaccharides form the prominent member due to its non-toxicity, biocompatibility, availability in large variety of composition and properties [6-9]. Biominerals formed by organic/inorganic composites resemble the materials obtained from geological processes [10]. Increasing interest has been shown in polymer composites as potential fillers of bone defects. The preparation of composite itself is a biomimetic approach [11].

Proceedings of PPS-30
AIP Conf. Proc. 1664, 070012-1–070012-5; doi: 10.1063/1.4918447

Bone, for example is made up of collagen, the principal organic component, hydroxyapatite, the inorganic mineral component, water and small amounts of other organic phases [11,12].The composites made from organic-inorganic hybrid material using biocompatible polymeric material in form of hydrogels and also biominerals like calcite or calcium phosphates show promising trend in the field of mineralization [12-14]. Mineralization within the hydrogel can be modified by various ways in presence of particular substrate. Furthermore, convenient scaffolds prepared by mineralization can better mimic the native microenvironment [12].

The significance in the present study lies in the fact that these hydrogels after mineralization can be utilized in biomedical applications like adynamic bone disorder. For accomplishing this task, firstly the mineralized hydrogel is prepared by liquid diffusion technique [3-5, 14]. PVP-CMC hydrogel was prepared by solvent casting technique, and then its dried film was used as a matrix for biomineralization for 90 min. Finally, the developed calcite filled polymeric composite material designated as mineralized polymeric composites (MPC), has been characterized following the established techniques. This paper presents about morphology, swelling trend and rheological behaviour of MPC.

EXPERIMENTAL

The dry PVP-CMC hydrogel [6-9] were used as a matrix, and the solutions of $CaCl_2.2H_2O$ (14.7%, pH=8.4) and Na_2CO_3 (10.5%, pH=7.4) were used for mineralization applying the liquid diffusion techniques. The PVP-CMC hydrogel was soaked in $CaCl_2.2H_2O$ and Na_2CO_3 solutions simultaneously after regular interval of 15 mins. The 90 min mineralized hydrogel were used for characterization, thus the samples washed in distilled water and incubated for freeze drying as well as air drying at room temperature respectively. The freeze dried MPC were used for morphological investigation, air dried MPC were used to evaluate the swelling capacity in physiological solution, and fresh/ before dry MPC were used for rheological measurements.

The morphology observation of calcite filled and unfilled PVP-CMC hydrogels was carried out using a scanning electron microscope (SEM) (VEGA II LMU (TESCAN)) ,which is operated in the high-vacuum/ secondary electron imaging mode at an accelerating voltage of 5-20 kV. The images were taken at the magnification of 100x-10kx.

The swelling studies of the MPC were carried out in the physiological solution (pH = 7.5 and temperature= 37^{o}C). The swollen gels in the physiological solution were taken out at regular time intervals, wiped superficially, weighed, and placed in the same bath. In this way, the cycle of swelling was repeated from 0-300 mins in the regular time interval of 30 mins each. The percentage of swelling was obtained using the following expressions where, W_s is for the swollen gel in physiological solution and W_d is the weight of dried MPC [6].

$$Swelling\ \% = [(Ws - Wd) + (Wd)] * 100$$

The Rheological properties (in the form of dynamic viscoelastic nature) of the MPC were investigated by using a parallel plate rheometer (ARES; Rhemetrics Scientifics, USA) testing with a "TA Orchestrator" software package. A 25mm diameter parallel plate measuring geometry with a gap of about 2-3 mm was used, employed at strain amplitude (1%) to maintain the measurement range within the linear viscoelastic region. Dynamic frequency sweep test were carried out at the temperature of 28^{o}C to observe the storage (G') and loss (G") moduli, and complex viscosity (η*) as a function of a wide range of angular frequency (ω: 0.1-100 rad/s).

RESULTS AND DISCUSSION

Morphology of MPC

Figure 1 shows the surface analysis of matrix (i.e. PVP-CMC hydrogel) and MPC (i.e. calcite filled PVP-CMC hydrogel matrix). It can be seen from the Figure 1(a) that several pores are exhibited within the 3-dimensional cross-linked structure of pure PVP-CMC hydrogel matrix. After liquid ($CaCl_2$ and Na_2CO_3) diffusion mineralization, conducted up to 90 min, the pores of hydrogel matrix first filled up with calcium ions and finally developed $CaCO_3$ crystal structures which can be visible clearly from Figure 1(b). In mesocystal theory, it is explained about the aggregation and development of crystals of calcite within the hydrogel matrix [14]. The crystals obtained on PVP-CMC hydrogel matrix are granular in shape and with increase in mineralization time, increases the size of crystals.

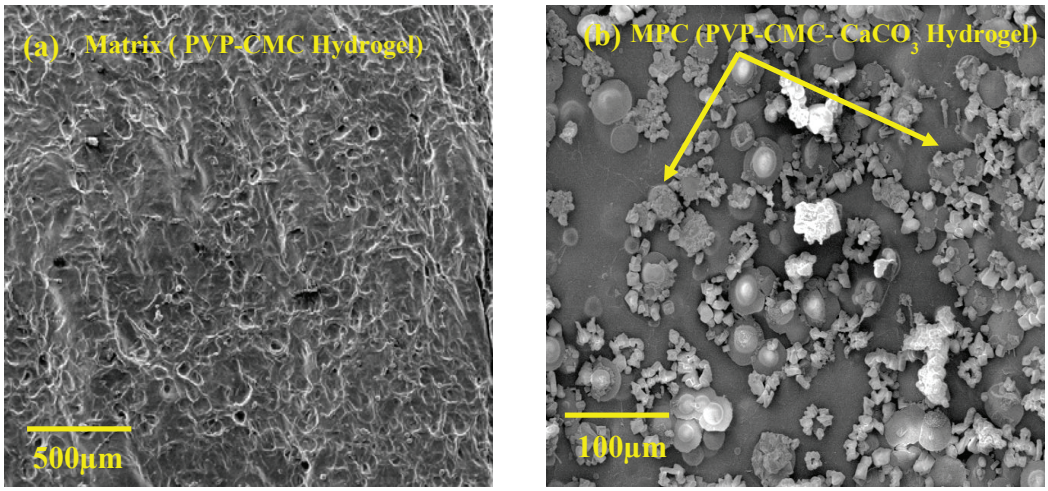

FIGURE 1: SEM Images of (a) Matrix (PVP-CMC hydrogel, surface image before mineralization) and (b) MPC (PVP-CMC-$CaCO_3$ hydrogel, surface image after 90 min mineralized)

Swelling of MPC

The swelling capacity of MPC was measured in physiological solution (0.9% NaCl) as body fluid, blood plasma contains about 90–92% of water. The osmotic pressure of the physiological solution is equivalent to human blood tissue [15]. Four different thicknesses (100,200,300,400µm) of mineralized hydrogels i.e. MPC were taken for investigation of the swelling property. It can be observed from Figure 2 that irrespective of thickness, in each case the initial rate of swelling is high, and the values of swelling ratio vary between 200-250. The swelling ratio of calcite filled polymer composite increases with time until 180 min but after that the values are getting more or less constant. This indicates that the degree of swelling value reaches to equilibrium condition after around 180 min.

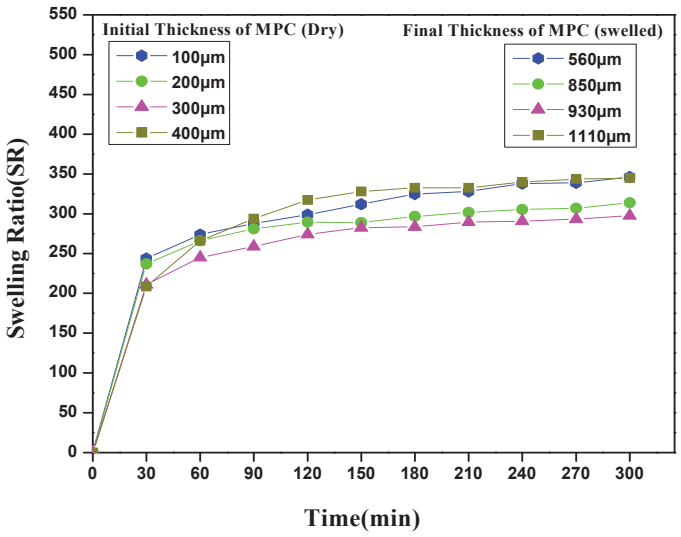

FIGURE 2: Swelling behaviour of MPC in physiological solution

Viscoelastic Behaviour of MPC

Figure 3 shows the viscoelastic properties of MPC (mineralized with $CaCO_3$ for 90 min). It can be observed through the figure that the values of G' is higher and ω independent in MPC at 1% strain compared to G'', which indicates the elastic behaviour is dominant to viscous nature. Moreover, the linear curve confirms the solidity of the material over wide range of deformation rate. But, when the strain increases up to 10%, the values of G' decrease to the level of G" which indicates the conversion of elastic nature of composite material to viscous nature. Moreover, the complex viscosity η* of MPC decreases linearly with increasing of ω in double-logarithmic coordinates, and also decreases with increasing of strain.

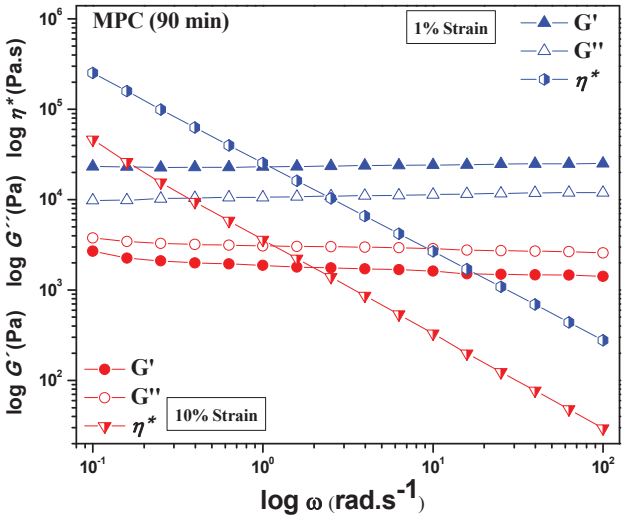

FIGURE 3: Effect of angular frequency (ω) at 1% and 10% strain on storage modulus (G', filled symbol), loss modulus (G", non-filled symbol) and complex viscosity (η^*, half-filled symbol) for mineralized polymer composite (90 min).

CONCLUSION

The liquid diffusion technique used for preparation of mineralized polymer composite (MPC).This MPC biomaterial can be utilized in biomedical applications like adyanamic bone disorder. The morphological image confirms the deposition of $CaCO_3$ into the matrix. The swelling study of MPC confirmed that irrespective of thickness the swelling ratio (SR) values are initially high until 30 min and thereafter gradually rises and get then more or less constant after 180 min. Further, the rheological properties of the MPC explained the elastic and viscous nature of the calcite filled biomaterial at low and high strain over wide range of deformation rate.

ACKNOWLEDGMENTS

The authors are thankful for the support of Research and Development for Innovation Operational Programme co-funded by the European Regional Development Fund (ERDF) and national budget of the Czech Republic within the framework of the Centre of Polymer Systems project (reg. number: CZ.1.05/2.1.00/03.0111) and the support of the "Education for Competitiveness" Operational Programme co-funded by the European Social Fund (ESF) and the national budget of the Czech Republic, within the "Advanced Theoretical and Experimental Studies of Polymer Systems" project (reg. number: CZ.1.07/2.3.00/20.0104). This work was also conducted within the framework of COST Action MP1301 "New Generation Biomimetic and Customized Implants for Bone Engineering" – www.cost.eu. Authors are also thankful to Internal Grant Agency (IGA/FT/2014/015) of Tomas Bata University in Zlin for providing financial support to carry out this research.

REFERENCES

1. A.A. Ashley and L. S. Nair, *Biomed. Mater*. **7,** 1-13 (2012)
2. S.K.Bajpai, *J.Appl.Poly.Sci*, **80**, 2782–2789 (2001)
3. R. Shah, N.Saha, T. Kitano, P.Saha, *J.Appl.Poly.Sci*,131(10) (2014) DOI: 10.1002/APP.40237
4. N.Saha, R. Shah, R.Vyroubal, T. Kitano, P.Saha, Novel Trends in Rheology V Book Series: American Institute of Physics 1526, 2013, pp. 292 – 300.
5. N.Saha, R.Vyroubal, R. Shah, T. Kitano, P.Saha, Novel Trends in Rheology V Book Series: American Institute of Physics 1526, 2013, pp. 301-309.
6. N.Roy, N.Saha, T.Kitano, P.Saha, *J. Appl. Polym. Sci*. **117**, 1703-1710 (2010).
7. N.Saha, A.Saarai., N.Roy., T.Kitano and P.Saha. *JBNB* **2**, 85-90 (2011).
8. P.Saha, N.Saha, N.Roy, Czech Republic, Patent No: CZ 302405B6 (4 May 2011).
9. N.Roy, N.Saha, T.Kitano, P.Saha, *Carbohydrate Polymers* **89**, 346-353, (2012).
10. L.A.Estroff and I. Cohen, *Nature Materials*, **10**, 810-811(2011).
11. H.E. Davis and J.K. Leach, *Hybrid and Composite Biomaterials in Tissue Engineering,* California, Ed. N Ashammakhi, 1999, pp.1-26.
12. N.H.Munro and K.M.McGrath, *Bioinsp. Biomime. and Nanobiomaterials*, **1**, 26-37 (2011).
13. T.Nonoyama, H.Ogasawara, M. Tanaka, M. Higuchi, T. Kinoshita, *Soft Matter* **8**, 11531–11536 (2012).
14. M. W. Rauch, M. Dressler, H. Scheel, D. V. Opdenbosch and C. Zollfrank, *Eur. J. Inorg. Chem,* 5192-5198 (2012).
15. N.Roy, N.Saha, T.Kitano, and P.Saha, *Soft Materials,* **8**(2), 130–148, (2010).

Paper IV

Stimuli responsive and biomineralized scaffold: an implant for bone-tissue engineering

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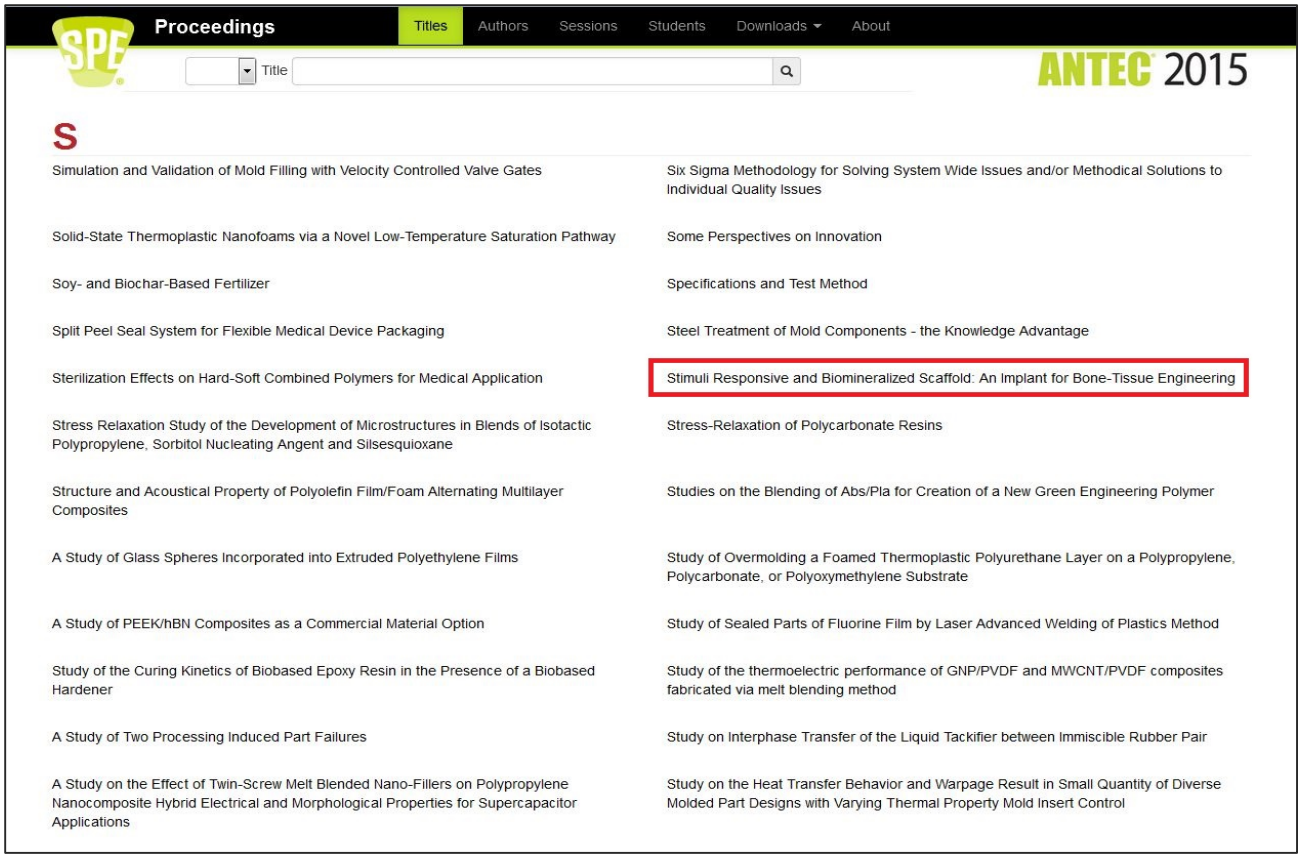
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9:30 AM–10:00 AM
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Development of bioactive and stimuli responsive materials in the form of implant/scaffold is the urgent need that mimics the natural bone structure and function. To achieve such novel *smart biomaterials* "PVP-CMC-CaCO3" scaffold was prepared using PVP-CMC hydrogel as a matrix, following the biomimetic mineralization process i.e. simple liquid diffusion technique. The main concern of this study is to establish its stimuli responsive nature as it is one of the important factor of biomaterials. The swelling-deswelling capacity of "PVP-CMC-CaCO3" scaffold was carried out at 37oC using physiological solution. The morphological changes which occurred in the biomineralized scaffold during swelling-deswelling-reswelling and deswelling steps were confirmed using scanning electron microscopy. The obtained results concerning *shape-size* and *thickness* indicated that PVP-CMC-CaCO3, a hydrogel based scaffold is a true stimuli responsive biomaterial.

Stimuli responsive and biomineralized scaffold: an implant for bone-tissue engineering

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Abstract

Development of bioactive and stimuli responsive materials in the form of implant/scaffold is the urgent need that mimics the natural bone structure and function. To achieve such novel *smart biomaterials* "PVP-CMC-$CaCO_3$" scaffold was prepared using PVP-CMC hydrogel as a matrix, following the biomimetic mineralization process i.e. simple liquid diffusion technique. The main concern of this study is to establish its stimuli responsive nature as it is one of the important factor of biomaterials. The swelling-deswelling capacity of "PVP-CMC-$CaCO_3$" scaffold was carried out at 37^{o}C using physiological solution. The morphological changes which occurred in the biomineralized scaffold during swelling-deswelling-reswelling and deswelling steps were confirmed using scanning electron microscopy. The obtained results concerning *shape-size* and *thickness* indicated that PVP-CMC-$CaCO_3$, a hydrogel based scaffold is a true stimuli responsive biomaterial.

Introduction

Current generation of engineered tissues deals with the development of material that is able to mimic the nature. However, the damaged musculoskeletal tissues or bones are becoming the main challenge in the field of bone-tissue engineering [1]. To replace and regenerate any bone defects inside the patient's body and to improve the quality of life, it becomes essential to develop smart biomaterial in the form of an implant that mimics the natural bone structure having the same chemical and physical composition. In scaffold / implant based on ceramic or polymeric, the incorporation of biomineral (calcium carbonate or calcium phosphate) in an inorganic phase strengthens the formation of composites with an advancement of novel functional biomaterials. Polymers, especially biodegradable ones, are widely used in the biomedical field because of the qualities they possess – such as their availability in wide amounts, good biocompatibility, flexible nature, light in weight etc. Among them hydrogels are favored significantly, as being hydrophilic in nature. They can form crosslink structures to develop an insoluble polymer matrix. Further, these hydrogels exhibit high liquid absorption capacity, are soft and flexible. Moreover, the bio-based hydrogels are biocompatible and also have structure similar to living tissues inside the body [2]. Hydrogels offer numerous application possibilities in the pharmaceutical and health care industries as smart diagnostics (i.e. in vivo delivery vehicles or as scaffolds in tissue engineering). [3]

The main objective of the present study is to evaluate the stimuli responsive behavior of the calcium filled biomineralized scaffold in presence of biological fluid (e.g. blood plasma), temperature and pH to confirm its ability to perform as an attractive biomaterial for medical applications. Generally, polymers that can

respond to external stimuli (pH and temperature) are of great interest in therapeutic aspect. Here, the polyvinylpyrrolidone (PVP) and carboxymethylcellulose (CMC) based hydrogel [4-6] was used as a matrix for the preparation of calcium carbonate ($CaCO_3$) filled biocomposites i.e. formation of a novel functional property based biomaterial [7-10]. As external stimuli (temperature, pH, ionic strength of solution etc.) are great interest in medicine, its effect on swelling- deswelling - reswelling and deswelling of PVP-CMC-$CaCO_3$ was conducted in presence of physiological solution (PS), at 37^{o}C and pH 7.5; to confirm its ability as an implant for bone tissue engineering.

The present paper is describing about the feasibility of the above mentioned calcium filled biomineralized scaffold in presence of PS (which is similar in nature of human plasma) and demonstrating its stimuli responsive nature in human body temperature (<35->40 ^{o}C) as well as in pH of body fluid (7.3-7.5).

