RESPONSE TO EXAMINER'S COMMENTS

Thesis Title: "Evaluation of bone repair using chitosan-hydroxyapatite biomaterial for Bone tissue engineering"

Student Name: Namrata Yadav

Supervisor Name: Prof. Pradeep Srivastava

Department: School of Biochemical Engineering

Response to Examiner-I

Suggestions:

Preface

Comment 1: Please refer to the sentence "The innate properties of the scaffold constituents, namely chitosan, gelatin and hydroxyapatite were modified by combining the by gelate freeze-drying." In page no 23 and correct the grammatical mistake.

Response: As per the suggestion, the grammatical mistake has been corrected.

Chapter 1. Introduction

Comment 1: Please refer to the sentence "This supports in reducing the mismatch between current in vitro and clinical approaches" in page no. 24 and specify what 'this' means.

Response: As suggested, 'This' has been changed to 'These techniques' as a follow up of the previous statement.

Comment 2: Please refer to the sentence "It lead the focus on alternate available procedures to reconstrucy bone after trauma, tumour resection, and congenital diseases" in page no. 25 and please correct the grammatical mistake.

Response: I acknowledge the suggestion, the error has been corrected.

Comment 3: Please refer to the sentence "Tissue-Engineering was given by Langer and Vacant, 1993 [12] states Tissue Engineering (TE) involves controlled stimulation of target cells via systematic combination of molecular and mechanical signals" in page no. 25 and correct grammatical mistake.

Response: As per the suggestion, the implied grammatical error has been corrected.

Comment 4: Please refer to the sentence “As a promising alternative, tissue engineering of bone evolved by involving materials that induce bone formation in response to the neigboring tissue” in page no. 25 and correct the grammatical mistake.

Response: As per the suggestion, the implied grammatical error has been corrected.

Comment 5: Please refer to the sentence “Sxaffold raw materials mimicking ECM component properties be an effective strategy in restoring majority of bone related deformities.” In page no. 26 and correct the grammatical mistake.

Response: As per the suggestion, the implied grammatical error has been corrected.

Chapter 2. Literature Review

Comment 1: Please note that the thesis suffers from usage of long sentences which reduces the focus of the statement. This also creates room for errors in tense in multiple places. For instance, please refer to the sentence “Nowadays, with the rise in incidences of facial trauma, the better understanding of the new procedural techniques and osteogenesis process had urged the need to optimize materials required for bone-defect healing” in page no. 28. The clarity of thought and inconsistency in tense can be takencare of by using short sentences. The style also will improve.

Response: The examiner has suggested correctly to limit the usage of long sentences. As per the suggestion, longer sentences have been shortened throughout the ‘Literature Review’.

Comment 2: Please refer to the “Figure 2.2 Materials classification on the basis of their chemical origin.” In page no. 30 and explain it elaborately. Please explain what do the start marks included in the figure indicates.

Response: As per the suggestion, usage of star marks have been included in the figure legend of figure 2.2.

Comment 3: Please refer to the sentence “The use of natural or synthetic polymer’s as three-dimentional (3D) structures for bone regeneration has been quite interesting quite interesting” in page no. 31 and remove the repetitive words.

Response: As per the suggestion, the repetitive words have been removed.

Comment 4: Please write “w.r.t” in the full form “with respect to” or include it in the list of abbreviations.

Response: As per the suggestion, “w.r.t” in the full form “with respect to” has been included in the list of abbreviations.

Comment 5: Please refer to “Figure 2.3 Parameters influenced from the biodegradation and biomineralization properties” and describe the figure briefly in legend of the figure.

Response: As per the suggestion, the figure legend has been re-written for Figure 2.3.

Comment 6: Please refer to the sentence “Osteoblasts found to be located along the surface of bone and show morphology characteristic to any protein synthesizing cell” in page no. 51 and correct the grammatical mistake.

Response: As per the suggestion, the indicated grammatical error has been corrected.

Chapter 3. Materials & Methods

Comment 1: Please refer to “Table 3.2 Composition of the prepared Simulated Body Fluid (SBF)” in page no. 64 and mention about concentration of 40 ml HCl.

