Gelatin Chitosan and Nano Bioactive Glass based Scaffold for Bone Tissue Engineering

Presented by

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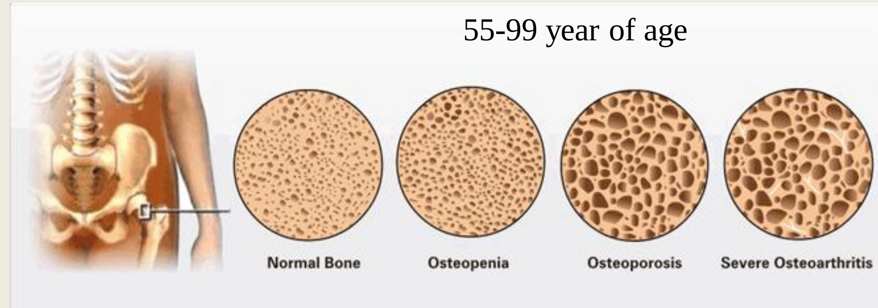
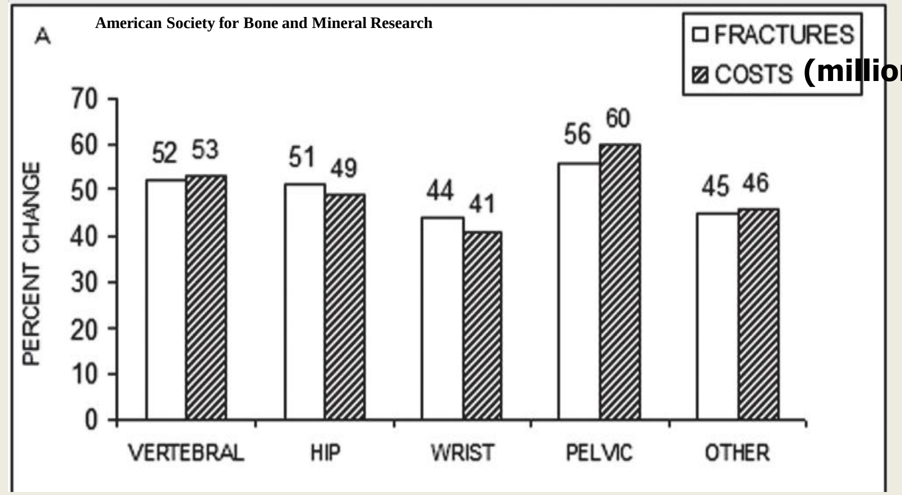
Results and Discussion

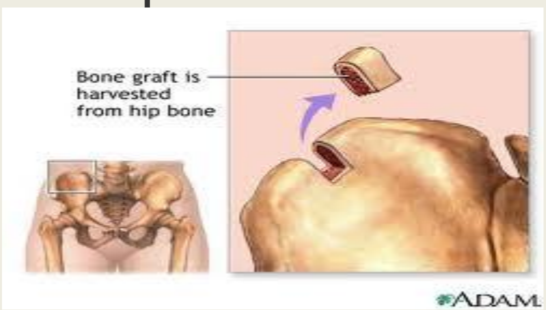
- Fabrication and characterization of Gelatin-chitosan-HAp composite scaffold(GCH).
- Fabrication and characterization of Gelatin-chitosan-58S Bioglass composite scaffold(GCB).
- Fabrication and characterization of Gelatin-chitosan-β-TCP composite scaffold(GCT).
- Comparative study between GCH30, GCB30, GCT30 composite scaffold.

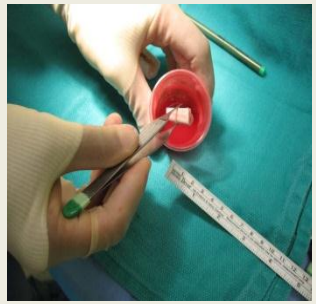
Introduction

In case of critical bone defects or patients suffering from osteopenia or osteoporosis tissue regeneration is required.

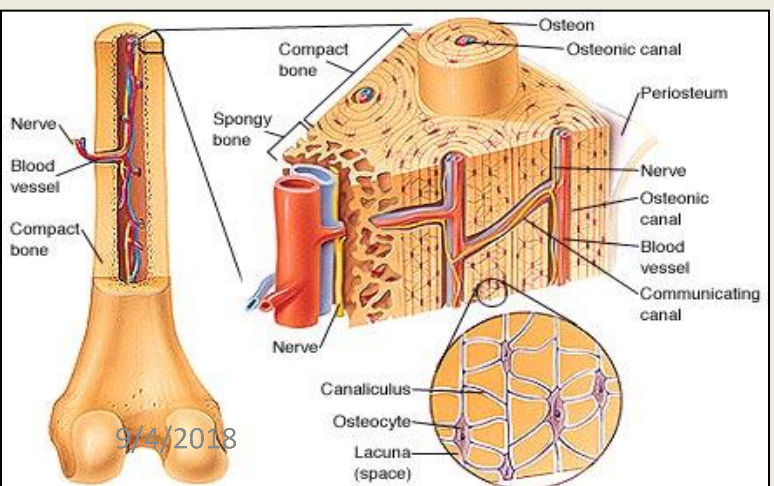


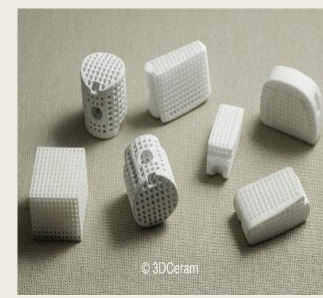






- Autografting ⟶ donor site limitations
- Allografting ⟶ tissue rejection and disease transfer



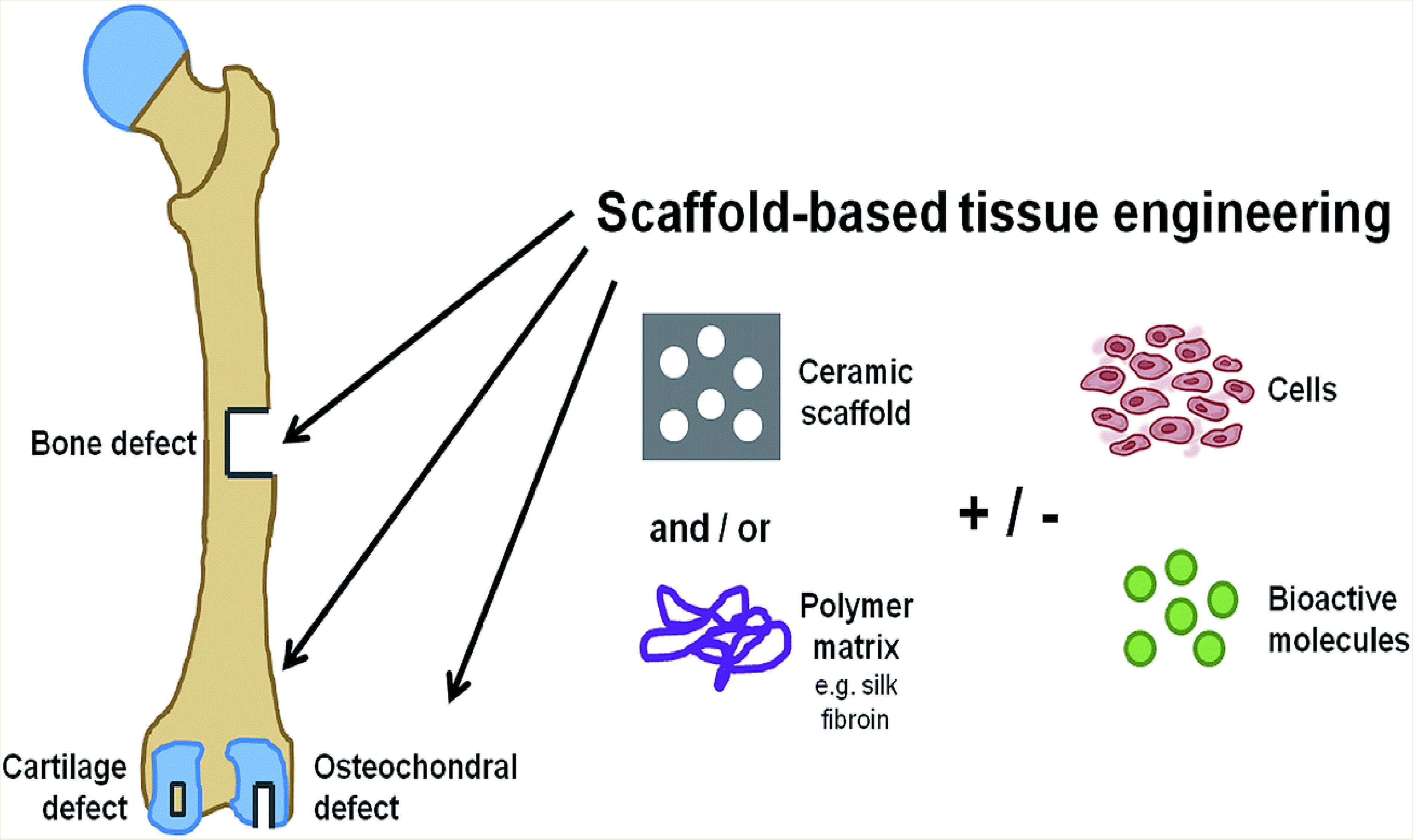


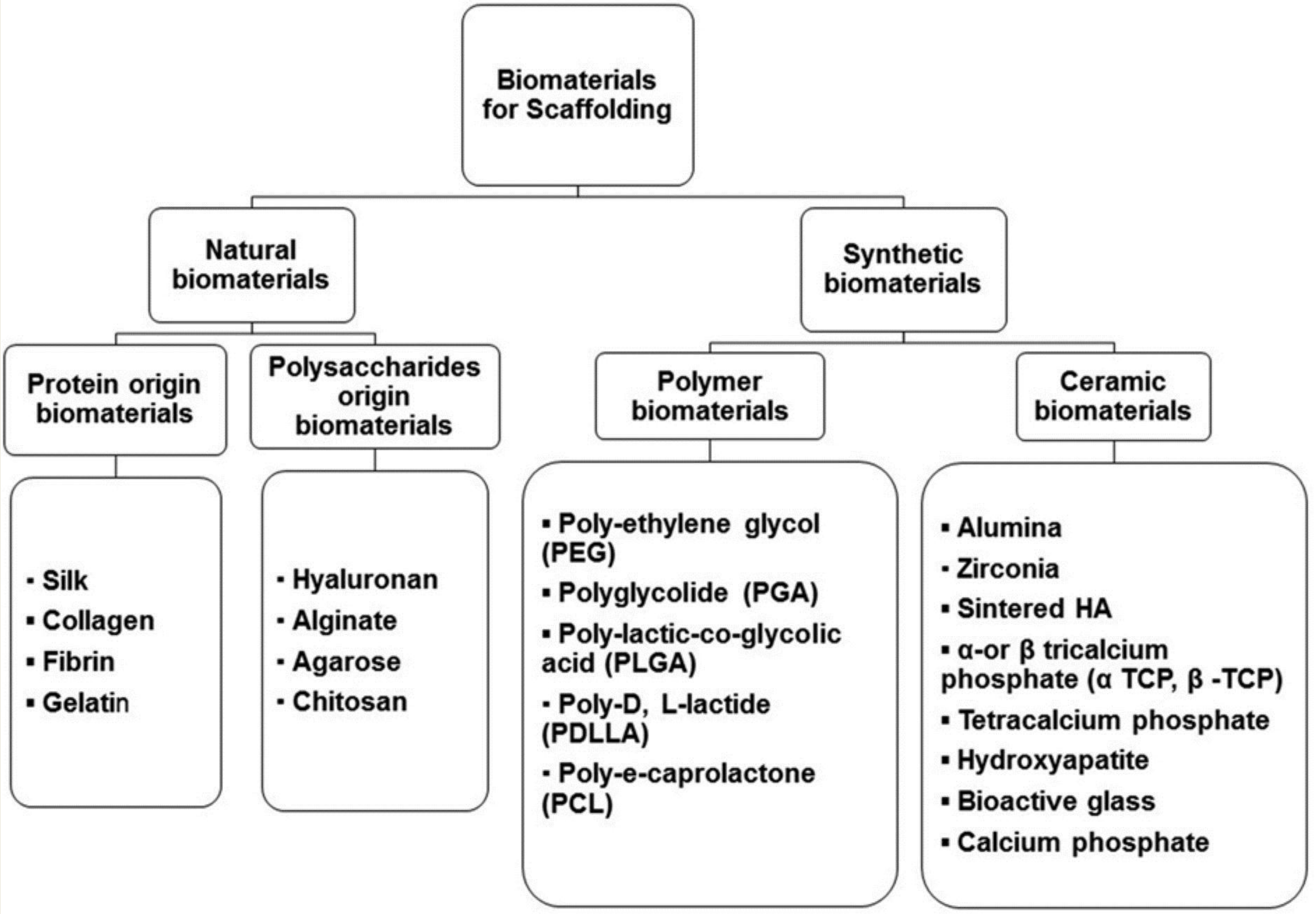
Bone is a bio inorganic-organic composite

About 60 % of bone graft substitute- Ceramic alone or in combination

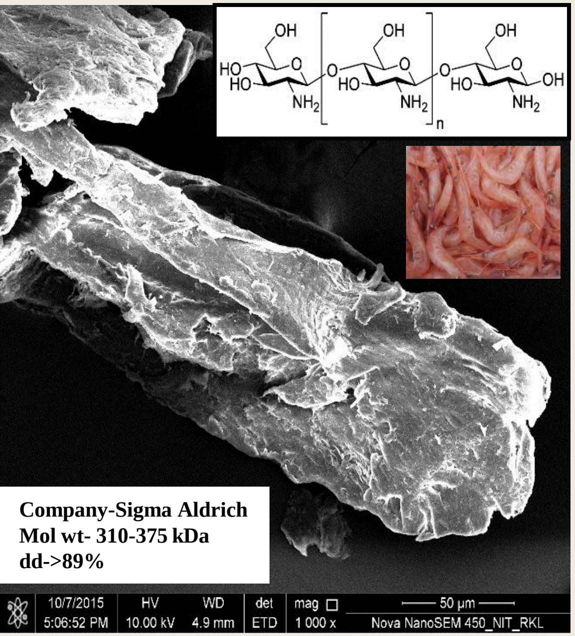
Ceramic such as: Calcium Sulphate, Calcium Phosphate, Bioactive Glass

Bone tissue Engineering using scaffolds



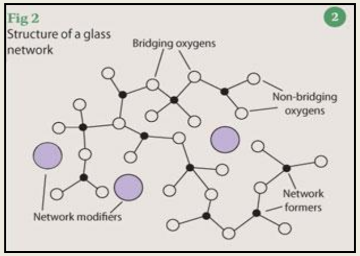

Biomaterials for Scaffolding
Natural biomaterials
Synthetic biomaterials
Protein origin biomaterials
Polysaccharides origin biomaterials
Polymer biomaterials
Ceramic biomaterials
▪ Silk
▪ Collagen
▪ Fibrin
▪ Gelatin
▪ Hyaluronan
▪ Alginate
▪ Agarose
▪ Chitosan
▪ Poly-ethylene glycol (PEG)
▪ Polyglycolide (PGA)
▪ Poly-lactic-co-glycolic acid (PLGA)
▪ Poly-D, L-lactide (PDLLA)
▪ Poly-e-caprolactone (PCL)
▪ Alumina
▪ Zirconia
▪ Sintered HA
▪ α-or β tricalcium phosphate (α TCP, β -TCP)
▪ Tetracalcium phosphate
▪ Hydroxyapatite
▪ Bioactive glass
▪ Calcium phosphate

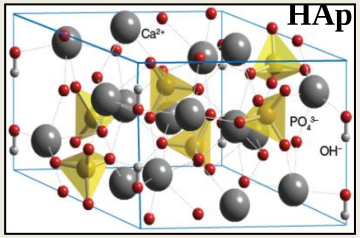
Motivation

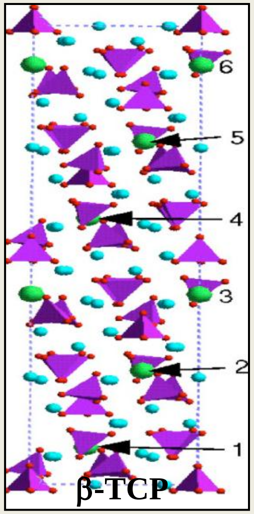


Chitosan

- ➢Biodegradability.
- ➢ Biocompatibility .
- ➢Antibacterial activity.
- ➢Wound healing properties.
- ➢Resembles ECM (GAGs)

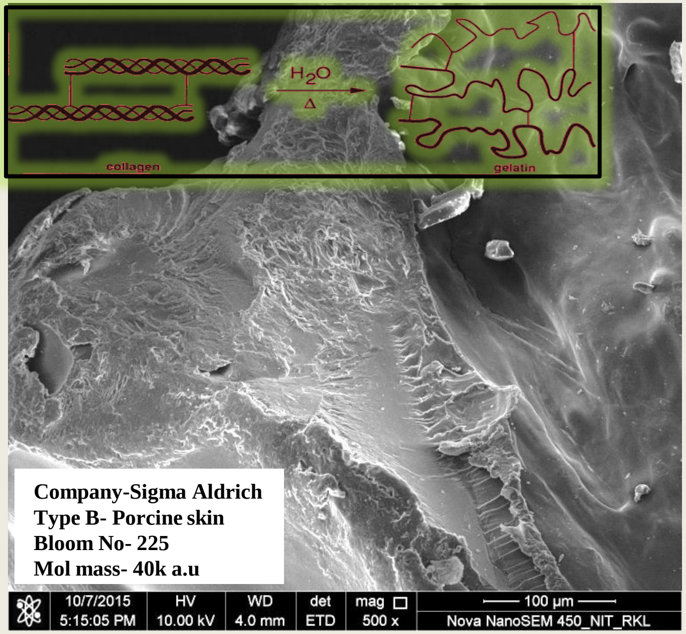






Calcium Phosphate bioactive ceramic

- ➢Resembles mineral component of natural bone
- ➢ Higher mechanical strength and bioactivity.
- ➢ osteoconductive and resorbable.



Gelatin

- ➢ Partially hydrolyzed product of collagen.
- ➢Supports osteoblast adhesion.
- ➢Supports osteoblast growth.
- ➢Biodegradable to generate natural ECM.
- ➢Cytocompatibility.