Methodology

Preparation of PVP-CMC-$CaCO_3$ Scaffold

To prepare the PVP-CMC-$CaCO_3$ scaffold, firstly PVP-CMC hydrogel was prepared by solvent casting method. The physical crosslinking agent (i.e. moist heat and pressure) was implemented to developed 3D structure within the scaffold [4-6]. The freshly prepared PVP-CMC hydrogel then dried at room temperature (24-25^{o}C) and subsequently followed by mineralization process using mineral solutions (1M Na_2CO_3 + 1M $CaCl_2$) [7-10]. The 90 minute calcite ($CaCO_3$) filled biomineralized scaffold was selected for this study where the simple liquid diffusion technique [7] was adapted during preparation of PVP-CMC hydrogel based biomineralized scaffold and designated as "PVP-CMC-$CaCO_3$". Finally, the air dried biomineralized scaffolds were used for carrying out the swelling-deswelling-reswelling studies.

Swelling-Deswelling-Reswelling-Deswelling studies of PVP-CMC-$CaCO_3$ Scaffold

"Swelling" refers to increase in the weight of hydrogel due to water absorption. Moreover, the percentage swelling of hydrogel is directly proportional to the amount of water imbibed inside the polymeric matrix of hydrogel and "de-swelling phenomenon refers to the loss of water/liquid from the swelled samples of polymeric material with time. Thus, to see the stimuli responsive behavior at human body temperature and its effect on structure of the PVP-CMC-$CaCO_3$ Scaffold, the swelling, de-swelling and re-swelling study was investigated at 37^{o}C in presence of PS: 0.9% NaCl solution with pH:7.5. The entire experiment was initiated using dry samples in triplets (i.e. PVP-CMC-$CaCO_3$, size: 25x25 mm and thickness: 0.4 mm). During swelling and reswelling phases of study, the experiments were performed until 180 mins. (as the saturation time of PVP-CMC-$CaCO_3$ hydrogel is around 150 min). The test samples were taken out after every pre-determined time interval of 15 mins and the surfaces of each sample were wiped off and the weight of the samples were undertaken.

The swelling percentage (%) for the PVP-CMC-$CaCO_3$ scaffold was calculated from the below mentioned equation, [11] wherein W_s = weight of swollen biomineralized scaffold in PS and W_d = weight of dry biomineralized scaffold (initial)

$$\text{Swelling \%} = (W_s - W_d/W_d) \times 100 \ldots\ldots [1]$$

The deswelling and re-deswelling study of biomineralized scaffold was carried out at room temperature (24-25^{o}C) using the scaffold, swelled until 180 min. Then, after every small time interval of 15 min, the loss in the weight of the samples was noted and continued until 180 min as well to maintain the time of swelling and de-swelling cycle constant. The said deswelled scaffold is apparently looking like as good as initial sample (dried) though it is not totally dried. This semi-dried PVP-CMC-$CaCO_3$ scaffold was then allowed to swell again in PS at 37^{o}C following the same cycle. The

deswelling or water retention (%) was obtained through the following equation, [11] wherein, W_t = weight of sample at the deswelling time, t, and W_{t0} = initial weight of the fully swollen sample.

De-Swelling % = $(W_t)/(W_{t0})$ x 100..... [2]

The results of whole cycle of swelling and de-swelling are depicted in figure 1 and 2.

Instrumental Analysis

Scanning electron microscopec (SEM) investigation was carried out to confirm the occurrence of internal structural changes into the biomineralized composites during swelling-deswelling and reswelling or so. The morphological changes within "PVP-CMC-$CaCO_3$" scaffold was analyzed using SEM, VEGA II LMU (TESCAN), which is operated in the high-vacuum/ secondary electron imaging mode at an accelerating voltage of 5-20 kV. The images were taken at the magnification of 100x-10kx. The visual images were carried out using Powershot 170IS Canon HD camera. The swelling- deswelling ratios were obtained by gravimetric measurements of the test samples at particular time intervals.

Results and Discussion

Swelling-Deswelling-Reswelling-Deswelling Behavior of PVP-CMC-$CaCO_3$ Scaffold

Stimuli response is an important character of hydrogel based scaffold. To confirm the stimuli responsive nature of PVP-CMC-$CaCO_3$ scaffold (initial thickness: 0.4mm), its swelling-deswelling-reswelling-deswelling capacity was investigated in presence of PS at 37^{o}C. PS was chosen as it is comparable to human body fluid (e.g. human blood plasma). However, as the human body temperature varies between >35^{o}C to >40^{o}C, [12] the entire swelling, de-swelling and re-swelling study of biomineralized scaffold was carried out at 37^{o}C and depicted in Figure 1 and 2. It is clearly visible from figure 1 that during swelling state, there is a quick uptake of solution in the initial stage of swelling, then increased slowly and then after 150 min of time interval it attains saturation state. It is required to mention that at the time of swelling phenomenon, PS needs to overcome the osmotic pressure inside the gel because when the osmotic pressure is low, any liquid (PS) permeates easily and pass inside the hydrogel based biomineralized matrix [13].

On the other hand, from the figure 2, it is visible that during de-swelling phase (temperature 24-25ºC) the weight of PVP-CMC-$CaCO_3$ scaffold was continuously decreased with increase of time and reached more or less to its initial thickness (i.e. 0.6 mm) within 180 min. But, when the same de-swelled sample was allowed to re-swell at the same conditions, there was increase in the uptake of the solution as well (figure 1) and the trend of uptake was similar as initial swelling stage of the sample. Analogous result has been noticed in the case of re-deswelling phase as well (figure 2). Here, the entire cycle of swelling-deswelling study is time dependent i.e.180 min, as saturated swelling point reaches within 150 min. Therefore, the de-swelling state never reaches to the zero level. Also, there was no sign of removal of $CaCO_3$ during swelling or de-swelling which was confirmed using Energy dispersive X-ray spectroscopy.

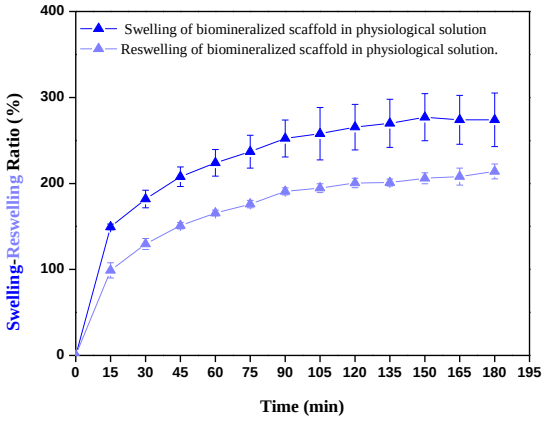

Figure 1: Swelling behavior of biomineralized scaffold in physiological solution (0.9%NaCl, 37ºC and pH=7.5)

Structural Properties of Stimuli Responsive PVP-CMC-$CaCO_3$ Scaffolds

Structural change (both external and internal) of biomaterials is connected with its stimuli responsive behavior while considering swelling -de-swelling-reswelling etc. Thus, the structure associated with the tested biomineralized scaffold was examined before and after undergoing swelling - deswelling - reswelling - redeswelling process and confirmed by SEM.

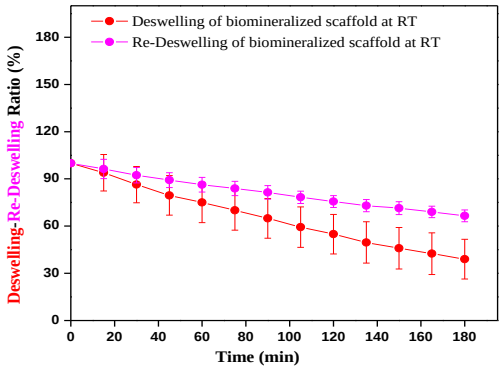

Figure 2: De-swelling behavior of biomineralized scaffold at room temperature.

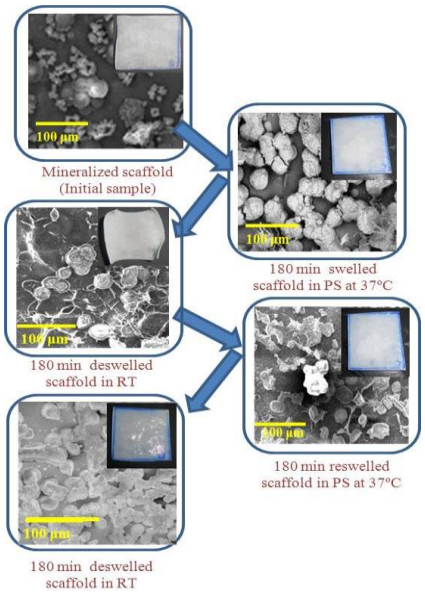

Figure 3: Surface view of biomineralized scaffolds after swelling-deswelling-reswelling - deswelling process in PS. (PS: physiological solution, RT: room temperature)

The visible changes in biomineralized scaffolds were evaluated based on texture, weight and thickness. It can be observed that initially the mineralized scaffold is rough and off-white in color due to the deposition of $CaCO_3$ biomineral. After undergoing swelling process in PS for 180 min, the sample becomes comparatively smooth, rubbery and white in color. Thereafter, during the de-swelling phenomenon the sample re-gains the features of the initial dry scaffold. When, again the re-swelling was done after de-swelling process the sample attains the similar features of the swelled scaffold samples. But, when this re-swelled sample is allowed to de-swelled, there is some noticeable change observed on the surface of the scaffold; it becomes little shiny. Overall, the size and shape of the scaffold remains the same throughout the process of swelling - deswelling - reswelling – redeswelling but the occurrence of noticeable internal structural changes in the of PVP-CMC-$CaCO_3$ (surface) can be seen from the figure 3. In addition, the apparent changes in thickness are depicted in Table 1.

Table 1: Effect of swelling-deswelling-reswelling-deswelling on thickness of the biomineralized scaffolds

Thickness* of Biomineralized Scaffolds

Time	Swelling (mm)	Deswelling (mm)	Reswelling (mm)	Deswelling (mm)
0	0.40	1.09	0.63	1.18
30	0.83	0.95	0.84	1.09
60	0.93	0.85	0.99	0.94
90	0.97	0.79	1.05	0.91
120	1.03	0.72	1.10	0.85
150	1.09	0.98	1.13	0.81
180	1.09	0.63	1.18	0.80

* Average data of three samples

Conclusions

✓ The preparation of PVP-CMC-$CaCO_3$ scaffold is simple. Solvent casting method and

liquid diffusion technique was applied to achieve a stimuli responsive biomineralized scaffold for biomedical applications.

✓ The stimuli responsive investigation of biomineralized scaffold was conducted in presence of various human body temperature (<35->40ºC) and pH (7.3-7.5) using the physiological solution which confirmed that the PVP-CMC-$CaCO_3$ scaffold is a smart biomaterial.

✓ The SEM analysis reveals the change in the morphological structure during swelling-deswelling-reswelling and deswelling process in the biomineralized composite which confirming that due to swelling and/or deswelling, there is no smear of major structural deformation, even in physical appearance of scaffold.

✓ Furthermore, it is important to mention that the PVP-CMC-$CaCO_3$ scaffold does not lose any $CaCO_3$ particle in the PS during swelling and re-swelling study. It seems, the $CaCO_3$ particles are strongly bonded with PVP-CMC hydrogel scaffold.

In conclusion it can be mentioned that PVP-CMC-$CaCO_3$ hydrogel scaffold is a true stimuli responsive biomaterial as it is not deformed, can be considered for utilizing in any biogenic application like bone tissue engineering or as dental implants.

Acknowledgement

The authors are thankful for the support of Research and Development for Innovation Operational Programme co-funded by the European Regional Development Fund (ERDF) and national budget of the Czech Republic within the framework of the Centre of Polymer Systems project (reg. number: CZ.1.05/2.1.00/03.0111), KONTAKT-II LH14050 (Czech-American Cooperation Project: 17140050302/2104). This work was also conducted within the framework of COST Action MP1301 "New Generation Biomimetic and Customized Implants for Bone Engineering" – www.cost.eu.

References

1. M. Dessı`, A. Borzacchiello, T.H.A Mohamed, I. Wafa, A. Fattah, L J. Ambrosio Biomed Mater Res Part A. **101A**, 2984–2993 (2013).
2. L.H Lim, Y. Hwang, M. Kar. S. Varghese. *Biomater. Sci.* **2**, 603–618, (2014).
3. D.S. Couto, Z. Hong, F. Mano, Acta Biomaterialia **5**, 115–123 (2009).
4. N. Roy, Saha N, Kitano T, Saha P. *J. Appl. Polym. Sci*. **117,** 1703-1710 (2010).
5. N. Saha, A. Saarai, N. Roy, T. Kitano, P. Saha: *J. Biomater. Nanobiotechnol*. **2**, 85-90 (2011).
6. P. Saha, N. Saha, N. Roy: "Hydrogel Wound Covering" Patent no: 302405, Czech Republic
7. M.W.Rauch, M Dressler, H Scheel, D.V Opdenbosch, C Zollfrank. *Eur. J. Inorg. Che.* 5192-5198, (2012).
8. N. Saha, R. Shah, R. Vyroubal, T. Kitano, P. Saha: *Novel Trends in Rheology V* Book Series: AIP Conference Proceedings 1526, 292 – 300. ISSN: 0094-243X, ISBN: 978-073541151-7 (2013).
9. N. Saha, R. Vyroubal, R. Shah, T. Kitano, P. Saha: *Novel Trends in Rheology V* Book Series: AIP Conference Proceedings 1526 301-309, ISSN: 0094-243X, ISBN: 978-073541151-7, (2013).
10. R. Shah, N. Saha, T. Kitano, P. Saha. *Journal of Applied polymer science*. **131** (10) (2014) DOI: 10.1002/APP.40237.
11. M. Sadeghi, H. Hosseinzadeh. Brazilian Journal of Chemical Engineering, **30**, 379-389, (2013)
12. Human Body Temperature http://en.wikipedia.org/wiki/Human_body_temperature (accessed on 28 September 2014)
13. Z.X Zhao, Z. Li ∗ , Q.B. Xia, E. Bajalis, H.X. Xi, Y.S. Lin. Chemical Engineering Journal 142, 263–270, (2008).

Paper V

Influence of Temperature, pH and Simulated Biological Solutions on Swelling and Structural Properties of Biomineralized ($CaCO_3$) PVP-CMC Hydrogel

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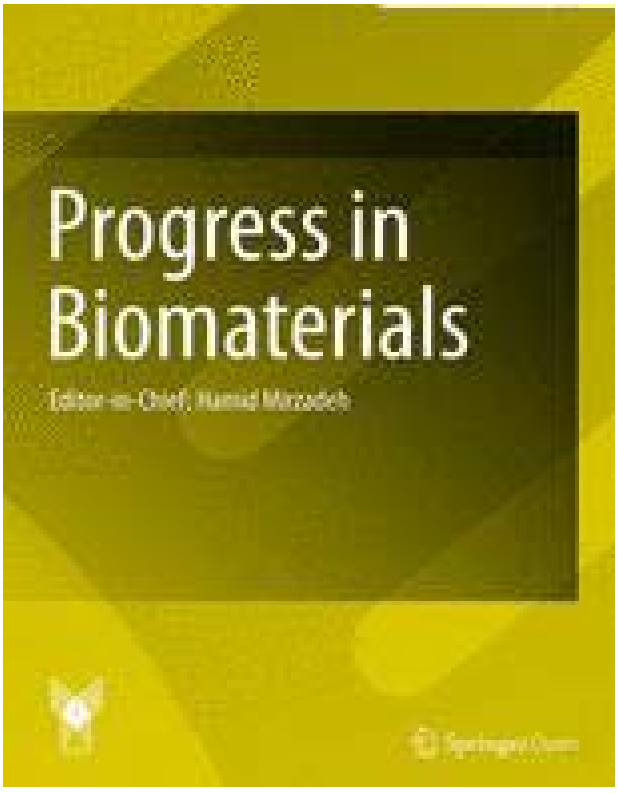
Progress in Biomaterial (a springer open journal)
2015, Vol.4, No.2, p.123-136



Prog Biomater (2015) 4:123–136
DOI 10.1007/s40204-015-0043-1

ORIGINAL RESEARCH

Influence of temperature, pH and simulated biological solutions on swelling and structural properties of biomineralized ($CaCO_3$) PVP–CMC hydrogel

Rushita Shah[1] · Nabanita Saha[1] · Petr Saha[1]

Received: 5 September 2015 / Accepted: 22 October 2015 / Published online: 2 November 2015

Abstract Biomaterials having stimuli response are interesting in the biomedical field. This paper reports about *swelling response* and *internal structural* of biomineralized ($CaCO_3$) polyvinylpyrrolidone (PVP) carboxymethylcellulose (CMC) hydrogel having various thicknesses (0.1–0.4 mm). Samples were tested in aqueous solution using temperature ranges from 10 to 40 °C; pH varies from 4 to 9, time 60 min. In addition, an experiment was conducted in the presence of simulated biological solutions (SBS): glucose (GS), physiological fluid (PS) and urea (US) at temperature 37 °C and pH 7.5 for 180 min. It is noticed that the maximum swelling ratio reached in 30–40 °C at pH 7 in aqueous solution. Among biological fluids, the swelling ratio shows: US > PS > GS at temperature 37 °C, pH 7.5, time 150 min. The equilibrium swelling ratio of the test sample in SBS and their non-reformative apparent structure confirm that biomineralized ($CaCO_3$) PVP–CMC hydrogel can be acclaimed for medical application like bone tissue engineering.

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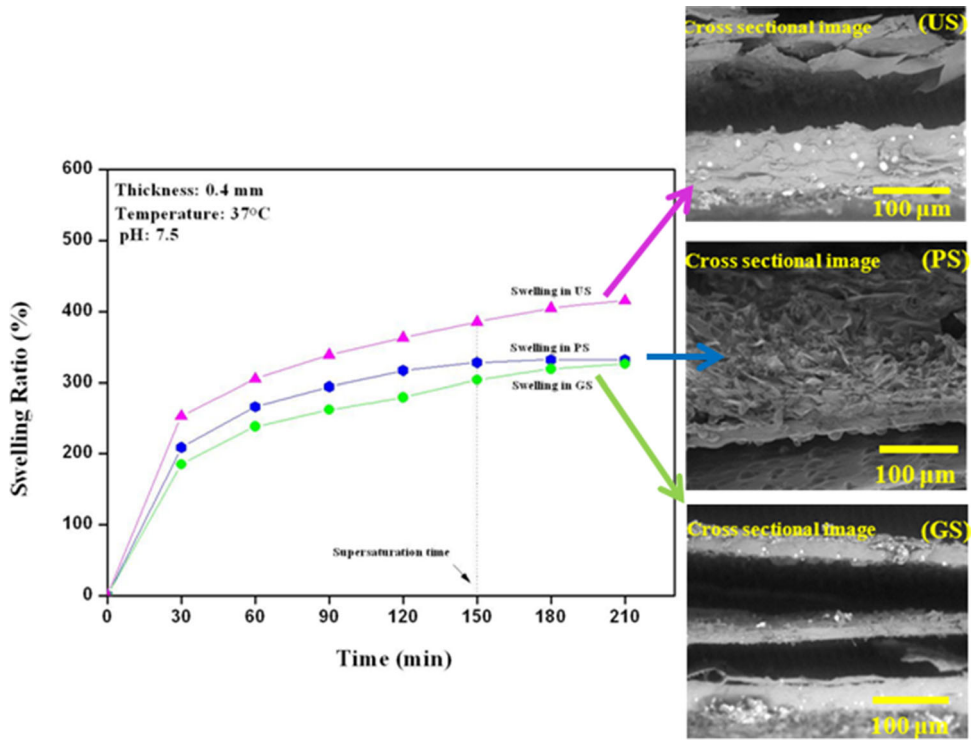
Graphical Abstract



Keywords Biomineralization · Swelling · Stimulus · Simulated biological solutions · Equilibrium swelling ratio

Introduction

In the new era of biomedical field of tissue engineering and regenerative medicines, the utilization of materials generally comes in contact with any biological resources, in the form of either cells, tissues/organs, biomolecules, physiological fluids, etc., which desire the interdisciplinary scientific approach that merges the field of material science and technology, basic science and life science (Mano et al. 2007). In general, a tissue engineering process begins with the fabrication of a biologically compatible scaffold that will support the living cells for their attachment, proliferation and differentiation, and thus promotes tissue regeneration both in vitro and in vivo (Thavornyutikarn et al. 2014). Thus, during present days, intense interest has been given in the biomedical field of tissue engineering wherein several new biomaterials are constructs in the form of scaffold (i.e., polymeric material, bioceramics, biocomposites) can be implanted in patients to replace failing or malfunctioning organs (Mano et al. 2007). Moreover, several materials have been proposed to have their applications in biomedical fields among which significant attention has been given on the use of polymers, especially biopolymers and bioactive polymers. Polysaccharides and their derivatives have attracted much interest, especially the blends of natural and synthetic polymers are used because of their special properties in the form of soft rubbery nature resembling the living tissues, elasticity and also low cost (Zhu and Marchant 2011; Karadag et al. 2005). Among various kinds of polymeric systems that have been utilized till today, hydrogels have gained noticeable interest by the material scientists and are interpreted from different point of view (Kim and Park 2014; Sadeghi and Hosseinzadeh 2010).