Response: As per the suggestion, the concentration of 40ml HCl has been included in the Table 3.2.

Comment 2: Please keep the page margins of page no. 71 as similar to other pages in the thesis.

Response: As per the suggestion, the page margin of page no. 71 has been aligned as same as to other pages in the thesis.

Response to the Examiner-II

Comment 1: The concept of using porous materials for bone tissue engineering has been reported for decades, the rational and the innovation of using the composition of gelatin, hydroxyapatite and chitosan need to be clearly justified. Yes, they are nature biomaterials, but what are the advantages of these materials compared to many other sophisticated nature materials? Such as biomechanic properties, the cost, the stabilities, etc.

Response: As per the suggestion, the rational and innovation of using the composition of gelatin, hydroxyapatite and chitosan have been mentioned in Chapter 3: Materials and Methods, Table 3.1. The justification has been included in the 'Chapter 2. Literature Review, 2.4 Recent advances in the Multi-component System.

Also, advantages of these material compared to other sophisticated natural materials (in term with their biomechanical properties, cost, stability) has been incorporated in the Table 2.2.

Comment 2: The innovation of the technical approach of "freeze-dried slurry and lyophilization" should be discussed in detail, by comparison with other common tissue engineering methods.

Response: As per the suggestion, the technical approach of scaffold fabrication 'freeze-dried slurry and lyophilization' have been discussed in terms of being innovative. Also, it has been compared with the existing common tissue engineering methods in the section '2.3.4 Comparative study on the methods of fabrication' in terms of being the innovative.

Comment 3: While the project attempted to mimic the in vivo microenvironment using the in vitro model, the factors tested in the thesis are limited to the characterization of scaffold composition and cell differentiation. As the matter of fact, the in vivo environment is way more complicated than the in vitro conditions the current project tests. In addition to the physical characterization and cell proliferation test, at least, the other considerable matrixes for evaluating cell-scaffold interaction should be discussed.

Response: I understand and acknowledge the comment. The *in vivo* microenvironment was restricted to the comparative analysis performed in the *in vitro* experiments. In addition, it could serve as the scope for the future work on the *in vitro* appropriate scaffold-cell combination.

Comment 4: in vitro osteogenesis and metabolic study of co-culture with HCG, rT over rM are limited at mineralization and osteogenic gene expression, discussion about other metabolism elements like ATP/ADP, glucose, NADH (if any) will provide complementary information.

Response: As per the suggestion, the study supporting metabolism elements have been included via the 'Glucose diffusion analysis' of the co-cultured samples in the 'Chapter 4, as section 4.2.11'.

NOTE: Necessary changes have been incorporated in the thesis. The same have been highlighted in red.

Chapter 2

Table 2.1 Comparative study of the raw materials used in this study w.r.t natural materials using matrix mapping.

Sr. no.	**Properties**	**C**	**H**	**G**	**Collagen**	**SF**	**A**	**HAA**
1.	Biomechanical [125,182,189]	2	3	2	1	**3**	1	1
2.	Cost [111,114]	1	2	1	**3**	2	1	**3**
3.	Stability [46,77,115]	2	3	2	2	2	-q	1
4.	**%Composition** [185-188]	**Nil**	**50**	**Nil**	**20**	**Nil**	**Nil**	**0.7**
5.	Biocompatibility	3	2	3	3	2	2	3

* Matrix score: 1-low, 2-intermediate, 3-high or strong.

Chitosan-C, Gelatin-G, Hydroxyapatite-H, Silk Fibroin-SF, Alginate-A, and Hyaluronic acid-HAA

Table 2.3 Comparative study on the methods of fabrication (in terms of being innovative)

Sr.no	**Fabrication Methods**	**State of scaffold**	**Contamination**	**Accessibility**	**Mechanical property**
1.	Lyophilization [22,23,29-30]	Rapid-solidification	Reduce chances	High	Superior
2.	Dry/wet-spinning based extrusion [57-60]	Amorphous consistency	Low	low	Inferior
3.	Gas-foaming [184-187]	Highly viscous	Relatively higher	intermediate	Intermediate
4.	Cryo-tropic gelation [111,125,187]	Interconnections not guaranteed	Intermediate	high	Inferior

Chapter 3

3.5.8.3 Glucose diffusion analysis

3.5.8.3b Measurement of the D value

Two slots in the 12-well cell culture plate were used for single-point data collection.

