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# Development of biocompatible coating on Ti6Al4V implant materials using chitosan extracted from shellfish waste

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ABSTRACT

The present study focuses on extracting chitosan from crustacean shells. The as-prepared chitosan was used to develop a uniform thin layer coating on titanium alloy (Ti6Al4V) implant material. Shellac, a natural resin was used intermediately for providing better adhesion. Taguchi optimization technique was used to optimize the control parameter values. Dipping duration and concentration of shellac were considered as control parameters and adhesion strength & thickness of the coating were considered as response characteristics. The concentration of shellac significantly affected the adhesion strength, whereas the thickness increased with an increase in dipping duration. Cytotoxicity tests and corrosion studies were performed on the samples coated with optimal values. The chitosan-coated sample was found as non-toxic and also showed better corrosion resistance.

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## 1. Introduction

Research for finding new biocompatible material for orthopedic applications is gaining momentum. A biocompatible material with decent durability and reliability still finds ample scope. Along with that, the metal implant industry is growing exponentially and the demands for long-lasting implants are on a high. Numerous numbers of studies have been carried out to develop a thin film of biocompatible coating on metal implants. Studies have shown that a metal implant coated with a thin film of biocompatible material promotes the osseointegration process [1,2]. Chitosan is a biocompatible material is gaining prominence very lately. The raw materials required for preparing chitosan are abundantly available. Chitosan is processed form of chitin. After cellulose, Chitin is considered as the second most abundantly available polysaccharide. Chitin $(C_8H_{13}O_5N)_n$ is a long-chain polymer of N-acetylglucosamine, a derivative of glucose and when deacetylated it becomes chitosan [3]. The major source of chitin is shellfish waste. Processed food related to shellfish is very prominent near the coastal belt regions. The processing of shellfish generates tons of waste. Only a small portion of the fish meat is used, the remaining is discarded as waste. These wastes are generally dumped in landfills or open spaces causing enormous health hazards to the human body. Obnoxious smell, pathogenic activity, and rodent attack are few other major problems because of Shellfish waste. Statistics show that the average shellfish waste generated per year is estimated to be around 80,000 tons [4].

Chitosan has many remarkable intrinsic properties. Its antibacterial, antimicrobial, and antifungal properties make it a suitable candidate for the medical purpose [5–7]. Studies have been carried to assess the biocompatibility of the material [8]. Many studies have analyzed chitosan's use as an effective biomaterial. Scaffolds with chitosan alone and a combination of other biocompatible materials with chitosan too have been synthesized and analyzed. Scaffolds in the form of hydrogels are prepared from chitosan and is used for repairing tissue damages. It is also capable of promoting cell attachment and proliferation [9]. Generally, PMMA (polymethylmethacrylate) is used as bio cement in orthopedic applications and it can cause damages to surrounding bone tissues. The high heat generated during the polymer hardening process causes tissue damage. PMMA can also cause aseptic loosening at the orthopedic joints. A bio-cement of tri-calcium phosphate and chitosan was prepared by the researchers to overcome these problems faced by usual types of cement [10]. Studies have shown that

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scaffold prepared by a combination of chitosan and alginate promotes cell growth and proliferation at a faster rate [11]. Composite scaffolds made of HA/β-TCP/chitosan were prepared by researchers with improved mechanical strength for more load-bearing orthopedic applications. The in-vivo and in-vitro tests confirmed that these composites too promote cell growth and proliferation [12].

Though many methods were incorporated to impart mechanical strength to the scaffolds prepared with chitosan alone and chitosan composite scaffolds, none of them could be used for high load-bearing orthopedic applications. Bio-compatible metal implants are coated with bioactive materials for promoting osseointegration. Chitosan-coated metal implants to have been studied extensively. Generally, metals like Stainless steel, Cobalt-Chromium alloys, commercially pure titanium, and titanium alloys are used as implants. These metals are having good mechanical properties and are biocompatible [13]. Studies have shown that chitosan-coated titanium implants showed little dissolution and promoted cell growth. Though the bond strength measured between the coating and the titanium substrate was much less when compared to other bioactive coatings developed, it still seemed to be a promising method [14]. Studies have shown that failure of implantation is more for diabetic patients and that can be overcome by using chitosan-coated porous titanium implants [15]. A bio-composite coating of chitosan/polyethylene oxide/bioactive nano-glass fiber was applied on titanium implants and studies showed that the antibacterial and osteoconductive properties of the coated implant improved significantly [16].