Hydrogels are a unique class of three-dimensional macromolecular network structure that is highly suitable for several biomedical and tissue engineering applications because of the resemblance of their structure to the biological tissues (Tomic et al. 2010; Roy and De 2014). Moreover, these hydrogels have a network structure which indicates that cross-links should be formed to avoid dissolving it in the solution and thus maintaining the structural integrity within the aqueous phase (Kaith et al. 2010). Further the hydrogels being hydrophilic in nature, they have the capacity to imbibe and store huge water content within them, thus maintaining their 3D structure (Zhao et al. 2006; Tyliszczak 2011; Ismail et al. 2012). The hydrophobicity nature of the hydrogels is because of the presence of chemical moieties like hydroxylic (–OH), carboxylic (–COOH), amidic (–CONH–), sulphonic (–

SO_3H), etc. and also the other functional groups present in the backbone of the polymers (Ganji et al. 2010). Another aspect of the hydrogels is that the changes observed with the variations seen in surrounding environment of pH, temperature, simulated biological solutions, ionic strength, electric field stimulus and so on (Roy and De 2014; Rodkate et al. 2015; Sadeghi and Koutchakzadeh 2007; Chang et al. 2010). Due to the presence of some functional groups within the background of the polymeric chain, hydrogels become sensitive to the environmental conditions (Nesrinne and Djamel 2013). Hydrogels can undergo swelling from ionic network when pH, temperature or any other stimulus become dependent. Here, the high swelling efficiency of hydrogels is concerned with the electrostatic repulsion within the ionic groups present in the polymers and also the osmotic pressure inside and outside the hydrogel (Roy and De 2014). This type of study becomes interesting in the scientific area to be utilized in the advanced technologies. These types of hydrogels are referred to as stimuli responsive and smart hydrogels (Rodkate et al. 2015). Till now, these hydrogels with unique characteristics have been implemented and given importance in several field of biomedical applications: wound healing, cell encapsulation, drug delivery, tissue engineering, etc. (Rodkate et al. 2015; Nesrinne and Djamel 2013). Further, uses of hydrogels have also been noticed in carrying out the biomineralization process in several in vivo environments (Kim and Park 2014).

Nowadays, researchers related to bioinspired and biomimetic biomineralization for the preparation of user friendly and cost effective functional biomaterial are giving much emphasis. Material scientists are focusing much on the use of polymeric-based matrices as a promising tool for the development, repair and regenerate the functional tissues, organs, etc. of the body (Rauch et al. 2012). In material science, mimicking the natural structure design offers the great advantage in developing hybrid materials that combine the properties of organic polymers and inorganic phase. The combination of organic–inorganic phase leads to strengthening the properties of the prepared material (Rauch et al. 2012). Numerous studies have been proposed till now on bioinspired matrix-based biomineralization. Mineralization carried out on the matrix, which are fibrous and porous in nature, can provide better framed structure on which growth and nucleation process of inorganic crystals can occur (Kim and Park 2014). However, to accomplish this process, several biominerals play an important role as they possess specific shape and properties. Further, most of the studies carried out till now are focused on the development of crystal formation on gels, fibers, or film (Kim and Park 2014). Also, it is interesting to understand the role of chemical motifs that are mediated by the matrix properties in the form of its pore size, its composition, functionalization with active groups present within the polymeric matrix and cross-linking density (Nindiyasari et al. 2015). Biominerals in the form of hydroxyapatite, calcium phosphate/carbonate are used to carry out the process of biomineralization, thus forming organic–inorganic hybrid materials (Rauch et al. 2012; Feng-ju et al. 2012). Among all the biominerals, calcium carbonate ($CaCO_3$ as calcite) is one of the most abundant minerals found in the nature produced by organisms. It has also several industrial applications like fillers in paints, plastics, or paper (Feng-ju et al. 2012). From the literature survey, it was found that mostly calcite crystal shows the crystallographic orientation within the hard tissues (Nindiyasari et al. 2015). Further, the formation of crystals within the matrix could be explained by the classical theory of crystallization which can be elaborately explained by the mesocystal theory (Rauch et al. 2012). Several studies have revealed the formation of crystals in various forms when comes in contact with the organic gel like matrixes.

In this aspect, it is already reported about the use of different kinds of matrixes which are mainly prepared with natural polysaccharides, i.e., alginate, cellulose, chitosan, gelatin, collagen, guar gum, dextran, etc. (Chunyu et al. 2011; Giridhar and Akanksha 2011) or peptoid nanosheets (Jun et al. 2015). Among all these, in our study cellulose (in derivative form) had been chosen as the best candidate to form hydrogel as cellulose is abundantly available in nature, biodegradable, having good water absorbing capacity and biocompatible too (Sadeghi and Hosseinzadeh 2010; Rodkate et al. 2015). In due course, to accomplish and maintain both hydrophilic and mechanical properties within the so-called "biobased hydrogel", PVP is added to form a blend of carboxymethylcellulose (CMC) and polyvinylpyrrolidone (PVP) within the hydrogel. The interesting properties of this PVP–CMC hydrogel have already been reported by the researchers of Tomas Bata University in Zlin (Roy et al. 2010, 2012; Saha et al. 2010, 2011). Because of adventitious properties of PVP–CMC hydrogel like: porous internal morphology, quite a good moisture/solvent/solution absorption capacity, flexible in nature for the preparation of sample for testing in different shape, size and thickness, this bio-based hydrogel has been chosen to be used as a matrix for the preparation of calcite ($CaCO_3$) incorporated hydrogel. Finally, this calcite-filled hydrogel termed as biomineralized ($CaCO_3$) PVP–CMC hydrogel which has been prepared following the liquid diffusion method (Rauch et al. 2012; Saha et al. 2013a, b; Shah et al. 2014, 2015a, b, c).

Swelling is one of the important properties of hydrogel, thus it is essential to investigate its absorption behavior in the presence of aqueous solution/fluid. Water absorption occurred due to the combination mechanism of hydration,

dissolution and thermodynamically expansion of the macromolecular chain restricted by crosslinkages of hydrogel like material (Ismail et al. 2012). Here, it is essential to mention that (to the best of our knowledge) till now no other group has been reported about the swelling behavior of biomineralized ($CaCO_3$) PVP–CMC hydrogel (Saha et al. 2013a, b, Shah et al. 2014, 2015a, b, c). Further, through study of swelling nature/features of this biomaterial was conducted in the presence of various stimuli: in different aqueous pH, different aqueous temperature and simulated biological solutions [SBS: glucose solution (GS), urea solution (US) and physiological solution (PS)]. Apart from this, considering the practical application of this biomaterial, investigation has also been conducted to see the influence of individual stimuli (temperature, pH and SBS: GS, US and PS) on the structural properties of the biomineralized ($CaCO_3$) PVP–CMC hydrogel. In this paper, emphasis has been given to understand and elucidate about changes in terms of *swelling* and *structure* of the said biomaterial in the presence of intracellular fluid or cytosol (liquid found inside cells), temperature: 37 °C and pH: 7.5. This information is important to evaluate the response of bone marrow-derived human mesenchymal stem cells in terms of cell proliferation and differentiation to the osteoblastic phenotype (which is responsible for bone formation) and to design the subsequent experiments.

Experiment

Chemicals

PVP K30 (PVP: molecular weight 40,000), polyethylene glycol 3000 (PEG: average molecular weight 2700–3300) and agar were supplied by Fluka, Switzerland; carboxymethyl cellulose (CMC) was purchased from Sinopharm Chemical Reagent Co-Ltd (SCRC), China; glycerin was obtained from Lachema, Czech Republic; calcium chloride ($CaCl_2$: molecular weight 110.99 g/mol, 97.0 %), Penta, Czech Republic; sodium carbonate-10-hydrate (Na_2CO_3: molecular weight 286.14 g/mol) was obtained from Sigma-Aldrich; D-Glucose was purchased from Lukes, Czech Republic, NaCl and Urea from Penta, Czech Republic.

Preparation of biomineralized ($CaCO_3$) PVP–CMC hydrogel

Biomineralized ($CaCO_3$) PVP–CMC hydrogel was prepared using a three-dimensional, crosslinked and porous matrix termed as "PVP–CMC hydrogel". This hydrogel was prepared following the solution casting technique and implemented only physical cross-linking agent (i.e., moist heat and pressure) to achieve soft, white PVP–CMC hydrogel. The polymer solution (20/30/40/50 ml) was poured into a Petri dish (diameter: 80 mm) and kept at room temperature for gel formation. Thereafter, the freshly prepared PVP–CMC hydrogel was allowed to dry at room temperature (25–26 °C). The dry "PVP–CMC hydrogel" was utilized to prepare calcite-filled biomaterial. To achieve a biomineralized hydrogel, the simple liquid diffusion technique was adopted where the dried PVP–CMC hydrogel was immersed in the ionic solutions of 1 M Na_2CO_3 and 1 M $CaCl_2$ simultaneously for 90 min. The obtained final form of biomaterial termed as "biomineralized ($CaCO_3$) PVP–CMC" hydrogel and placed overnight for air drying. The dried samples were used for further investigations (i.e., swelling study). The physical appearance of the biomineralized ($CaCO_3$) PVP–CMC hydrogel and their characteristics are presented in Fig. 1 and Table 1 respectively.

Swelling studies

The swelling study was performed using a dry sample (diameter: 25 mm × 25 mm and thickness: 0.1–0.4 mm) of biomineralized ($CaCO_3$) PVP–CMC hydrogel. Gravimetric technique has been used to evaluate the swelling performance of biomineralized PVP–CMC hydrogel. Further, the influence of reaction parameters such as variation of pH, temperature in aqueous solution (water) and simulated biological solutions [i.e., glucose solution (GS), urea solution (US) and physiological solution (PS)] was investigated. The percentage of swelling ratio was determined using equation (Giridhar and Akanksha 2011; Fathi et al. 2013)

$$\text{Swelling Ratio}\,(\%) = (W_s - W_d/W_d) \times 100 \quad (1)$$

Swelling studies at different temperature of aqueous solution

A uniform sized (25 mm × 25 mm) biomineralized ($CaCO_3$) PVP–CMC hydrogel samples with a diverse thickness (i.e., 0.1, 0.2, 0.3 and 0.4 mm) were immersed in the 50 ml distilled water (aqueous solution, pH = 7) and then incubated in different temperature (i.e., 10, 20, 30 and 40 °C) for 60 min. Then, the weight of each set of swelled sample with different thickness was noted and their percentage of swelling at different temperature was calculated as per Eq. (1). Three replicas for each set of sample were prepared for estimation.

Swelling studies at different pH of aqueous solution

Similarly, biomineralized ($CaCO_3$) PVP–CMC hydrogel samples (size: 25 mm × 25 mm; thickness: 0.1, 0.2, 0.3 and 0.4 mm) were immersed in 50 ml solution of distilled

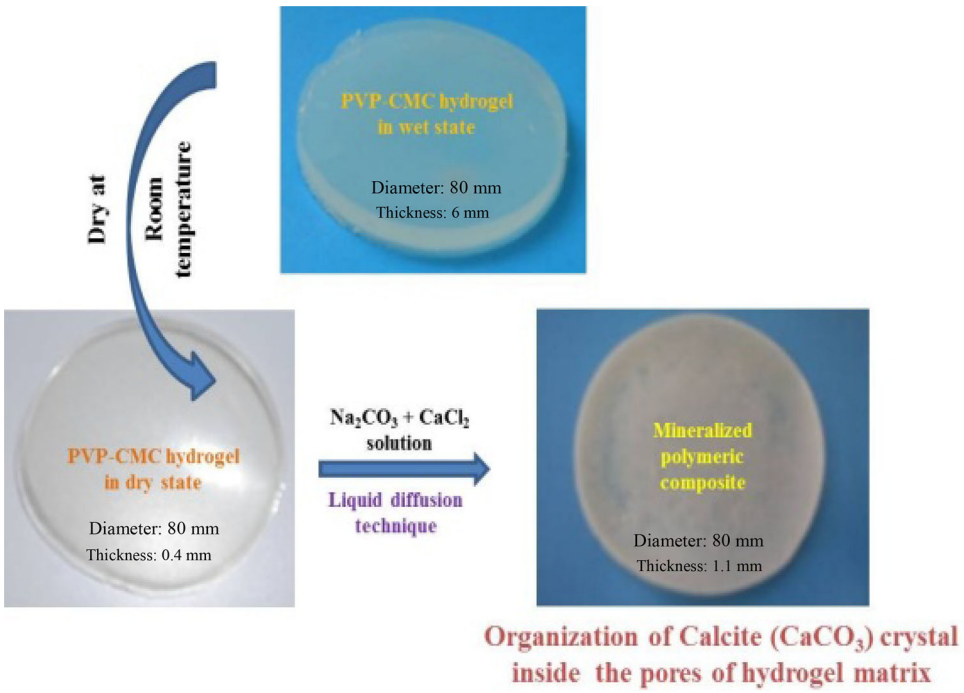


Fig. 1 The physical appearance (*optical view*) of the biomineralized ($CaCO_3$) PVP–CMC hydrogel before and after mineralization

water (aqueous solution having pH of 4.0, 5.0, 7.0, 8.0, and 9.0) and then incubated at room temperature (25–26 °C) for 60 min. The pH values were precisely noted using the pH meter from company Sension TM$^+$. Finally, the percentage swelling at different pH was calculated as per Eq. (1). Three replicas for each set of sample were prepared for estimation.

Swelling studies in simulated biological solutions

Glucose solution (GS, 5 g in 100 ml distilled water), urea solution (US, 5 g in 100 ml distilled water) and physiological solution (PS, 0.9 g in 100 ml distilled water) are considered as simulated biological solutions. Biomineralized ($CaCO_3$) PVP–CMC hydrogel samples (size: 25 mm × 25 mm; thickness: 0.1, 0.2, 0.3 and 0.4 mm) were immersed individually in each simulated biological solution (pH: 7.5) and incubated at 37 °C. In each case, the observed physical changes (occurred due to swelling) were noted in every 30 min interval and the experiment was carried out until 180 min. The percentage swelling was calculated using same Eq. (1) for the case of simulated biological solutions as well. Three replicas for each set of sample were prepared for estimation.

Instrumental analysis

For structural analysis of polymeric hydrogel scaffold (PVP–CMC–$CaCO_3$) always lyophilized (swelled samples were freeze dried under –81 °C for 72 h and then lyophilized for 24 h to produce porous scaffold) samples were used. The morphology of swelled biomineralized ($CaCO_3$) hydrogel in the presence of GS, US and PS was investigated using scanning electron microscopy (SEM: Phenom world Pro) which is operated in the high vacuum/secondary electron imaging mode at an accelerating voltage of 5–20 kV). All the images were taken at the magnification of 100×–10k× and always freeze drying samples were used for cross-sectional studies.

Fourier transform infrared spectroscopy (FTIR) was used to determine physical–chemical structure of the biomineralized ($CaCO_3$) PVP–CMC hydrogel after swelling in GS, US and PS. The spectra were obtained at wave number of 2000–600 cm^{-1} at room temperature with uniform resolution of 2 cm^{-1}. Attenuated total reflectance ATR-FTIR was used with NICOLET 320 FTIR Spectrophotometer with "Omnic" software package.

Results and discussion

Swelling behavior of biomineralized ($CaCO_3$) PVP–CMC hydrogel

Swelling capacity usually becomes prime significance in many practical applications of biomedical field that includes either personal hygiene products, drug delivery systems or in tissue engineering and also in agriculture as water releasing or storing system. This swelling capacity is said to be affected when comes in contact with external

Table 1 Physical characteristics of biomineralized ($CaCO_3$) PVP–CMC hydrogel

Initial polymer solution	PVP CMC Hydrogel					Biomineralized ($CaCO_3$)PVP CMC Hydrogel					
	Before dry		After dry			Before dry		After dry			
Volume (ml)	Thickness (mm)	Weight (gm)	Thickness (mm)	Weight (gm)		Thickness (mm)	Weight (gm)	Thickness (mm)	Weight (gm)		
20	4.10–4.15	19–20	0.10–0.15	1.00–1.03	Off–white, transparent smooth and flexible Size (diameter: 80 mm)	0.40–0.45	6.60–6.70	0.11–0.15	1.10–1.30	Off–white, opaque, coarse and Brittle, Size (diameter: 80 mm)	
30	4.90–4.95	24–25	0.21–0.25	1.40–1.50		0.55–0.65	7.50–7.60	0.21–0.26	1.50–1.80		
40	5.90–5.95	32–35	0.30–0.35	2.00–2.01		0.80–0.90	9.70–9.80	0.31–0.36	2.40–2.70		
50	6.40–6.50	42–45	0.40–0.45	2.15–2.5		1.00–1.15	9.90–9.95	0.40–0.45	2.75–3.00		

solutions like different ions, salt concentrations and valencies. The favorable property dealing with the hydrogel is the ability to swell when kept in any solvent. One of the best theories that explains the swelling mechanisms is the Donnan equilibrium theory which states that there always exist electrostatic interactions and osmotic pressure between inside and outside of the gel (Sadeghi and Hosseinzadeh 2010). When any hydrogel comes in contact with the solvent molecules, the solvent tries to attack the hydrogel surface and penetrate within the polymeric network structure. The hydrogel possesses any of the acidic or basic pendant groups within its polymeric backbone structure. If the acidic group is present, then the H^+ ions move out and combine with the OH^- ions and form H_2O. In this way, charge neutrality gets balanced. If the cation concentration rises, then there is an increase in the osmotic pressure in the gel system which finally causes shrinkage in it. However, the equilibrium stage is achieved within the swelling phenomenon when the elastic force within the network structure of the gel as well as osmotic pressure outside balances (De et al. 2002). In this series of experiment, swelling studies of biomineralized ($CaCO_3$) PVP–CMC hydrogel were performed using different stimulus like: temperature, pH, and several simulated biological solutions which has been explained below.

Effect of temperature on swelling

The effect of temperature on swelling of biomineralized ($CaCO_3$) PVP–CMC hydrogel is depicted in Fig. 2. It is noticed that irrespective of sample thickness (varies between 0.1 and 0.4 mm), the swelling capacity rises with increase of temperature, even though at lower temperature (10 or 20 °C), 0.1 and 0.2 mm thick samples showed lower range of swelling capacity. But, in the case of higher range

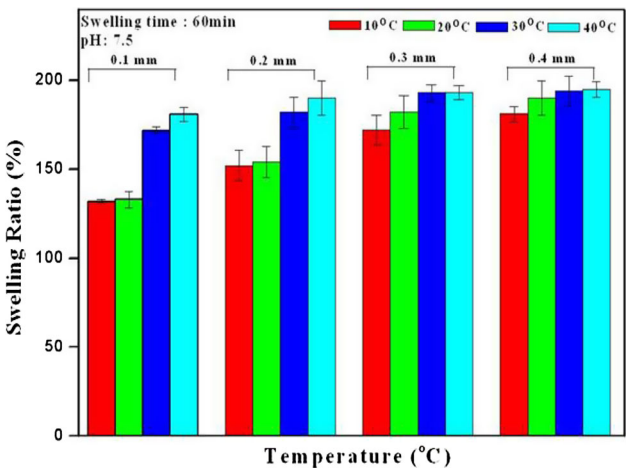


Fig. 2 Effect of temperature on swelling of biomineralized ($CaCO_3$) PVP–CMC hydrogel

of temperature, i.e., 30 and 40 °C, it shows more or less comparable range of swelling ratio similar to thick (i.e., 0.3 and 0.4 mm) samples. The reason behind the increase of swelling ratio of PVP–CMC–$CaCO_3$ hydrogel with rise in temperature from 10 to 40 °C is basically due to the nature of internal polymeric interactions as well as the elasticity nature within the matrix. Moreover, at higher temperature the chain mobility increases which facilitates the network expansion and leads to increase in the ratio of swelling capacity (Gupta and Shivakumar 2012).

Effect of pH on swelling

The effect of pH on swelling of the biomineralized ($CaCO_3$) PVP–CMC hydrogel was investigated in acidic (4–5), neutral (7) and basic (8–9) range of pH and the results are shown in Fig. 3. Slight change in swelling ratio of the biomineralized hydrogels has been noticed when placed in various pH of the aqueous solution. It can also be noticed that irrespective of sample thickness at neutral pH = 7, all samples show a higher value of swelling ratio. This could be due to the presence of carboxylate (COO–) and amide groups in cellulose and PVP polymers. In pH = 7, water molecules undergo H_2 bonding so generates more space for the molecules to penetrate inside, thus swells more. But, there was not much difference noticed when the biomineralized ($CaCO_3$) PVP–CMC hydrogel is kept in the acidic and basic pH. Also, with the increase in pH above 7, the rate of swelling is lowered as there is dissociation observed in COOH– group present is cellulose, which gradually increases the movement of mobile ions that reduces the osmotic pressure. However, a little fluctuation has been noted in the swelling ratio with the increase in sample thickness. This indicates that change in the thickness of the samples slightly alters the absorbing capacity of the material (Kaith et al. 2010).