Each well was 7.3cm^3 in volume (dimension of the cylindrical well: 2.2cm diameter X 1.9 cm depth). Each well can hold up to 1.2ml media volume.

The wells were incubated with cell culture media saturated 'cell-scaffold' combination. The set-up was kept at 37ºC. Glucose at 1mg/ml was added to saturation in one in every two wells. The 12 well plates were incubated in the CO_2 incubator.

As per Fick's law, Diffusion flux, J is directly proportional to the concentration gradient of the particle ($d\Phi/dx$). Provided there was no change in the volume of the diffusion cell.

$$J = -D \frac{d\Phi}{dx}$$

$$= -DA \Phi_d - \Phi_r / x$$

J is the diffusion flux, mass transfer through an area per unit time

Φ is the concentration of the diffusing solute

x is the scaffold thickness,

A is the area of the scaffold

D is the effective diffusivity of glucose in the seeded scaffold

V is the volume of the well

D was calculated by fitting the experimental values into the equation for J. All experiment was performed at n=3. Also, no significant deviation among the data values were recorded.

3.5.8.3b Measurement of D value for cell-scaffold saturated cell culture media

A UV spectrophotometer was used to record the change in glucose concentration w.r.t time at 190nm. Each well was filled to saturation using 1.2 ml of glucose (1mg/ml). Sampling was done from both the wells, with and without cell-scaffold saturated with water after 1,2,4,6 and 8 hours. All the readings were recorded in triplicate.

Chapter 4

Results and Discussion

4.3.7.2 Determination of the Glucose Diffusion Coefficient (D)

The varying surface and core morphology tend to influence the diffusion of the glucose moieties. Hence, the value of D is directly correlated to both, the porosity and morphology.

Figure illustrates the changes in glucose concentration over a period of eight hours from 0 to 27 culture days.

The GH based scaffold depicts lesser degree of diffusion than the CGH scaffold. Also, rTCGH displayed maximum glucose diffusion, independent to its rate of biodegradation.

Also, the rT seeded groups (rTGH and rTCGH) displayed marginally lower resistance to glucose diffusion over the rM seeded groups (rMGH and rMCGH).

The effective glucose diffusion coefficient was higher for CGH groups than the GH groups.

From the study done by H.Suhaimi et.al. the diffusion coefficient for the scaffold material increases at 37ºC.

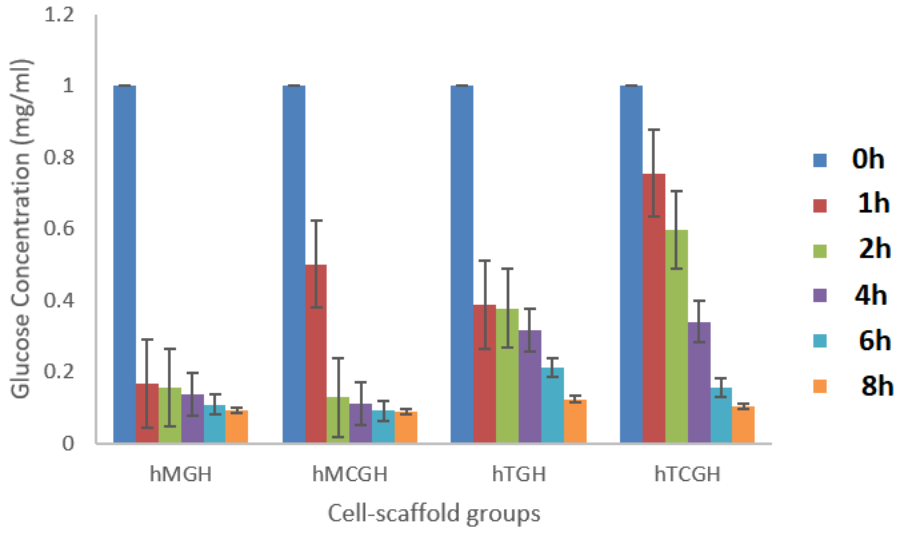

Figure 4.42 Variation in the glucose concentration w.r.t time factor among the four cell-scaffold groups: hMGH, hMCGH, hTGH and hTCGH

Table 4.11 Effective diffusion coefficient with standard deviation for glucose across cell-scaffold saturated with cell culture media.

