On 3D printed scaffolds for orthopedic tissue engineering applications

Nishant Ranjan^{1,2} · Rupinder Singh¹ · I. P. S. Ahuja² · Ranvijay Kumar^{1,2} · Jatenderpal Singh^{1,3} · Anita K. Verma⁴ · Ankita Leekha⁴

Received: 10 May 2019 / Accepted: 3 January 2020 / Published online: 11 January 2020
© Springer Nature Switzerland AG 2020

Abstract

This paper outlines an in vitro evaluation of 3D printed scaffold of polylactic acid (PLA) blended with hydroxyapatite (HAp) and chitosan (CS) for orthopedic tissue engineering applications. In the first stage, selected composition/proportion of PLA–HAp–CS (based on melt flow-ability, mechanical and thermal properties) was 3D printed with fused deposition modeling (FDM) process. The 3D printed scaffolds were used for process capability analysis to ascertain the industrial usability of PLA–HAp–CS composite scaffold for batch production (especially in assembly applications). Further, in the second stage, an in vitro evaluation was performed to investigate the linkages of fibroblast cells for 3D printed scaffold. The results of study outline the rapid increase in growth of fibroblast cells for FDM-printed scaffolds of PLA–HAp–CS thus ensuring its capability of supporting cell adhesion and cell proliferation. Further, the dimensional variations and Shore *D* hardness of 3D printed scaffolds are under statistically control, with process capability indices (C_p and $C_{pk} \geq 1$).

Keywords Scaffolds · Tissue engineering · 3D printing · Composite material · HAp · PLA

1 Introduction

PLA is one of the commonly used polymers in field of tissue engineering for fabrication of biomedical scaffolds and implants [1]. Biocompatible and biodegradable grades of PLA are available in crystalline as well as amorphous form depending upon relative levels of optical isomers present in the molecules [2, 3]. The reported literature outlined that for fast growth of bones/tissues, matrix of PLA is usually blended with biocompatible as well as bioactive reinforcements [4]. The HAp- and CS-based ceramic reinforcements have been used extensively as reinforcements for fabrication of biomedical implants/scaffolds [5]. These reinforcements are useful biomaterials with potential orthopedic, dental and tissue engineering applications due to properties like: bioactivity, biocompatibility and osteo-conductivity [6–8]. As a composite material PLA,

HAp and CS have number of applications in bone regeneration [9–11].

Usually, a biocompatible substance is defined as “inserts/implants” having no reaction with tissue for imprecise periods of time. This may be due to the formation of a tough layer within implants [12]. Polymers are classified based on the presence or absence of degradation phenomena in a biological environment that is dependent on their belongings to the second- or third-generation biomaterials [13–16].

Non-degradable polymers are the essential materials which are largely applicable for preparations of medicine as fillers materials, orthopedic implants/scaffolds, ocular lens, heart valves, bone cements and vascular grafts for long-term devices [17]. Sometimes degradable polymeric biomaterials are selected for manufacturing therapeutic provisional devices for prostheses, resorbable 3D porous structures as scaffolds for drug release [18–20]. Tissue

✉ Rupinder Singh, rupindersingh78@yahoo.com | ¹Department of Production Engineering, Guru Nanak Dev Engineering College, Ludhiana, India. ²Department of Mechanical Engineering, Punjabi University, Patiala, India. ³Department of Defence Production, DGQA, SQAE(A), Jabalpur, India. ⁴Nanobiotech Lab, Kirori Mal College, University of Delhi, New Delhi, Delhi, India.

engineering takes advantage of 3D structures that provide a pattern for cells to join, reproduce, preserve their differentiated function and organize in order to restore structure and function to damaged tissues [21–23]. Essentially, scaffolds act as a synthetic analogue of the natural extracellular matrix [15, 24–27]. Some studies in recent past have reported Hap- and CS-coated PLA nanofibers for bone regeneration [28–34].