Effect of simulated biological solutions (SBS) on swelling

To broaden the application of the biomineralized ($CaCO_3$) PVP–CMC hydrogel and to examine the influence of simulated biological fluids on its swelling ratio, the swelling study was performed until 180 min in the presence of three different biological fluids: GS, PS and US holding the same environmental conditions of pH (7.5) and temperature (37 °C). The purpose of this investigation, i.e., exposure of the biomineralized ($CaCO_3$) PVP–CMC in simulated biological fluids or simulated biological solution, is to understand the biomaterial condition when it comes in contact with biological/body fluid considering its application as a scaffold for bone tissue engineering or drug delivery. It is understandable from the literature that generally in the case of most healthy people, the body fluid maintains pH 7.5, temperature 37 °C and sugar level maintains the normal range which is as good as similar to the physiological solution but in the case of diabetic patient, the sugar level of body fluid will be little higher than healthy people or in the case of hyperuricemia patient, uric acid level will be excess in the blood and so on. Thus, considering the status of body fluid (healthy and un-healthy people), the effect of SBS on swelling of biomineralized ($CaCO_3$) PVP–CMC hydrogel has been conducted. The observed results are depicted in Figs. 4, 5, and 6, respectively. In all the three simulated biological fluids, biomineralized ($CaCO_3$) PVP–CMC hydrogel exhibited interesting results with their swelling behavior which indicates that this calcite-filled biomaterial will be helpful

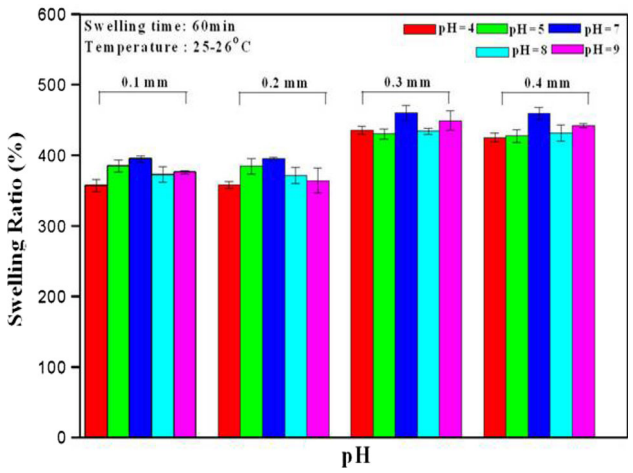


Fig. 3 Effect of pH on swelling of biomineralized ($CaCO_3$) PVP–CMC hydrogel

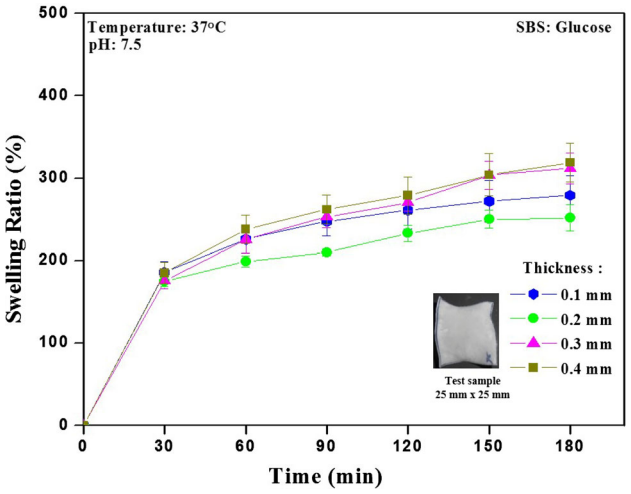


Fig. 4 Swelling behavior of biomineralized ($CaCO_3$) PVP–CMC hydrogel in glucose solution (GS)

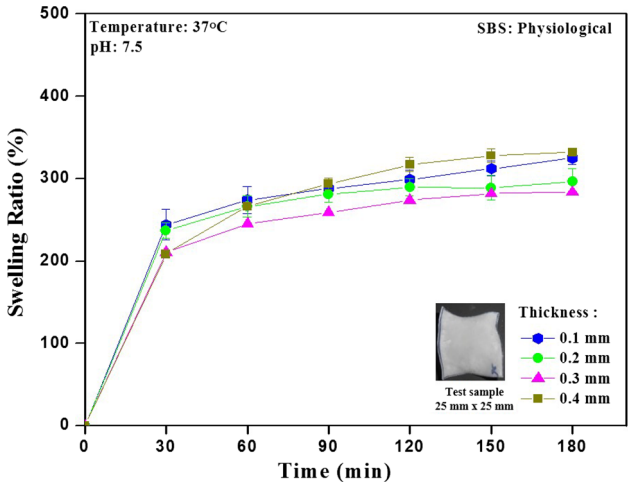


Fig. 5 Swelling behavior of biomineralized ($CaCO_3$) PVP–CMC hydrogel in physiological solution (PS)

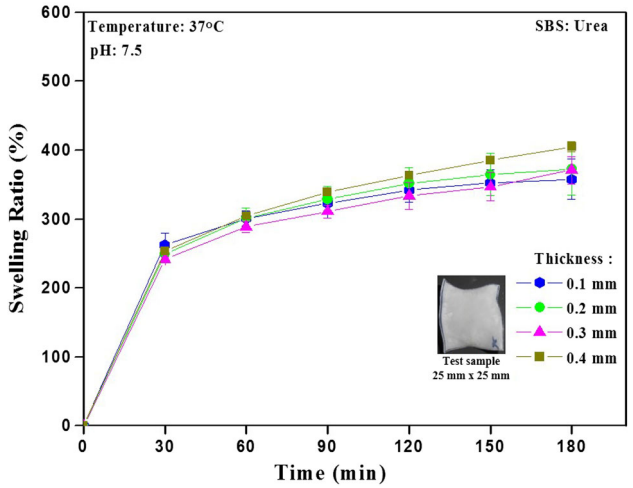


Fig. 6 Swelling behavior of biomineralized ($CaCO_3$) PVP–CMC hydrogel in urea solution (US)

for cell proliferation which is in connection with bone tissue engineering. It can be seen in Figs. 4, 5 and 6 that the swelling capacity of hydrogel is gradually increased with the increase in time, though the values of swelling ratio vary for GS, PS and US. The reaction between a variety of fluids and composition of the test sample of biomaterial has great influences concerning uptake and swelling behavior.

Figure 4 represents the swelling trend of the biomineralized ($CaCO_3$) PVP–CMC hydrogel in the presence of GS. Here, it can be revealed that the binding of glucose moieties with the mineralized hydrogels gradually increases the charge density within the hydrogels, hence enhances the hydrophilicity within the hydrogel which finally leads to the rise in the swelling capacity (Kim et al. 2014).

Figure 5 represents the swelling trend of the biomineralized ($CaCO_3$) PVP–CMC hydrogel in the presence of PS.

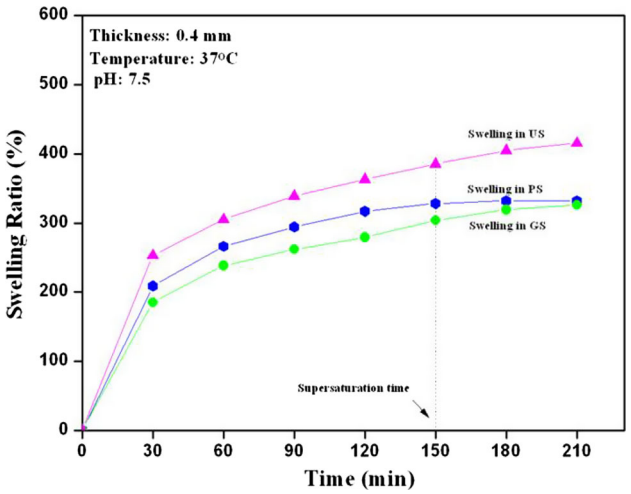


Fig. 7 Swelling behavior and super saturation time of biomineralized ($CaCO_3$) PVP–CMC hydrogel in simulated biological solutions

Like GS, in the physiological solution (0.9 % NaCl), there is a continuous uptake of PS which has been noticed by the hydrogel, and after certain time interval the absorption becomes stable. Here, during swelling studies, PS needs to overcome the osmotic pressure within the gel, and as osmotic pressure becomes lower water permeates inside the gels fast which leads to expansion of the gel (Sadeghi and Hosseinzadeh 2010).

Like GS and PS, the same trend of swelling behavior is observed in US which is depicted in Fig. 6. In urea solution, there is the presence of more hydrophilic sites like NH_2^+ and C=O and also urea being weak base can interact readily with COOH group of cellulose present in the hydrogel. Therefore, when urea reacts with water, it gains more hydrophilicity (Karadag et al. 2005; Kundakci et al. 2011). Thus, an increase in the hydrophilic groups within the urea aqueous solution will ultimately increase the swelling of biomineralized ($CaCO_3$) PVP–CMC hydrogel.

Further, the super-saturation swelling time (data represented in Fig. 7) has been identified from the swelling period (0–210 min) of biomineralized ($CaCO_3$) PVP–CMC hydrogel when conducted the swelling study in the presence of GS, US and PS. It can be perceived from the Fig. 7 that the super saturation is attained in the time period of 150 min. After this, the stability in the swelling ratio has been noticed. It is well known that the swelling of any hydrogel is governed by the electrostatic repulsion of the ionic charges within the polymeric network structure. The more the hydrophilic groups present in the matrix, the more will be the swelling capacity. As far as the swelling behavior is concerned, two main important phenomenons are focused: (1) Donnan osmotic pressure and (2) elastic property within the polymeric network structure. When these two phenomena become equal, no further uptake of

the solution takes place by the material and hence super-saturation stage is attained (Kim et al. 2014).

The super-saturation point of the biomineralized ($CaCO_3$) PVP–CMC hydrogel (thickness = 0.4 mm) with respect to the test sample of biological solutions (GS, PS, US, etc. which is usually present in cytosol/intracellular fluid) is shown in Fig. 8. Finally, at super-saturation point, the value of the equilibrium swelling ratio of biomineralized ($CaCO_3$) PVP–CMC hydrogel (thickness: 0.1–0.4 mm) has been identified in the presence of simulated biological solutions (SBS: GS, PS and US). The values of equilibrium swelling ratio in terms of thickness are depicted in Fig. 8. The order of the swelling ratio obtained is as follows: US > PS > GS irrespective of sample thickness. The observed variation in the equilibrium swelling ratio may be due to the presence of non-uniform porous internal morphology of the biomineralized matrix/non identical filler ($CaCO_3$) content inside the calcite-filled biomaterial. The equilibrium swelling value of biomineralized ($CaCO_3$) PVP–CMC hydrogel is higher in US compared to PS and GS. The overall results obtained through this study indicated that there exists a strong electrostatic interaction within the biomineralized ($CaCO_3$) PVP–CMC hydrogel. Moreover, apparently no deformation observed within the biomaterial due to the occurrence of swelling phenomenon (except increase of thickness).

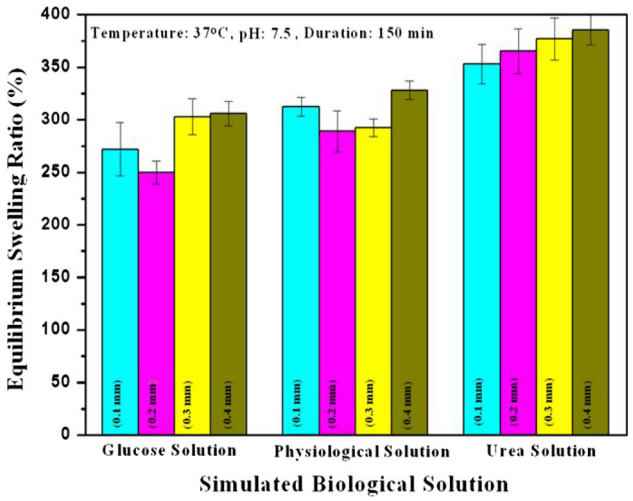


Fig. 8 Equilibrium swelling ratio of biomineralized ($CaCO_3$) PVP–CMC hydrogel in simulated biological solutions

Morphology of biomineralized ($CaCO_3$) PVP–CMC hydrogel (after swelling)

Effect of temperature

SEM generally provides the information about internal morphology, pore size and homogeneity/heterogeneity of the material. Figures 9 and 10 represent the SEM images of biomineralized ($CaCO_3$) PVP–CMC hydrogel which are

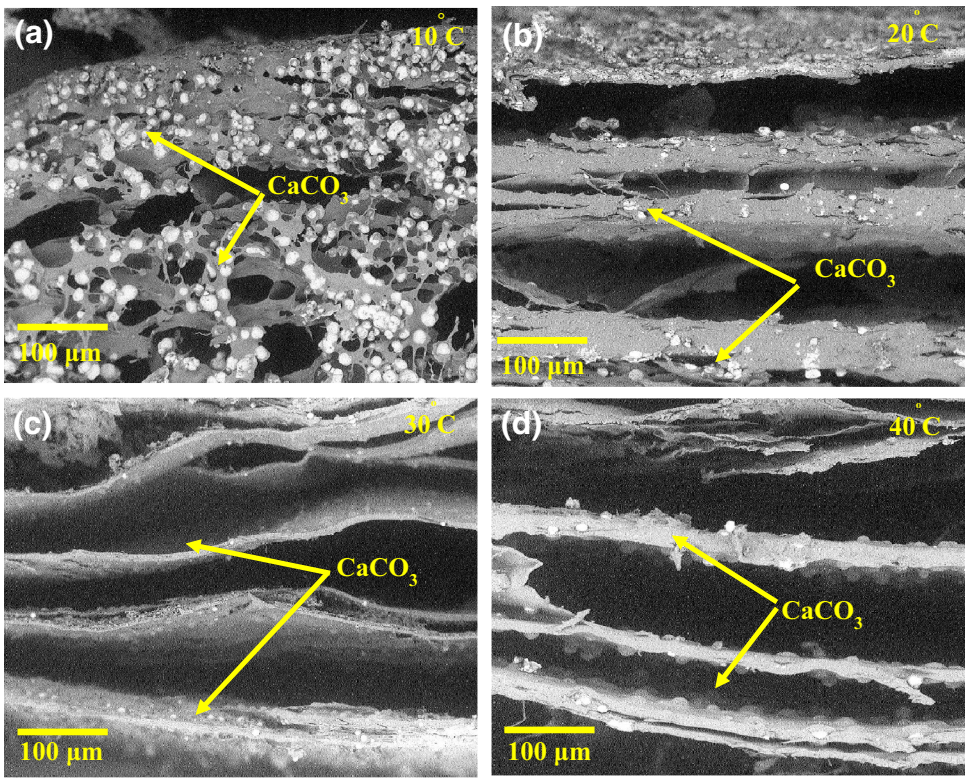


Fig. 9 SEM Images of biomineralized ($CaCO_3$) PVP–CMC hydrogel (thickness: 0.4 mm). Swelled in different temperatures: **a** 10 °C, **b** 20 °C, **c** 30 °C, **d** 40 °C

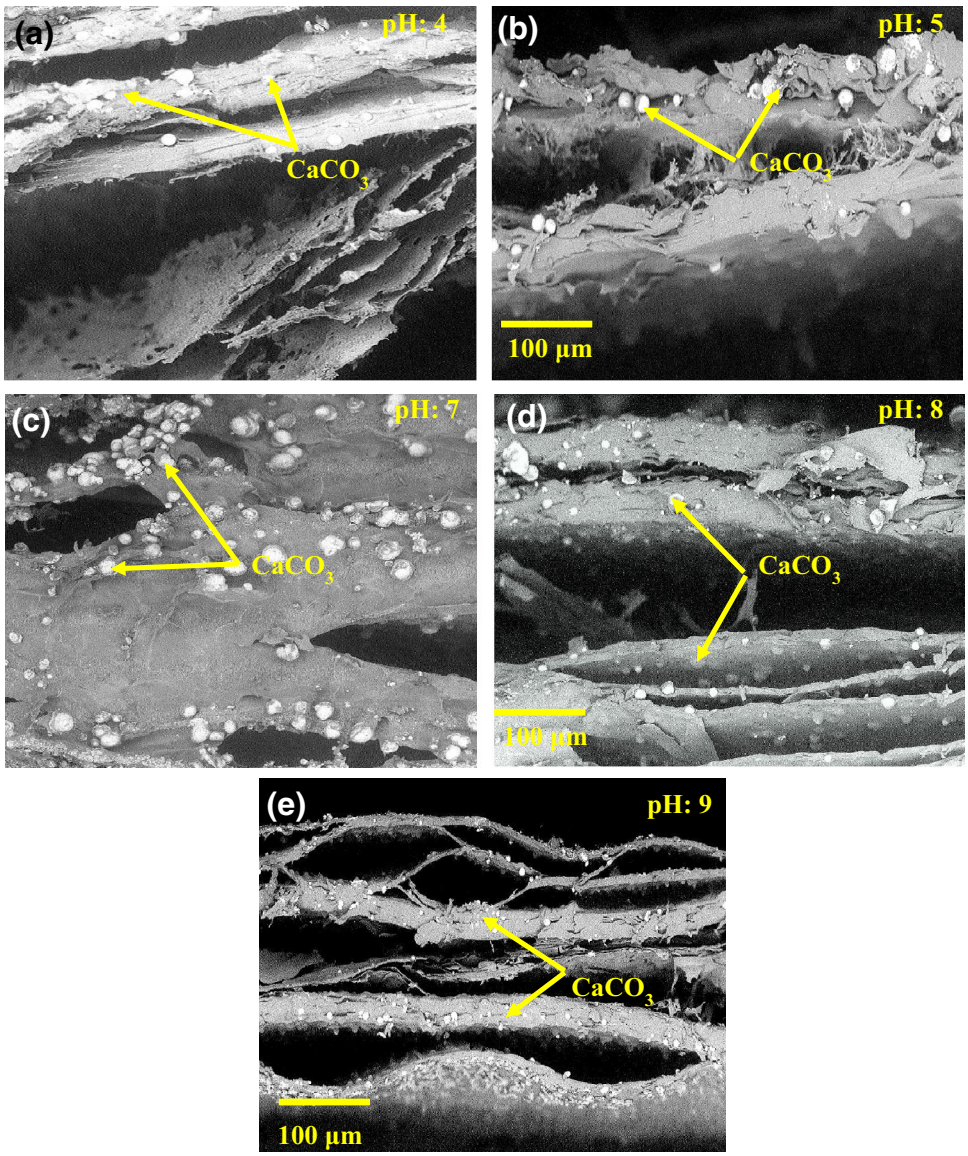


Fig. 10 SEM Images of biomineralized ($CaCO_3$) PVP–CMC hydrogel (thickness: 0.4 mm) swelled in different pH: **a** pH = 4, **b** pH = 5, **c** pH = 7, **d** pH = 8, **e** pH = 9

swelled in different temperatures (10, 20, 30 and 40 °C) and pH (4, 5, 7, 8, and 9). From the images of swelled hydrogels, one thing is commonly noticed that they exhibits discontinuous morphology with two phases, wherein polymer phase gets separated by the presence of internal unequal spaces between them (Ceylan et al. 2006). Moreover in all the figures, it is clearly visible the difference about the formation of flakes like structures due to swelling in which the crystals of $CaCO_3$ are already embedded within the PVP–CMC hydrogel. Even though the thickness (i.e., 0.4 mm) of the biomineralized hydrogel sample is same, the increase in the temperature not only enhances the swelling ratio but also changes the internal structure of the biomineralized hydrogel.

Effect of pH

Like temperature, pH also exhibited the difference in internal morphology of the biomineralized ($CaCO_3$) PVP–CMC hydrogel. It can be seen from the Fig. 10 that pH plays a significant role during swelling as well as the internal morphology of the biomineralized, which is different from the effect of temperature. More fractured planes and honey comb-like structures are developed within the hydrogel matrix due to swelling. The $CaCO_3$ crystals (as droplets) are embedded tightly inside the hydrogel matrix throughout the surface of the planed cross-sectional structure.

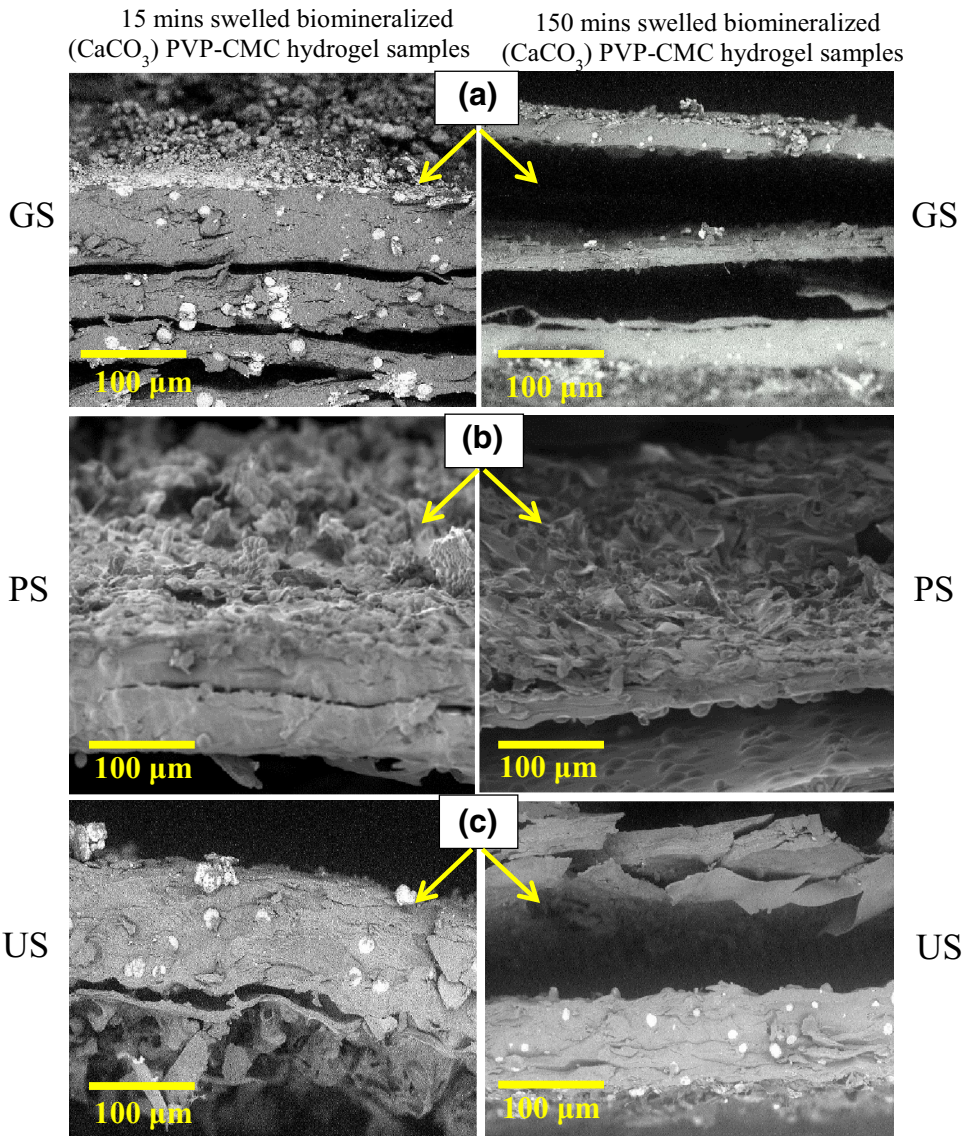


Fig. 11 SEM Images of biomineralized ($CaCO_3$) PVP–CMC hydrogel swelled in simulated biological solutions: **a** Glucose solution (GS), **b** physiological solution (PS) and **c** urea solution (US)

Effect of simulated biological solutions (SBS)

SEM micrographs (cross-sectional view) of the biomineralized ($CaCO_3$) PVP–CMC hydrogel swelled in simulated biological solutions, i.e., GS, PS and US, are shown in Fig. 11. The results obtained are credited with the previously shown fact in case of swelling capacity wherein the presence of electrostatic interactions as well as more hydrophilic group increases the capacity of the sample to swell more. Therefore, in the SEM image of swelled biomineralized ($CaCO_3$) PVP–CMC hydrogel, numerous bulked flakes-like structure with embedded calcium carbonate ($CaCO_3$) is recognized in the case of GS and US except PS. Moreover, it can be seen from the Fig. 11 that not much difference is visible in terms of internal morphology with an increase in swelling time (between 15 and 150 min); however, the density increases within the hydrogel.