Sr. no.	Cell-scaffold group	Std Deviation (σ)	D (m^2/s) After 27 culture days
1.	hMGH	$2 \pm 2.67 * 10^{-10}$	$2.05 \pm 0.77 * 10^{-10}$
2.	hMCGH	$2.05 \pm 0.11 * 10^{-11}$	$1.05 \pm 0.1 * 10^{-11}$
3.	hTGH	NA	$1.011 \pm 2.66 * 10^{-10}$
4.	hTCGH	$9.01 \pm 0.66 * 10^{-9}$	$7.05 \pm 0.12 * 10^{-9}$

Dedicated

to

My Beloved Parents

ACKNOWLEDGEMENT

Any accomplishment requires a combined effort of many individuals and my project is no different. I would like to express my gratitude towards a number of people whose help has been instrumental for the success of my Ph.D. study. So it is with deep gratitude that I express my appreciation to the following people for their contribution.

First of all I would like to express my sincere gratitude to my supervisor Professor Pradeep Srivastava, School of Biochemical Engineering, IIT (BHU) for his valued guidance and instruction at every step which were truly enlightening. I am grateful for his wholehearted support and innovative suggestions. I have benefited greatly from his imperial style of developing innovative ideas and the implementation of those ideas. I sincerely show profound sense of veneration for his helping attitude, prospective comments, constant help, moral support, and suggestions throughout the period of the project. I would not have imagined a better supervisor and advisor for my Ph.D. work.

I wish to express my profound gratitude again to Prof. Pradeep Srivastava, Co-ordinator, School of Biochemical Engineering, IIT (BHU), Varanasi, for providing effective management, necessary facilities and valuable suggestion for the completion of my work.

I thank my doctoral evaluation committee (RPEC Members), Prof. S.K. Srivastava, Prof. P.K.Mishra, and Dr.(Mrs.) Abha Mishra, for their constant monitoring and valuable suggestions which helped in completion of the Ph. D. work successfully.

I also take this opportunity to express my heartfelt thanks and respect to all respect to all the faculty members of my School, for their indispensable suggestions, help and support. Prof. Subir Kundu (Retd.), Prof. S.K. Srivastava (Retd.), Prof. R.M. Banik, Prof. Mira (Debnath) Das, Prof. Vikas Kumar Dubey, Dr. Ashish Kumar, Dr. Vishal Mishra, and Dr. Sanjay Kumar, School of Biochemical Engineering, IIT-BHU, Varanasi for their indispensable suggestions and encouragement throughout my Ph..D. work.

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I would also like to put in words my gratitude to my mother, to whom I owe my success for being the inspiration, my father for the abundant blessings and unconditional support, my darling husband for being my pillar of strength, my brother and my baby daughter for being the cordial. I extend my gratitude to my new found family in my in-laws.

I am indebted to many more people that could possibly not be mentioned in this brief space. So my sincere apologies are offered to those who contributed significantly but whose names regretfully do not appear here.

Finally, I thank to GOD for giving me the strength and wisdom to do this work and complete it successfully.

Namrata Yadav

(Roll No.-13011004)

CONTENTS

List of Abbreviations

ßGP	Beta-Glycerophosphate
δ	Chemical Shift
%	Percent
µg	micro gram
µl	micro litre
µM	Micro molar
^{0}C	degree Celsius
AB	Alamar Blue
AFM	Atomic Force Microscopy
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
ARS	Alizarin Red S
CLSM	Confocal Laser Scanning Microscope
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffer Saline
ECM	Extracellular Matrix
EDTA	Ethyl di-amine tetra acetic acid
EtBr	ethidium bromide
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
FC	Flow Cytometry
FESEM	Field Emission Scanning Electron Microscope
FITC	Fluorescein Isothiocyanate
FTIR	Fourier Transform Infrared Spectroscopy
h	hours
MNCs	Mononuclear Cells
MSCs	Mesenchymal Stem Cells