Objectives

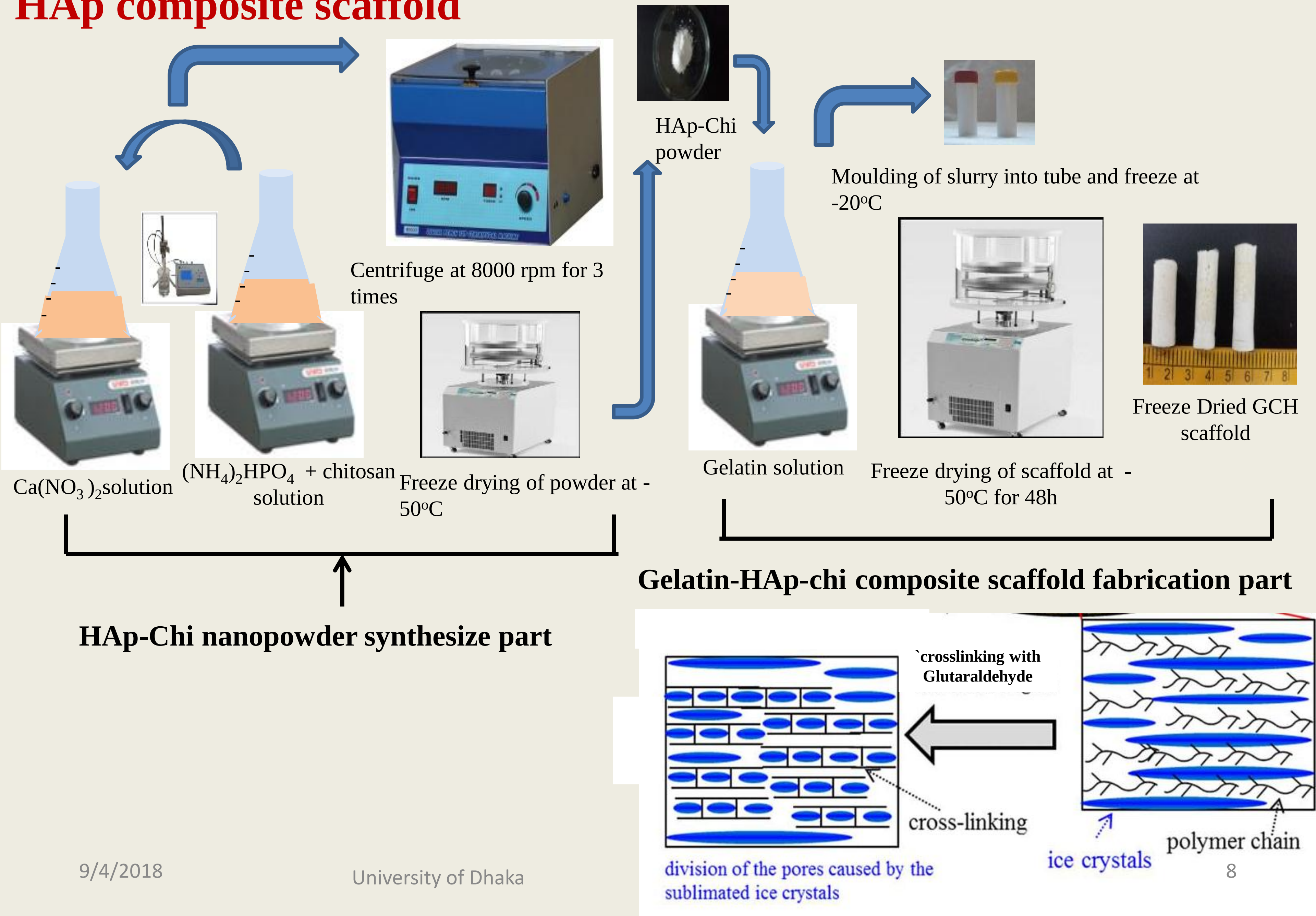
i. To develop **Gelain-Chitosan-Hydroxyapatite/ β-TCP / 58S Bioglass** based composite scaffolds with improved biological and mechanical properties.

ii. Systematic investigation on the **effects of compositional variation** on the microstructure, mechanical strength of different scaffolds.

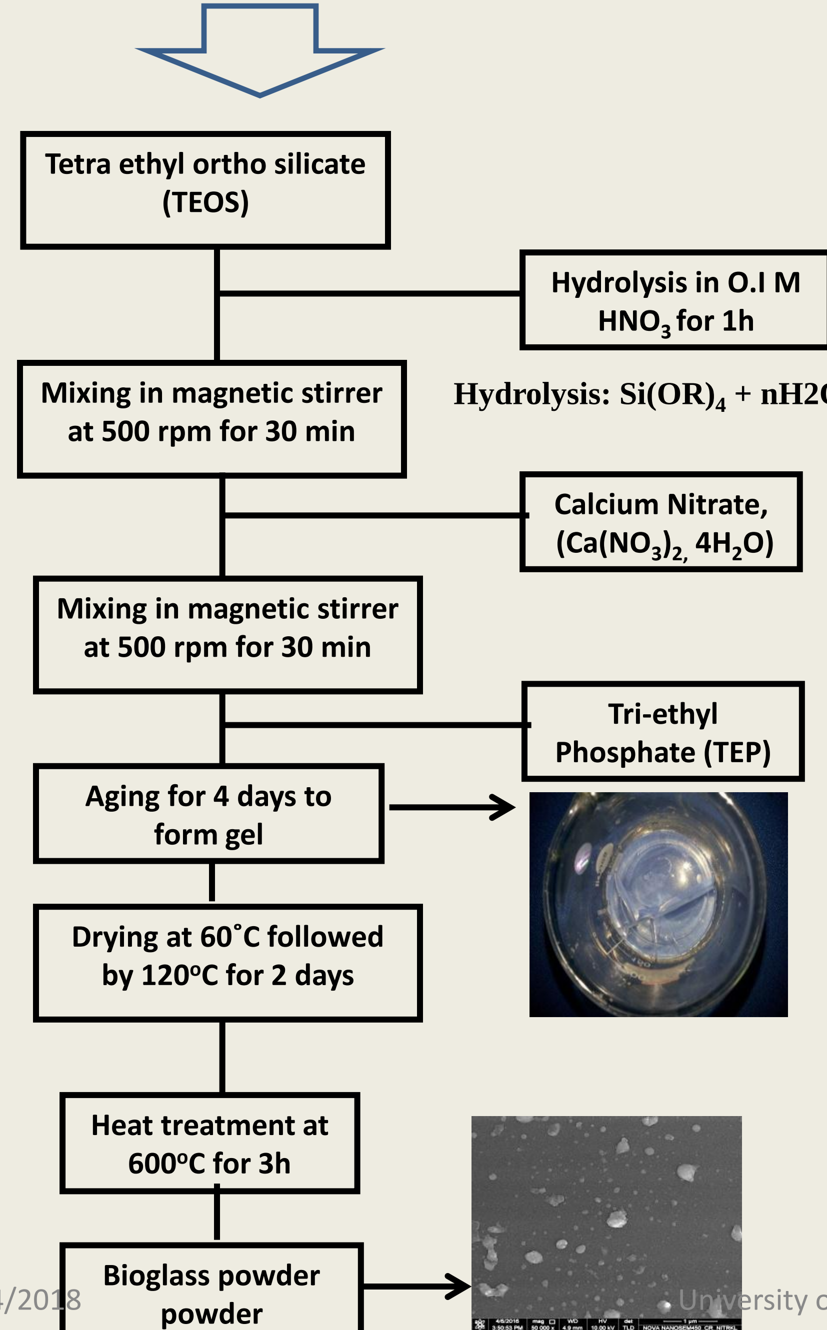
iii. Study on **physico-chemical**, **mechanical** and **in-vitro biological** properties of the prepared composite scaffolds with variation in bioactive ceramic phase content.

iv. Comparative ***in vitro*** and ***in vivo*** analysis on biocompatibility and **osteogenic potentiality** of the developed composite scaffolds prepared using nano-phasic hydroxyapatite, β-Tricalcium phosphate and 58S bioglass as reinforcing particulate in gelatin, chitosan matrix.

Schematic presentation of the fabrication process of Gelatin/Chi-HAp composite scaffold



Flow diagram of synthesized 58Sbioactive glass powder using sol-gel method



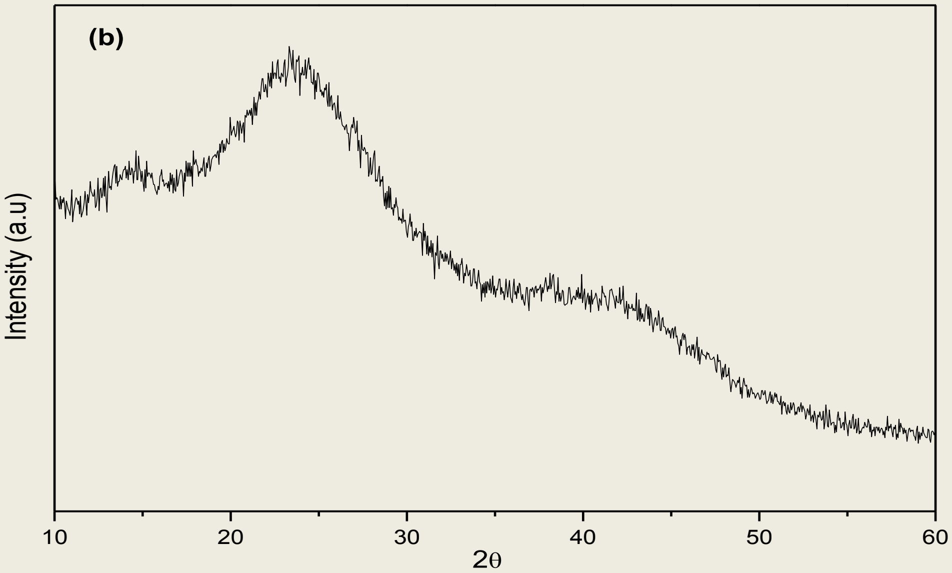
Many groups have reported the controlled nucleation and growth of nHA crystals from organic templates in vitro [18–20]. These studies suggested that the anionic groups on the surface of organic templates are nucleation sites of crystalline. The anionic groups exposed in solution tend to enrich the cations resulting in local supersaturation followed by nucleation of crystalline

Hydrolysis: $Si(OR)_4 + nH2O \rightarrow (OH)nSi(OR)_{4-n} + nROH$

Precursor Material for synthesizing 58s Bioglass

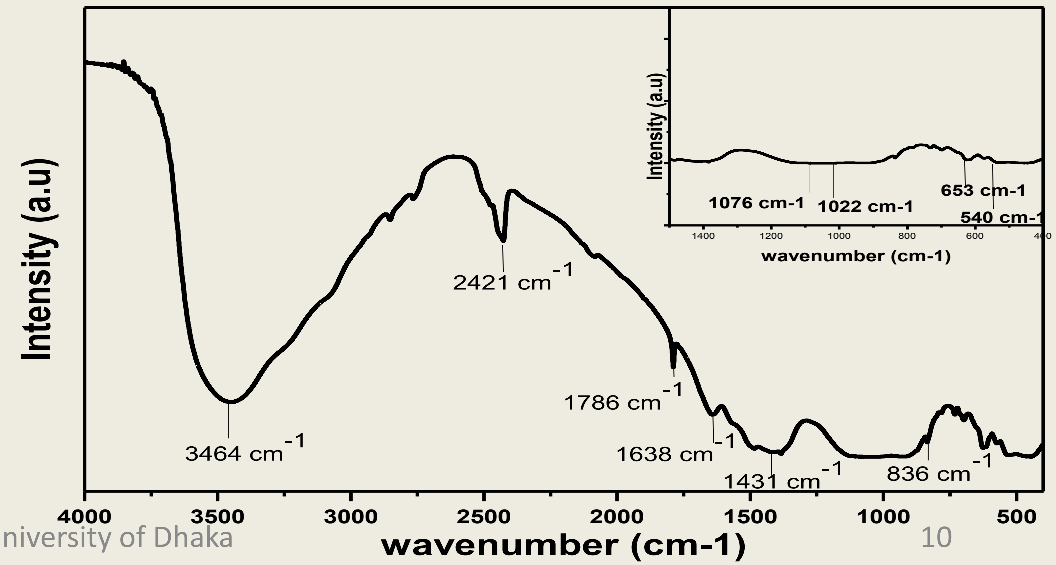
Calcium Nitrate	Tetra Ethyl Ortho silicate (TEOS)	Tri Ethyl Phosphate (TEP)
$Ca(NO_3)_{2,}4H_2O$	$Si(OC_2H_5)_4$	$(C_2H_5)_3PO_4$
Ca(33 mol%)	Si(58 mol%)	P(9 mol%)

XRD pattern of 58SBioglass powder



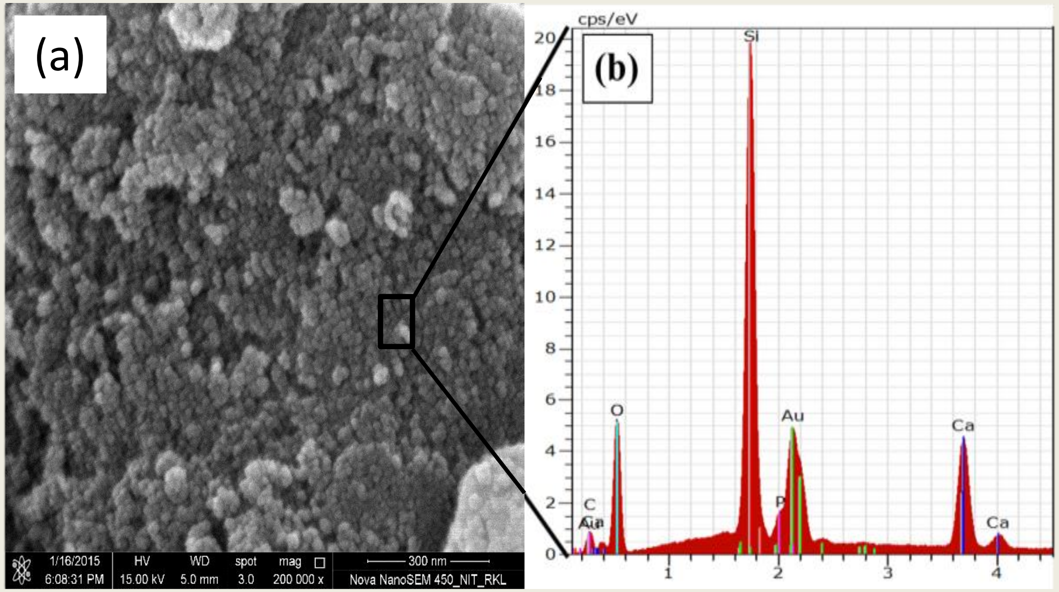
- Phase analysis of the 58s nano-powder was performed using XRD.
- clearly indicate the amorphous nature of the synthesized bioglass powder.

FTIR spectrum of 58SBioglass powder



Wavenumver (cm^{-1})	Assignment
836	Si-O group in Bioglass
1076,540	Si-O-Si stretching and bending
1022,653	PO_4^{-3}
1431	Adsorb water
1638	CO_3^-

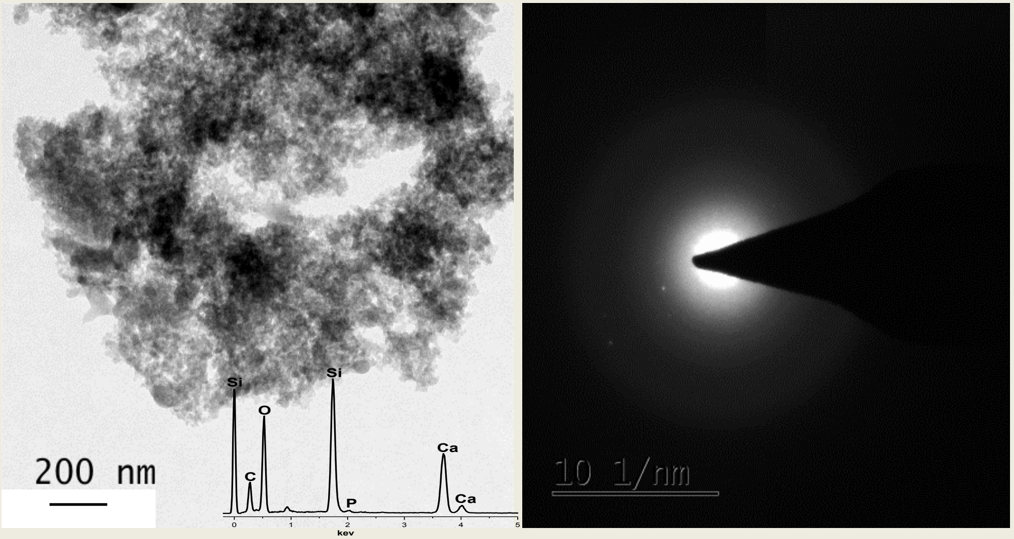
FESSEM micrograph of synthesized bioglass nanopowders



- Particle size of 50 nm was obtained from the FESEM mesurement.
- EDX analysis confirmed the presence of Si , Ca and P in synthesize 58s nanopowder.
- EDS analysis suggests that the synthesized powder with a composition of Si:Ca:P=36:21:7 closely resembled the theoretical composition.

TEM analysis of synthesized bioglass nanopowders

- particle size of 80 nm was obtained from the TEM analysis.
- EDX analysis confirmed the presence of Si , Ca and P in synthesize 58s nanopowder.
- SADE pattern confirmed the amorphous nature of synthesized 58s glass nanopowders.