Physical and chemical nature of biomineralized ($CaCO_3$) PVP–CMC hydrogel (after swelling)

Besides observed changes in swelling ratio in the presence of simulated biological solutions (GS, PS and US), the FTIR assay of biomineralized ($CaCO_3$) PVP–CMC hydrogel sample (before and after swelling) has been performed to confirm the changes and shown in Fig. 12. The FTIR spectra of PVP–CMC hydrogel have been placed as a control. It can be seen from the figure that the strong absorption band of CO_3^{2-} is present in all the peaks except pure PVP–CMC hydrogel with the range around 1410 and 871 cm^{-1} which confirms the deposition of calcite within the matrix (Shah et al. 2014; Ma et al. 2013). Further, it can be seen in all the spectra that a broad band of peak in the range of 3332 and 2923 cm^{-1} corresponds to hydrogen bonding forming –OH group of the polymer glycoside ring

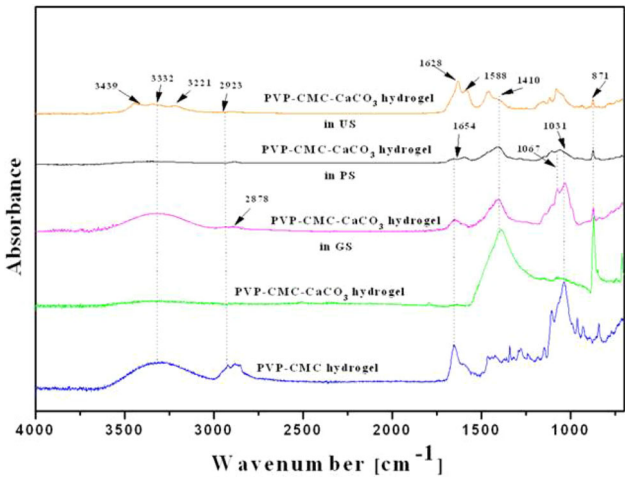


Fig. 12 FTIR spectra of PVP–CMC, PVP–CMC–$CaCO_3$ and swelled sample of PVP–CMC–$CaCO_3$ in simulated biological solutions: GS, PS, US

and the –OH stretching of water free and involved in hydrogel bonds. The peak showing around 1600 cm^{-1} corresponds to the amide I, amide II and free carboxylate groups of the CMC polymer. It is interesting to obtain the band at a range of 3439 and 3221 cm^{-1} in case of hydrogel when swelled in US. This peak corresponds to –N–H group of amide linkage which is developed due to the presence of urea in the swelled sample. It seems that during the swelling of PVP–CMC–$CaCO_3$ hydrogel in the presence of US, no chemical reaction occurs between the existing –OH and –NH group after uptake of urea. Also, in the same urea swelled PVP–CMC–$CaCO_3$ hydrogel, there are peaks seen at 1628 and 1588 cm^{-1}, which are of amide –N–H and carbonyl group –C=O of urea. In the case of hydrogel samples swelled in the presence of GS, the peaks seen at 2878 cm^{-1} corresponds to the presence of –C-H group existing in its chemical structure. Further, in case of peaks in PS and GS, there is the presence of bands at a range of 1067 and 1031 cm^{-1} which are of –C–O– and –C–C– groups. All these observations confirming that even though the PVP–CMC hydrogel is filled with $CaCO_3$, still the biomineralized sample has enough absorption capacity.

Conclusions

In this work, a series of experiments concern with the swelling study of the biomineralized biomaterial (i.e., $CaCO_3$–PVP–CMC hydrogel) was performed as "swelling nature of a material" is an important parameter of any biomaterials for biomedical or pharmaceutical application point of view. Different stimulus like temperature, pH, and simulated biological solutions like glucose (GS), physiological (PS) and urea (US) solution was used to investigate their response/changes in the form of swelling ratio and structure. The "physiological solution" was selected as its osmotic pressure is considered equivalent to human tissue fluids. "Urea solution" was chosen as urea is one of the important fluids produced in the body though kidney and also used in regeneration systems of artificial kidney machines wherein it is produced as toxic waste in the dialysate solution during the hemodialysis process of the patients. Furthermore, it is present in the blood in the amount of 6-20 milligrams per deciliter mg/dl as blood urea nitrogen (BUN) (Kamal 2014). Swelling test was also performed in the presence of "Glucose solution" because like urea, glucose is also present in the blood (an example of body fluid) less than 100 mg/dl (Dixon 2015). However, this level of glucose swings within the body throughout the day. In addition, it is important to mention that in all cases the swelling study of biomineralized ($CaCO_3$) PVP–CMC hydrogel was performed at temperature 37 °C (as it is the normal temperature of internal body fluid) and pH 7.5 (as cell growing state pH remains little higher range than usual pH range, i.e., 7.0–7.4).

In conclusion, it can be mentioned that the biomineralized ($CaCO_3$) PVP–CMC hydrogel is a smart biomaterial which showed an active response (i.e., change in swelling ratio) in all types of stimulus (i.e., Temperature, pH and SBS solutions). Certain changes in the internal structure of the biomaterial (during the swelling phenomenon) have also been observed. Moreover, through the entire experiment accomplished, there was no deformation noticed in the test material. As a whole, the aforementioned biomineralized ($CaCO_3$) PVP–CMC hydrogel gives the fact that it has stimuli responsive behavior and can be recommended for its potential applications in several biomedical fields after performing the cytotoxicity and cell proliferation assay using several kinds of osteoblastic or stem cell, etc. which is in progress.

Acknowledgments This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic—Program NPU (LO1504). The presented work was also conducted within the framework of COST Action MP1301 "New Generation Biomimetic and Customized Implants for Bone Engineering" www.cost.eu.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

Ceylan D, Ozmen MM, Okay O (2006) Swelling-Deswelling Kinetics of Ionic Poly (acrylamide) Hydrogels and Cryogels. J Appl Polym Sci 99:319–325

Chang C, Duan B, Cai J, Zhang L (2010) Superabsorbent hydrogels based on cellulose for smart swelling and controllable delivery. Eur Poly J 46:92–100

Chunyu C, Meng H, Zhou J, Zhang L (2011) Swelling behaviors of pH- and salt-responsive cellulose-based hydrogels. Macromolecules 44:1642–1648

De SK, Aluru NR, Johnson B, Crone WC, Beebe D, Moore J (2002) Equilibrium swelling and kinetics of pH-responsive hydrogels: models, experiments, and simulations. J Microelectromech Syst 11(5):544–555

Dixon A (2015) Normal sugar glucose levels in a man. http://www.livestrong.com/article/296203-normal-sugar-glucose-levels-in-a-man/. Accessed 4 Sep 2015

Fathi M, Entezami AA, Pashaei-Asl R (2013) Swelling/deswelling, thermal, and rheological behavior of PVA-g-NIPAAm nanohydrogels prepared by a facile free-radical polymerization method. J Polym Res 20:125

Feng-ju Z, Xin-guo Y, Yu Z, Kun-kun G, Ming-jun W, Wen-feng W (2012) Crystallization of calcium carbonate in hydrogels in presence of meso-tetrakis (4-hydroxylphenyl) porphyrin. J Cent South Univ 19:1802–1807

Ganji F, Farahani SV, Farahani EV (2010) Theoretical description of hydrogel swelling: a review. Iran Polym J 19(5):375–398

Giridhar RS, Akanksha SP (2011) Swelling behavior of calcium-ions crosslinked bipolymeric sodium alginate-lignosulphonic acid blends. Int J Poly Mater 60:1123–1129

Gupta VN, Shivakumar HG (2012) Investigation of swelling behavior and mechanical properties of a ph-sensitive superporous hydrogel composite. J Pharma Res 11(2):481–493

Ismail H, Irani M, Ahmad Z (2012) Starch-based hydrogels: present status and applications. Int J Poly Mater Poly Biomater 62:411–420

Jun JMV, Altoe MVP, Shaul A, Zuckermann RN (2015) Peptoid nanosheets as soluble, two-dimensional templates for calcium carbonate mineralization. Chem Commun. doi:10.1039/C5CC03323C

Kaith BS, Jindal R, Mittal H, Kumar K (2010) Temperature, pH and electric stimulus responsive hydrogels from Gum ghatti and polyacrylamide-synthesis, characterization and swelling studies. Der Chem Sinica 1(2):44–54

Kamal A (2014) Estimation of blood urea (BUN) and Serum creatinine level in patients of renal disorder. Indian J Fundam Appl Life Sci 4(4):199–202

Karadag E, Uzum OB, Saraydin D, Guven O (2005) Dynamic swelling behavior of-radiation induced polyelectrolyte poly(AAm-co-CA) hydrogels in urea solutions. Int J Pharma 301:102–111

Kim SY, Park JS (2014) Biomineralized hyaluronic acid/poly (vinylphosphonic acid) hydrogel for bone tissue regeneration. J Appl Polym Sci. doi:10.1002/APP.41194

Kim A, Mujumdar SK, Siegel RA (2014) Swelling properties of hydrogels containing phenylboronic acids. Chemosensors 2:1–12

Kundakci S, Karadağ E, Üzüm OB (2011) Investigation of swelling/sorption characteristics of highly swollen AAm/AMPS hydrogels and semi IPNs with PEG as biopotential sorbent. J Encap Adsorp Sci 1:7–22

Ma Y, Feng Q, Bourrat X (2013) A novel growth process of calcium carbonate crystals in silk fibroin hydrogel system. Mater Sci Eng, C 33:2413–2420

Mano JF et al (2007) Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends. J R Soc Interface 4:999–1030

Nesrinne S, Djamel A (2013) Synthesis, characterization and rheological behavior of pH sensitive poly(acrylamide-co-acrylic acid) hydrogels. Arab J Chem. doi:10.1016/j.arabjc.2013.11.027

Nindiyasari F et al (2015) Effect of hydrogel matrices on calcite crystal growth morphology, aggregate formation, and co-orientation in biomimetic experiments and biomineralization environments. Cryst Growth Des. doi:10.1021/cg5018483

Rauch MW, Dressler M, Scheel H, Opdenbosch DV, Zollfrank C (2012) Mineralization of calcium carbonates in cellulose gel membranes. Eur J Inorg Chem 2012:5192–5198

Rodkate N, Rutnakornpituk B, Wichai U, Ross G, Rutnakornpituk M (2015) Smart carboxymethylchitosan hydrogels that have thermo- and pH-responsive properties. J Appl Polym Sci. doi:10.1002/APP.41505

Roy SG, De P (2014) Swelling properties of amino acid containing cross-linked polymeric organogels and their respective polyelectrolytic hydrogels with pH and salt responsive property. Polymer 55:5425–5434

Roy N, Saha N, Kitano T, Saha P (2010) Novel hydrogels of PVP–CMC and their swelling effect on viscoelastic properties. J Appl Polym Sci 117:1703–1710

Roy N, Saha N, Kitano T, Saha P (2012) Biodegradation of PVP–CMC hydrogel film: a useful food packaging material. Carbohydr Polym 89:346–353

Sadeghi M, Hosseinzadeh H (2010) Synthesis and super-swelling behavior of a novel low salt-sensitive protein-based superabsorbent hydrogel: collagen-g-poly(AMPS)H. Turk J Chem 34:739–752

Sadeghi M, Koutchakzadeh G (2007) Swelling kinetics study of hydrolyzed carboxymethylcellulose-poly (sodium acrylate-co-acrylamide) superabsorbent hydrogel with salt-sensitivity properties. J Sci I A U (JSIAU) 17(64):19–26

Saha P, Saha N, Roy N (2010) Hydrogel wound covering. Patent no: 302405, 2010, Czech Republic

Saha N, Saarai A, Roy N, Kitano T, Saha P (2011) Polymeric biomaterial based hydrogels for biomedical applications. J Biomater Nanobiotechnol 2:85–90

Saha N, Shah R, Vyroubal R, Kitano T, Saha P (2013) Morphology, absorptivity and viscoelastic properties of mineralized PVP–CMC hydrogel, novel trends in rheology. V Book Series: AIP Conference Proceedings 1526, pp 292–300. ISSN: 0094-243X, ISBN: 978-073541151-7

Saha N, Vyroubal R, Shah R, Kitano T, Saha P (2013) Effect of strain on viscoelastic behavior of fresh, swelled and mineralized PVP–CMC hydrogel, novel trends in rheology. V Book Series: AIP conference proceedings, 1526, pp 301–309. ISSN: 0094-243X, ISBN: 978-073541151-7

Shah R, Saha N, Kitano T, Saha P (2014) Preparation of $CaCO_3$-based biomineralized polyvinylpyrrolidone–carboxymethylcellulose hydrogels and their viscoelastic behavior. doi:10.1002/APP.40237

Shah R, Saha N, Kitano T, Saha P (2015a) Influence of strain on dynamic viscoelastic properties of swelled (H_2O) and biomineralized ($CaCO_3$) PVP–CMC hydrogels. Appl Rheol 25:33979. doi:10.3933/ApplRheol-25-33979

Shah R, Saha N, Zuckermann RN, Saha P (2015) Stimuli responsive and biomineralized scaffolds: an implant for bone-tissue engineering. SPE-ANTEC 2015, conference proceedings Florida, USA. http://legacy.4spe.org/conferences/antec2015/titles.html

Shah R, Saha N, Kitano T, Saha P (2015) Mineralized polymer composites as biogenic bone substitute material. PPS-30, AIP conference proceedings 1664, 070012. doi:10.1063/1.4918447. ISBN: 978-0-7354-1309-2

Thavornyutikarn B, Chantarapanich N, Sitthiseripratip Thouas GA, Chen Q (2014) Bone tissue engineering scaffolding: computer-aided scaffolding techniques. Prog Biomater 3:61–102

Tomić SL, Micić MM, Dobić SN, Filipovic JM, Suljovrujic EH (2010) Smart poly (2-hydroxyethyl methacrylate/itaconic acid) hydrogels for biomedical application. Radiat Phys Chem 79:643–649

Tyliszczak B (2011) PAA/PVA matrix for biomedical application. Czasopismo Techniczne Chemia 108:219–223

Zhao Y, Kang K, Tan T (2006) Salt-, pH- and temperature-responsive semi-interpenetrating polymer network hydrogel based on poly (aspartic acid) and poly (acrylic acid). Polymer 47:7702–7710

Zhu J, Marchant RE (2011) Design properties of hydrogel tissue-engineering scaffolds. Expert Rev Med Devices 8:607–626

Paper VI

Properties of biomineralized ($CaCO_3$) PVP-CMC hydrogel with reference to the cytotoxicity test using fibroblasts cells

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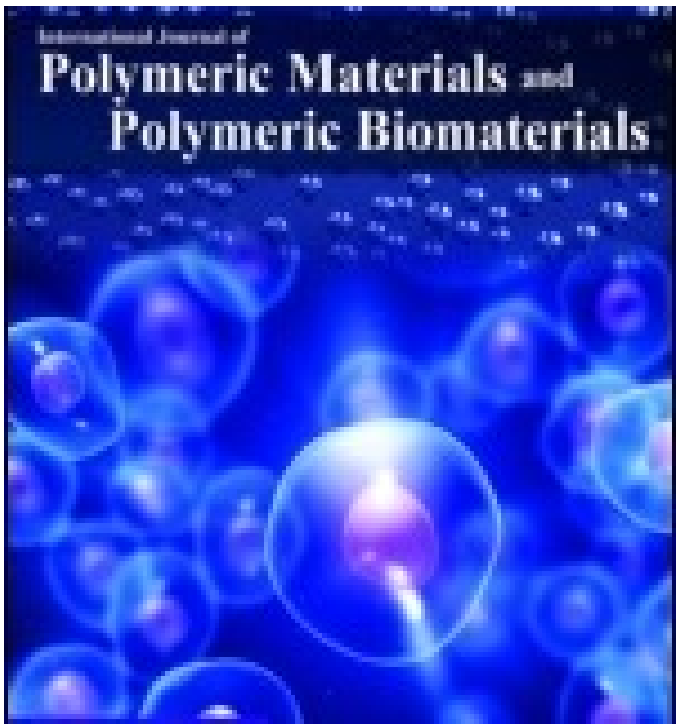
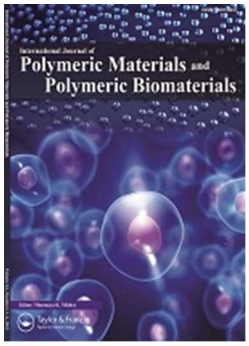
International Polymeric Materials and Polymeric Biomaterials

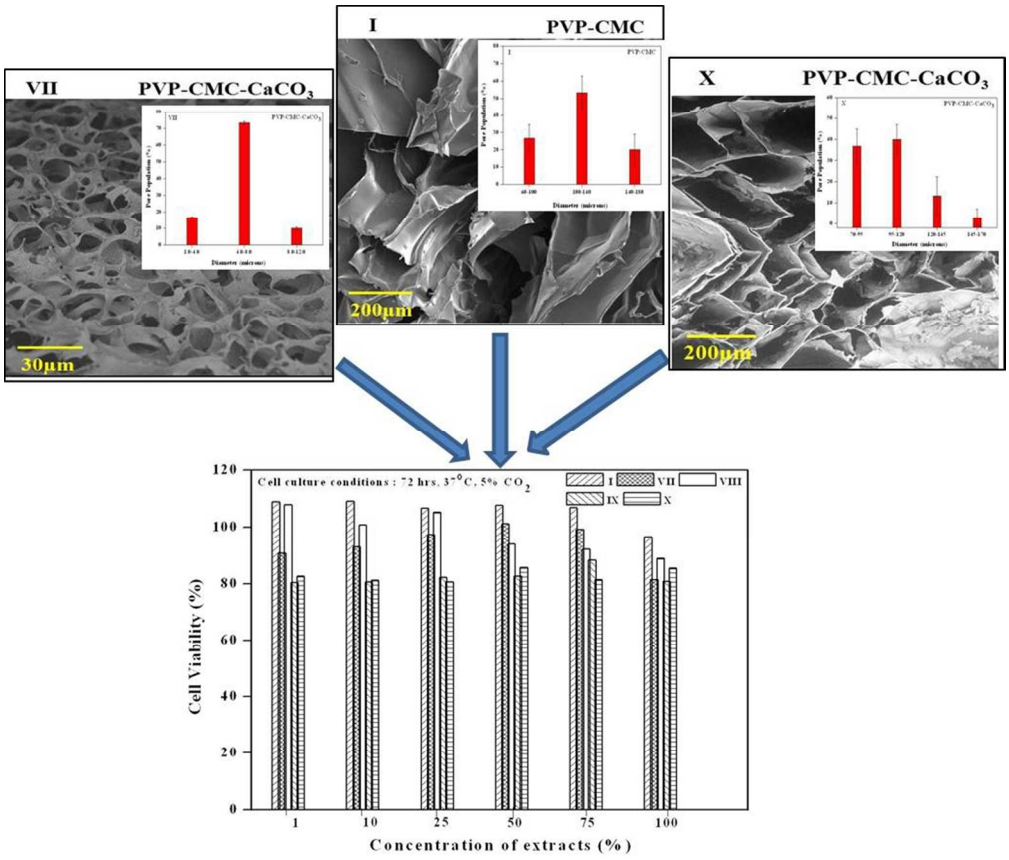
[Submitted in October 2015]





Properties of biomineralized (CaCO3) PVP-CMC hydrogel with reference to the cytotoxicity test using fibroblasts cells

Journal:	*International Journal of Polymeric Materials*
Manuscript ID	GPOM-2015-3200
Manuscript Type:	Full Research Paper
Date Submitted by the Author:	05-Oct-2015
Complete List of Authors:	Shah, Rushita; Tomas Bata University in Zlin, Center of Polymer Systems Saha, Nabanita; Tomas Bata University in Zlin, Faculty of Technology Kuceková, Zdenka; Tomas Bata University in Zlin, Center of Polymer Systems Humpolichek, Petr; Tomas Bata University in Zlin, Center of Polymer Systems Saha, Petr; Tomas Bata University in Zlin, Center of Polymer Systems
Keywords:	biomineralized, cytotoxicity, PVP-CMC, modulus, CaCO3



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Properties of biomineralized ($CaCO_3$) PVP-CMC hydrogel with reference to the cytotoxicity test using fibroblasts cells

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ABSTRACT

This article focus on properties of biomineralized ($CaCO_3$) PVP-CMC hydrogel (designated as I-X) including cytotoxicity assay using mouse embryonic fibroblasts. The biomineralized samples (VII-X) showed > 80 % cell viability, was selected for further characterizations. FTIR and XRD indicate deposition of $CaCO_3$ within the PVP-CMC hydrogel matrix; SEM signifying morphological changes and pore diameter (VII and VIII: 1-12 µm, IX: 10-70 µm and X: 70-170 µm); TGA determines the decomposition scenario of $CaCO_3$; and tensile strength of samples (VII-X) ranged between 0.04 -1.0 GPa which practically corresponds to the modulus of cancellous bone.