The literature review reveals the studies on fabrication of biomedical scaffolds by using biocompatible and biodegradable polymers with reinforcement of bioactive ceramics [3, 28, 30, 32, 35, 36]. But hitherto little has been reported on fit and tolerance of 3D printed scaffolds especially in assembly applications, their process capability analysis and surface roughness (Ra) profile of fibroblast cells (for in vitro analysis). This paper is an extension of work reported for fabrication of biomedical scaffolds [37, 38]. In this study, the selected composition/proportion of PLA–HAP–CS as 91–8–1 (by weight percentage) was further used for 3D printing of biomedical scaffolds (in the first stage), and its process capability analysis followed by in vitro evaluation (in the second stage) was supported with rendered images of fibroblast cells and their Ra profiles.

2 Materials and methods

During pilot experimentation, effort has been made to select suitable material composition/proportion for preparations of biocompatible and degradable scaffolds. Initially, blends of PLA, HAP and CS (biocompatible and bioactive materials) were prepared in different weight proportions. In next step, melt flow index (MFI) characterization was performed to investigate flow continuity for feedstock filaments preparations (as per ASTM D1238), followed by investigations of statistical process capability analysis, mechanical and thermal properties for selections of best set of input process parameters of twin screw extruder [37, 38]. For preparations of feedstock filaments of 3D printing, HAAKE Mini CTW, (Thermo Fisher, Germany) was used. The previous studies have reported parametric optimizations of twin screw extruder (for establishing composition/proportion of HAP and CS in PLA) followed by parametric optimization of fused deposition modeling (FDM) setup from mechanical properties view point [37, 38]. These recommended settings were further used for printing of benchmark (flexural sample as per ASTM D790-17) in the present case study. It should be noted that for commercial applications, the customized biocompatible scaffolds must be dimensionally accurate with acceptable hardness. So, experimental study was conducted to establish the dimensional features of the scaffold prepared

(from commercial 3D printing view point). The standard dimensions for benchmark were: full length (FL) 125 mm, full width (FW) 12.7 mm and thickness (H) 3.2 mm. Figure 1a–c shows pictorial view of commercial FDM printer, 3D printed flexural samples and scanning electron microscopy (SEM) images of 3D printed fractured samples at different magnifications. It has been reported that best settings for printing of flexural samples on FDM are: Layer height is 0.2 mm, and raster angle is 30° (for tensile properties) and 45° (for flexural properties) with infill percentage of sample which was 100% [37, 38]. These settings were further used to ascertain the dimensional variations and surface hardness of scaffolds. Figure 2 shows process flow diagram for investigations of biocompatible/bioactive customized scaffolds (blends of PLA, HAP and CS).

3 Results and discussion

As per process flow diagram (Fig. 2), Tables 1 and 2 show the standard deviation (σ) for measured dimensions (D_{JM}) and Shore D hardness of flexural samples (ten different samples) at 95% confidence level which were 3D printed at best composition/proportion (of PLA–HAP–CS) and other input parametric settings suggested in previous studies [37, 38].

In the first stage, based on Table 1, international tolerance (IT) grades for different dimensions were calculated (see Table 2). As observed from Table 2, IT grades for D_{JN} was 12.70 mm, and for all samples, IT grades lie in tolerance zone (IT06–IT12) acceptable for assembly purposes. Further based on results given in Table 1, the process capability analysis has been performed to investigate that whether process is statically capable or not for industrial utility. For given D_{JN} , the number of tolerance unit can be calculated as [39]:

$$N = \frac{1000(D_{JN} - D_{JM})}{i} \quad (1)$$

where

$i = 0.45\sqrt[3]{D} + 0.001D$ is the tolerance factor and D is the geometric mean of generic data.