Flow diagram of fabrication of GCB scaffolds using Freeze Drying Technique

58s bioglass nanoparticles

Chitosan solution (2 wt%)

Mixing in magnetic stirrer for 1h

Gelatin solution (4 wt%),

Mixing in magnetic stirrer at 4°C for 30 min

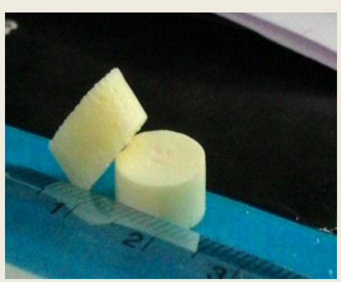
Glutaraldehyde solution (0.25%)

Moulding and Freezing at -20°C

Freeze Drying at -50˚C for 48h

Washing with $NaBH_4$, NaOH, PBS

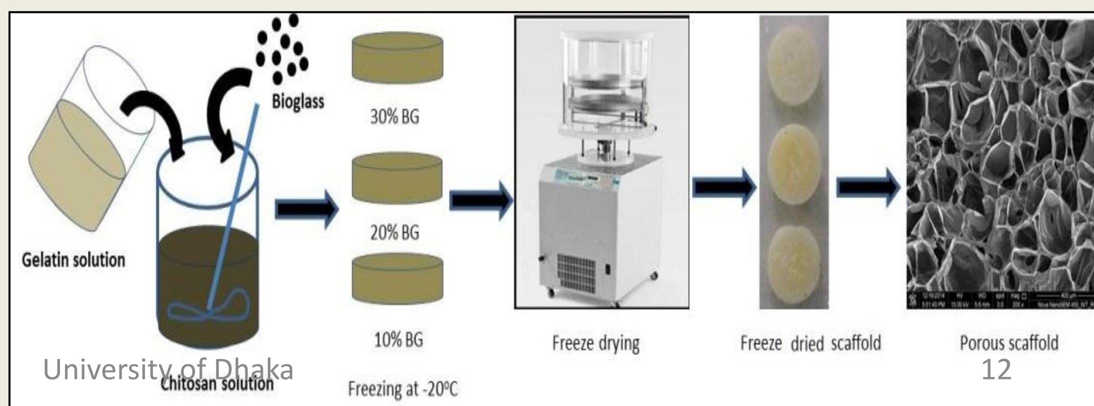
Freeze dried GCB scaffold



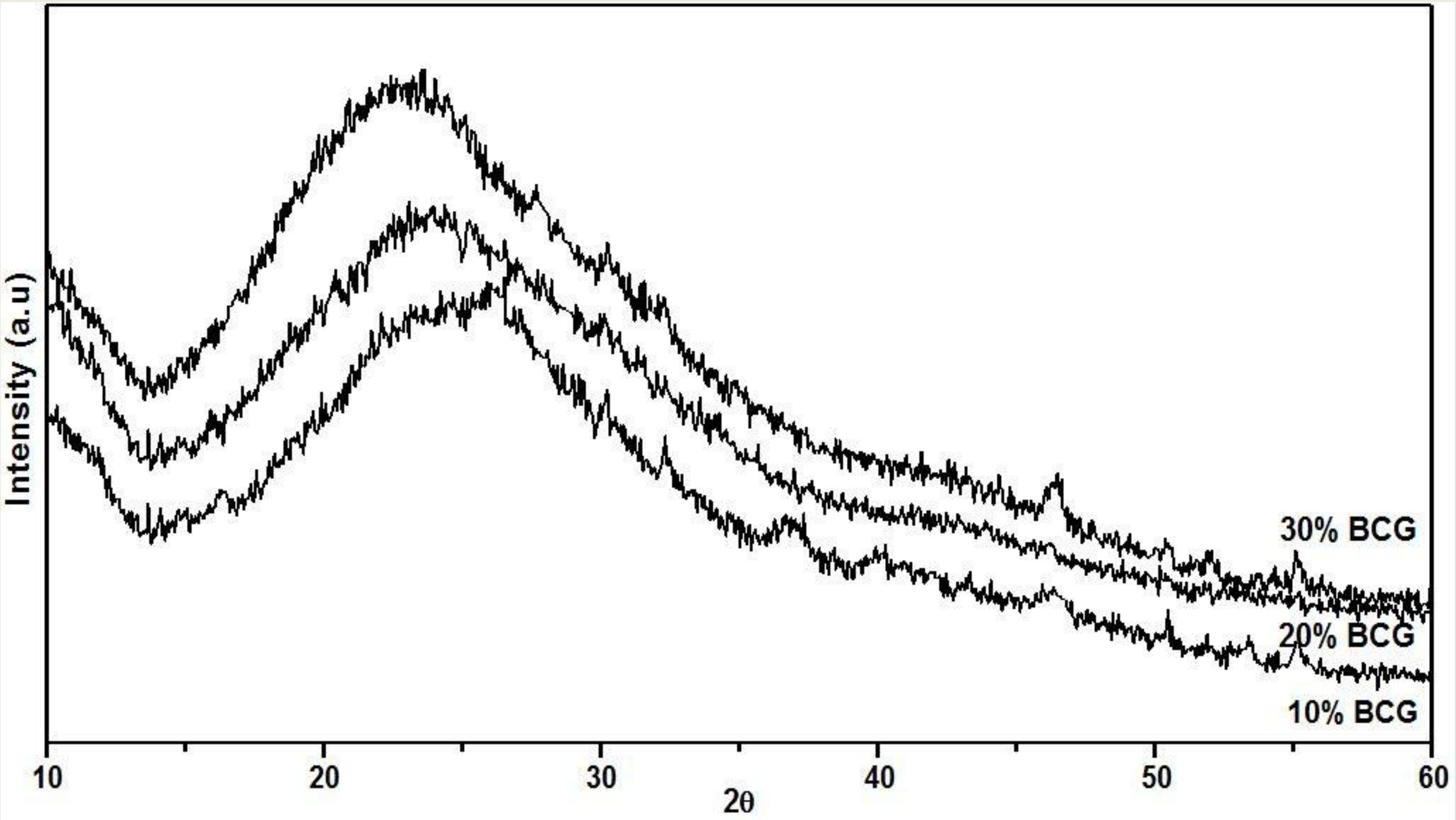
Material composition of GCB scaffolds

Specimen name	Gel concentration (w/w)%	Chitosan Concentration (w/w %)	CS-Gel/BG Ratio (w/w)%
GCB-0	30	70	100/0
GCB-10	30	60	90/10
GCB-20	30	50	80/20
GCB-30	30	40	70/30

Schematic of Fabrication of the GCB scaffolds

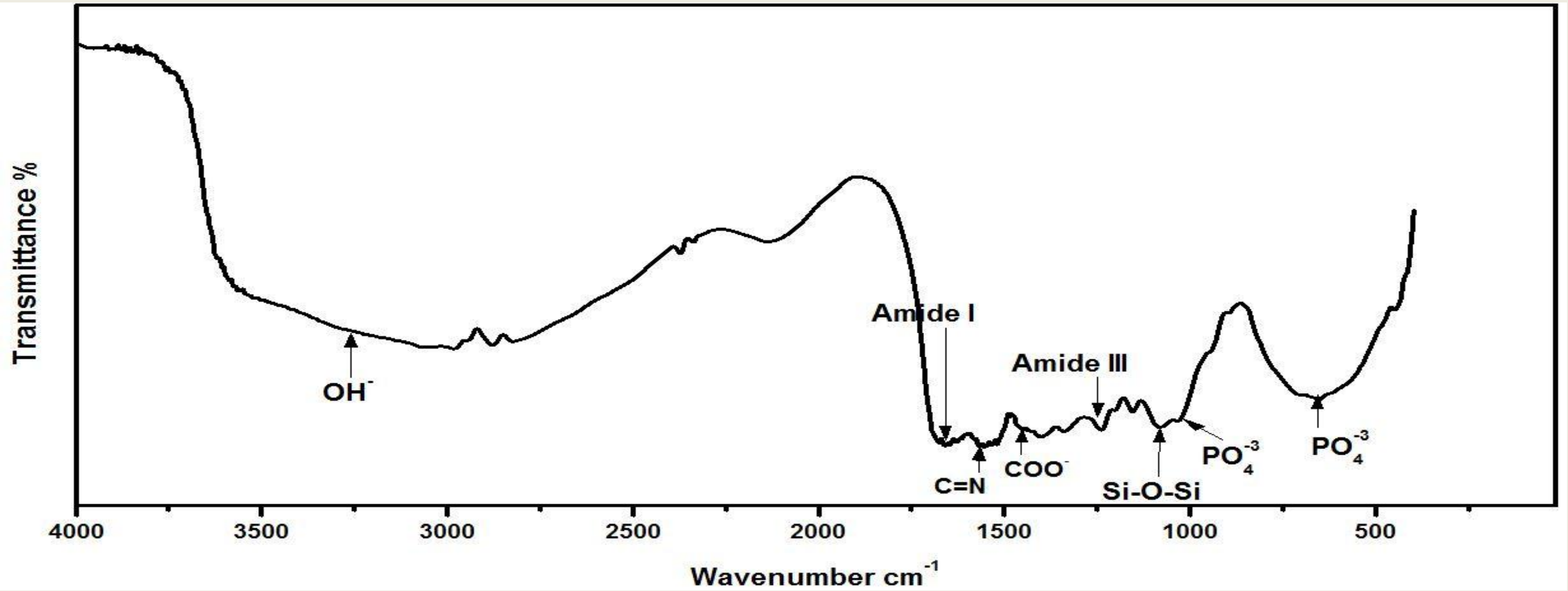


XRD analysis of GCB composite scaffold



- The characteristic diffraction peaks for both chitosan and gelatin were suppressed by the huge amorphous peak of bioglass observed in the range between 2θ equals to 20^{o}-40^{o}.
- The broad amorphous peaks of bioglass confirmed that the synthesized scaffolds were predominantly amorphous.

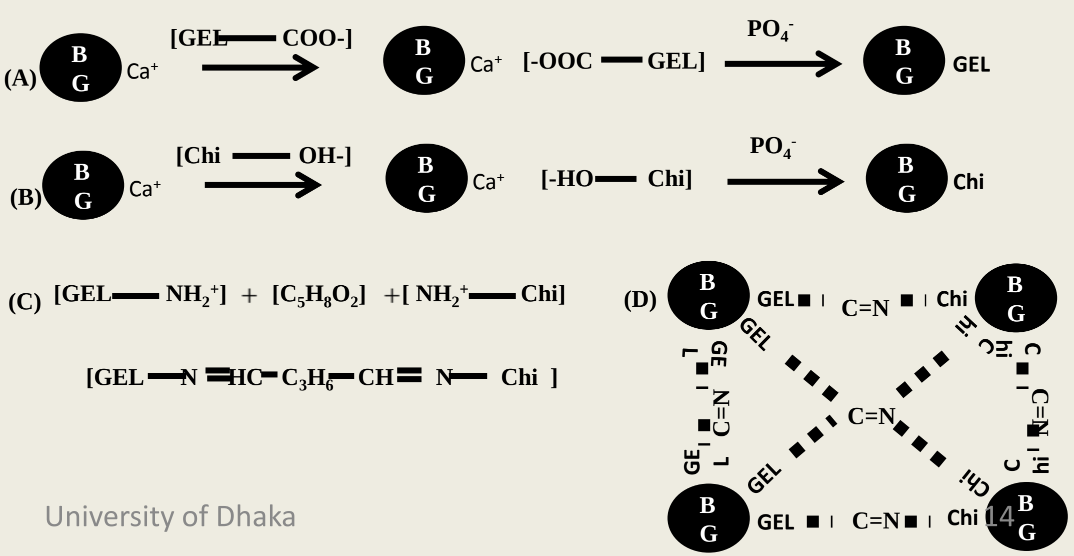
FTIR study of GCB composite scaffold



FTIR Peak Assignment

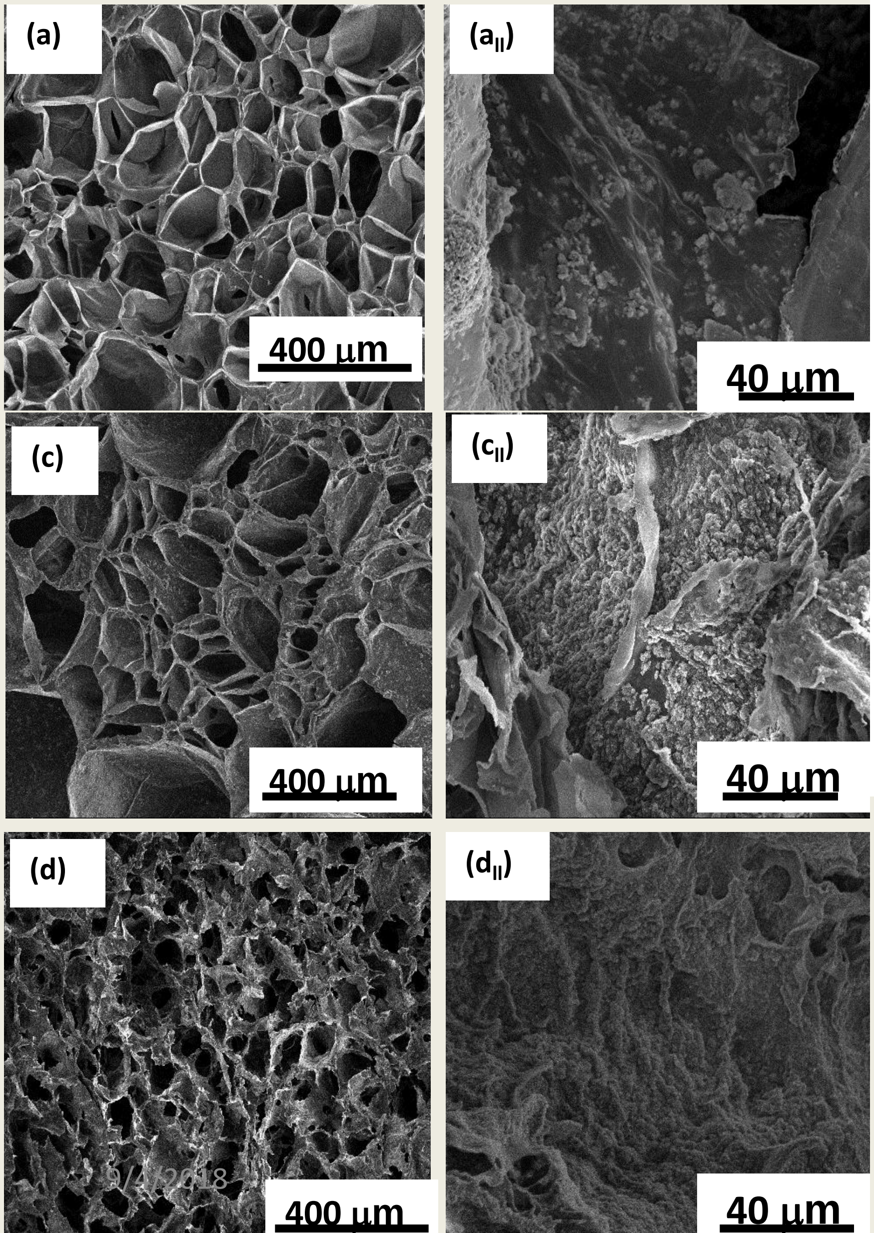
Wavenumver (cm^{-1})	Assignment
1440	Ca+------COO^-
1076,540	Si-O-Si stretching and bending
1022,653	PO_4^{-3}
1664-1640	Amide I
3266	-OH

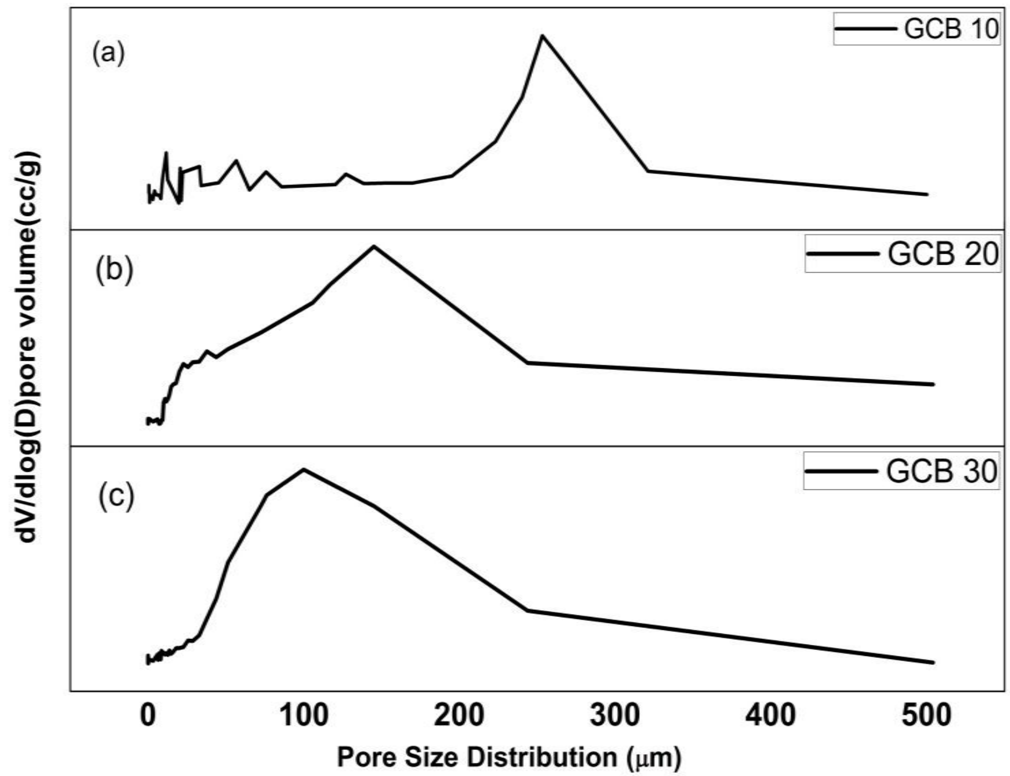
Bonding Mechanism



FESEM microstructure of fabricated GCB scaffold

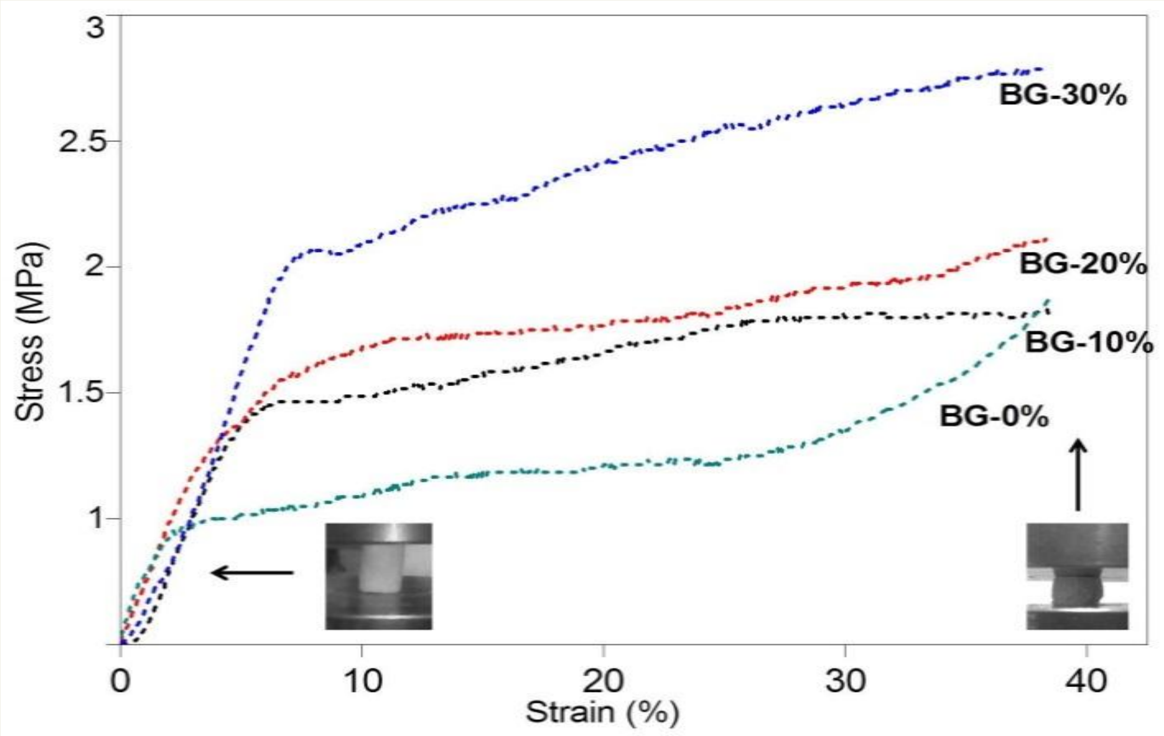
Mercury porosimetry plot of fabricated GCB scaffold





Sample name	Average Pore Diameter(mm)	Porosity
BCG 10	250 ± 26.3	82.4 ± 5.0
BCG 20	160±17.8	80.8 ± 3.3
BCG 30	100 ±25.9	81.3 ± 6.1