Keywords: biomineralized, cytotoxicity, PVP-CMC, modulus, $CaCO_3$.

Introduction

Growing evidence in the regenerative medicines which combines different principles of material science, lifescience and medicines leads to the development of several tissue-engineered products with better biological structure and their functionality [1,2]. Todays scientists are more oriented in developing mostly every tissue of human body [3]. The focus has been given in preparing scaffolds in the form of the matrix to be used inside the human body that can serve in soft and hard tissue regeneration. Mineralized tissues such as bone and dentin have structure of organic matrix impregnated with carbonated apatite crystals [4]. Biomineralization is an important attention in today's generation as this is a biological process in which living organisms, produces minerals with proper control [5]. Mineralization offers advantages in both clinical as well as in basic science. This mineralization can be successfully accomplished in several ways like the addition of inorganic particles, creation of nucleation sites by biomimetic methods or derivatization of hydrogel backbones with specific functional groups etc. With respect to the application to prepare scaffolds to be used in biomedical field, template mediated mineralization is mostly preferred wherein the extracellular matrix can be useful in the regeneration of the tissues/organs of the body [6]. The significance of using template mediated mineralization lies in the fact that it can control the crystallization from the beginning. The formation of initial crystalline phase will have same structure maintained thoughout till the last stage of forming crystals. During this process several phase transformation occurs [7]. Further, the incorporation of additives or biominerals like hydroxyappatite/ calcium carbonate ($CaCO_3$) / calcium phosphate ($CaPO_4$) influences the properties of the matrix and can serve as promising materials for biomedical application especially in bone-tissue regeneration [8]. Among the several biominerals used, $CaCO_3$ is found to be abundantly available in nature. Moreover, it

exists in three different forms calcite, vaterite and aragonite. This $CaCO_3$ is biocompatible, osteoinductive in nature, can be a suitable candidate to be used as a coating, or as additives in scaffolds, etc. However, such biominerals incorporation is usually carried out either on gels, scaffolds, films or fibers [8-12].

In biological systems any mineral deposition takes place in gel-like extracellular matrices. For the gel like system, hydrogels are considered among the best and suitable to be used as a scaffold matrix based implant for the human body [4]. These hydrogels can be prepared either from natural or synthetic polymers by physical or chemical crosslinking. Being hydrophilic in nature, when hydrogels comes in contact with water they form insoluble three dimensional network and they possess huge water holding capacity as well as any biological fluids can be absorbed without being dissolved in that [13-17]. Also, they easily allow the movement/diffusion of nutrients or by-products of cell metabolisms, promote cell migration, angiogenesis etc. Further, such hydrogels or polymers that mimic the extracellular matrix (ECM) are of great importance because they possess same surface chemistry, mechanical property, storage and controlled release of growth factors, drugs etc. like that of ECM [9,10,18]. Several natural based polymers like collagen, cellulose, gelatin, fibrin, alginate etc. have already been utilized in the field of tissue regeneration, but they suffered drawback related to infection during clinical/in-vivo trials. So, to avoid this synthetic polymers are also preferred to precisely control the toxic effect or risk of any infection [19]. Till now mostly there are reports showing application of hydrogels in soft-tissue regeneration, but since last few years, there is divergence of interest to use such polymer based hydrogels in hard-tissue regeneration [20]. However, some difficulties are still faced in applying such hydrogels as scaffolds for hard tissue regeneration like limited bioactivity, mechanical weakness or poor mineralization of hydrogels during the time of implantation [21].

Hence, to overcome these drawbacks, proper strategies are applied to prepare biomaterials for the purpose of hard-tissue regeneration [19,22]. Now any biomaterial when prepared for the purpose to be utilized for diagnostic, prosthetic or therapeutic application, should possess host response with respect to toxicity, carcinogenecity or inflammatory [23,24]. The success of any biomaterial after its implantation in the human body depends on biocompatibility. Apart from considering the fact that biocompatibility focuses on having non-toxic effect on any biological substitutes, it also needs to have a proper host response to satisfy the performance of that particular biomaterial prior to its application. In vitro biocompatibility test includes cytotoxicity, antibacterial and antifungal tests, irritation, sensitization, acute inflammation and systemic toxicity etc. [25-27]. As per the ISO norms, any prepared biomaterial can be evaluated in three ways, a) biological safety of ingredients used for manufacturing basic material b) biological safety of the leachable or degradable product and c) biological safety of final biomaterial [28].

The current study focuses on the development of novel scaffolds in the form of biomineralized hydrogel using biomimetic approach. Polyvinylpyrrolidone (PVP) as synthetic polymer and carboxymethylcellulose (CMC) as natural polymer, together after blending forms the matrix for mineralization. The "PVP-CMC hydrogel" (used as a matrix for biomineralization) is the work, already reported by the researchers of Tomas Bata University in Zlin [13-16]. Further, to achieve a novel biomaterial for bone tissue engineering, the said "PVP-CMC hydrogel" has been utilized as a matrix to incorporate calcium carbonate ($CaCO_3$) to accomplish a novel "biomineralized hydrogel scaffold" using a simple liquid diffusion technique [29]. This work served as an important step towards the development of hydrogel based biomaterial obtained through biomimetic approach. The thorough study of such novel biomaterial in the form of structural (i.e. in the form of physical and chemical analysis), viscoelastic nature and also the change in

swelling behavior in presence of intracellular fluids (present inside the body) have already been reported in our previous articles [30-35]. Now, considering the role of these novel biomaterial and predicting their application in bone-tissue regeneration, this paper reports about the biocompatibility (in terms of cytotoxicity) including some of its specific properties (like: morphology, FTIR, XRD, TGA and mechanical properties). Therefore, cytotoxicity and cell viability of primary fibroblast cells were examined in the presence of biomineralized ($CaCO_3$) PVP-CMC-hydrogel (sample index II-X, depicted in Table I) and PVP-CMC hydrogel (sample index-I, depicted in Table I) as a control set. It is important to mention that higher to lower concentration of ionic solutions (**Na_2CO_3:**10.5-0.07g/100ml and **$CaCl_2$:**14.7-0.07g/100ml) were used during the preparation of mineralized hydrogel scaffold and designated as I-X. This wide range of ionic solution concentration has been chosen in order to validate the exact range of $CaCO_3$ concentration in PVP-CMC hydrogel wherein the test cell (i.e. fibroblast cells) remained mostly viable so that its applicability can be decided. It is also important to focus on mechanical strength of hydrogel based biomaterial, if it is decided to use in any orthopedic field i.e. for bone tissue engineering. Thus, assessment of the mechanical behavior of the said biomineralized ($CaCO_3$) PVP-CMC-hydrogel is interesting and challenging too, before recommend for its application either as soft tissue or hard tissue regeneration.

EXPERIMENTAL

Materials

For Biomineralized ($CaCO_3$) PVP-CMC hydrogel

PVP K30 (PVP: molecular weight 40,000), polyethylene glycol 3000 (PEG: average molecular weight 2700-3300) and agar were supplied by Fluka, Switzerland; carboxymethyl cellulose (CMC) was purchased from Sinopharm Chemical Reagent Co-Ltd (SCRC), China; glycerin was

obtained from Lachema, Czech Republic; calcium chloride ($CaCl_2$: molecular weight 110.99 g/mol, 97.0%), Penta, Czech Republic; sodium carbonate-10-hydrate (Na_2CO_3: molecular weight 105 g/mol) was obtained from Sigma Aldrich.

For Cytotoxicity Assay

Mouse embryonic fibroblasts, Dulbecco's Modified Eagle's Medium (PAA Laboratories GmbH, Austria) containing 20 % of calf serum (BioSera, France), Penicillin/Streptomycin (100 U mL^{-1}) from BioSera, France, Mercaptoethanol (7µl L^{-1}) from Serva, Germany, Tetrazolium from Duchefa Biochemie, Netherlands , 96-well microtiter tissue culture plates.

Biomineralized ($CaCO_3$) PVP-CMC hydrogel preparation

Preparation of PVP-CMC hydrogel

The matrix of PVP-CMC hydrogel was prepared following solution casting technique. To prepare this hydrogel, PVP (0.2g), CMC (0.8g), PEG (1.0g), Agar (2.0g), Glycerin (1g), and water (95 ml) was taken. The polymer solution was treated by applying moist heat and pressure to construct three dimensional porous structures within the soft PVP-CMC hydrogel. This hydrogel was prepared in round sterile polystyrene petri plates with a diameter 80 mm using 50 ml polymer solution in each and incubating 10-15 mins at room temperature. Thereafter, the fresh hydrogel samples were allowed to air drying for 48-72 hrs at room temperature (25-26°C).

Preparation of biomineralized hydrogel

The dried PVP-CMC hydrogel was further used to undergo the mineralization process using a simple liquid diffusion technique. Two different ionic solutions (i.e. Na_2CO_3 and $CaCl_2$) were used in different concentration ratios to prepare final biomineralized ($CaCO_3$) PVP-CMC hydrogel as listed in the Table 1. Initially nine different concentrations of ionic solutions (i.e.

Na_2CO_3 and $CaCl_2$) were prepared which ranges from higher to lower values as depicted in Table 1. Finally, cytotoxicity study of the novel biomaterial (i.e. biomineralized hydrogels (II - X) and PVP-CMC hydrogel (I) were carried out individually using fibroblast cell lines.

Table 1: Brief information of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Sample Index	Concentration of Ionic Solution (g/100ml)		Biomineralized ($CaCO_3$) PVP-CMC hydrogel (Diameter = 80 mm)			
			Wet Sample		Dry Sample	
	Na_2CO_3	$CaCl_2$	Weight (gm)	Thickness (mm)	Weight (gm)	Thickness (mm)
*I	-	-	43.762	6.88	2.507	0.41
II	10.50	14.70	9.450	1.15	2.681	0.46
III	5.25	7.35	9.802	1.21	2.289	0.44
IV	4.20	5.88	9.989	1.28	2.239	0.43
V	2.10	2.94	12.033	1.37	1.990	0.41
VI	1.05	1.47	13.105	1.42	1.810	0.40
*VII	0.55	0.55	17.320	1.59	1.757	0.40
*VIII	0.27	0.27	20.327	1.87	1.664	0.37
*IX	0.13	0.13	22.16	2.14	1.558	0.34
*X	0.07	0.07	24.16	2.31	1.518	0.34

Note: * - These samples are used for detail investigation and characterization

Preparation of extract from biomineralized ($CaCO_3$) PVP-CMC hydrogel

The extract from biomineralized ($CaCO_3$) PVP-CMC hydrogel was prepared according to ISO standard 10993-12. According to the standards, 0.1g (i.e. 100 mg) of the hydrogel was taken in 1ml of DMEM medium with calf serum and incubated for 24 hours at 37ºC with stirring. Next day, the parent extracts (100 %) were then diluted in culture medium (DMEM) to obtain a series of dilutions with concentrations of 75, 50, 25, 10, and 1 % which has been used further for cell viability test.

Test on Mouse embryonic fibroblast cells

The 96-well microtiter tissue culture plates were used and the selected cell line i.e. mouse embryonic fibroblast cells were seeded in the suspension of $1x10^5$ cells/ml of DMEM. This plate was thereafter incubated for 24 hrs. During next day, after a pre-incubation period, the old medium was replaced with the new medium and the extract from biomineralized ($CaCO_3$) PVP-CMC hydrogel was added and kept in an incubator for 24 hrs. 37°C and 5% CO_2. Now to determine the toxicity range / cell viability of fibroblast cells, the extract was cultivated in DMEM medium for three days and simultaneously after every 24 hrs MTT assay were performed. In MTT assay, in the medium with the extract after incubation for 24 hours at 37°C and 5% CO_2, MTT reagent was added and kept for 4hrs. Thereafter, the absorbance was noted down at 570 nm range.

Mouse embryonic fibroblast cells Morphology

Growth of mouse embryonic fibroblast cell was examined individually a sterile petri plate using hydrogel samples (15 mm diameter) in presence of DMEM medium. The morphology of Mouse embryonic fibroblast cells from each culture plate of test sample (I-X) of biomaterials along with reference (i.e. only fibroblasts cells) was observed using an inverted phase contrast microscope

(Olympus). The morphology of viable cells was observed in presence and absence of hydrogel samples with varying concentrations of parent extract (i.e. 100, 75, 50, 25, 10, and 1 %) from 24-72 hrs.

Scanning Electron Microscopy (SEM)

Morphological structures of selected biomineralized ($CaCO_3$)-PVP-CMC hydrogel samples (VII-X) were examined by scanning electron microscopy. The samples prior to studying its structural changes, were lyophilized using lyophilizator instrument Christ Alpha 1–4 (Christ, Osterode, Germany), at a temperature of - 40ºC and pressure p ~ 12 kPa. The entire process of lyophilizing the samples takes two days (i.e. ~48 hrs). The SEM observation was carried out on (SEM: Phenom world Pro) operating in the high-vacuum/ secondary electron imaging mode at an accelerating voltage of 5-15 kV. The images were taken at a magnification of 100x-10kx. The macroporous structure of all the mineralized hydrogel samples was found using Image J software. The statistical analysis (in sort of a histogram) and pore diameter of the examined biomineralized samples were counted

Apparent Bulk Density

The apparent density of biomineralized ($CaCO_3$) PVP-CMC hydrogel samples were examined by soaking in distilled water up to the saturation time at room temperature (25-26^{O}C). The apparent density was determined using the equation as below, (17)

$$\rho = 4m/\pi d\text{^}2h$$

Here, ρ = apparent density of material (g/cm^3), m=mass (gm) of hydrogel after incubating in water till the saturation time (i.e 90 mins), d= diamter (cm) of hydrogel after incubation, h=height (cm) of hydrogel after incubation in water.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of the selected biomineralized ($CaCO_3$) PVP-CMC hydrogel samples were obtained at wave number of 2000-600 cm-1 at room temperature with uniform resolution of $2cm^{-1}$. For this, attenuated total reflectance ATR-FTIR was used with NICOLET 320 FTIR Spectrophotometer with "Omnic" software package.

X-ray Diffraction (XRD)

The XRD patterns of the selected biomineralized ($CaCO_3$) PVP-CMC hydrogels were studied using X´Pert PRO X-ray diffractometer (PANalytical, The Netherlands) with Cu Kα radiation of α = 0.1540598 nm. The operating voltage and current were 40 kV and 30 mA, respectively. Samples were scanned from 5 to 90º.

Thermo-Gravimetric Analysis (TGA)

The TA Q500 apparatus (TA Instruments, US) was used for thermogravimetric analysis (TGA). This analysis was performed at the constant heating rate of 10ºC/min from temperature range of 25-700°C under Nitrogen atmosphere. The amount of each selected sample was approximately 10 mg.

Tensile Tests

The tensile strengths of biomineralized ($CaCO_3$) PVP-CMC hydrogels were performed in a tensile testing machine with model number M350-5CT with Testometric type – DBBMTCL-10kgF and serial number- 35018. The cross-head speed was kept 20 mm/min. All the samples (in dry state) were initally cut in dumb-bell shaped by means of tensile clamps and allowed to dry at toom temperature for 24 hrs. The length of the samples was 22 mm, width 4 mm and thickness 0.21 mm. The sliding in the clamps was prevented by using tape. The Young's modulus (MPa),

tensile strength at break (MPa) was determined. Here, the average values of five samples were taken for each test samples (VII-X).

Results and Discussion

Cytotoxicity/Biocompatibility

The main objective of this study is to investigate the biocompatibility/acceptable limit of cytotoxicity of the prepared novel biomaterial (i.e. biomineralized ($CaCO_3$) PVP-CMC hydrogel as listed in Table 1. that has promising future in biomedical applications. Different assays are known to be used for measuring cytotoxicity and cell viability of novel biomaterials. Among different assays used, MTT is popular approach to determine the toxicity of any novel biomaterial like hydrogel. In MTT assays, generally the color of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) solution gets reduced to purple formazan in living cells which confirms the percentage cell viability [26].

In this study, it was found that the test samples (II-VI) comprising of higher ionic concentration, the toxicity was higher in the case of fibroblast cells thus the viability of the cells were not visible. Therefore, in this article, focus was given to investigate the properties of biomineralized ($CaCO_3$) PVP-CMC hydrogel samples prepared with the lower ionic concentration. Also, it is essential to mention here that biomaterials when constructed from the view of its application in the biomedical field should be totally non-toxic in nature. Keeping the application of this calcite filled novel biomaterial, into consideration; the further analysis of biomineralized samples was tested utilizing a sample with lower ionic concentration range (i.e. samples VII-X). The cell viability was performed until 72 hrs for the biomineralized ($CaCO_3$) PVP-CMC hydrogel samples by taking their extract. The cell viability was calculated every 24 hrs interval and the

results are depicted in Figure 1-3. As an evidence of fibroblast cell growth in the presence of calcite filled hydrogel is depicted in Figure 1a. It can be understood from the Figure (1-3) that the viability of cells is above >80% in all the extract concentration of hydrogels after 24, 48 and 72 hrs of incubation and carrying MTT assay respectively.

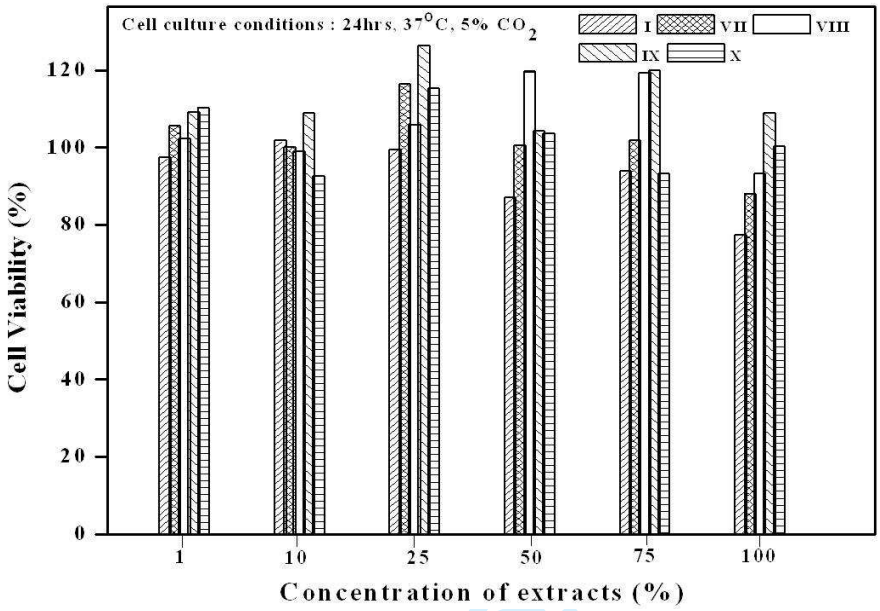

Figure 1: Cell viability of fibroblast cells in presence of biomineralized ($CaCO_3$) PVP-CMC hydrogel extracts (24 hrs).