In this case study, for FW of flexural sample, $D_{JN} = 12.70$ mm (see Table 1). Further, the values of D and i can be evaluated as:

$$D = \sqrt{(10 \times 18)} = 13.416 \text{ mm [39] and } i = 1.082964.$$

For flexural sample dimension FW by following Eq. (1):

$$N = 120.$$

The evaluation studies were performed by selecting the ten samples of measured values of “FW.” The outcome of the study is considerable for industrial utility if and only if

Fig. 1 **a** Operational view of FDM. **b** 3D printed flexural samples. **c** Top and fractured view of flexural sample at different magnifications

Fig. 2 Process flow for 3D printing of scaffolds by using PLA–HAp–CS composite

Table 1 Standard deviation and margin of error for D_{JM} of flexural samples (as per ASTM D790-17 standard)

Nominal dimensions (D_{JN})	125 (FL)	12.7 (FW)	3.2 (H)
Standard deviation (σ) for measured dimensions (D_{JM})	0.85	0.05	0.02
Margin of error at confidence level of 95%	126.328 ± 0.528	12.751 ± 0.0365	3.243 ± 0.0154

Table 2 Standard deviation and margin of error for Shore D hardness of flexural samples

Mean Shore D hardness	65.25
Standard deviation (σ) for observed Shore D hardness	1.41
Margin of error at confidence level of 95%	65.25 ± 0.879

Table 3 IT grades for D_{JN} of width 12.70 mm and $i = 1.082964$

S. no	D_{JM}	N	IT grade
1	12.83	120	IT 12
2	12.63	65	IT 10
3	12.72	19	IT 08
4	12.79	83	IT 11
5	12.78	74	IT 11
6	12.69	10	IT 06
7	12.81	102	IT 11
8	12.80	93	IT 11
9	12.73	28	IT 09
10	12.73	28	IT 09

all the measured values with considerable standard deviation exist in between the upper and lower critical limit [40, 41]. The term process capability indices (C_p , C_{pk}) are used to summarize a system's ability to meet two-sided specification limits (upper and lower). Table 3 shows output result of process capability wizard software for nominal dimension 12.70 mm.

As per observations of Table 4, statistical process capability analysis calculations have been made for Shore D hardness value of the flexural sample. Table 5 shows output result of process capability for Shore D hardness value for biomedical scaffolds printed by using FDM.

In order to evaluate the biocompatibility of PLA, PLA-HAp, PLA-HAp-CS, an in vitro evaluation was designed to investigate the growth of fibroblast cells in 3D printed scaffolds (with macro-pores 150–300 μm , interconnected with micro-pores < 50 μm with average 10% porosity as per ASTM B276) in the second stage. For the experimentation purpose, 5 mm × 0.5 mm size of scaffold was primarily sterilized in autoclave environment under 103,421 N mm⁻² pressure at 121 °C temperature for 15 min time interval and further, samples were

Table 4 Output result of D_{JN} 12.70 mm of biomedical scaffolds

Output	Result	Output	Result
Readings	10	Maximum (in mm)	12.83
USL (in mm)	13	Average (X-Bar) (in mm)	12.75
Target (in mm)	12.7	Minimum (in mm)	12.63
LSL (in mm)	12.4	Data Range	0.20
Tolerance (in mm)	0.6	Median	12.755
AD test	Passed	Std. deviation (σ)	0.065012
C_p	1.538	P_p	1.611
C_{pk}	1.277	P_{pk}	1.337

USL upper specification limit, LSL lower specification limit, AD Anderson–Darling, P_p process performance, P_{pk} process performance index

Table 5 Output result of Shore D value for biomedical scaffolds

Output	Result	Output	Result
Readings	10	Maximum	68
USL	70	Average (X-Bar)	65.30
Target	–	Minimum	63.50
LSL	60	Data range	4.5
Tolerance	10	Median	65.00
AD test	Passed	Std. deviation	1.495363
C_p	1.128	P_p	1.115
C_{pk}	1.072	P_{pk}	1.059

cured at an ultraviolet light for 30 min before start of actual in vitro evaluation. For this study, fibroblasts cells of mouse embryonic (NIH3T3) were exposed in adherent cultures. Cells of 90% confluency were trypsinized and re-suspended in serum-containing medium, and these first entry cells were seeded at a thickness of 1×10^5 cells/well in twenty-four plates for planned examples and were kept in a 5% CO₂-humidified 37 °C in CO₂ incubator (Thermo, USA). Initially, cells were grown in a standard culture medium, i.e., in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 g/ml streptomycin). After the cells were allowed to adhere on the scaffolds, the samples were rinsed to remove the unattached cells. The scaffolds with attached cells were incubated for 2 weeks, and the medium was replaced every 3 days. The