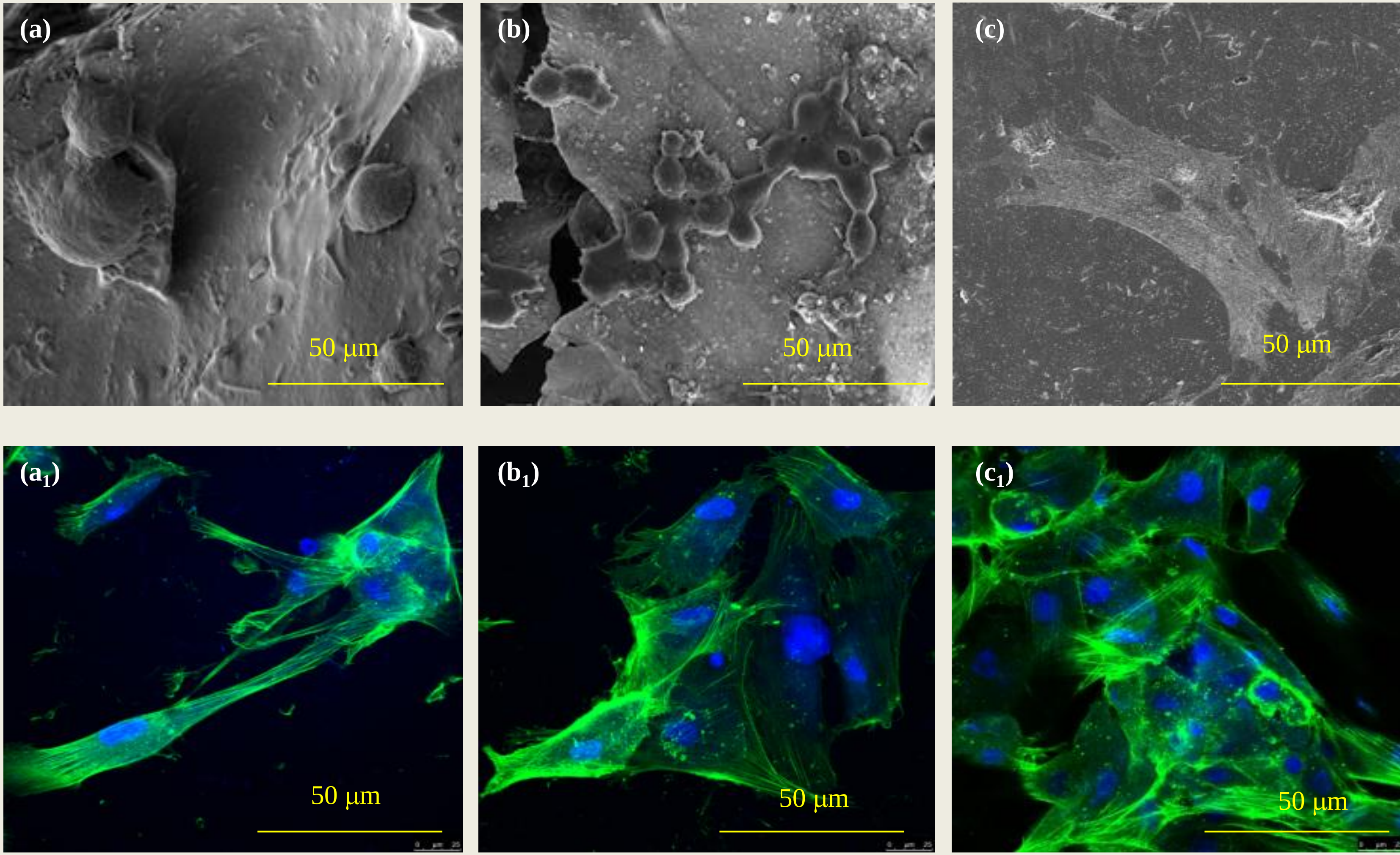
Mechanical properties of GCB scaffolds



Bioglass scaffolds specimens	Porosity (%)	Mechanical Properties
		Compressive Strength* (MPa)
BG 0%	89.3 ± 7.8	0.8 ± 0.16
BG 10%	82.4 ± 5.0	1.2 ± 0.01
BG 20%	80.8 ± 3.3	1.6 ± 0.01
BG 30%	81.3 ± 6.1	2.2 ± 0.02

*P<0.05, by student's t-test, n=5, all values in each mechanical property category were found to be significantly different from each other.

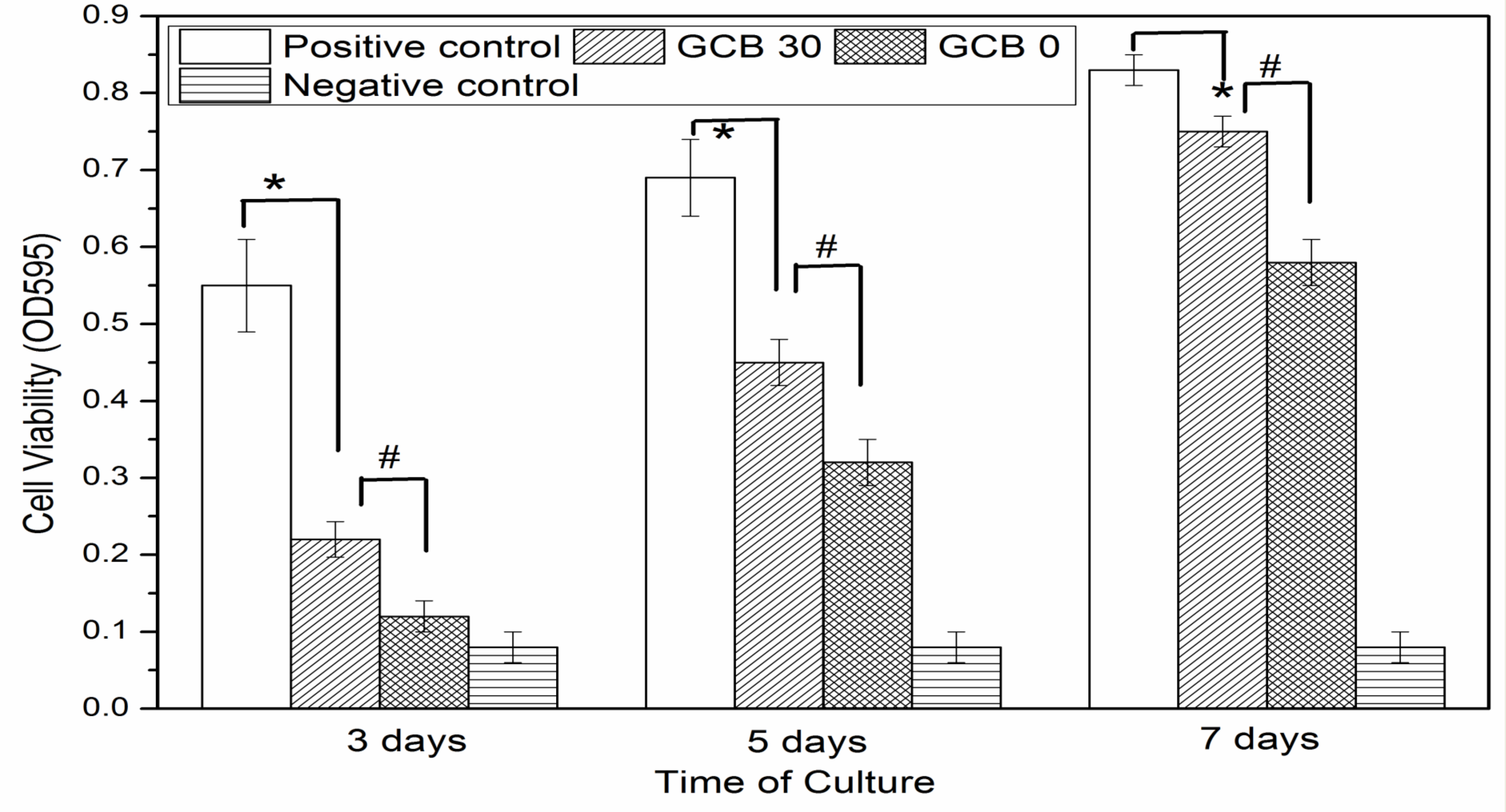
Cell attachment study on scaffolds



GCB30, 1d **GCB30,3d** **GCB30,14d**

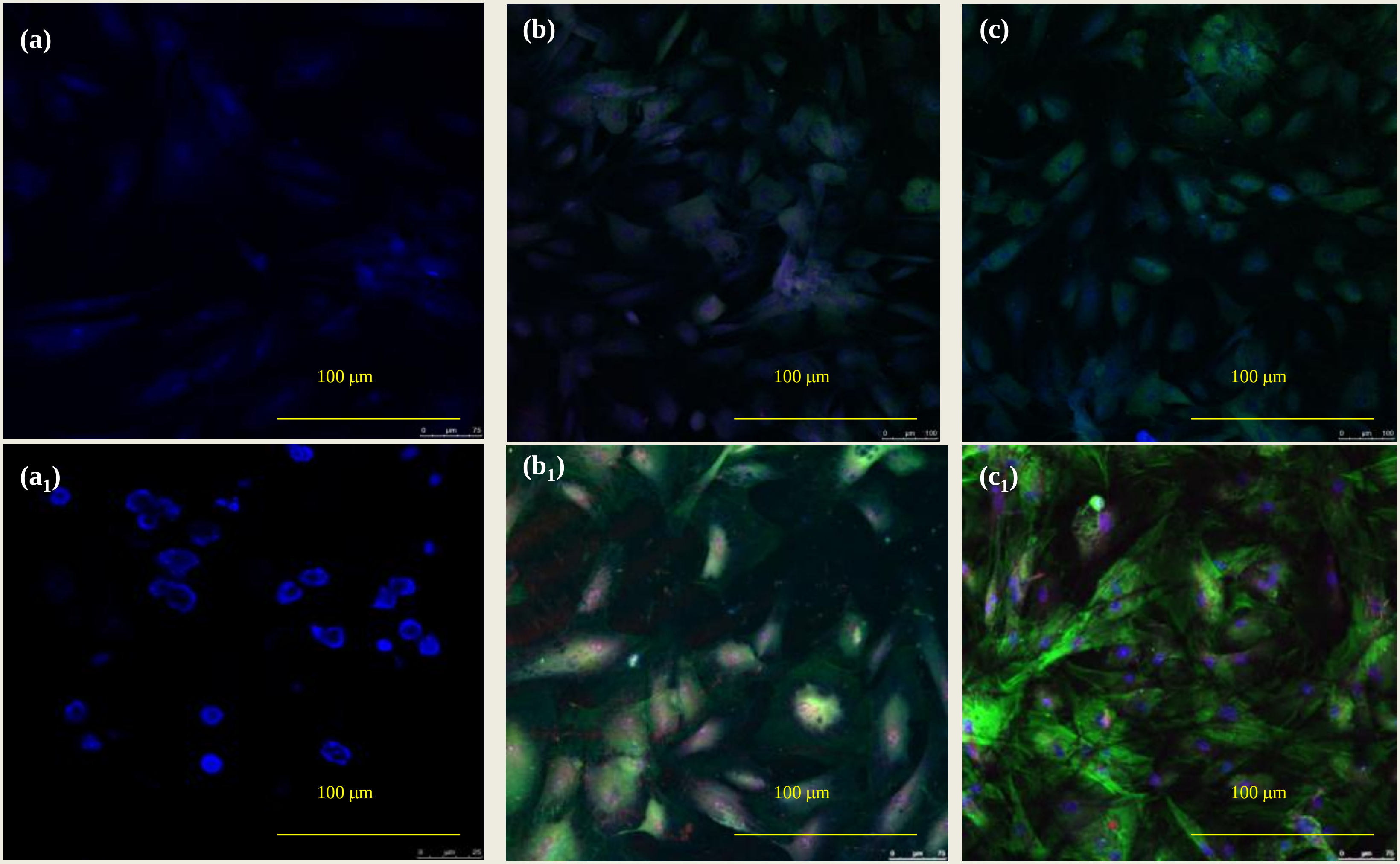
- Extensive networks of polymerized *β*-tubulin and F-actin filaments as well as multiple cell–cell contacts indicates a higher degree of active cell spreading, movement, and signalling events with progress in cell culture.
- All the results revealed higher proliferation of MSCs on GCB 30 scaffolds after 14 days of cell culture.

Viability of pre-osteoblasts on GCB30 composite scaffolds measured by MTT assay



For all incubation periods, GCB 30 presented significantly higher ($p = 0.026$) cell viability than that on GCB 0 scaffold which suggests that addition of 58S bioglass nanoparticles in the scaffold promoted better cell adhesion and proliferation.

Confocal study of RUNX2 and Osteocalcin expression in GCB scaffold



GCB30,1d **GCB30,7d** **GCB30,14d**

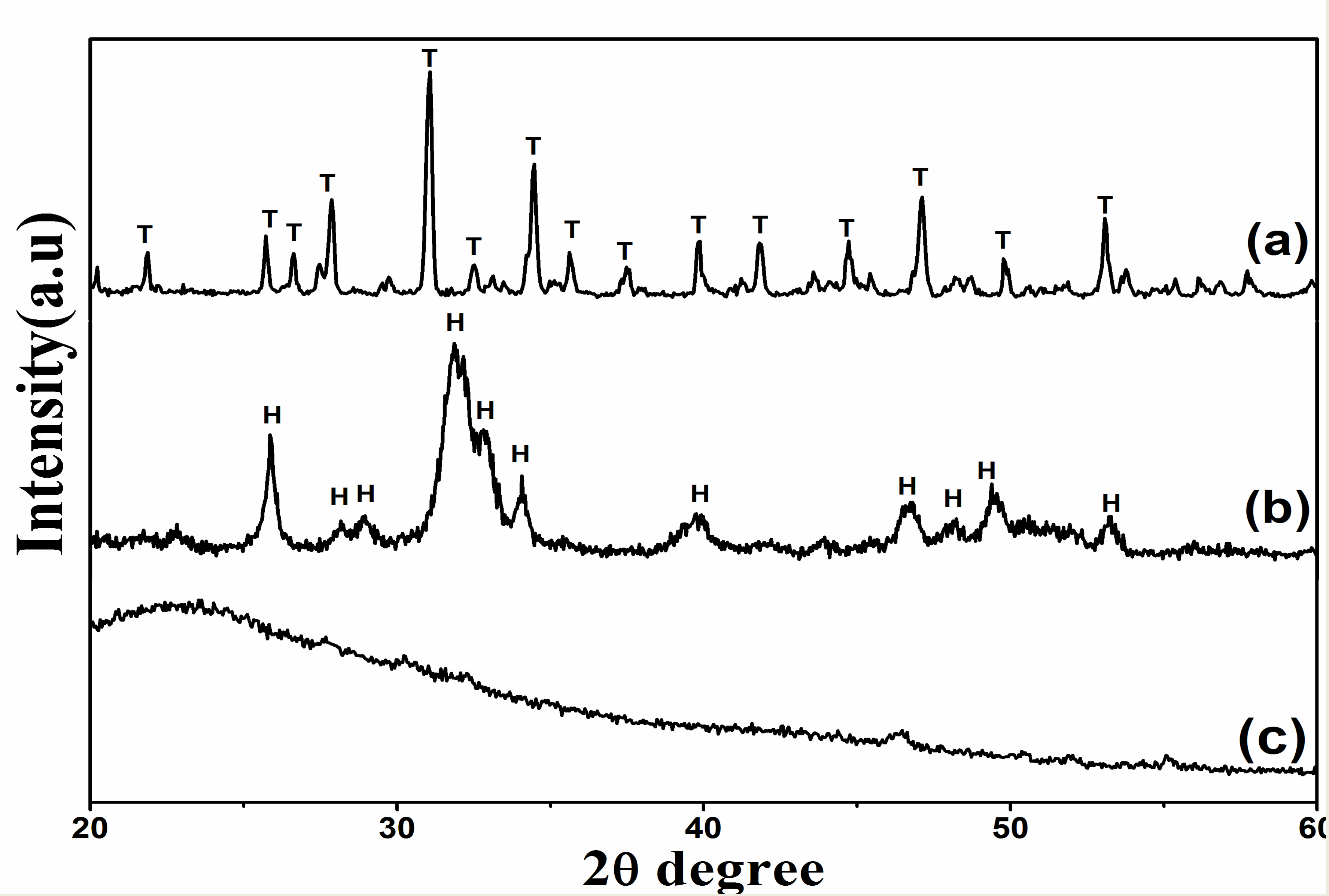
- an increase in the specific activity of RUNX2 with progress in cell culture time indicates a corresponding shift to a more differentiated state .
- Greater osteocalcin (Green) deposits were seen in scaffolds of 14 days cell culture indicating a higher amount of new bone formation with progress in cell culture time.

In summary

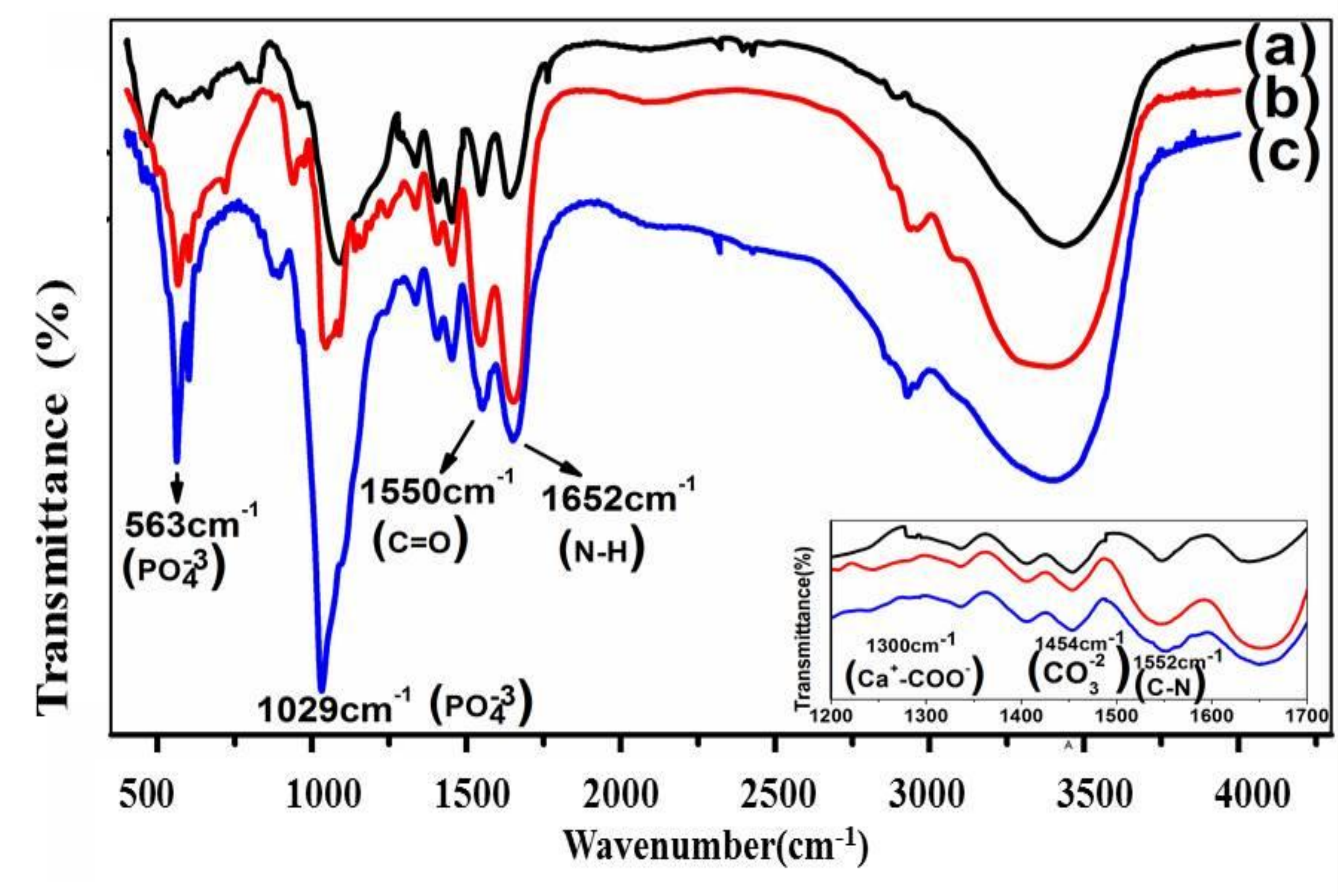
- we have successfully fabricated GCB nanocomposite scaffolds using Freeze drying method.
- The scaffolds were highly porous with total porosity of about 80% and average pore size in the scaffold fell to nearly 100 μm from 250 μm with increase in bioglass content from 10 wt% to 30 wt% in the gelatin –chitosan matrix.
- The bioglass particles (BG) were well distributed in gelatin-chitosan matrix, significantly improving the compressive strength. Thus, GCB 30 scaffold showed a compressive strength value comparative to that of natural cancellous bone.
- It was found that the swelling behaviour of the scaffolds was reduced on the increase in 58S-BG nanopowder content in the scaffold. Biodegradation test in PBS showed that the increase in 58S-BG content resisted the biodegradability of the scaffold.
- Preliminary results on cell culture using MSCs suggested that cells could adhere, spread, proliferate and differentiate very well onto GCB 30 scaffolds.
- MSCs were also found to transform into the new bone within 14 days of cell culture on the GCB 30 scaffold making them promising artificial bone grafts.

