Structural, mechanical and in vitro studies on pulsed laser deposition of hydroxyapatite on additive manufactured polyamide substrate

Kuppuswamy Hariharan* and Ganesan Arumaikkannu*
Department of Manufacturing Engineering, College of Engineering, Guindy, Anna University, Chennai, India

Abstract: Additive manufacturing (AM) is an emerging field that merges engineering and life sciences to produce components that can effectively act as a replacement in the human body. This AM encompasses biofabrication using cells, biological or biomaterials as building blocks to fabricate biological and bio-application oriented substance, device and therapeutic products through a broad range of engineering and biological processes. Furthermore, bioactive coating on BAM surface facilitates biological fixation between the prosthesis and the hard tissue which increases the long term stability and integrity of the implant. In this paper, hydroxyapatite (HA) powder was coated over AM polyamide substrate using pulsed laser deposition. Coating morphology was characterised using scanning electron microscope (SEM) analysis and observed that the coating was dominated by the presence of particle droplet with different sizes. Compounds like tricalcium phosphate and a few amorphous calcium phosphates were found along with HA which was confirmed by X-ray diffraction (XRD) analysis. Fourier transform infrared spectroscopy (FTIR) techniques shows the presence of phosphate and carbonate groups in the HA structure. Nano-indentation and pull-out test reveals that the layer was strong enough and withstands higher load before it peels off. In vitro analysis was evaluated with human osteosarcoma MG-63 cells with respect to the cell viability and results shows that the good viability was observed on coated surface due to combinational effect of Ca²⁺ and PO₄³⁻ ions. The multitude of characterisation conducted on the coating has established that coating polyamide with HA results in a positive combination for an implant.

Keywords: bioadditive manufacturing, hydroxyapatite, polyamide, pulsed laser deposition, characterisation, cell line studies

*Correspondence to: Kuppuswamy Hariharan and Ganesan Arumaikkannu, Department of Manufacturing Engineering, College of Engineering, Guindy, Anna University, Chennai, India; E-mail: hariharancim28@gmail.com and arumai@annauniv.edu

Received: April 27, 2016; Accepted: June 13, 2016; Published Online: June 24, 2016


http://dx.doi.org/10.18063/IJB.2016.02.008.

1. Introduction

Material which is intended to interface with the living tissue to evaluate, treat or replace any tissue, organ or function of the body is known as a biomaterial[1]. The success of biomaterials in the body depends on factors such as surgical techniques, individual activities and conditions[2], the most important aspect of this biomaterial is biocompatibility, which is defined as the ability of the material to form a biological bond with the host tissue intended for a specific application[3]. The application of biomaterials is to fabricate 3D scaffolds for tissue engineering orthopaedic joints, soft contact lens, drug delivery and...
The success of implantation is reliant on the chosen biomaterial, design, mechanical properties and chosen method of fabrication. Conventional processes suffer from the following drawbacks: higher relative cost, inability to produce complex designs, poor customisation, immune rejection and difficulties in shaping bone grafts for bone defects. Current biomaterial research has focused on developing implants that are both customised and surface engineered to improve bone healing. An ideal implant necessitates customisation because it can mimic the original anatomy as closely as possible and increases osseointegration. The above said requirements of implants can be obtained by (i) direct designing of implants with data procured from CT/MRI scan, which provides a realistic designing of the implants and spatially correct images for preoperative diagnosis and surgical planning; (ii) fabricating such custom design model with many computer assisted manufacturing technology without any tooling or process planning.

Additive manufacturing (AM) is becoming an increasingly preferred technique in the field of medicine due to certain unique merits such as absence of physical tooling or process planning. The materials used for fabrication of bioproducts can be classified as biometals, bioceramics, biopolymers and biocomposites. Among these, biopolymers are most intensively investigated particularly for extracellular matrix tissue engineering, prosthetic devices and drug delivery applications. Polystyrene (PE), polyurethane (PU), polyamide (PA), polytetrafluoroethylene (PTFE), poly(methyl methacrylate) (PMMA), etc., are in biopolymers, since they have a wide range of mechanical, syntheses technique, degradable property and versatility. They are rapidly replacing other groups of biomaterials such as metals and ceramics. Among various polymeric materials, polyamide is an inert biocompatible polymer which is generally used to make 3D scaffolds, sutures and wound dressings. Although this material contains the same amide linkage found in polypeptides, their rate of biodegradation is so low that it is often reported as non-biodegradable. To improve the bioactivity of polyamide, bioceramic materials can be physically blended with polyamide or coated over the surface of the scaffold or implant made with polyamide. Selective Laser Sintering (SLS) of AM technique has an advantage of building implants from a variety of biomaterials especially biopolymers, as the process uses lasers that sinters selectively thin layers of powder according to the CAD data.