Fig. 3 Estimation of biocompatibility of **a** PLA, **b** PLA + HAp, **c** PLA + HAp + CS on NIH-3T3 cell line

cells were observed under the inverted microscope (Olympus CKX41). The specimen was transplanted to another well-enhanced with complete media (second entry) and checked for expansion. This was performed to gauge the quantity of follower cells in a culture well to assess the extent of the surface which is secured by cells. Figure 3 shows expansion of NIH3T3 cells in considered samples. The results of this study reveal the growth of fibroblast cells. It has been observed that fibroblast cells were grew rapidly over the scaffolds. This confirms the biocompatibility of the samples in underneath cell bond and cell propagation. Further, photomicrographs of fibroblast cells were processed with image processing software to see the rendered images (for substrate (A) PLA, (B) PLA + HAp, (C) PLA + HAp + CS on NIH-3T3 cell line) and corresponding surface roughness (Ra) value (Fig. 4). As observed from Fig. 4, Ra value was lowest for PLA + HAp + CS on NIH-3T3 cell line; hence, this may be considered as best scaffold material. However, the growth of fibroblast cells was acceptable for other two cases also. These results indicate prepared scaffolds by 3D printing are suitable for in vivo use in tissue engineering applications.

Further, in order to evaluate the serum stability of PLA, PLA-HAp, PLA-HAp-CS, a serum stability study was conducted to ensure the stability of serum with the fabricated samples. For the experimentation purpose, the 5 mm × 0.5 mm size of scaffold was primarily sterilized in autoclave environment under 103,421 N mm⁻² pressure at 121 °C temperature for 15 min time interval and further, samples were cured at an ultraviolet light for 30 min before start of actual experimentation. It should be noted that glass transition temperature of PLA lies in range of

65–70 °C; therefore, while autoclaving at 121 °C temperature, there will be structural changes. But in scaffold application, the actual temperature will not rise above 40 °C. In the present in vitro testing, autoclaving was performed at 121 °C temperature as a standard procedure being followed in laboratory environment. Fetal bovine serum (FBS) was diluted to 10% using autoclaved phosphate-buffered saline (PBS). The weights of the fabricated samples were taken prior to the start of the experiment. The specimens were kept in six-well plate, and 3 ml of 10% FBS was added to each well. The experiment was continued up to 1 month so as to see the changes in the weight of the fabricated samples. Post 1 month of incubation, the experiment was terminated and again the weight of fabricated samples was calculated and percentage changes in weight were measured (see Table 6). As observed from Table 6, percentage reduction in weight in serum stability test is minimum for PLA + HAp + CS-based samples. Hence, these thermoplastic composites are acceptable in tissue engineering applications.

4 Conclusions

Following conclusions have been drawn from the present study of in vitro evaluation of 3D printed scaffolds:

1. The proposed 3D printing route for preparation of functional prototypes is statistically controlled. (As observed from Cp and Cpk, values are ≥ 1 .) Further, these functional prototypes are acceptable for assembly applications as the IT grades are $\leq IT12$.

Fig. 4 Rendered images of fibroblast cells and Ra profile **a** PLA, **b** PLA + HAp, **c** PLA + HAp + CS on NIH-3T3 cell line

Table 6 Percentage reduction in weight (based upon serum stability test)

Sample type	%Reduction in weight
PLA (virgin)	6.7
PLA + HAp	3.04
PLA + HAp + CS	0.12

- The PLA–HAp–CS-based 3D printed functional prototypes are acceptable from biocompatibility and bioactivity view point (as observed from Ra profile, serum stability test) and can be gainfully employed for commercial production in tissue engineering applications. Further, it has been ascertained that in place of PLA, PLA–HAp, synergic combination of PLA–HAp–CS is better solution from biocompatibility view point of functional prototypes.