Comparative study between GCH, GCB & GCT composite scaffold

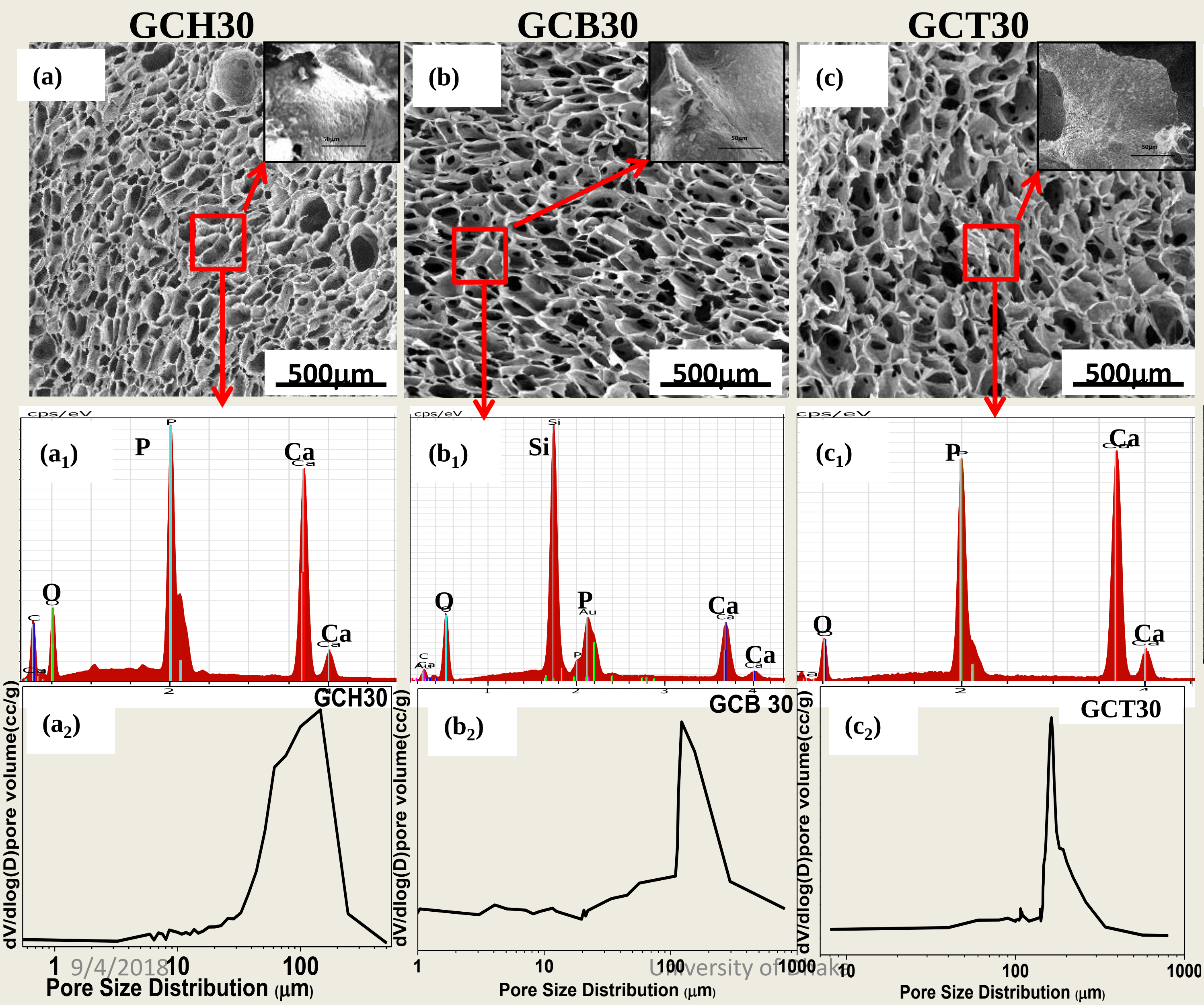
XRD pattern of composite scaffold (a) GCT30, (b) GCH30, (c) GCB30

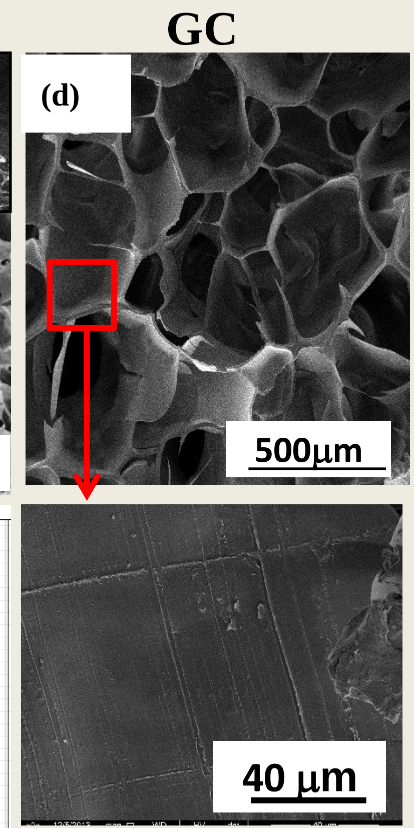


FTIR-ATR analysis of (a) GCH30 scaffold (b) GCT30 scaffold, (c) GCB30 scaffold



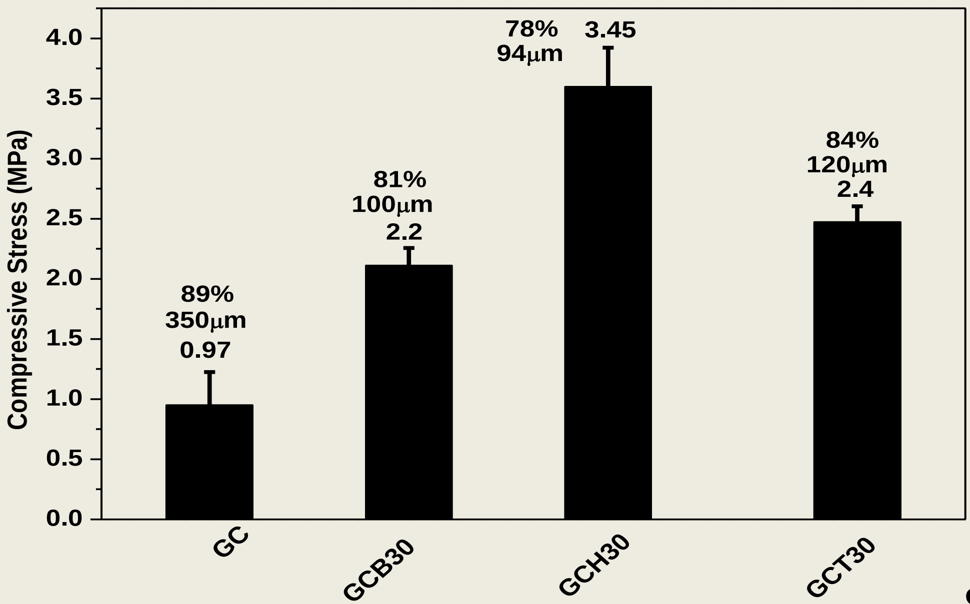
FESEM micrograph of pore distribution in the cross-section of the composite scaffolds



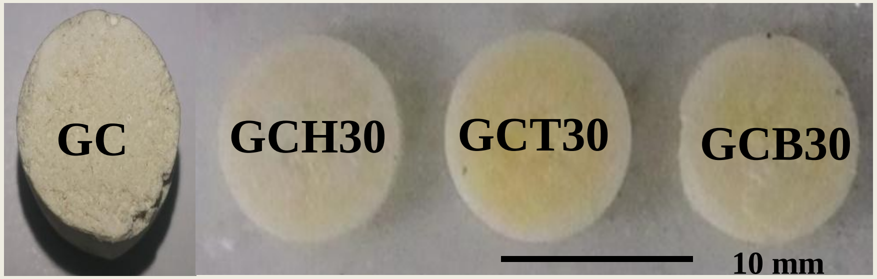


Sample name	Average Pore Diameter(µm)
GC	350
GCH 30	94
BCG 30	100
GCT 30	120

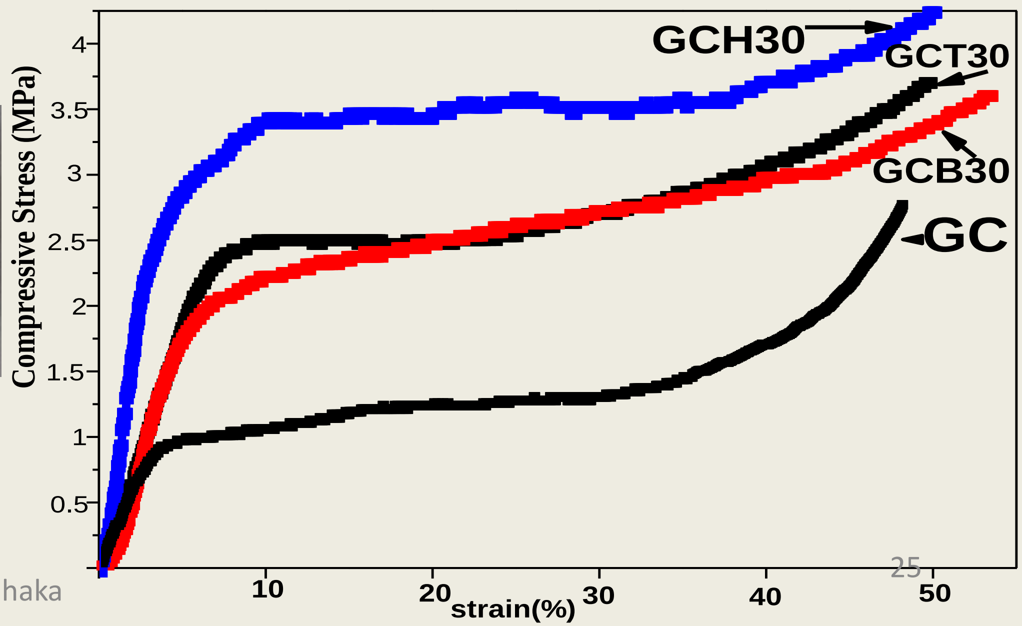
Mechanical Property of composite scaffold

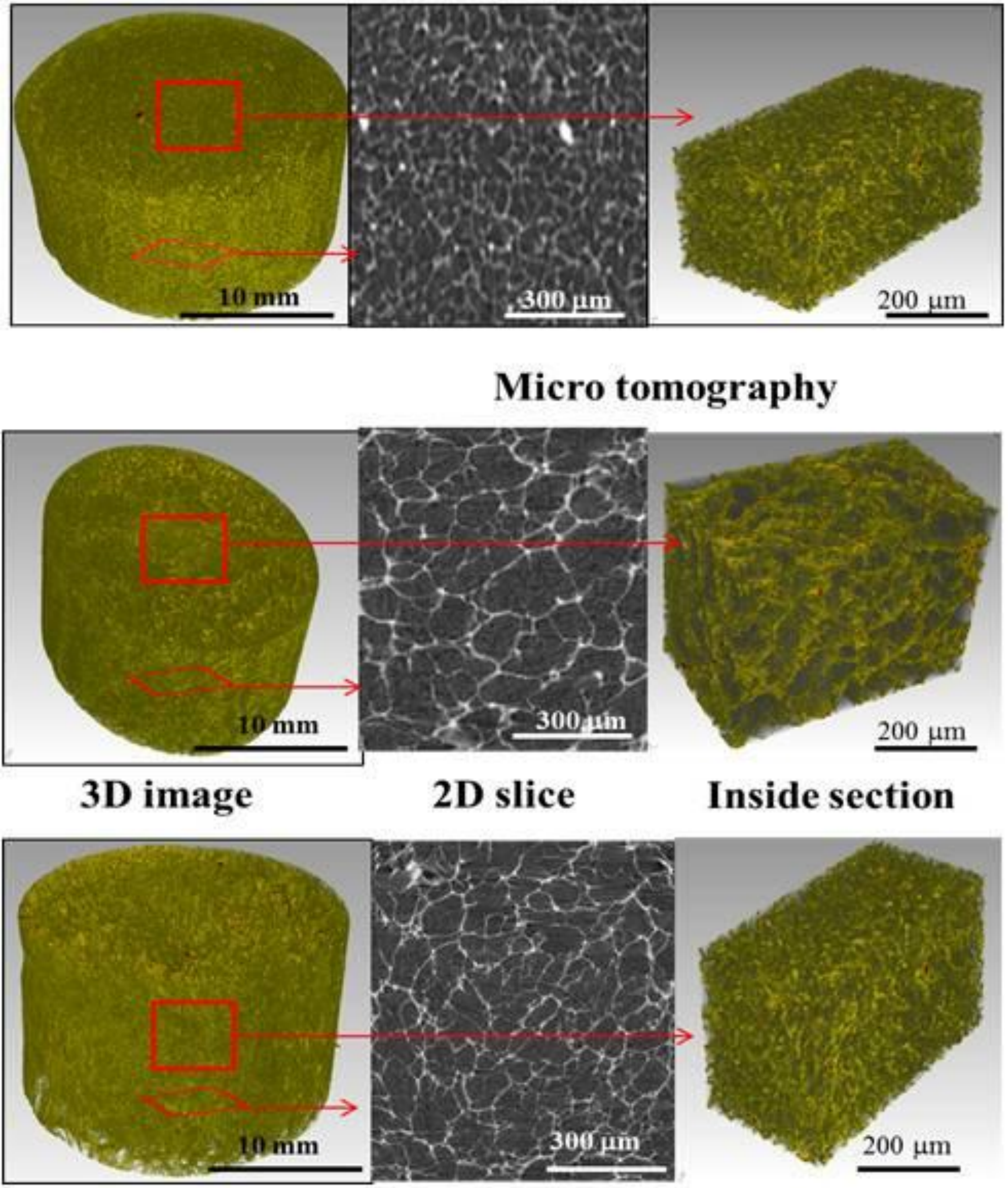


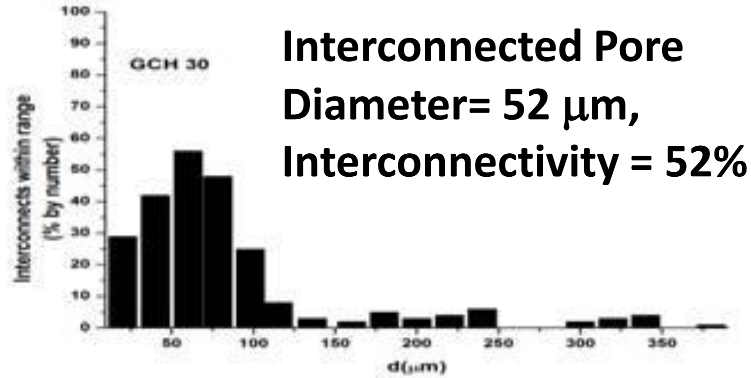
The compressive strengths of the three types of scaffolds were in the range of 1–3.6 MPa .
It is clear that the all composite scaffolds (~3.5 MPa) have a significantly higher compressive strength than pure gelatin-chitosan scaffolds (~0.97 MPa, data not shown)



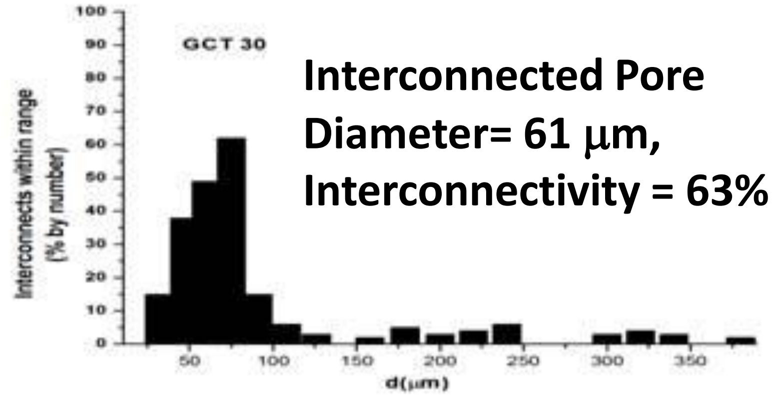
Stress-strain plot of composite scaffold