The surface of the implant is the first part which interacts with the host tissue, hence surface modifications are very essential to enhance the biocompatibility. Various techniques have been developed to enhance the compatibility of implants with the bone, such as grit blasting and acid etching of implant surface and coating with bioactive materials which is a more popular technique. Hydroxyapatite (HA) is a commonly used bioactive material to promote the compatibility of implants since it has proven that the material will provide good biocompatibility and good osseointegration. Coating with HA can be done by many techniques with desired features like surface chemistry, energy, roughness, morphology and crystallinity, which influences the cellular response to biomaterials. Techniques like plasma spraying, flame spraying, ion-beam process, biomimetic coating and a combination of these techniques are commonly used. However, these techniques are unable to fulfil the necessary features for enhanced cell growth, but pulsed laser deposition (PLD) yields almost high quality HA coating with uniform and dense layer and high adhesion to the substrate. The cell has a capacity to sense features like surface chemistry, size, elasticity, and topography of the implant surface and shows a change in proliferation and cellular differentiation. Cell behaviour can be enhanced by generating 3D surface features in the form of micro/nano grooves, pits, pin holes and random surface roughness which could stimulate cell attachment. The presence of functional groups like carboxylic acid (-COOH), amine (-NH2), hydroxyl (-OH), carbonate (-CO3), phosphate (-PO3), etc. at the surface favours the adsorption of the protein. In addition to other surface properties, mechanical properties of the thin film also play a significant role in vivo as most implant materials are found to fail due to cracking and decohesion leading to detachment. Such interaction between the implant and the surrounding environment occurring at the interface is investigated via the in vitro test. Cell line studies are commonly accepted for such investigations. Primary culture of cells on the surface is able to multiply or expand in vitro and can also be differentiated to synthesise an extracellular matrix which substantiates the biological investigation.

In this study, HA was successfully deposited on AM polyamide substrate using pulsed Nd:YAG laser in
infrared range (\(\lambda = 1060 \text{ nm}\)). Surface features like microstructure, topography, crystal structure, functional groups and mechanical properties like hardness, Young’s modulus and adhesion strength were investigated along with cell response to the coated surface. Generally large sized particles and hard coating will be evident when the film was formed using Nd:YAG laser in infrared range, which in turn favours the biocompatibility by bone-implant anchoring\[44\].

2. Materials and Methods

2.1 Fabrication of Substrate

A 3D CAD model with a dimension of 25 \(\times\) 25 \(\times\) 3 mm was designed and the data was sliced into layers because AM fabricates parts in layer by layer. The part has been built using SLS technique (EOS FORMIGA P100). SLS fuses thin layers of polyamide powder (EOSINT P/PA2200)\[45\] which has been spread across the build area using a counter-rotating powder leveling roller or blade. The part building process takes place inside an enclosed chamber and to minimise oxidation and degradation due to atmospheric gases, nitrogen was allowed to flow inside the chamber. The powder in the build platform was maintained at a temperature just below the melting point or glass transition temperature of the polyamide material and it was preheated using infrared heater which was placed above the build chamber as well as the powder feed chamber. This heater was generally used to maintain the elevated temperature throughout the fabrication process, which minimises the laser power requirements of the process, with pre-heating, less laser energy is required for fusion and could prevent warping of the part during the build due to non-uniform thermal expansion and contraction (curling). Once appropriate preheating of the powder was done, a focused laser beam is directed onto the powder bed and moved according to the CAD design to thermally fuse the material to form the sliced cross-section. Surrounding un-sintered powders act as a support structure to the part and eliminate the external secondary powder to use as a support material. After completing a layer, the build platform is lowered according to the defined layer thickness and a new layer of powder is laid and levelled using the counter-rotating roller. The laser beam scans the subsequent slice cross-section. This process repeats until the complete part is built. Once the part has been completed, a cool-down period is usually required to allow the parts to uniformly draw closer to a low and adequate temperature that they can be handled and exposed to ambient temperature and atmosphere. Finally, the parts are removed from the powder bed and loose powder is cleaned off from the parts\[46,47\].