Acknowledgements The authors are grateful to SERB under AISTDF Secretariat (File No. IMRC/AISTDF/R&D/P-10/2017, Dated 01-02-2018).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR (2006) Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. *Biomaterials* 27(18):3413–3431
- Harris AM, Lee EC (2008) Improving mechanical performance of injection molded PLA by controlling crystallinity. *J Appl Polym Sci* 107(4):2246–2255
- Gupta AP, Kumar V (2007) New emerging trends in synthetic biodegradable polymers—polylactide: a critique. *Eur Polym J* 43(10):4053–4074
- Gupta B, Revagade N, Hilborn J (2007) Poly (lactic acid) fiber: an overview. *Prog Polym Sci* 32(4):455–482
- Habraken WJEM, Wolke JGC, Jansen JA (2007) Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering. *Adv Drug Deliv Rev* 59(4–5):234–248
- Misra SK, Nazhat SN, Valappil SP, Moshrefi-Torbati M, Wood RJ, Roy I, Boccaccini AR (2007) Fabrication and characterization of biodegradable poly (3-hydroxybutyrate) composite containing bioglass. *Biomacromolecules* 8(7):2112–2119
- Poinern GEJ, Brundavanam RK, Fawcett D (2013) Nanometre scale hydroxyapatite ceramics for bone tissue engineering. *Am J Biomed Eng* 3(6):148–168
- Tayyebi S, Mirjalili F, Samadi H, Nemati A (2015) A Review of synthesis and properties of hydroxyapatite/alumina nano composite powder. *Chem J* 5(2):20–28
- Yang S, Leong KF, Du Z, Chua CK (2001) The design of scaffolds for use in tissue engineering. Part I. Traditional factors. *Tissue Eng* 7(6):679–689
- John KRS (2007) Biocompatibility of dental materials. *Dent Clin North Am* 51(3):747–760
- Wulf K, Teske M, Löbner M, Luderer F, Schmitz KP, Sternberg K (2011) Surface functionalization of poly (ϵ -caprolactone) improves its biocompatibility as scaffold material for bioartificial vessel prostheses. *J Biomed Mater Res Part B Appl Biomater* 98(1):89–100
- Ellingsen JE, Thomsen P, Lyngstadaas SP (2006) Advances in dental implant materials and tissue regeneration. *Periodontology* 2000 41(1):136–156
- Ramakrishna S, Mayer J, Wintermantel E, Leong KW (2001) Biomedical applications of polymer-composite materials: a review. *Compos Sci Technol* 61(9):1189–1224
- Caterson EJ, Nesti LJ, Li WJ, Danielson KG, Albert TJ, Vaccaro AR, Tuan RS (2001) Three-dimensional cartilage formation by bone marrow-derived cells seeded in polylactide/alginate amalgam. *J Biomed Mater Res* 57(3):394–403
- Salgado AJ, Coutinho OP, Reis RL (2004) Bone tissue engineering: state of the art and future trends. *Macromol Biosci* 4(8):743–765
- Chen H, Yuan L, Song W, Wu Z, Li D (2008) Biocompatible polymer materials: role of protein–surface interactions. *Prog Polym Sci* 33(11):1059–1087
- Andrady AL (1994) Assessment of environmental biodegradation of synthetic polymers. *J Macromol Sci Part C Polym Rev* 34(1):25–76
- Shastri VP (2003) Non-degradable biocompatible polymers in medicine: past, present and future. *Curr Pharm Biotechnol* 4(5):331–337
- Nair LS, Laurencin CT (2007) Biodegradable polymers as biomaterials. *Prog Polym Sci* 32(8–9):762–798
- Williams DF (2014) The biomaterials conundrum in tissue engineering. *Tissue Eng Part A* 20(7–8):1129–1131
- Andersson H, Van Den Berg A (2004) Microfabrication and microfluidics for tissue engineering: state of the art and future opportunities. *Lab Chip* 4(2):98–103
- Metcalfe AD, Ferguson MW (2007) Tissue engineering of replacement skin: the crossroads of biomaterials, wound healing, embryonic development, stem cells and regeneration. *J R Soc Interface* 4(14):413–437