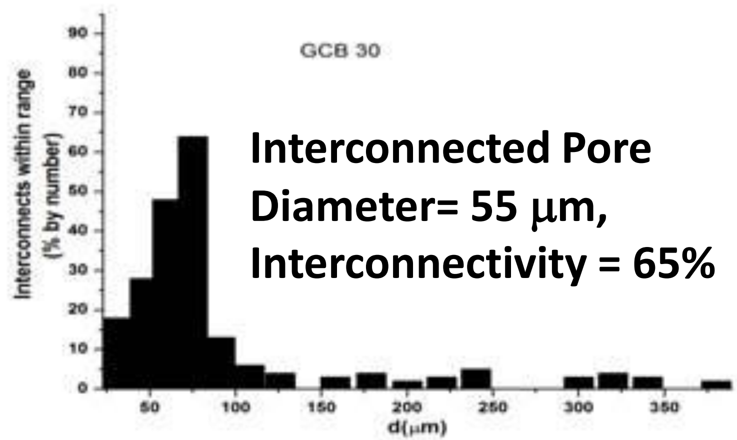




Interconnected Pore Diameter= 52 µm, Interconnectivity = 52%

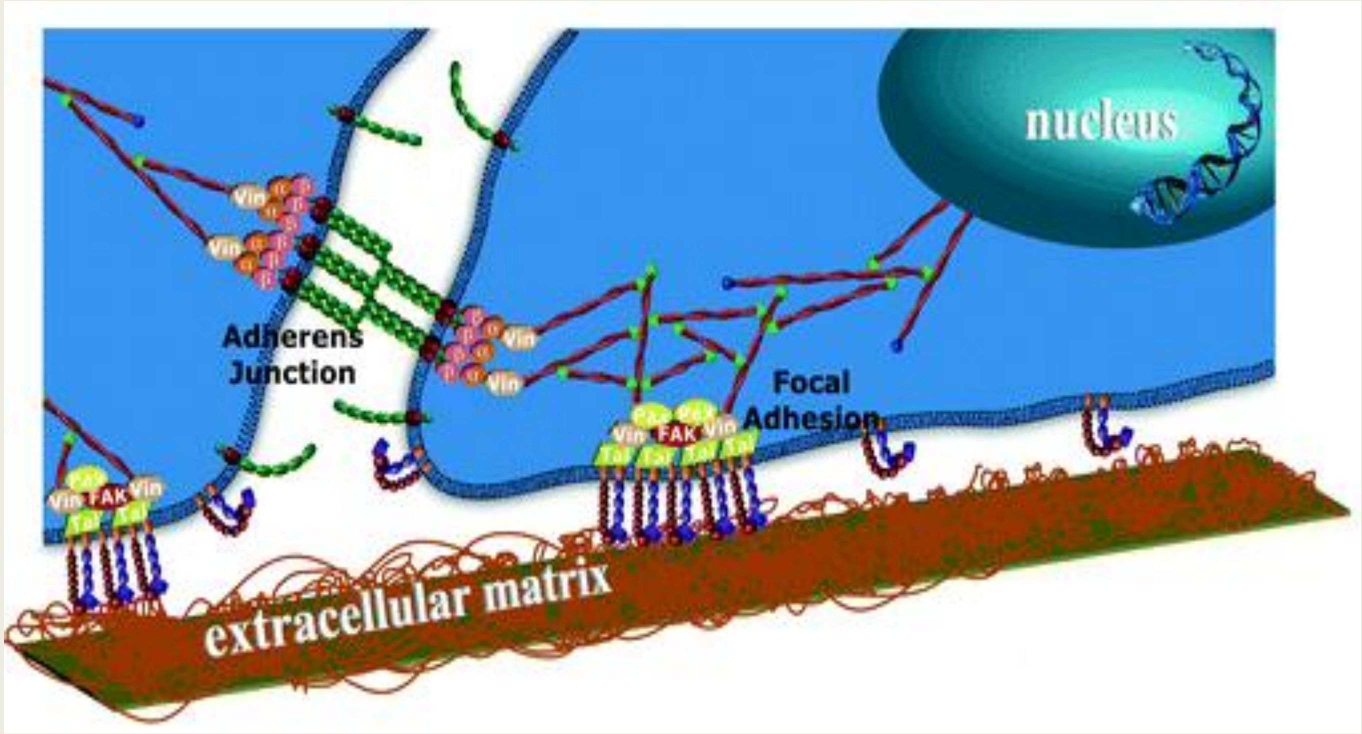


Interconnected Pore Diameter= 61 µm, Interconnectivity = 63%



Interconnected Pore Diameter= 55 µm, Interconnectivity = 65%

Osteoblast Adhesion

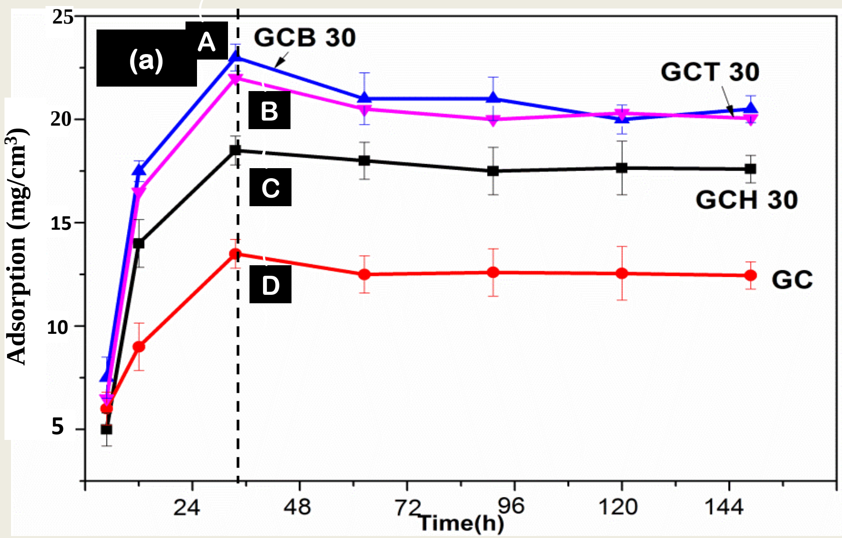


www.bioscience.org/2009/v14/af/3293/figures.htm

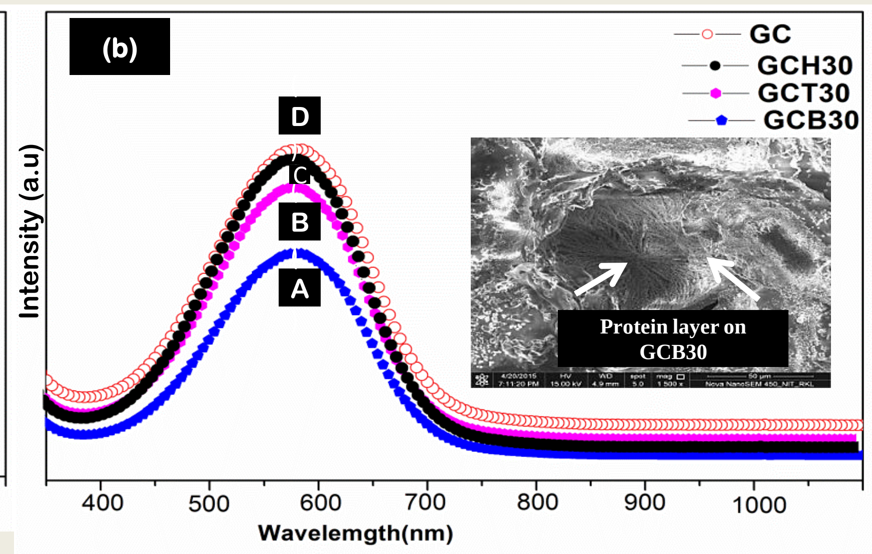
- Integrin receptors bind ECM proteins *via* their extracellular domains, while their cytoplasmic domains are associated with a supramolecular plaque containing talin (Tal), vinculin (Vin), paxillin (Pax), focal adhesion kinase (FAK), *etc*.

BSA Protein adsorption Study

Protein adsorption by Scaffold

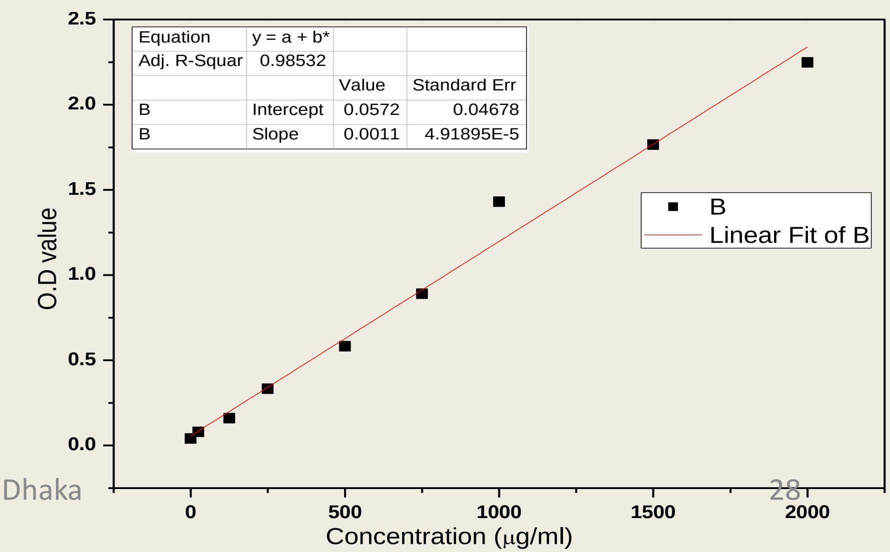


UV-visible of BSA adsorbed by composite scaffold

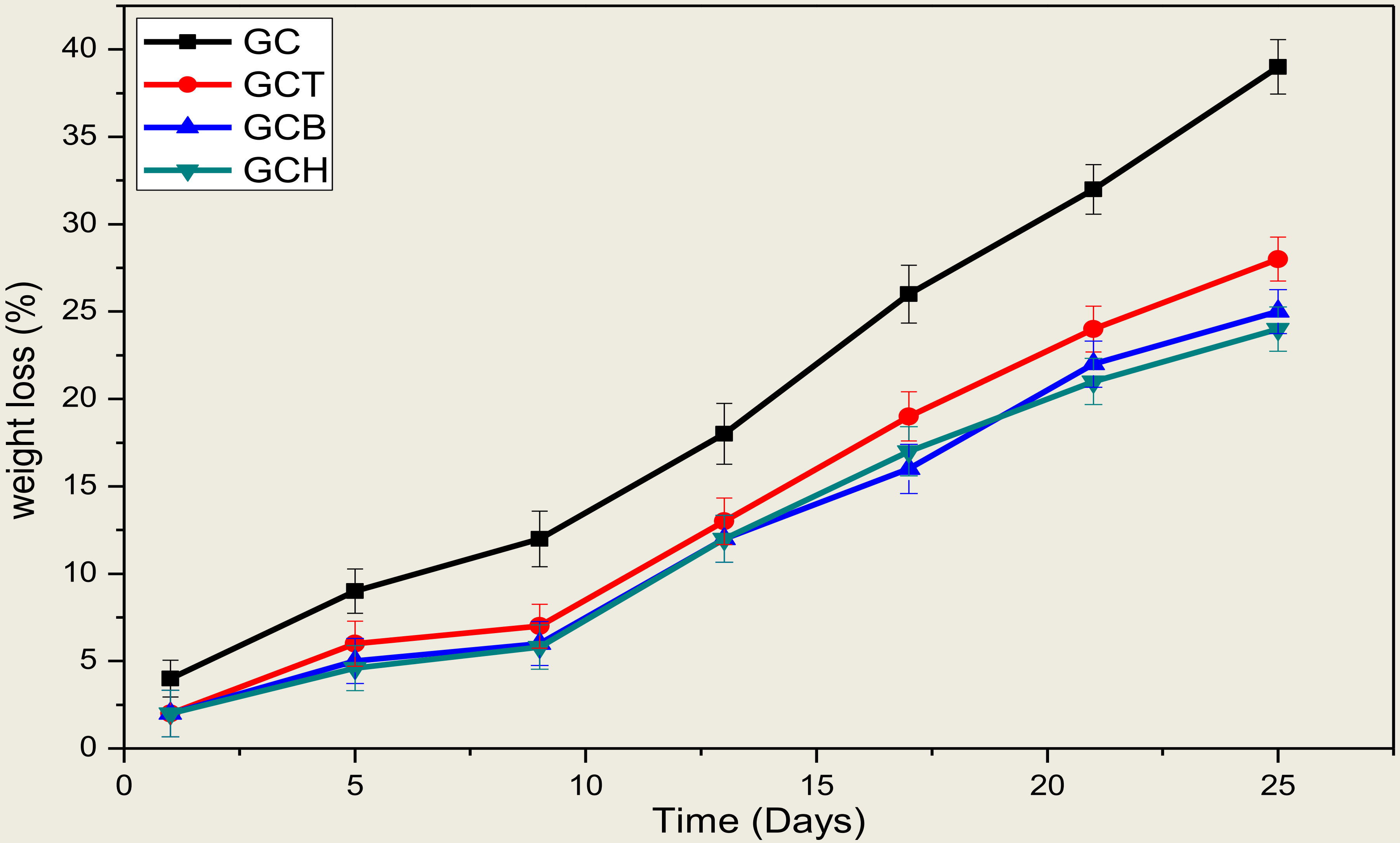


Amount of BSA uptakes by composite scaffolds are calculated as 45 mg/g, 42 mg/g ,38 mg/gm and 27 mg/g for GCB 30, GCT 30, GCH 30 and GC, respectively

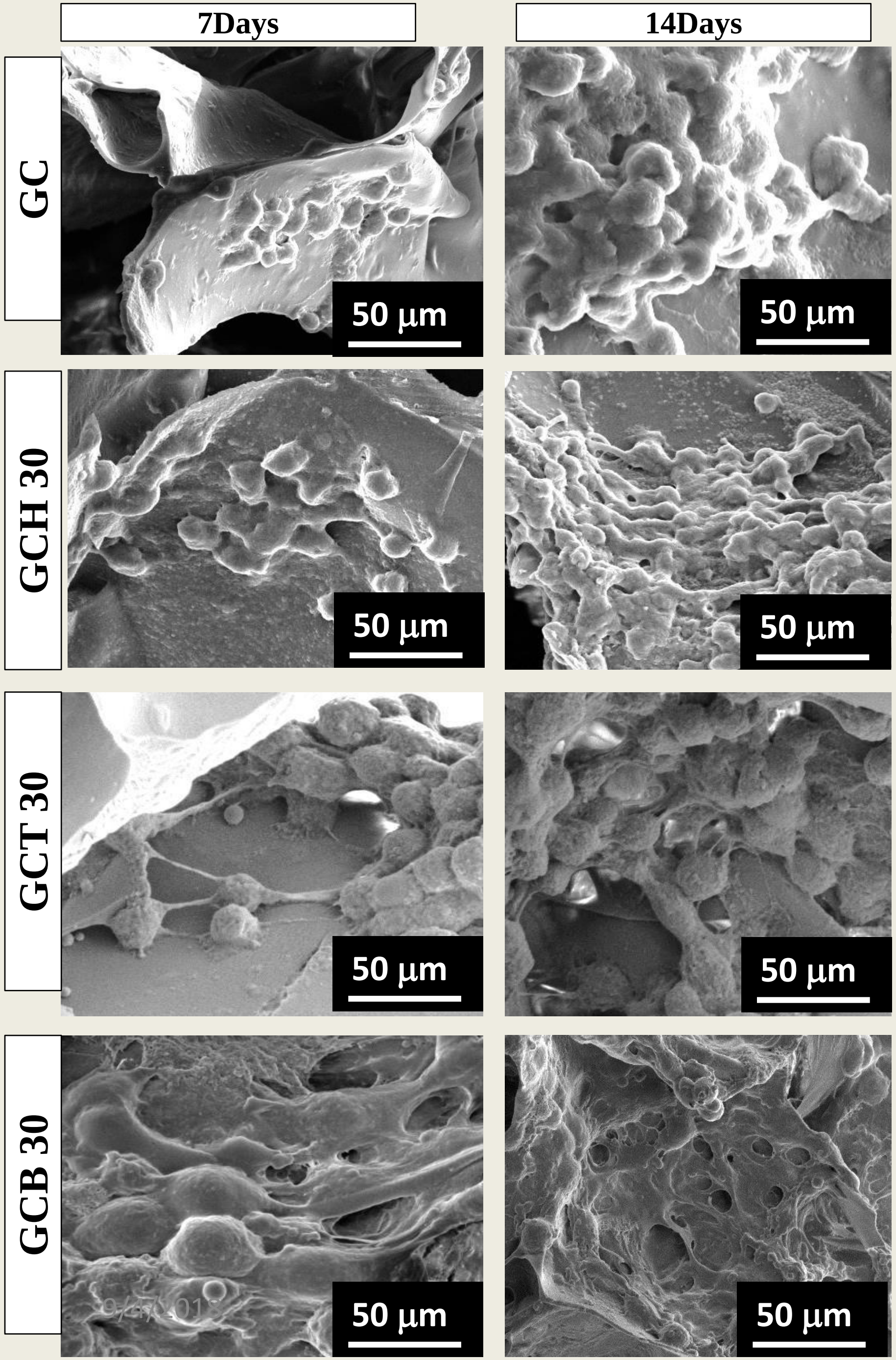
BSA protein standard curve



In vitro Biodegradation study of composite scaffold



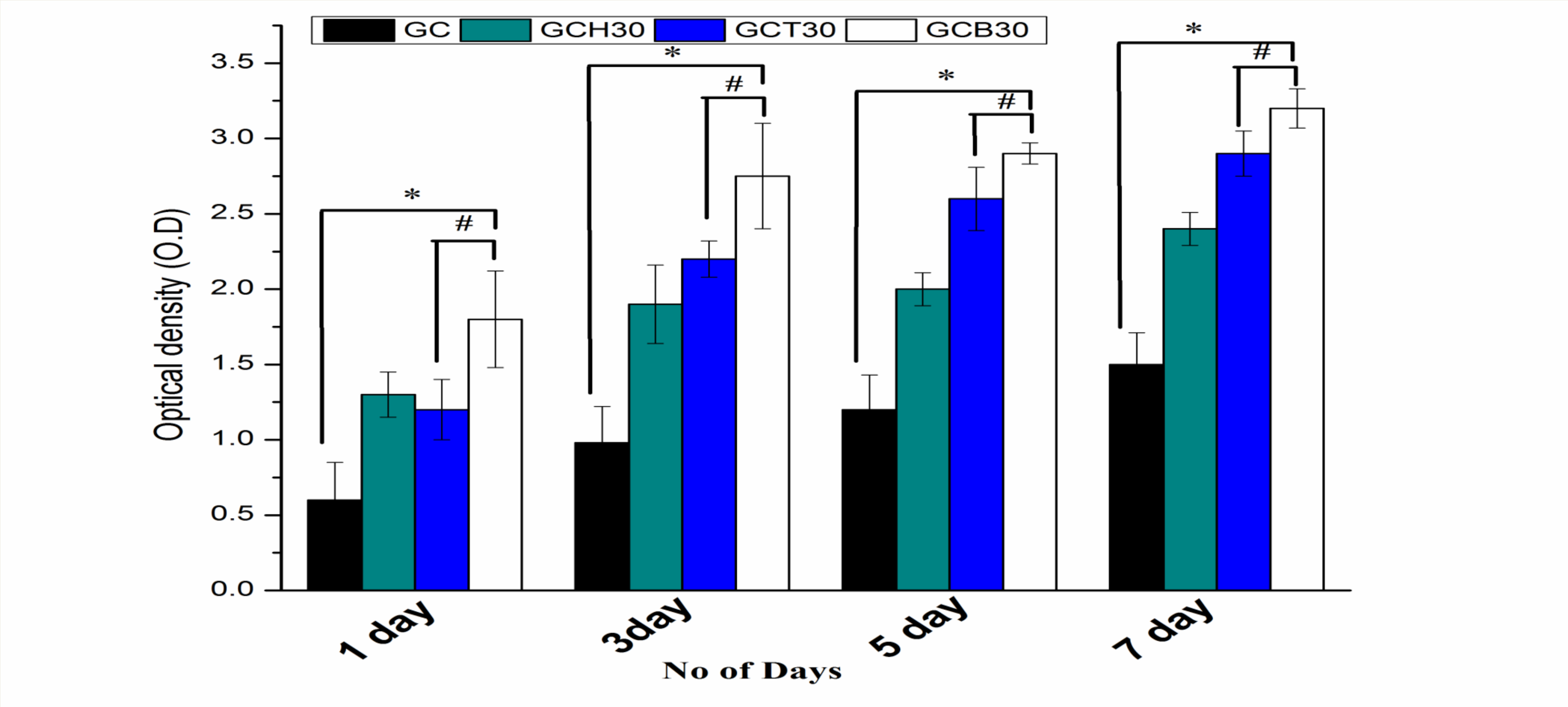
- Inclusion of Bioactive Ceramic particle decrease the degradation % in a significant manner.
- 26 wt% degradation was recorded for all composite scaffold after immersion in PBS solution up to 25 days.