n	Number of experiments
$NaHCO_3$	Sodium Bicarbonate
Na_2SO_4	Sodium Sulphate
pH	Hydrogen ion concentration
PBS	Phosphate Buffer Saline
Q-PCR	Real Time Polymerase Chain Reaction
rOb	Rabbit osteoblast
RPM	Revolutions per minute
RH	Relative Humidity
SAED	Selected Area Electron Diffraction
SD	Standard Deviation
SEM	Scanning Electron Microscope
$t_{1/2}$	half life
TE	Tissue Engineering
TEM	Transmission Electron Microscope
v/v	Volume per unit volume
w/v	Weight per unit volume
XRD	X-ray Diffraction

List of Figures

Chapter 4

PREFACE

Despite great progress in the available bone healing procedures. Large scale production had been lagged by the mismatch between the demand and requirement. Over a decade tissue engineering have evolved to cater substitutes for damaged tissue/organ. Bone related degeneration could be reduced in the elderly by utilizing scaffolds when seeded and cultured with osteoblast. Combining technologies in engineering scaffolds which can mimic the extracellular matrix of the bone in terms of the morphological and mechanical properties, directly influences the healing step employing bone tissue engineering. The scaffold was evaluated both before and after seeding them with either rabbit osteoblast followed by the human osteoblast, derived from both bone tissue and bone marrow mesenchymal stem cells.

The innate properties of the scaffold constituents, namely chitosan, gelatin and hydroxyapatite were modified by combining them by gelate freeze-drying. The obtained scaffold variations were characterized by studying their morphology, chemical structure, crystallinity and mineral content, degradation to mineraliztion behaviour, antibacterial response and mechanical to porosity parameter. The most bone extracellular biomimic scaffolds were studied *in vitro* at different time-points to scrutinize the osteoblast-scaffold combination for further *in vivo* implantation studies. Data suggested that the osteoblast-scaffold when used together as bone extracellular matrix substitute can have great potential in promoting bone repair during clinical practices. The presented study also highlighted the areas on which research is needed with relevance to enhance the understanding of the complex role of scaffold and osteoblasts in bone tissue engineering.

CERTIFICATE

It is certified that the work contained in the thesis titled ″Evaluation of bone repair using chitosan-hydroxyapatite biomaterial for Bone tissue engineering″ by Namrata Yadav has been carried out under my supervision and that this work has not been submitted elsewhere for a degree.

It is further certified that the student has fulfilled all the requirements of Comprehensive Examination, Candidacy and SOTA for the award of Ph.D. Degree.

Prof. Pradeep Srivastava
(Supervisor)
School of Biochemical Engineering
Indian Institute of Technology,
Banaras Hindu University
Varanasi-221005

DECLARATION BY THE CANDIDATE

I, **Namrata Yadav** certify that the work embodied in this thesis is my own bona fide work and carried out by me under the supervision of **Prof. Pradeep Srivastava**, from July 2013 to July 2019, at the School of Biochemical Engineering, Indian Institute of Technology (Banaras Hindu University), Varanasi. The matter embodied in this thesis has not been submitted for the award of any other degree/diploma. I declare that I have faithfully acknowledged and given credits to the research workers wherever their works have been cited in my work in this thesis. I further declare that I have not wilfully copied any other's work, paragraphs, text, data, results, *etc.*, reported in journals, books, magazines, reports, dissertations, theses, *etc.*, or available at websites and have not included them in this thesis and have not cited as my own work.

Date: Signature of the student

Place: Varanasi **(Namrata Yadav)**

CERTIFICATE BY THE SUPERVISOR(S)

It is certified that the above statements made by the student is correct to the best of my knowledge.

Prof. Pradeep Srivastava
(Supervisor)
School of Biochemical Engineering
Indian Institute of Technology
Banaras Hindu University
Varanasi-221 005

(Co-ordinator)

School of Biochemical Engineering
Indian Institute of Technology
Banaras Hindu University
Varanasi-221005

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