- Zhao C, Tan A, Pastorin G, Ho HK (2013) Nanomaterial scaffolds for stem cell proliferation and differentiation in tissue engineering. *Biotechnol Adv* 31(5):654–668
- Hutmacher DW (2001) Scaffold design and fabrication technologies for engineering tissues-state of the art and future perspectives. *J Biomater Sci Polym Ed* 12(1):107–124
- Biondi M, Ungaro F, Quaglia F, Netti PA (2008) Controlled drug delivery in tissue engineering. *Adv Drug Deliv Rev* 60(2):229–242
- Gloria A, Causa F, Russo T, Battista E, Della Moglie R, Zeppetelli S, De Santis R, Netti PA, Ambrosio L (2012) Three-dimensional poly (ϵ -caprolactone) bioactive scaffolds with controlled structural and surface properties. *Biomacromol* 13(11):3510–3521
- Billiet T, Vandenhaute M, Schelfhout J, Van Vlierberghe S, Dubrue P (2012) A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. *Biomaterials* 33(26):6020–6041
- Faulkner A (2008) Medical technology into healthcare and society: a sociology of devices, innovation and governance. Springer, Berlin
- Lopes MS, Jardini AL, Maciel Filho R (2012) Poly (lactic acid) production for tissue engineering applications. *Procedia Eng* 42:1402–1413
- Vaezi M, Seitz H, Yang S (2013) A review on 3D micro-additive manufacturing technologies. *Int J Adv Manuf Technol* 67(5–8):1721–1754
- Jose RR, Rodriguez MJ, Dixon TA, Omenetto F, Kaplan DL (2016) Evolution of bioinks and additive manufacturing technologies for 3D bioprinting. *ACS Biomater Sci Eng* 2(10):1662–1678
- Singh R, Ranjan N (2017) Experimental investigations for preparation of biocompatible feedstock filament of fused deposition modeling (FDM) using twin screw extrusion process. *J Thermoplast Compos Mater*. <https://doi.org/10.1177/0892705717738297>
- Miar S, Shafiee A, Guda T, Narayan R (2018) Additive manufacturing for tissue engineering. In: Ovsianikov A, Yoo J, Mironov V (eds) 3D printing and biofabrication. Reference series in biomedical engineering, Springer, Cham, pp 3–54
- Roy A, Saxena V, Pandey LM (2018) 3D printing for cardiovascular tissue engineering: a review. *Mater Technol* 33(6):433–442
- Athanasios KA, Agrawal CM, Barber FA, Burkhart SS (1998) Orthopaedic applications for PLA–PGA biodegradable polymers. *Arthroscopy* 14(7):726–737
- Shalumon KT, Sowmya S, Sathish D, Chennazhi KP, Nair SV, Jayakumar R (2013) Effect of incorporation of nanoscale bioactive glass and hydroxyapatite in PCL/chitosan nanofibers for bone and periodontal tissue engineering. *J Biomed Nanotechnol* 9(3):430–440

37. Ranjan N, Singh R, Ahuja IPS (2018) Development of PLA-HAp-CS-based biocompatible functional prototype: a case study. *J Thermoplast Compos Mater*. <https://doi.org/10.1177/0892705718805531>
38. Ranjan N, Singh R, Ahuja IS, Singh J (2019) Fabrication of PLA-HAp-CS based biocompatible and biodegradable feedstock filament using twin screw extrusion. In: AlMangour B (ed) *Additive manufacturing of emerging materials*. Springer, Cham, pp 325–345
39. DeVor RE, Chang TH, Sutherland JW (1992) *Statistical quality design and control: contemporary concepts and methods*. Macmillan, New York, pp 542–544
40. Singh R (2011) Comparison of statistically controlled rapid casting solutions of zinc alloys using three dimensional printing. *Int J Autom Mech Eng* 3:293–305
41. Singh JP, Singh R (2008) Investigations for reducing wall thickness in low brass rapid casting using three dimensional printing. In: *Proceedings of international conference on advances in mechanical engineering (AME 2008)*, India, pp 878–883

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.