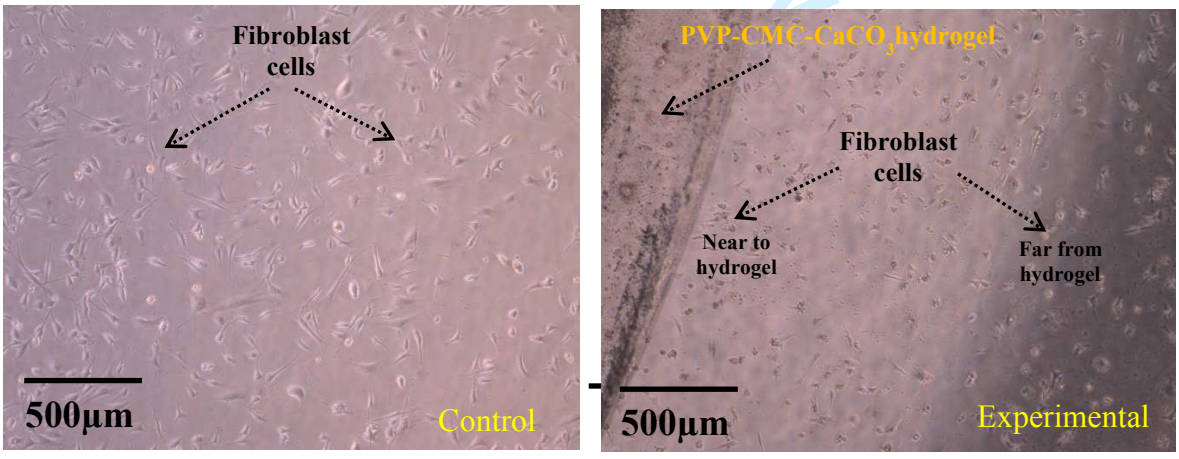

Figure 1a.Fibroblast cell growth in presence of biomineralized ($CaCO_3$) PVP-CMC hydrogel

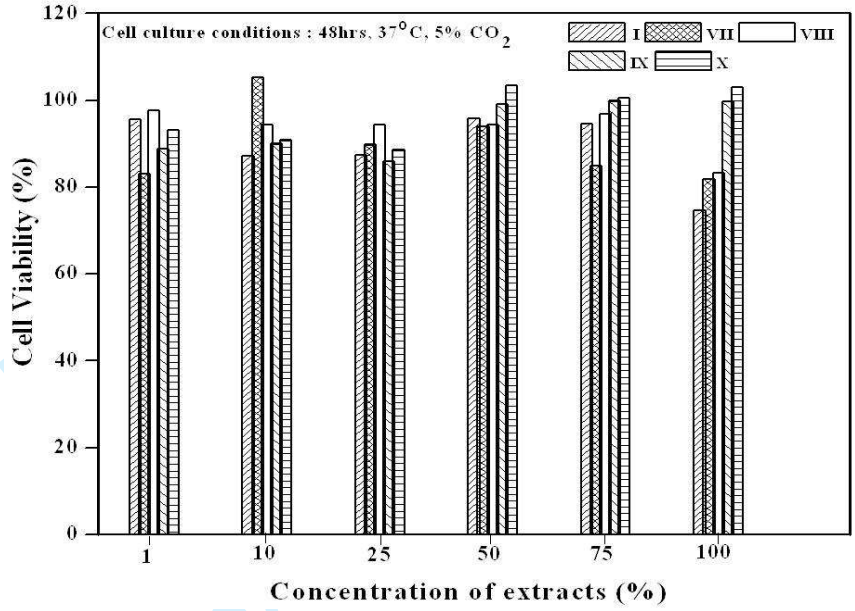

Figure 2: Cell viability of fibroblast cells in presence of biomineralized ($CaCO_3$) PVP-CMC hydrogel extracts (48 hrs).

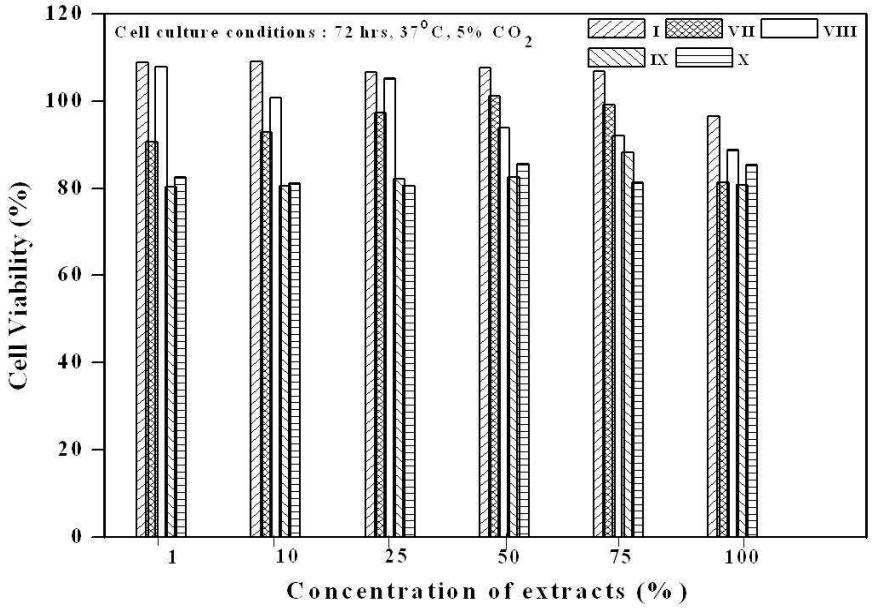

Figure 3: Cell viability of fibroblast cells in presence of biomineralized ($CaCO_3$) PVP-CMC hydrogel extracts (72 hrs).

SEM Micrographs

Biomineralized sample having crystal growths of $CaCO_3$ within the three dimensional PVP-CMC hydrogels were prepared using a liquid diffusion technique and its sound structure

was examined by SEM. Figure 4 shows the surface position of the selected biomineralized ($CaCO_3$) PVP-CMC hydrogel samples (VII, VIII, IX and X) which shows cell viability within the acceptable limits along with pure PVP-CMC (I) as a control set.

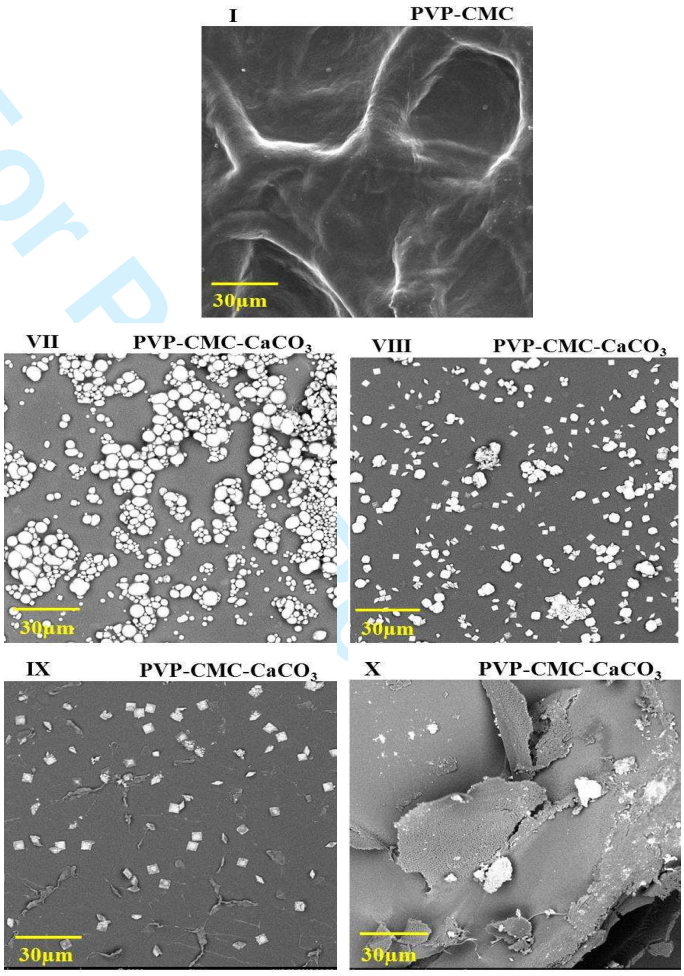

Figure 4: SEM micrographs (surface view) of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Pure PVP-CMC hydrogel clearly shows the formation of pores as well as cross-linked structure within the hydrogel. Nevertheless, on that point is no uniformity seen within the size of these pores formed within the ground substance. On the other hand, as soon as crystallization of $CaCO_3$ occurs within the PVP-CMC hydrogel matrix, the pores get filled up with the calcite.

When mineralization occurs in the pure PVP-CMC hydrogel matrix with the help of ionic solutions, the Ca^{+2} ions initially gets inside the matrix and thereafter the process of nucleation followed by the formation of carbonate ions occurs. Therefore, the calcite in form of crystals grown over the matrix could be distinctly seen through Figure 4. However, with the decrease in ionic concentration, the formation of calcite within the matrix also reduces. It can be visibly noticed that the development of crystals in sample VII and VIII is more as compared in sample IX and X. whereas in sample IX and X, small formation of crystals in their initial stage is seen, but they are non-homogenously spread out in the entire hydrogel matrix.

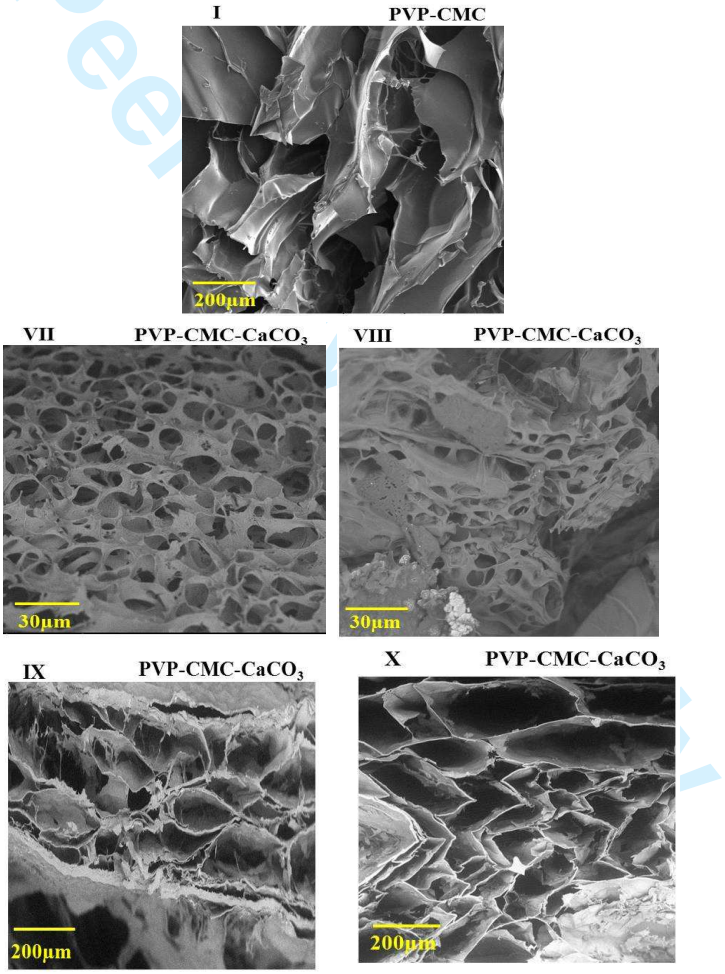

Figure 5: SEM micrographs (cross sectional view) of biomineralized ($CaCO_3$) PVP-CMC hydrogel

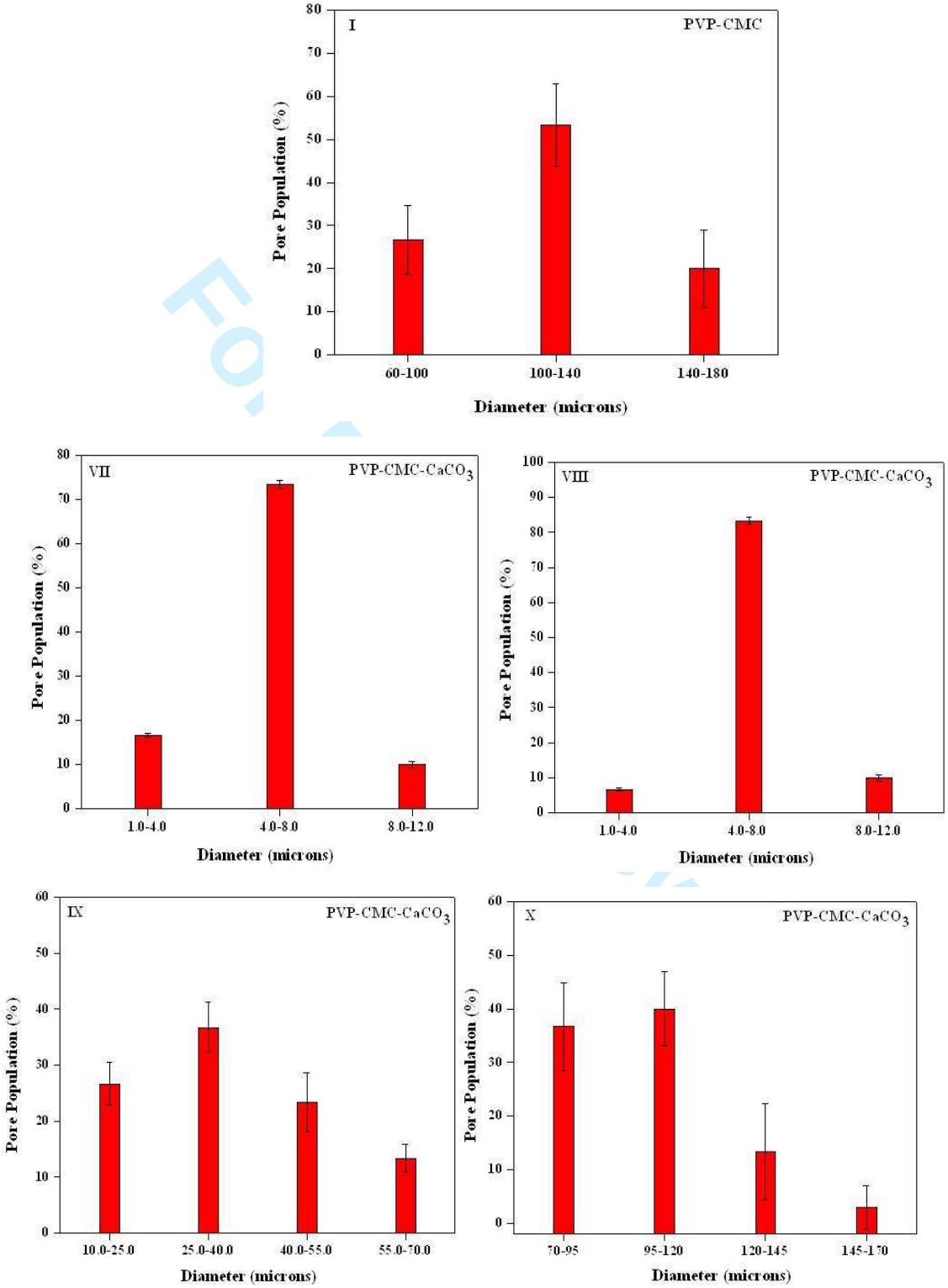

Figure 6 Statistical Analysis of Pore population distribuation in PVP-CMC and biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 5 and 6 shows the microstructure of the selected biomineralized ($CaCO_3$) PVP-CMC hydrogel in the course of their cross-sectional view. Several interconnected pores can be visualized in biomineralized ($CaCO_3$) PVP-CMC hydrogel as well as pure PVP-CMC hydrogel matrix. The pore diameter ranges from 60-180 µm for the pure PVP-CMC hydrogel matrix. Later on the calcite being incorporated inside the hydrogel matrix, the pore size and diameter varies depending on its absorption. In sample VII and VIII, the pore diameter ranges between 1-12 µm. In sample IX, the pore diameter ranges between 10-70 µm whereas in sample X, it is between 70-170 µm. The statistical analysis of the pore diameter size is depicted in all the graphs presented. Further, it can be stated that the pore size related to the biomineralized ($CaCO_3$) PVP-CMC hydrogels is inversely proportional to the ionic concentration. To fall in concentration of calcite, more space in form of pores is noticed within the hydrogel matrix conforming the development of cross-linked structure. Whereas, in high concentration of $CaCO_3$, all the pores get filled up and so there is a decrease in the pore size of the hydrogel based matrix.

Apparent Bulk Density

Figure 7 shows the apparent bulk density of the selected samples (VII-X) of biomineralized ($CaCO_3$) PVP-CMC hydrogel. It can be clearly seen all through the figure that bulk density gradually decreases from samples VII-X i.e. on decrease in concentration of ionic solutions used to prepare calcite incorporated hydrogels; the bulk density of particular hydrogels gets lowered. This study particularly provides the data about the porous structure, permeability or the presence of structural defects present in the polymer and also it acts upon the mechanical strength of the material. When considered the SEM micrographs of the selected biomineralized ($CaCO_3$) PVP-CMC hydrogel samples, the pore size are found to be higher from samples VII to X. This

however influenced the apparent density by the fact that the decrease in biomineral concentration decreases the bulk density but increases the porosity gradually.

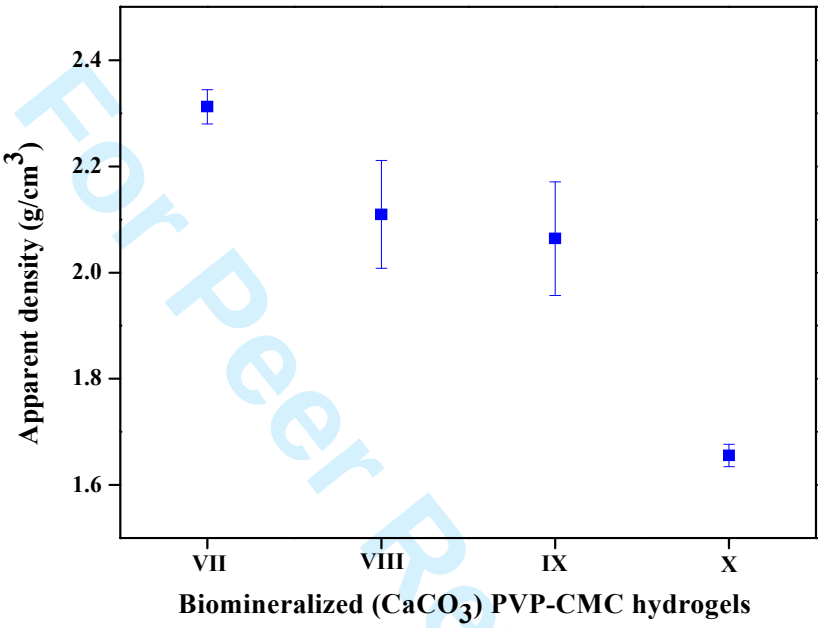

Figure 7: Apparent density of biomineralized ($CaCO_3$) PVP-CMC hydrogel

FTIR Analysis

The confirmation of presence of calcite i.e. $CaCO_3$ in the selected samples of biomineralized ($CaCO_3$) PVP-CMC hydrogel (i.e. sample VII-IX) was confirmed using FTIR spectroscopy. It can be noted from Figure 8 that there exists the peak related to CO_3^{2-} at 1400 cm^{-1} and 871 cm^{-1} in the samples of biomineralized ($CaCO_3$) PVP-CMC hydrogel, which confirms that $CaCO_3$ had got successfully incorporated within the PVP-CMC matrix [30,32]. At the same time, it is noticed that the intensity of the peak corresponding to $CaCO_3$ decreases from sample VII to X. This is due to the decrease in concentration of ionic solutions (i.e. sample VII to X) used to

prepare biomineralized hydrogel. Further, the peak at 1071 cm^{-1} corresponds to –C-O is stretching of CMC and the intensity of this peak gradually increases from sample VII to X which indicates clear visibility of one of the constituent of PVP-CMC matrix, which is of CMC polymer [36]. Further, the band at region 1654 cm^{-1} relates to the presence of C=O stretching of pyrrolidone present in PVP [37]. The peak noticed in between the range of 2800-3000 cm^{-1} is for the -C-H asymmetric stretching of CH_2 absorption band present in all the spectra of biomineralized ($CaCO_3$) PVP-CMC hydrogel samples and pure PVP-CMC hydrogel matrix. Moreover, the peak around 3400 cm^{-1} in all the spectra shows the hydroxyl group (-OH) of bound water present in all the selected biomineralized ($CaCO_3$) PVP-CMC hydrogel samples. All this observation, however confirmed the successful incorporation of calcite within the PVP-CMC polymeric matrix.

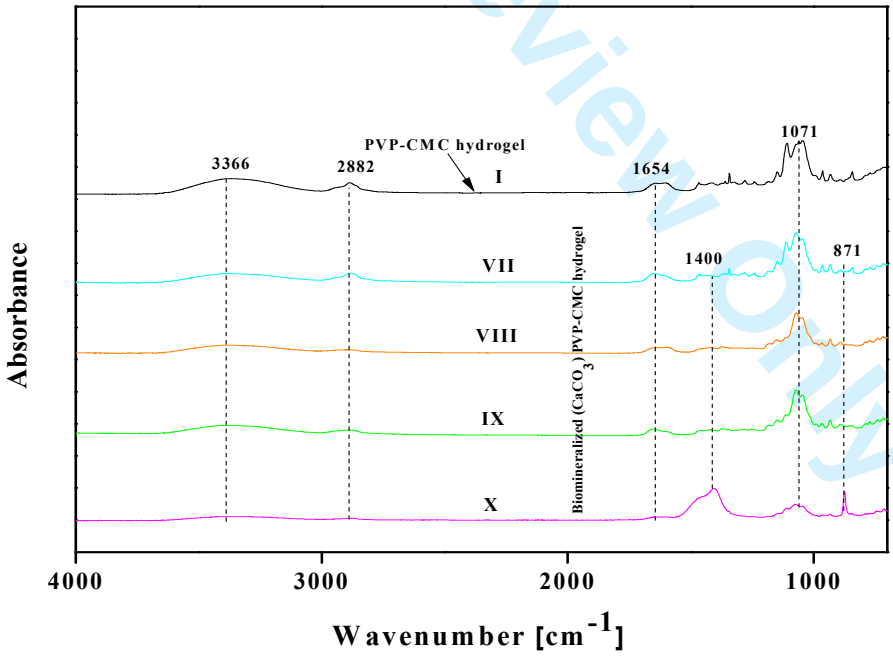

Figure 8: FTIR spectra of biomineralized ($CaCO_3$) PVP-CMC hydrogel

XRD Analysis

Test of the internalization of the calcite formation within the pure PVP-CMC hydrogel matrix was confirmed using XRD as shown in the Figure 9. The pure PVP-CMC hydrogel matrix shows the peak around 2θ = 32 which is due to the blending of PVP-CMC. Normally, the peak ranging between 2θ = 20-30 shows the presence of PVP and CMC polymer. Further, the XRD pattern exhibited peaks at 2θ = 23.10 (012), 29.46(104), 36.03(110), 39.48(113), 43.24 (202), 47.62(018), 48.61 (116) corresponds to the presence of calcite within the PVP-CMC hydrogel matrix [38, 39]. Here, it could be understood clearly that samples VII and VIII, the peaks for calcite are prominent whereas in samples IX and X they are not clearly visible because of the lower concentration of calcite within the PVP-CMC hydrogel.