2.2 Preparation of Hydroxyapatite Target for Coating

The chosen coating material, HA, was synthesised via wet chemical precipitation process. It involves stirring 0.5 mol/dm\(^3\) of calcium hydroxide [Ca(OH)\(_2\)] for a period of 30 min in 1000 mL distilled water. Ammonium phosphate [(NH\(_4\))\(_3\)PO\(_4\), 0.3 mol/dm\(^3\)] is stirred in the same manner in another solution of 1000 mL distilled water and this is added in drops to the Ca(OH)\(_2\) solution. After a couple of hours of preparation, maintained at a pH level at 7 and above, a gelatinous precipitate is obtained. In order to extract the HA powder, the precipitate is calcined at 100°C for 5 h\[48\]. The powder is then hydraulically pressed at 400 MPa to form a 25 mm diameter deposition target that is further furnace sintered at 800°C for a period of 5 h.

2.3 Pulsed Laser Deposition

Polyamide was pulsed laser deposited with HA using Nd:YAG laser (Quanta Laser, USA) consisting a wavelength (\(\lambda\)) of 1064 nm and energy of 135 mJ. After evacuating the deposition chamber with a base pressure of 10\(^{-5}\) Torr, the laser beam was brought to focus on the rotating target at an incident angle of ~45° for deposition. The laser beam scans the continuously rotating target to serve three purposes: (a) minimisation of craters formation, (b) reduction of undesirable melting and (c) reduction in re-solidification. The rotation of the target also helps enhance the erosion rate. While maintaining the substrate at a distance of 4.5 cm from the target and at a temperature of 150°C the HA layer deposition took place at a period of 30 min.

2.4 Film Characterisation

The microstructure of the coated surface was examined using field-emission scanning electron microscopy (CARL-ZEISS Supra 40VP, FE-SEM) and energy dispersive X-ray spectroscopy (EDX) (Oxford Instruments). Analysis was carried out to find the stoichiometry of the coated layer for 60 s and electron beam energy of 15 keV. Atomic force microscope (AFM) (NTMDT, Ireland) analysis was carried out at an ambient pressure, room temperature and humidity. AFM images were prepared with non-contact tapping.
mode and the maximum scanning area was $5 \times 5 \, \mu m$ and $10 \times 10 \, \mu m$. The X-ray diffraction (PANalytical X-Pert Pro) measurement was performed to observe the crystalline phases in the coated layer. The presence of functional groups was detected using a FTIR Spectrometer (Josco Instruments, Japan). The hardness and Young’s modulus of HA layer was identified using nanoindentation (CSM instruments, USA). According to ASTM F1501-94, pull-out based adhesion strength of the HA layer was observed using a DeFelsko pull-off adhesion tester.

2.5 In Vitro Analysis

2.5.1 Cell Culturing

The cellular response of coating was assessed in terms of cell viability and proliferation to the surface. MG-63 osteosarcoma cell was used in this study and it is suitable for screening a large number of samples for cytotoxic compound and also used in the rapid evaluation of the biomaterial surface qualities. Cells were grown in 75 cm$^2$ flasks containing Dulbecco’s modified Eagle’s medium (DMEM; Sigma). The media were supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1.5 g/L sodium bicarbonate, 10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B. Cells were cultured as monolayers in culture flasks at 37°C under a humidified atmosphere of 5% CO$_2$.

2.5.2 Cell Seeding on Hydroxyapatite Coated Substrate

The coated substrate was sterilised with 70% ethanol for 30 min and autoclaved for 30 min, followed by drying at room temperature for 2 h. Cells were seeded approximately $1 \times 10^5$ cells/sample. Cell proliferation and viability was assessed and observed at specific time periods at 1, 7 and 15 days respectively.

2.5.3 Cell Viability

The viability of cells was assessed by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Only live cells are able to take up the tetrazolium salt. The enzyme (mitochondrial dehydrogenase) present in the mitochondria of the live cells converts internalised tetrazolium salt to formazan crystals, which are purple in colour. Then the cells were dissolved in DMSO solution. The colour developed is then determined in an ELISA reader at 570 nm of UV-absorbance wavelength.

Data were analysed using the student $t$-test. Results were considered statistically significant when the $P$ value was less than 0.05 ($P < 0.05$). Results were displayed as the mean ± standard deviation.

3. Results and Discussion

3.1 SEM with EDX Analysis

From Figure 1A, it was evident that the layer was composed of dense droplet-like spherical particles of non-uniform size. Some isolated particles were also identified on the layer, and this was due to scattering or spurious melting of target material resulting in globular particles present on the coating. At higher magnification a cluster (agglomeration) of nanoparticles (Figure 1B) can also be observed. At 135 mJ of laser energy, particles were ablated explosively and there are possibilities to ablate bigger particles directly from the target [49] and forming a layer with some isolated pin holes and voids which in turn results in a heterogeneous rough surface. Few literatures demonstrated that having a rough surface will stimulate better osteointegration [50,51].