This paper mainly focuses on developing a chitosan coating on Ti6Al4V alloy using a dip-coating process. Chitosan coating developed on metal implants has poor adhesion strength. Another ceramic coating attains better adhesion strength while exposed to high temperatures. Chitosan on the other hand starts degrading at a temperature more than 150 °C [17]. And so a unique method of improving adhesion strength of the developed chitosan coating using an intermediate layer of shellac is investigated in this paper. Shellac is a natural bio-resin secreted by a female lac bug found commonly on trees of forests in India and Thailand [18].

## 2. Materials & methodology

The chitosan required in this research work was extracted from crustacean shells. The other raw materials required for the preparation of chitosan powders are HCl and Caustic soda. Crustacea consists of aquatic animals living in fresh, brackish, and marine environments. Some are active and nektonic, while others are benthic, living on sea bed. Certain crustaceans are parasitic. In the commercial production of chitin, wet and dried exoskeletons of crustaceans alone are used. The commercial extraction of chitosan is a removal process of constituents other than chitin from the crustacean waste. Three stages of chitosan preparation are given below.

De-mineralization: Removal of carbonates and phosphates from the raw shell

De-deproteinization: Removal of protein from the demineralized shell to get Chitin

De-acetylation: Removal of an acetyl group from chitin.

The Crystallinity of the as-prepared chitosan was analyzed using XRD (Bruker AXS D8) machine. The diffraction pattern was recorded over a 2θ range of 5° to 85°. For preparing the chitosan solution (0.01% m/v) 0.1 g of the as-prepared chitosan was mixed with 10 ml of acetic acid and 1L of distilled water. Magnetic stirring was performed for approximately 1 hr [19]. The Shellac solution was prepared by dissolving natural shellac flakes in pure ethanol. Three different concentrations of shellac solutions were prepared. 12.5%, 13.0% and 13.5% (w/w) of shellac was used to prepare the solution. The dip-coating process was performed on a custom-made dip coating apparatus. The sample was first dipped in the shellac solution. The dipping duration was varied from 15 to 35 s. Subsequently, the sample was dipped in the chitosan solution for 15 s, which was kept constant throughout the experiment. The range for coating parameters was fixed after the pilot study.

Optimization of the coating parameters was carried out using the Taguchi optimization technique. An L9 array was selected for this purpose. Dipping duration and shellac concentration was fixed as the control factors and adhesion strength of the developed coating and thickness of the coating was selected as the response. Table 1.Table 2. The adhesion strength was measured as per ASTM D 7234 standard and the thickness was measured as per IS- 3203–2001. Signal-to-noise ratio, Analysis of Variance (ANOVA), and regression analysis were performed to find out the optimal solution. *Larger-the-better* was selected for adhesion strength response and a *smaller-the-better* was selected for thickness response characteristics as the objective is to maximize the adhesion of the coating onto the substrate by keeping the thickness of the coating minimal.

Samples developed by optimal coating parameters were subjected to cell viability and corrosion studies. Cell viability studies of the coated samples were carried out. The samples were ETO sterilized and the entire study was performed as per ISO 10993–12. MG-63 human osteosarcoma cell lines were used for the study. The cell lines were procured from National Centre for Cell Science (NCCS, Pune, India). As per the standard protocol, the cells were cultured with Dulbecco's modified eagle medium (DMEM), 10% fetal bovine solution (FBS), and incubated at 37 °C in 5% $CO_2$ for 24 hrs. Sterilized samples were immersed in the medium without shaking for 72 h and maintained at 37 °C to prepare the extract medium. This medium was then diluted with the culture medium at 12.5%, 25%, 50%, and 100%. MTT assay was used to determine the in vitro cytotoxicity and a 96 well plate was used for the same. After the cell attachment process was complete, the medium was treated with 100 μL of serially diluted extract solution.