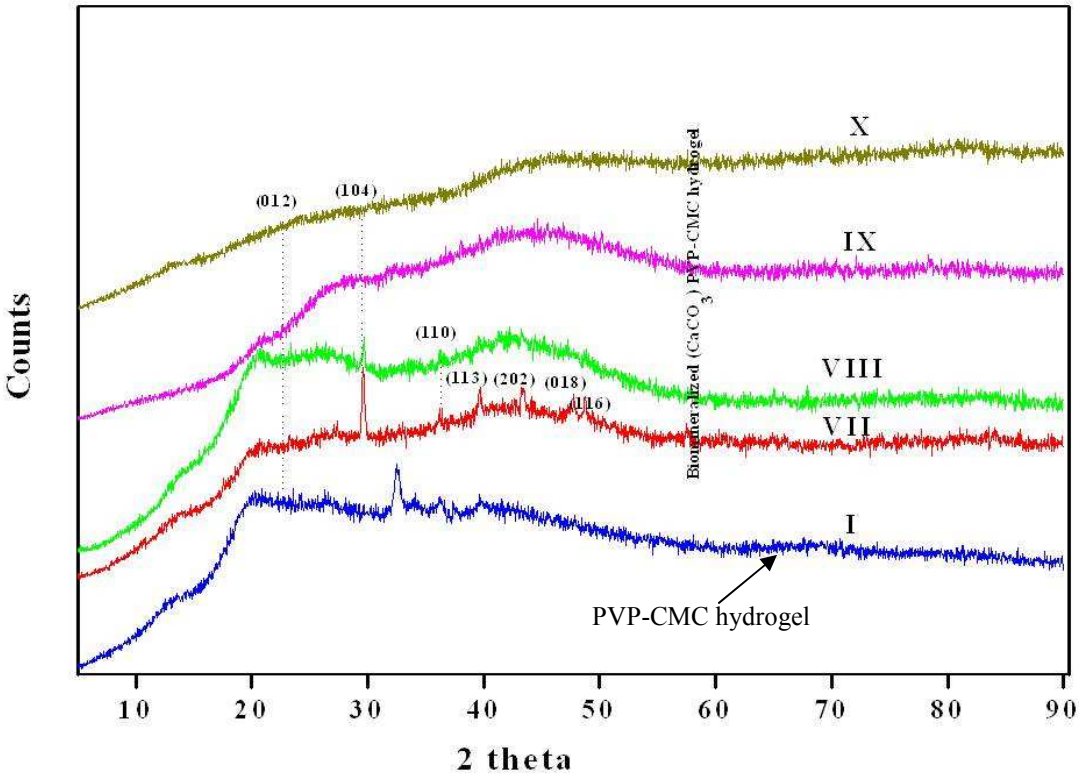

Figure 9: XRD Analysis of biomineralized ($CaCO_3$) PVP-CMC hydrogel

TGA Analysis

Thermal stability is one of the important behavior need to be studied for application of calcite incorporated in PVP-CMC hydrogel matrix. The thermal behavior of selected biomineralized ($CaCO_3$) PVP-CMC hydrogel (VII-X) obtained by means of TGA under nitrogen atmosphere is depicted in the Figure 10a. Usually, the first stage decomposition around 150°C corresponds to the bound water molecule within the hydrogel. Further, any polysaccharides start its decomposition above 250°C [40]. The CMC has COO- group and the decarboxylation takes place above 250°C with the formation of CO, CO_2 and CH_4 [40,41]. Now, the degradation concerned with the pure PVP is mostly above 300°C [42]. There exist several inorganic compounds like silicates, $BaSO_4$ and $CaCO_3$ which are used as fillers or additives. Such compounds/fillers possess stability upto 1000°C (maximum temperature for polymers in TGA) except $CaCO_3$ which starts its decomposition above 600°C. Here, in the case of selected biomineralized ($CaCO_3$) PVP-CMC hydrogel (VII-X), during first stage decomposition starts with the bound water molecules within a polymer matrix. Between 250-300 °C, the second stage of degradation starts which is of cellulose moieties. As the temperature rises the rate of weight lost increases. The weight lost seen above 300°C shows the degradation of PVP present in the hydrogel blend.

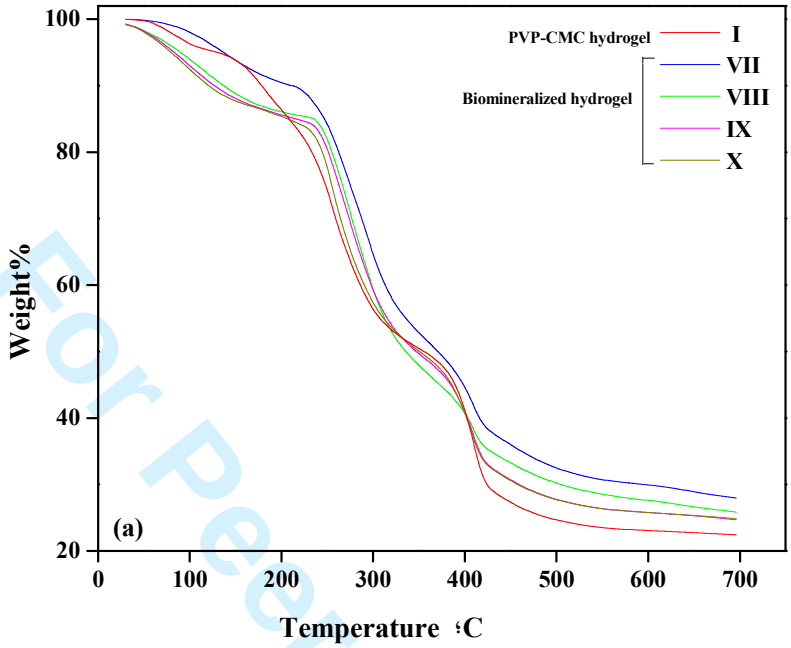

Figure 10a: Thermal Analysis of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Ultimately, the main aspect to be considered in all such hydrogels is the decomposition of inorganic matter i.e. $CaCO_3$ present in the polymer matrix of PVP-CMC. The weight loss observed beyond 600 °C in the selected samples of biomineralized ($CaCO_3$) PVP-CMC hydrogels shows the incorporation of calcite within the matrix. Here, from Figure 10a it clearly indicates the decrease in weight loss from samples VII to X. Also, separately the percentage weight loss of $CaCO_3$ within the PVP-CMC hydrogel matrix is also shown in Figure 10b. The reason behind this is, as the initial absorption of ionic solution chosen to prepare biomineralized hydrogel is decreased from samples VII-X, the formation of $CaCO_3$ within the PVP-CMC hydrogel matrix is also lowered, thus the TGA curve shows the percentage weight loss which gets lower down.

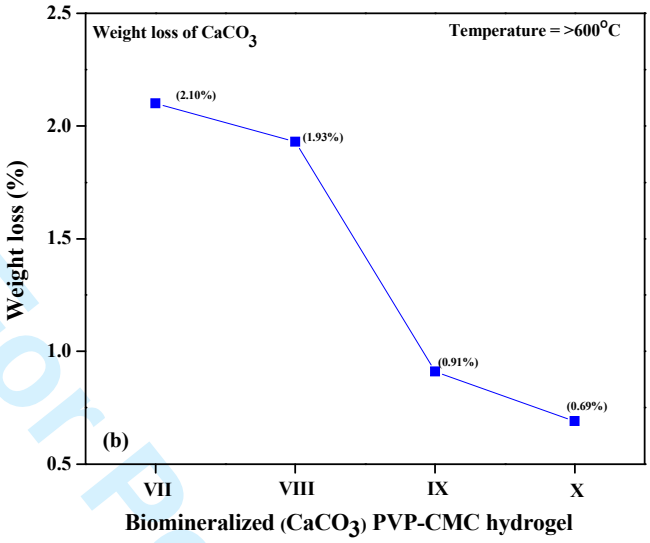

Figure 10b: Weight loss (%) scenario of $CaCO_3$ present within biomineralized hydrogel

Tensile Test

The benefits and drawbacks dealing with any material under consideration with the aspect to their biological and mechanical properties are important prior to use as implants inside the body. An ideal material should not draw out the toxic response after being embedded inside the human body, should not undergo degradation or give inflammatory response, and mechanically should be stiffer and strong enough to resist the load of the particular organs or tissues that are being replaced or repaired.

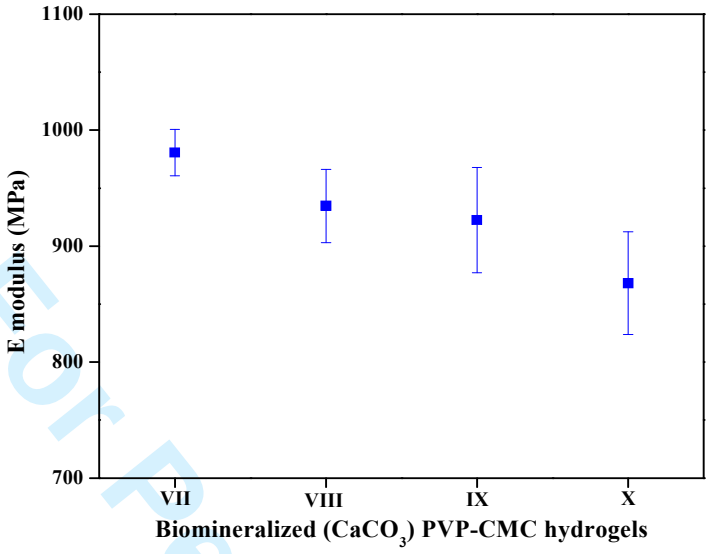

Figure 11: Elastic Modulus/Young Modulus of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 11 indicates the Young modulus / E modulus for the selected biomineralized ($CaCO_3$) PVP-CMC hydrogel set prepared with varying the concentration of ionic solutions. Here, it is observed that by decreasing the absorption of ionic solutions, the formation of calcite within the pure PVP-CMC hydrogel matrix decreases thus mechanical strength also reduces gradually. However, it is always expected that the addition of fillers/inorganic particles within the matrix improves its mechanical properties due to high mechanical strength as well as interaction with the surface energy generated between the matrix and reinforcement. As a further advantage of the incorporating calcite as inorganic filler within the PVP-CMC matrix, the modulus obtained with such novel scaffolds can be compared with the modulus related to the cancellous bone present inside the body. The modulus of such type of bones ranges between 0.04 -1.0 GPa (i.e. 40 -1000MPa) strength [43]. Here, in our case, the prepared novel biomaterial (designated as VII-X) is exhibiting approximately the similar modulus values i.e. ranging between 868-980

MPa, which is fitted within the E-modulus values of cancellous bones. The details about the young modulus as well as tensile strength values of novel biomaterial (i.e. biomineralized ($CaCO_3$) PVP-CMC hydrogel) are depicted in the Table 2.

Table 2: Mechanical property of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Sample Index	Young Modulus (MPa)	Tensile Strength at Break (MPa)
VII	980.6	49.088
VIII	934.6	47.282
IX	922.2	47.482
X	868	47.032

CONCLUSION

The present work emphasized the biocompatibility study using Mouse fibroblast cells for the novel biomaterial i.e. biomineralized ($CaCO_3$) PVP-CMC hydrogel. MTT assay was performed for determining the viability range of the cells when comes in direct contact with the prepared test samples. Concerning the application aspect for such novel biomaterial to be utilized as scaffolds/implants in hard tissue regeneration (especially in bone-tissue), the mechanical properties in form of modulus were determined that corresponds to the modulus range of cancellous bones which are inside the body. Cancellous bone can also be referred to as trabecular bone which is not so hard as compact bone, but spongy in nature and protects the bone marrow. It is less flexible in nature as well as jointed with some bones for e.g. of arms, legs (in the pelvis

region) as well as the spine. This type of bones is not easily regenerated after a certain age in human-beings thus experiencing long term healing effects during its fracture [44].

Through the entire work performed in terms of biocompatibility/cytotoxicity study and different characterizations done with such novel biomineralized ($CaCO_3$) PVP-CMC hydrogel, it can be suggested to use such material in the field of bone-tissue regeneration. Likewise, it is necessary to examine the cytotoxicity of such biomaterial using osteoblasts cell lines (*bone forming cells*) which is in progress and soon be reported.

ACKNOWLEDGEMENTS

This work was conducted within the framework of COST Action MP1301 "New Generation Biomimetic and Customized Implants for Bone Engineering" – www.cost.eu. And the work was supported by the Ministry of Education, Youth and Sports of the Czech Republic - NPU Program I (LO1504).

REFERENCES

1. Liuyun, J.; Yubao, L.; Chengdong, X. *Jour. of Biome. Sci*, **2009**, 16:65, doi:10.1186/1423-0127-16-65
2. Shi, J.; Xing, M.M.Q and Zhong, W. *Membranes.* **2012**, 2, 70-90.
3. Ozkan, S.; Kalyon, D. M.; Yu, X. *Jour.of Biomed Mater Resear*. A. **2009,** 1007-1018.
4. Zhuwei, D.; Cuixiang, L.; Haoran. L.; Dingjie, L. *Chin. J. Chem.* **2009**, 27, 2237-2241.
5. Carrillo, N.A.; Quitral, V.P.; Pedram, Y.M.; Arias, L.J. *Eur.Poly.Jour.* **2010**, 46, 1184–1193.
6. Gkioni, K.; Leeuwenburgh, S.C.G.; Douglas, T.E.L.; Mikos, A.G.; Jansen, J.A. *Tissue Engineering*: B, **2010,** 16 (6), 577-586.

7. Naka, K. (2007). Biomineralization II Mineralization Using Synthetic Polymers and Templates, (Springer, Verlag-BerlinHeidelberg) ISBN 978-3-540-46378-8, 2007.
8. Liu, Y.; Jiang, T.; Zhou, Y.; Zhang, Z.; Wang, Z.; Tong, H.; Shen, X.; Wang, Y. *Mater. Sci. and Eng.* **2011,** C31, 1055–1061.
9. Douglas, T.E.L et.al. *Macromol. Biosci.* **2012**, 12, 1077–1089.
10. Pasqui, D.; Torricelli, P.; Cagna, M. De.; Fini, M.; Barbucci, R. *J.of. Biomed.Mater.Reser.A.* **2014,** 102A (5), 1568-1579.
11. Azami, M.; Moosavifar, M.J.; Baheiraei, N.; Moztarzadeh, F. *J.of. Biomed.Mater.Reser. A*. **2012**, 100 (5), 1347-1355.
12. Song, J.; Saiz, E.; Bertozzi, C.R. *J. Am. Chem. Soc.* **2003,**125, 1236-1243.
13. Roy, N.; Saha, N.; Kitano, T.; Saha, P. *J. Appl. Polym. Sci.* **2010**, *117*, 1703-1710.
14. Saha, N.; Saarai, A.; Roy, N.; Kitano, T.; Saha P. *J. Biomater. Nanobiotech*. **2011**, 2, 85-90.
15. Saha, P.; Saha, N.; Roy, N. "Hydrogel Wound Covering" Patent no: 302405, **2010,** Czech Republic
16. Roy, N.; Saha, N.; Kitano, T.; Saha, P. *Carbohy. Polym.* **2012**, *89*, 346-353.
17. Derakhshan, Z.H et.al. Inter. *Jour. of Polym. Mater. Poly. Biomater*. **2015**, 64: 919–926.
18. Nanda, S.; Sood, N.; Reddy, B.V.K.; Markandeywar, T.S. *Ind.Jour.of Mater. Sci.* **2013**. Article ID 516021.
19. Kun Na et. al. *Biomaterials.* **2007,** 28, 2631–2637.
20. Guarino, V.; Gloria, A.; Raucci, M.G.; Ambrosio, L. *Polymers,* **2012**, 4, 1590-1612; doi: 10.3390/polym4031590.

21. Killion, J.A.; Geever, L.M.; Devine, D.M.; Farrell, H.; Higginbotham, C.L. *Jour. of Polym. Mater. Poly. Biomater,* **2014**, 63: 641–650.

22. Chen, Q.; Roether, J.A.; Boccaccini, A.R. (2008). Tissue Engineering, Vol. 4. Eds. N Ashammakhi, R Reis, & F Chiellini.

23. Torres, F.G.; Commeaux, S.; Troncoso, O.P. J. *Funct. Biomater.* **2012**, 3, 864-878; doi:10.3390/jfb3040864

24. Roy, N.; Saha, N.; Humpolicek, P.; Saha, P. *Soft Materials*, 8(4), **2010**, 338-357.

25. Biazar, E. et.al. *Jour.of Biomed. and Biotech*. **2012**, Article ID 343989.

26. Pal, K.; Banthia, A.K.; Majumdar, D.K. *Designed monomers and polymers*, **2009**, 12 197-220.

27. Kim, S. et. al. *Jour.of Biomed.Resear.B: Appl.Biomater*, **2014**, 102(7), 1393-1406.

28. Groot, C.J.De et. al. *Biomaterials*. **2001**, 22 1197-1203.

29. Rauch, M.W.; Dressler, M.; Scheel, H.; Opdenbosch, D.V.; Zollfrank, C. *Eur. J. Inorg. Che*. **2012**, 5192-5198.

30. Saha, N.; Shah, R.; Vyroubal, R.; Kitano, T.; Saha, P. Novel Trends in Rheology V Book Series: *AIP Conf. Proc*. **2013**, *1526*, 292 – 300. ISSN: 0094-243X, ISBN: 978-073541151-7.

31. Saha, N.; Vyroubal, R.; Shah, R.; Kitano, T.; Saha, P. Novel Trends in Rheology V Book Series: *AIP Conf. Proc.* **2013**, *1526*, 301-309. ISSN: 0094-243X, ISBN: 978-073541151-7.

32. Shah, R.; Saha, N.; Kitano, T.; Saha, P. *J. Appl. Polym. Sci.* **2014**, DOI: 10.1002/APP.40237.

33. Shah, R.; Saha, N.; Kitano, T.; Saha, P. *Appl. Rheol.* **2015**, *25, 33979* DOI: 10.3933/ApplRheol-25-33979.

34. Shah, R.; Saha, N.; Kitano, T.; Saha, P. *AIP Conf. Proc*. **2015,** 1664, DOI: 10.1063/1.4918447.

35. Shah, R.; Saha, N.;Zukermann R.N.; Saha, P. SPE ANTEC 2015 *Conf. Proc*, Orlando, Florida, USA.

36. Pasqui, D.; Torricelli, P.; Cagna, M D; Fini, M ; Barbucci, R.; *Jour. of Biomed. Mater. Resear. A*, **2013,** 00A, 00, 1-12.

37. Sivaiah K.; Kumar, N.K.; Naresh, V.; Buddhudu, S. *Mater. Sci. and Appli*. **2011**, 2, 1688-1696.

38. Ma, Y.; Feng, Q.; Bourrat, X. *Materials science and Engineering C*, **2013**, 33, 2413-2420.

39. Fu, L.H.; Dong, Y.Y.; Maa, M.G.; Li, S.M.; Sun, R, C. *Ultrasonics Sonochemistry*, **2013**, 20, 839–845.

40. H.L. Abd El-Mohdy Poly. Engineer and Sci. 2014, 2753-2761. DOI 10.1002/pen.23831

41. Xiao, C.; Lu, Y.; Liu, H.; Zhang, L. *Jour.of Appl. Poly.Sci*. **2001**, 80, 26–31.

42. Gasaymeh, S.S.; Radiman, S.; Heng, L.Y.; Saion, E.; Saeed, G.H.M. *African Physical Review*, **2010** 4:0006, 31-41.

43. Bureau, M.N, Legoux, J.G, Denault, J. Implantable biomimetic prosthetic bone .WO 2006074550 A1, 2006.

44. **What is Cancellous Bone**? http://www.wisegeekhealth.com/what-is-cancellous-bone.htm

Caption , Tables and Figures of the article

Properties of biomineralized ($CaCO_3$) PVP-CMC hydrogel with reference to the cytotoxicity test using fibroblasts cells

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Captions:

Table 1: Brief information of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Table 2: Mechanical property of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 1: Cell viability of fibroblast cells in presence of biomineralized ($CaCO_3$) PVP-CMC hydrogel extracts (24 hrs).

Figure 1a: Fibroblast cell growth in presence of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 2: Cell viability of fibroblast cells in presence of biomineralized ($CaCO_3$) PVP-CMC hydrogel extracts (48 hrs).

Figure 3: Cell viability of fibroblast cells in presence of biomineralized ($CaCO_3$) PVP-CMC hydrogel extracts (72 hrs).

Figure 4: SEM micrographs (surface view) of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 5: SEM micrographs (cross sectional view) of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 6: Statistical Analysis of Pore population distribution in PVP-CMC and biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 7: Apparent density of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 8: FTIR spectras of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 9: XRD Analysis of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 10a: Thermal Analysis of biomineralized ($CaCO_3$) PVP-CMC hydrogel

Figure 10b: Weight loss (%) of $CaCO_3$ from biomineralized ($CaCO_3$) PVP-CMC hydrogel by Thermal Analysis

Figure 11: Elastic Modulus/Young Modulus of biomineralized ($CaCO_3$) PVP-CMC hydrogel