Field Emission Scanning electron microscope (FESEM) images of human mesenchymal stem cells (hMSCs) grown on GC, GCH30, GCT30 and GCB30 scaffolds .

- The MSCs cultivated onto GCB30 showed higher number of lamellipodia and filopodia extensions to adhere to the scaffold surface after 7 days of culture.
- Compare to other scaffold, cells cultured in GCB30 scaffold stretched fully and cover the surface with lamallipodia extension , whereas in other scaffold cells were cling together

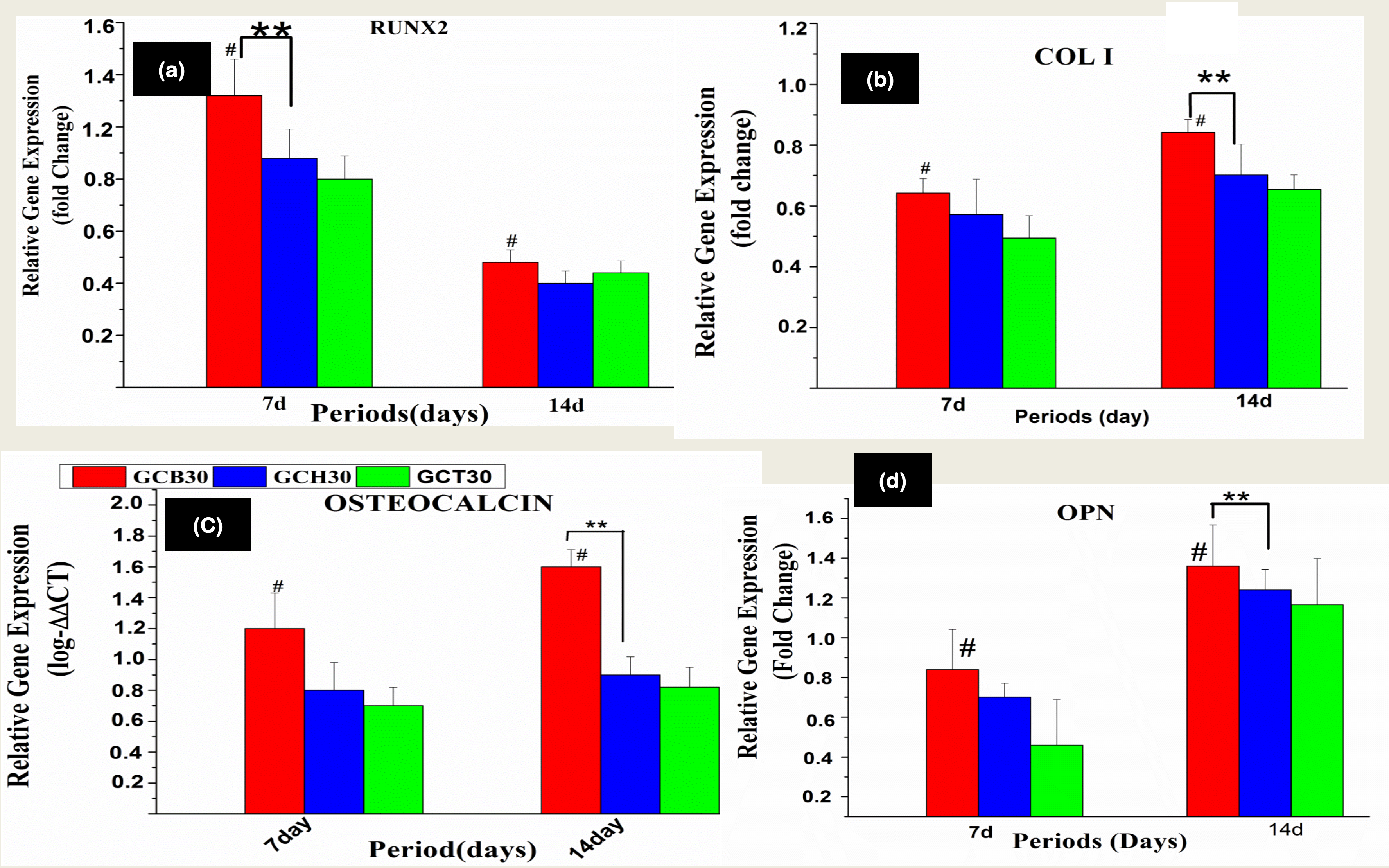
MTT assay of GCT composite scaffold

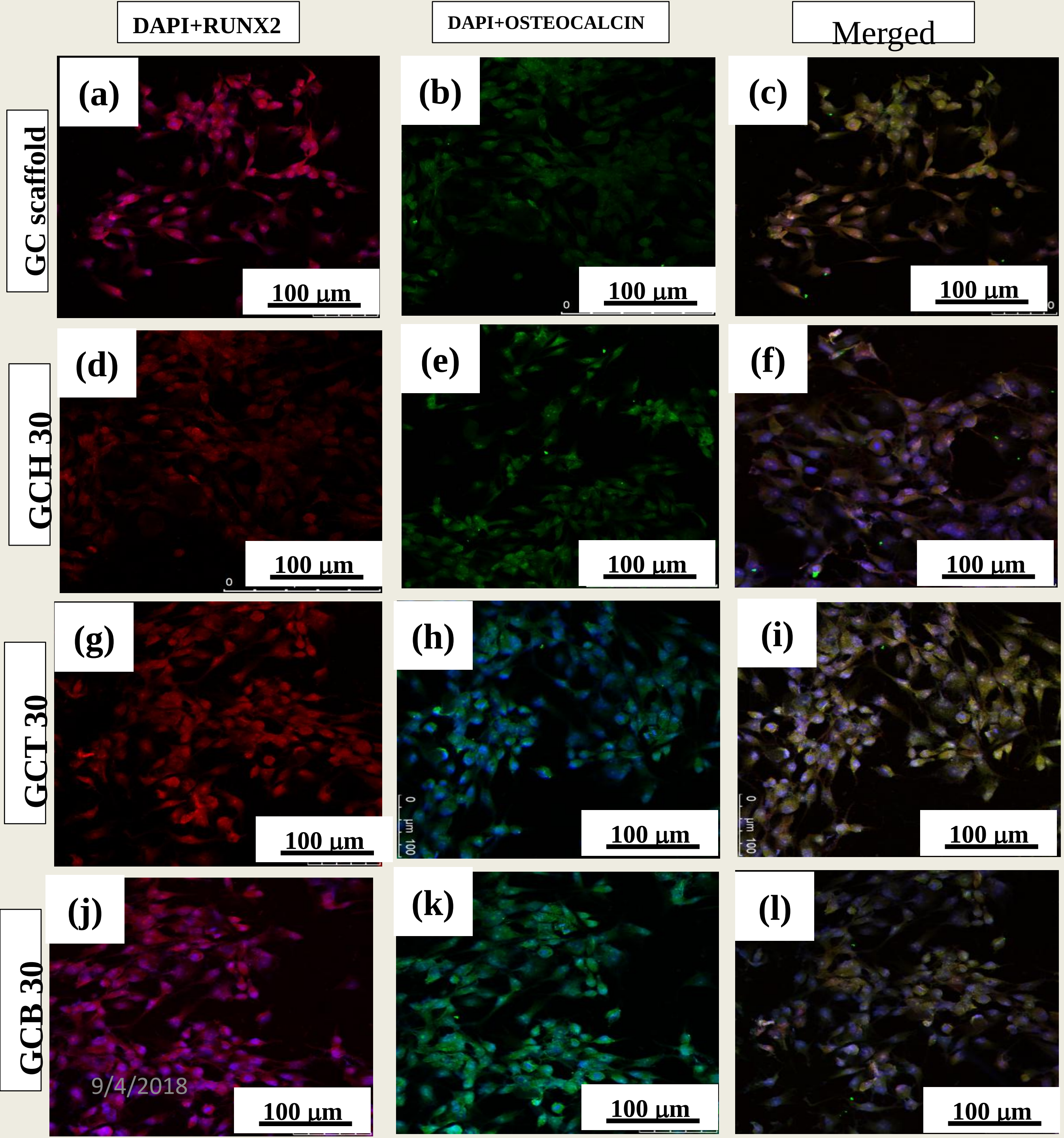


- The cell number increased with the culture time in all the group.
- Incorporation of bioactive calcium phosphate ceramic nanoparticle dramatically increase the cell proliferation in composite scaffold.
- GCB30 scaffold shows significant higher amount of cell proliferation compared to other scaffold.

*P<0.05, by student's t-test, n=5, all values in category were found to be significantly different from each other.

In vtro Osteogenic Gene Expression Study



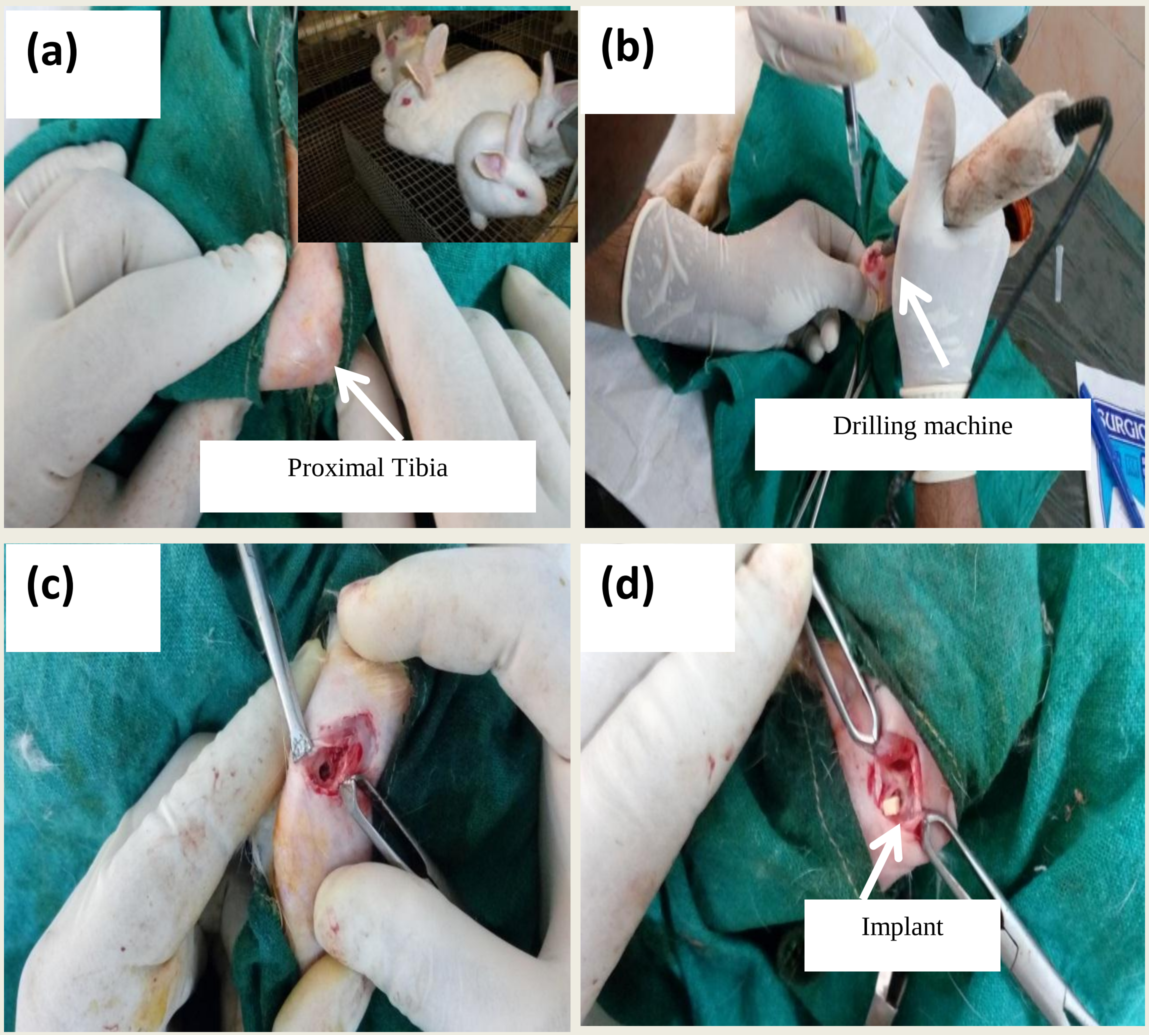


The expression of MSC-specific marker RUNX2 and osteocalcin by the MSCs cultured in the GC,GCH30,GCT30 and GCB30 for 14 days of culture.

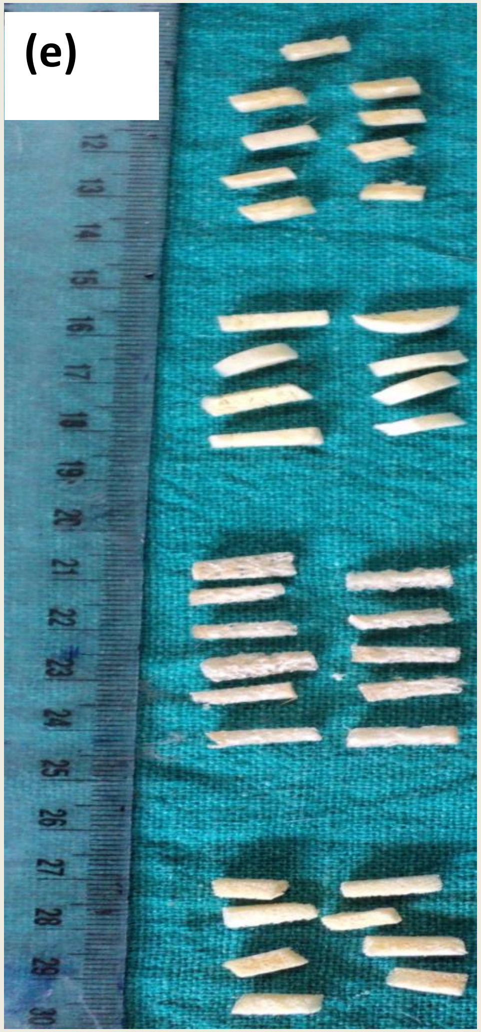
In GCB30 scaffold more cells express osteocalcin markers indicating that the differentiation is higher in these scaffolds than in GC, GCH30 and GCT30 scaffold , which is in the line with the gene expression study