The 96 well plates were then incubated at 37 °C for 24hrs. 20 μL of MTT reagent and 180 μL of complete medium were used for further incubation. The plates were then again incubated for 4 hrs in 5% $CO_2$ incubator. After the entire incubation process was completed 100 μL of DMSO (solubilizing agent) & 100 μL of absolute ethanol were slowly added to each well by micro-pipet. 20 min time period was provided for the formazan crystals to dissolve. The TECAN microplate reader (Thermo Fisher Scientific) was used to measure the absorbance value at 595 nm.

A potentiodynamic polarization test was used for analyzing corrosion. The corrosion test was conducted in the simulated body fluid (SBF). The SBF was prepared using kokubo methodology. The bare Ti6Al4V metal implant sample (uncoated) and the chitosan-coated sample were analyzed in the simulated body fluid. Inductively Coupled Plasma –Mass Spectrometry (ICP-MS) analysis was used to measure the ion concentration (in ppm) of SBF.

The electrochemical behavior was measured following the ASTM standard G5-94. A three-electrode electrochemical cell of 250 ml capacity was used for in vitro potentiodynamic corrosion tests. Platinum was used as the counter electrode, saturated calomel electrode (SCE) as the reference electrode, and the samples were kept as the working electrode. Some other studies have explored the scope of using Ag/AgCl as a reference electrode. The as-prepared SBF is used as the electrolyte and during the corrosion test, the temperature of SBF was maintained at 37 °C to simulate the body conditions. 1 $cm^2$ area of samples was exposed to the electrolyte. The samples are kept in contact with the solution till a steady open circuit potential (OCP) was obtained. The electrode

**Table 1**
Control factors and its different levels.

| Control Factors | Denoting symbols | Experiment Levels | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| Dipping Duration (Seconds) | $y_1$ | 15 | 25 | 35 |
| Concentration of Shellac (w/w %) | $y_2$ | 12.5 | 13.0 | 13.5 |

**Table 2**
Standard L9 orthogonal array.

| Sample Code | | |
|---|---|---|
| Experiment Levels | 1 | |
| 1 | 1 | |
| 2 | 1 | 2 |
| 3 | 1 | 3 |
| 4 | 2 | 1 |
| 5 | 2 | 2 |
| 6 | 2 | 3 |
| 7 | 3 | 1 |
| 8 | 3 | 2 |
| 9 | 3 | 3 |

**Fig. 1.** Chitosan powder extracted from crustacean shells.

potential was raised from −1000 mV to 1000 mV. The scanning rate was 0.005mVs$^{-1}$. The potentiodynamic polarization curve was constructed for the coated and uncoated samples and the equivalent corrosion current densities were determined by Tafel extrapolation.

## 3. Results & discussions

Fig. 1 shows the powder extracted from crustacean shells and it was observed that the powder was having an off-white color. The powder thus extracted was found to be crystalline as can be seen in Fig. 2. The chitosan peaks were identified between 2θ value of 19° and 20°. Previous studies conducted on chitosan too confirm this result [20]. A few studies have shown that chitosan tends to exhibit an amorphous structure as well [21].

The results of adhesion strength and thickness of the chitosan-coated samples by varying the dipping duration and concentration of shellac are presented in Table 3. From the mean effects plot shown in Fig. 3, it can be observed that sample 6 (with 25 s dipping duration and 13.5% w/w of shellac concentrations) gives the maximum adhesion strength and Sample 1 (with 15 s dipping duration and 12.5% w/w of shellac concentrations) gives the minimum thickness. From the results and the graph, we can infer that with increasing dipping duration the thickness to increases.