Figure 2A shows a typical texture of deposited surface. The layer of HA has randomly distributed globular particles with a thickness of 1.6 µm and has a growth rate of 53.34 nm/min (as per Equation 1). Generally, at higher laser energy, the kinetic energy of ablated atom increases and the number of surface nucleating atom also increases and hence it shows the faster rate of deposition. Figures 1C and 2B shows the EDX spectrum and EDX mapping of surface and cross section respectively. The spectrum reveals the presence of calcium and phosphate at a ratio of 1.7 which is nearer to the ratio of standard HA. Moreover, the dense distribution of such ions was observed in the EDX mapping at the cross section.

$$\text{Growth rate} = \frac{\text{Layer Thickness (nm)}}{\text{Coating Duration (min)}}$$  \hspace{1cm} \text{Equation (1)}

3.2 AFM Analysis

The 3D morphology of HA layer obtained by AFM is shown in Figure 3. A rougher texture relating heterogeneity can be observed in Figure 3A, due to a mixed mode of particle size and agglomeration of larger particles in a number of valleys and some sharp tiny
peaks were observed with an average surface roughness (Ra) of 51.38 nm. If a scanning area was increased to 10 × 10 µm (Figure 3B), a bright plateau like structure can be seen. Moreover, a clear picture of randomly agglomerated particle can be observed in this 3D morphology which accruing for rougher texture (peaks and valley height) with a roughness of 124.35 nm. This increase in roughness clearly gives an idea that the coating was non-uniform as attributed in Figure 2.

### 3.3 XRD Analysis

The crystalline structure and the phase composition of the layer were assessed using XRD (Figure 4). The diffraction pattern [Figure 4 (inset)] confirms that the formation of HA with sharp diffraction at a 2θ value of 32.62°, 34.58° and 36.47° corresponds to the relative intensity of diffraction of standard data (ICSD 087727). Furthermore, sharper and narrow peaks indicate that the layer reaches maximum crystallinity.
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Figure 3. AFM image of HA layer. (A) 5 × 5 µm scanning area; (B) 10 × 10 µm scanning area.

Figure 4. XRD diffraction.

Figure 5. FTIR spectra of HA coating.

which may facilitate better bone-tissue integration and osseointegration [52]. Substrate effect has been observed at 2θ = 21.24° due to some voids and pores on the coated surface.

3.4 FTIR Analysis

The FTIR spectra of HA deposited on polyamide by PLD is shown in Figure 5. As there is no standard IR spectra for HA coating, the spectra was obtained between 400 to 4000 cm⁻¹. The observed bands in the spectra are due to the vibration mode of phosphate (PO₄³⁻) groups from 596 to 1038 cm⁻¹. Asymmetric bending (ν₄) and stretching (ν₃) of phosphate group appears at 596 cm⁻¹ and 1038 cm⁻¹ respectively, whereas a symmetric stretching (ν₁) appeared at 1011 cm⁻¹. For carbonate (CO₃²⁻) group, two bands at 1462 and 1536 cm⁻¹ were observed for asymmetric stretching (ν₃) and a strong peak at 716 cm⁻¹ corresponding to asymmetric bending (ν₂). The polyamide substrate gives three bands that appear arbitrarily at 1646, 2843 and 2945 cm⁻¹ [53]. The sharp water band at 3745 cm⁻¹ was observed in the IR spectra. A shoulder peak at 610 cm⁻¹ corresponds to the hydroxyl (-OH) stretching frequency indicating the presence of hydroxyl group in the laser deposited HA coating.

3.5 Nanomechanical Analysis

3.5.1 Nanoindentation

Hardness (H) and Young’s modulus (E) of deposited HA layer was measured using nanoindentation. Such technique is capable of measuring the hardness at loads in micron-Newton range. The indentation test was carried out with loads of 0.5 mN, 1 mN, 3 mN and 5 mN. The load displacement curve (Figure 6) was recorded for calculating hardness (H) and Young’s modulus (E). Penetration depth at submicron level was observed for HA coating at various loading conditions. Sharp loading and unloading curve was seen for all the loading conditions but at a high range of 3 mN and 5 mN loads, some pop-in was observed. It is believed that this is due to the formation of lateral cracks, voids or pores in the surface. A typical displacement was observed in 3 mN curves which were attributed to linear strain during the indentation. Furthermore, at 5 mN loading, the displacement was minimal when
compared to the 3 mN load and shows the perfect brittle nature (refer to E/H ratio in Table 1). E/H ratio is the ratio of Young’s modulus to hardness of the coating. This ratio tells about the brittle and ductile nature of the coated film. Experimentally determined H and E were listed in Table 1.