In vivo study in rabbit model

Surgical Procedure



Sample Size



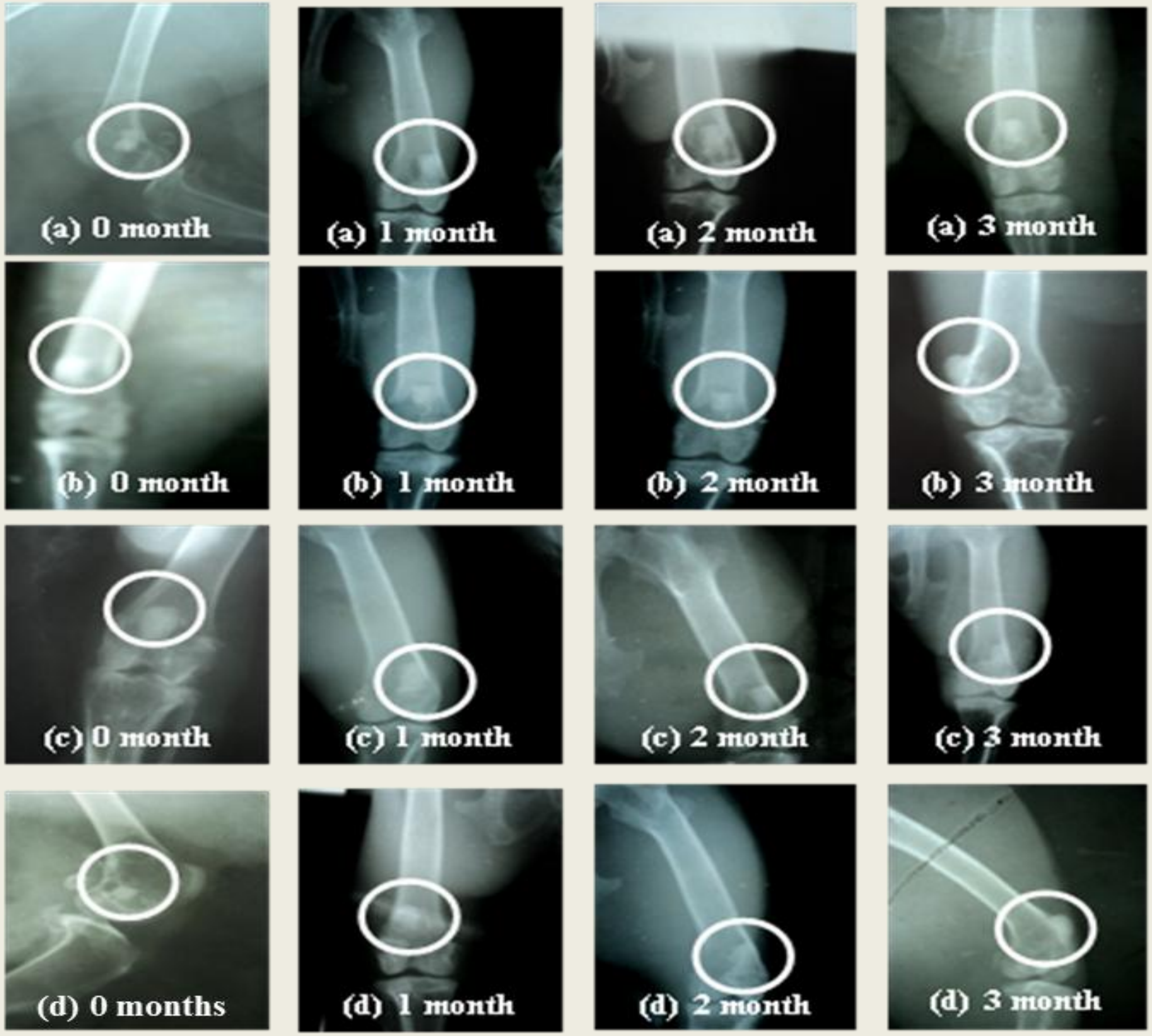
GCB30

GCT30

GCH30

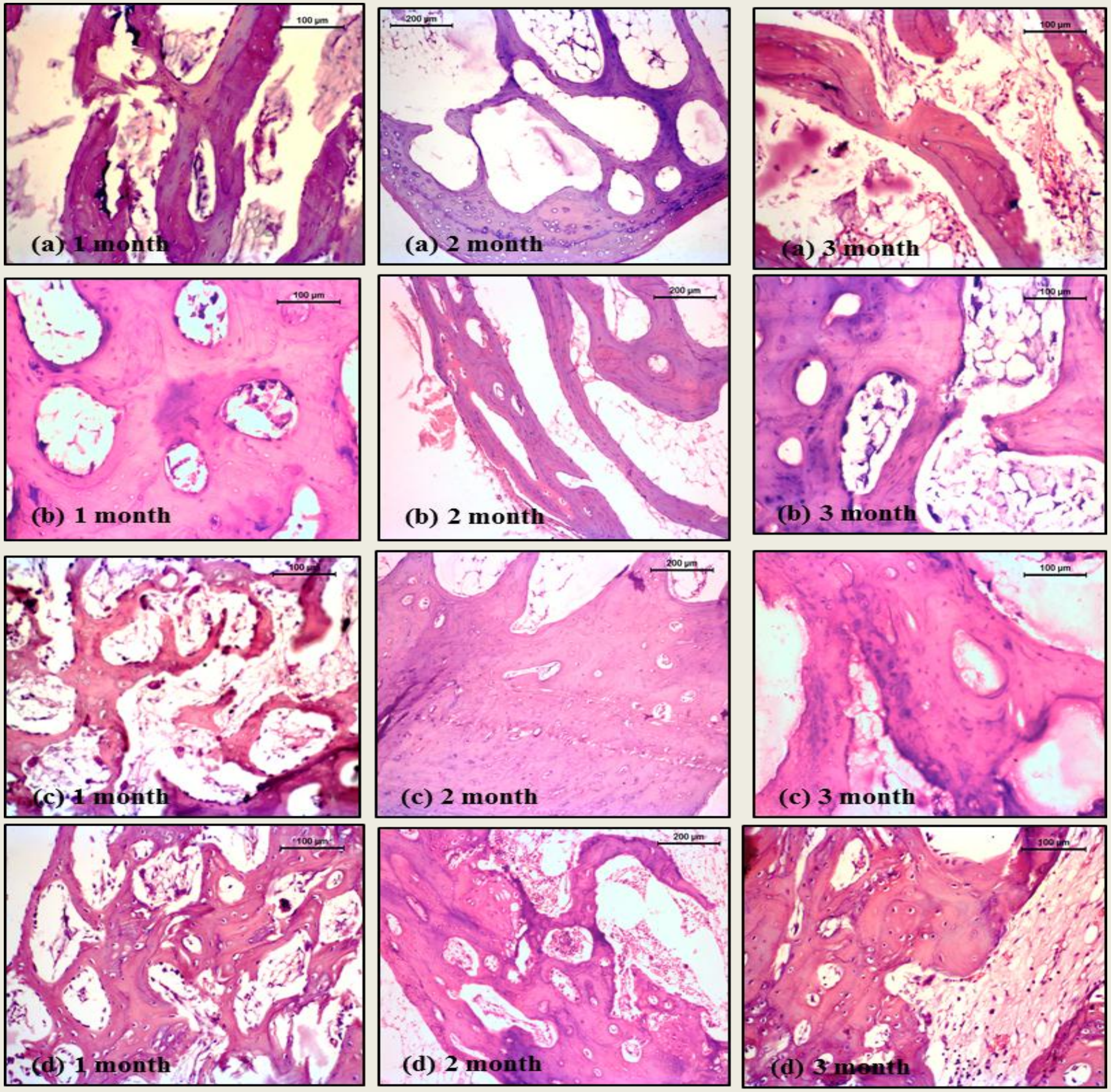
GC

X-Ray image of implant after 0,1,2,3 months of observation in rabbit

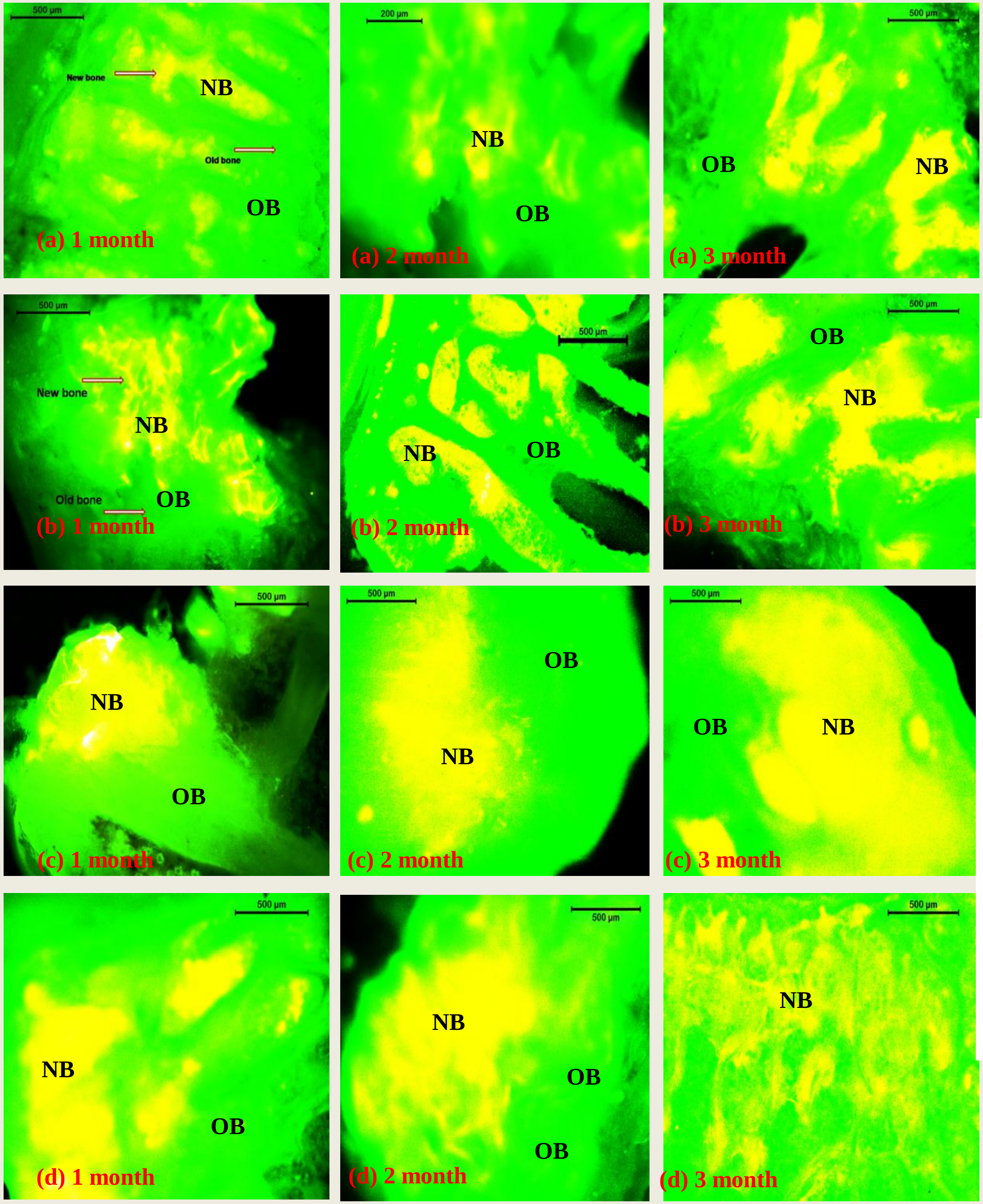


Radio density of material is decreasing with increasing time of implantation in all the scaffold (a) GC (b) GCH 30 (c) GCT 30 (d) GCB 30.

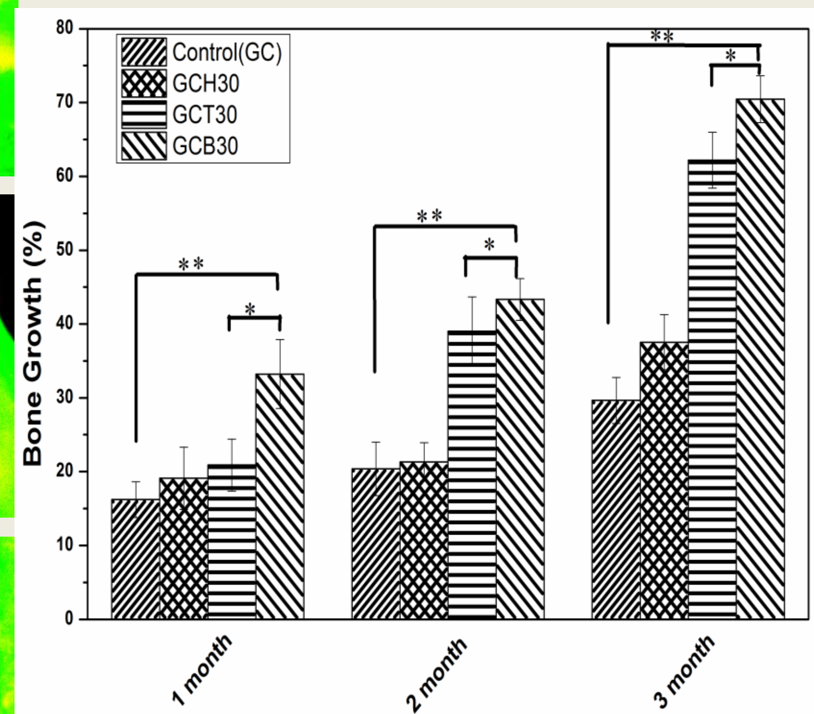
H E staining image of 1,2 and 3 month in (a) GC (b) GCH 30 (c) GCT 30 (d) GCB 30



- More cellular activity(Osteoblast & Osteoclast) has been seen in GCB 30 implant after three month study.
- Angiogenesis is visible in all the implant but more pronounce than control after three month of study.

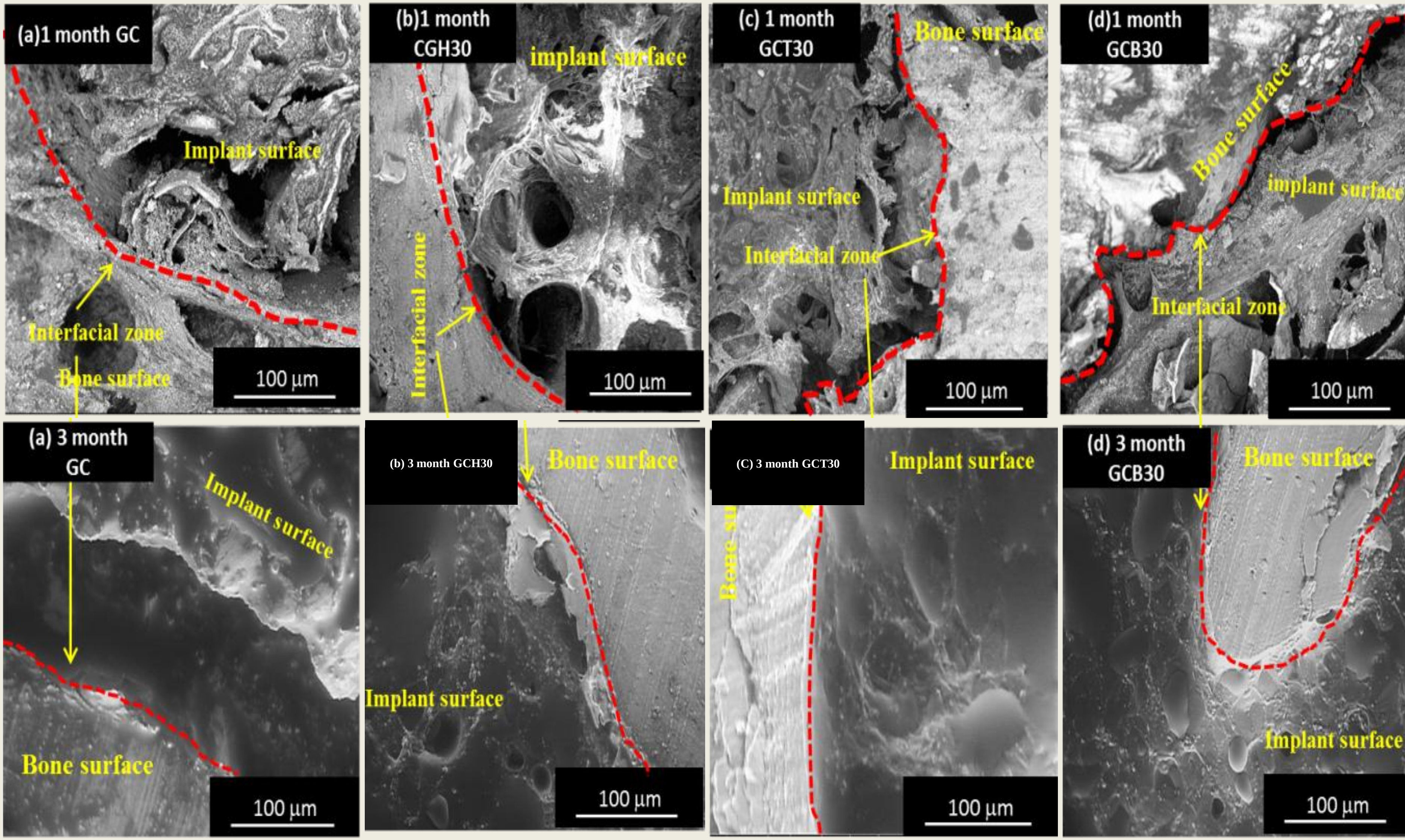


Flurochrome labelling images taken after 1,2 and 3 months post-operatively implanted in (a) GC (b) GCH 30 (c) GCT 30 (d) GCB 30



Bone ingrowth in GC, GCH 30, GCT 30 and GCB 30 composite scaffolds after 1,2 and 3 months of implantation

ESEM image of host implant interface in (a) GC, (b) GCH 30, (c) GCT 30 and (d) GCB 30 scaffolds post operatively after 1 month



Conclusions

- Gelatin-chitosan based biopolymer matrix reinforced with nanoparticulate bioactive ceramic in the form of composite scaffold has been successfully prepared and characterized both *in vitro* and *in vivo* .
- The optimization of phase composition in the composite scaffold to obtain desired physiochemical mechanical and biological properties in the scaffold was performed with the help of different characterization techniques such as mercury porosimetry , mechanical strength, *in vitro* and *in vivo* bioactivity studies..
- 30wt% ceramic reinforced scaffold having composition HAp:Chi:gel (28:42:30) showed the maximum compressive strength of 3.26MPa while a lowest average compressive strength of 2.2MPa was recorded for bioglass based scaffold having composition gelatin:chi:bioglass equal to (30:40:30).
- In general with increasing bioactive ceramic phase content from 10-30 wt% bioactivity and mechanical property of all the scaffold(GCH,GCB,GCT) increased in a significant manner.
- Micro-CT analysis demonstrated the interconnected porous structure of all the scaffold with a highest of 73% interconnectivity shown by GCB30 scaffold.
- Among the GCH30, GCT30 and GCB 30 scaffold, the later showed the highest protein adsorption capacity of 45 mg/gm and lowest of 27 mg/gm exhibited by GCH 30 scaffold.
- Based on the histological, radiological and fluorochrome labelling results, 58s bioglass reinforced GCB30 composite scaffold showed enhanced early-stage bone formation at the defect site in rabbit tibia.

Future Scope

- Fabrication of freeze drying scaffold with varying Gelatin, Chitosan molecular weight and freeze drying parameters.
- Study on the effect of viscosity of slurry on pore morphologies and compressive strength.
- Future studies will focus on the ability to functionalize the surfaces of 58s bioglass/polymer composites with conjugate with other protein and study of their adsorption/release characteristics.
- To check the ability of composite scaffold in drug delivery system.
- Evaluate the bone growth phenomenon inside the implanted scaffold using Micro-CT technique.
- There is a lack of current understanding in the literature regarding the long-term in vitro and in vivo characterization of the porous 3D scaffold composites .

Publication

1.**Kanchan Maji**, Sudip Dasgupta "Hydroxyapatite-chitosan and gelatin based scaffold for bone tissue engineering" ,Transaction of Indian ceramic society,2014;73:110-114.

2.**Kanchan Maji**, Sudip Dasgupta, Biswanath Kundu, Akalabya Bissoyi " Development of Gelatin-Chitosan-Hydroxyapatite Based Bioactive Bone Scaffold with Controlled Pore Size and Mechanical Strength-. Journal of Biomaterials Science, Polymer Edition, 2015;26(16):1190-1209.

3.**Kanchan Maji**, Sudip Dasgupta "Bioglass and biopolymer based composite scaffold for bone regeneration", Transaction of Indian ceramic society,2015: 74(4):1-7.

4.**Kanchan Maji**, Sudip Dasgupta, Krishna Pramanik, Akalabya Bishoyi**,**"Preparationand evaluation of novel chitosan-gelatin-nano-Bioglass 3D porous scaffold for Bone Tissue Engineering"-International Journal Of Biomaterials,2016,14.

5. Soumini Mondal, Sudip Dasgupta, **Kanchan Maji"**MgAl-layered double hydroxide nanoparticles for controlled release of salicylate**" Material science and enginerring C,** 68, 2016, 557–564.

6. **Kanchan Maji**, Sudip Dasgupta, Krishna Pramanik, Akalabya Bishoyi ," Development of Gelatin-Chitosan-β-TCP 3D porous scaffold for orthopaedic application" Journal of Material Science and Engineering C, Under Review.

6. **Kanchan Maji**, Sudip Dasgupta, Samit Nandy, Akalabya Bishoyi, "*In vitro* and *In vivo* Comparative study of Gelatin-Chitosan-Bioactive ceramic composite scaffold for orthopaedic application" Manuscript under preparation.

7.Sudip Dasgupta, **Kanchan Maji** "comparative study on mechanical strength of macroporous Hydroxyapatite-biopolymer based composite scaffold"- (International Conference on Advances in Engineering and Technology (ICAET'2014) March 29-30, 2014 Singapore) .

8.Sudip Dasgupta, Debosmita Pani, **Kanchan Maji "**Reinforcement of Calcium Phosphate Cementwith E-Glass Fibre**"** International Science Index Vol:9, No:9, 2015 waset.org/Publication/10002520.

Acknowledgement

Ceramic Engineering (CR)
All Faculty member and Technical stuff

Biotechnology and Medical Engineering (BM)
Cell culture group

Department of Chemistry (CY)
Freeze Drying Facility

CSIR-*central Glass and Ceramic Research Centre*
Bioceramic and coating division

Raja Raman Centre for Advanced Technology
Computed Tomography facility

West Bengal University of animal and Veterinary Science
In-vivo facility

Thank You