Table 4 & 5 shows the analysis of variance (ANOVA). From the ANOVA table, it is seen that concentration of shellac plays a dominant role in the case of adhesion, whereas thickness is affected by dipping duration. The contribution of concentration of shellac towards adhesion is 77.46% and the contribution of dipping duration towards thickness is 94.97%. The P-value in the ANOVA table signifies the contribution of each control parameter.

between the control parameters and response characteristics. This model could also be used to predict the optimal values within a range of control parameters. Following are the regression equation for each response characteristic.

$$AdhesionStrength = -40.03 + 0.0467\ y_1 + 3.333\ y_2 \quad (1)$$

$$Thicknessum = -66.0 + 12.00\ y1 + 5.00\ y2 \quad (2)$$

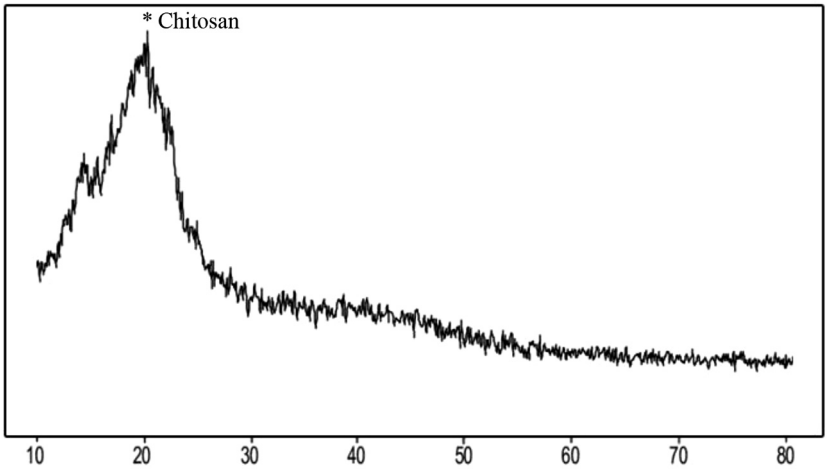


**Fig. 2.** XRD of as-prepared Chitosan.

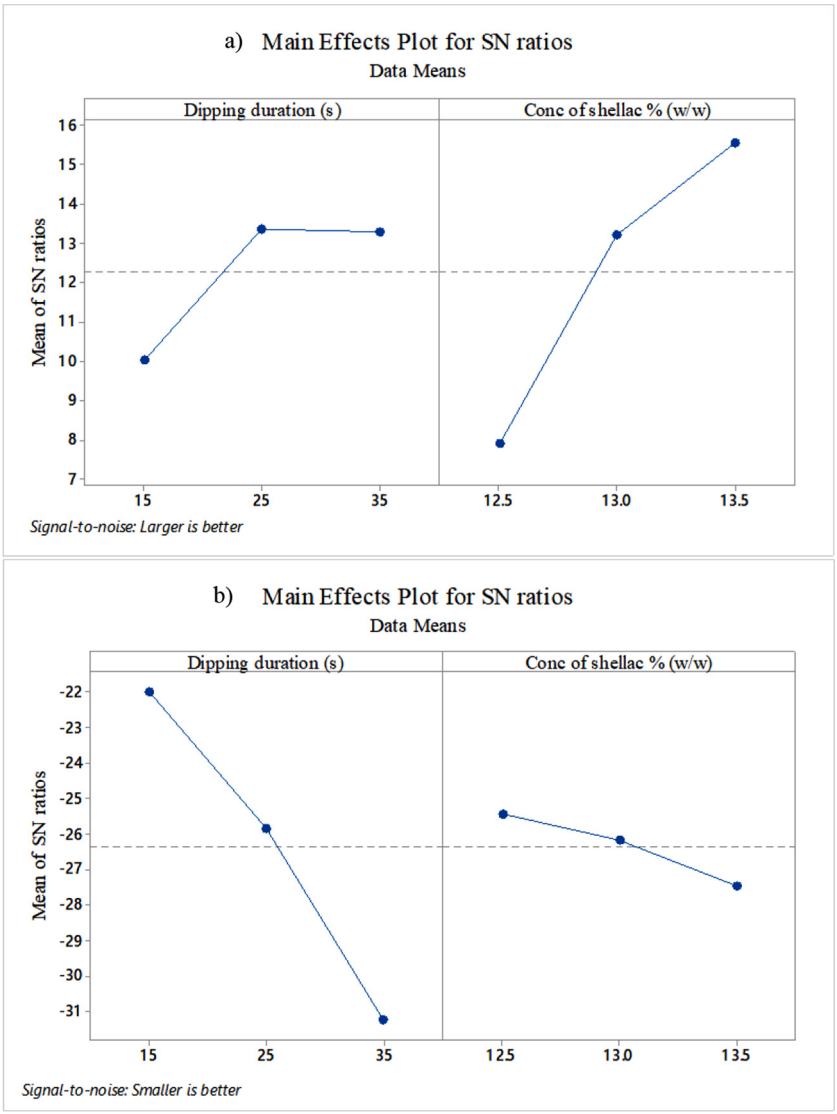


**Fig. 3.** Effect of coating parameters on a) Adhesion strength b) Coating Thickness.

**Table 3**
Experimental results with signal-to-noise ratio (S/N) ratio.

| Sample Code | Experimental results | | S/N Ratio | |
|---|---|---|---|---|
| | Adhesion Strength (Mpa) | Thickness (μm) | Adhesion Strength (Mpa) | Thickness (μm) |
| 1 | 0.9 | 8 | 2.278 | −20.827 |
| 2 | 4.1 | 9 | 13.064 | −21.583 |
| 3 | 5.1 | 12 | 14.807 | −23.521 |
| 4 | 2.8 | 15 | 10.103 | −25.105 |
| 5 | 3.7 | 15 | 13.064 | −25.575 |
| 6 | 6.3 | 17 | 17.025 | −26.848 |
| 7 | 2.9 | 28 | 11.364 | −30.370 |
| 8 | 4.0 | 32 | 13.624 | −31.364 |
| 9 | 4.8 | 28 | 14.96376 | −32.669 |

**Table 4**
ANOVA results for adhesion strength.

| | Degrees of Freedom | Sum of Squares | Mean Square | F-Value | P-Value |
|---|---|---|---|---|---|
| Dipping duration (Sec) | 2 | 2.287 | 1.1433 | 1.77 | 0.282 |