### Table 1. Young’s modulus and hardness of HA coating

<table>
<thead>
<tr>
<th>Load (mN)</th>
<th>Young’s Modulus (E) (GPa)</th>
<th>Hardness (H) (GPa)</th>
<th>E/H ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mN</td>
<td>9.66</td>
<td>1.72</td>
<td>5.61</td>
</tr>
<tr>
<td>1 mN</td>
<td>8.881</td>
<td>1.55</td>
<td>5.72</td>
</tr>
<tr>
<td>3 mN</td>
<td>7.28</td>
<td>1.22</td>
<td>5.96</td>
</tr>
<tr>
<td>5 mN</td>
<td>6.22</td>
<td>1.46</td>
<td>4.26</td>
</tr>
</tbody>
</table>

#### 3.5.2 Pull-out Adhesion Test

In the view of successful implantation and long term stability of any implants, mechanical properties are very important because the implant requires stable adhesion strength to withstand the interlocking of bone and implant. According to ASTM F1501-94, pull-out based testing method was used to determine the tensile adhesion behaviour of coating. The component to be tested was bonded with a polymeric adhesive (in this work, epoxy was used) and left to cure for 24 h, as was shown in Figure 7A. Tensile load was applied normally to the plane of coating and the adhesion strength was measured till the coating gets peeled off from the substrate and the load determines the maximum adhesion strength of coating. Figure 7B shows the adhesion strength for HA coating and five measurements have been made to avoid the random error and the average adhesion strength was found to be 13.34 ± 2 MPa. Most of the samples seem to be peeled off in the epoxy itself (glue failure) and some got struck with the polyamide layer, which penetrate in pin holes and increases the adhesion strength.

#### 3.6 In Vitro Cell Response

*In vitro* analysis of HA coated substrate was carried out by measuring the viable cells seeded on the substrate at a time interval of 1, 7 and 15 days. Figure 8 shows that the cells can grow more effectively on the coated surface. From the day 1 observation, cell growth on the coated substrate was slow due to low cell-substrate interaction. The amount of cells on the coated substrate increases significantly from 38% to 76% from the 1st to the 15th day. The better cell viability may be attributed to the combination effect of Ca²⁺ and PO₄³⁻ ions, surface roughness and crystallinity of the HA layer and it is understood that the cell-substrate interaction directly corresponds to that of organic and inorganic interface.

#### 4. Conclusion

In this experimental work, synthesised HA powder was successfully deposited over AM polyamide substrate using PLD technique. Several characterisation
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Studies reveal that the layer was composed of spherical shaped particles of different sizes. Due to scattering or spurious melting of the target, most of the particles are deposited as droplets and an agglomeration of nanocrystalline HA was seen. This heterogeneity increases the surface roughness. The agglomerated rough particles favour the better bone tissue integration. A higher percentage of crystalline HA was also observed along with the substrate peak. Ca/P ratio of the deposited HA was reasonably close to that of standard HA. Phosphate and carbonate content were induced during the deposition process along with some organic groups. The layer shows considerably higher hardness and modulus value with good adhesion property. In vitro results reveal that the HA coated layer shows a better cell viability. These coated implants are safe, efficacious and cost effective and they can be used in orthopaedic and dental application for fixing fractures, spinal reconstruction and soft tissue anchorage.

References


http://dx.doi.org/10.1111/j.1600-0501.2009.01777.x


http://dx.doi.org/10.1016/S1534-5807(04)00075-9


http://dx.doi.org/10.1016/j.cell.2006.06.044


http://dx.doi.org/10.1038/nmat2013


http://dx.doi.org/10.1007/978-0-387-98161-1_1


http://dx.doi.org/10.1073/pnas.0603216103


http://dx.doi.org/10.5402/2012/208760


http://dx.doi.org/10.1016/j.polymertesting.2009.06.010


http://dx.doi.org/10.1016/j.matchemphys.2004.02.009


http://dx.doi.org/10.1016/j.apsusc.2012.10.072


http://dx.doi.org/10.1007/s10856-011-4342-3


http://dx.doi.org/10.1016/j.biomaterials.2011.01.029


http://dx.doi.org/10.3390/ma3073994


http://dx.doi.org/10.1590/S1516-14392009000200012