| Concentration of shellac (% w/w) | 2 | 16.747 | 8.3733 | 12.95 | 0.018 |
| Error | 4 | 2.587 | 0.6467 | | |
| Total | 8 | 21.620 | | | |

**Table 5**
ANOVA results for coating thickness.

| | Degrees of Freedom | Sum of Squares | Mean Square | F-Value | P-Value |
|---|---|---|---|---|---|
| Dipping duration | 2 | 130.400 | 65.1999 | 493.41 | 0.000 |
| Concentration of shellac (% w/w) | 2 | 6.373 | 3.1863 | 24.11 | 0.006 |
| Error | 4 | 0.529 | 0.1321 | | |
| Total | 8 | 137.301 | | | |

The $R^2$ value of Eq (1) & (2) is 77.51% & 92.40% respectively. The $R^2$ value is an indication of the capability of the mathematical model developed. Generally for an experimental study $R^2$ value greater than 75% is considered good. The normal probability plots are plotted for analyzing how residual errors are distributed. From Fig. 4 it is observed that most of the residual errors are lying near the straight line indicating that the coefficients of the mathematical model are significant.

Contour plots are shown in Fig. 5. It explains the relationship between the control parameters and response characteristics. From the plots, it can be inferred that better adhesion strength can be obtained if we dip the sample for 20 to 30 s and if the shellac concentration is kept at 13.5% w/w. In the case of adhesion, it is seen that minimum thickness can be obtained when we reduce the dipping duration as well as the shellac concentration.

The samples developed with optimal control parameters were then made to undergo cytotoxicity test as well as the corrosion test. The cell viability was calculated for different proportions as can be observed in Fig. 6. It is observed that cell viability is more than 50% at dilution proportions of 12.5%, 25%, 50%, and 100%. The cell attachment and proliferation were

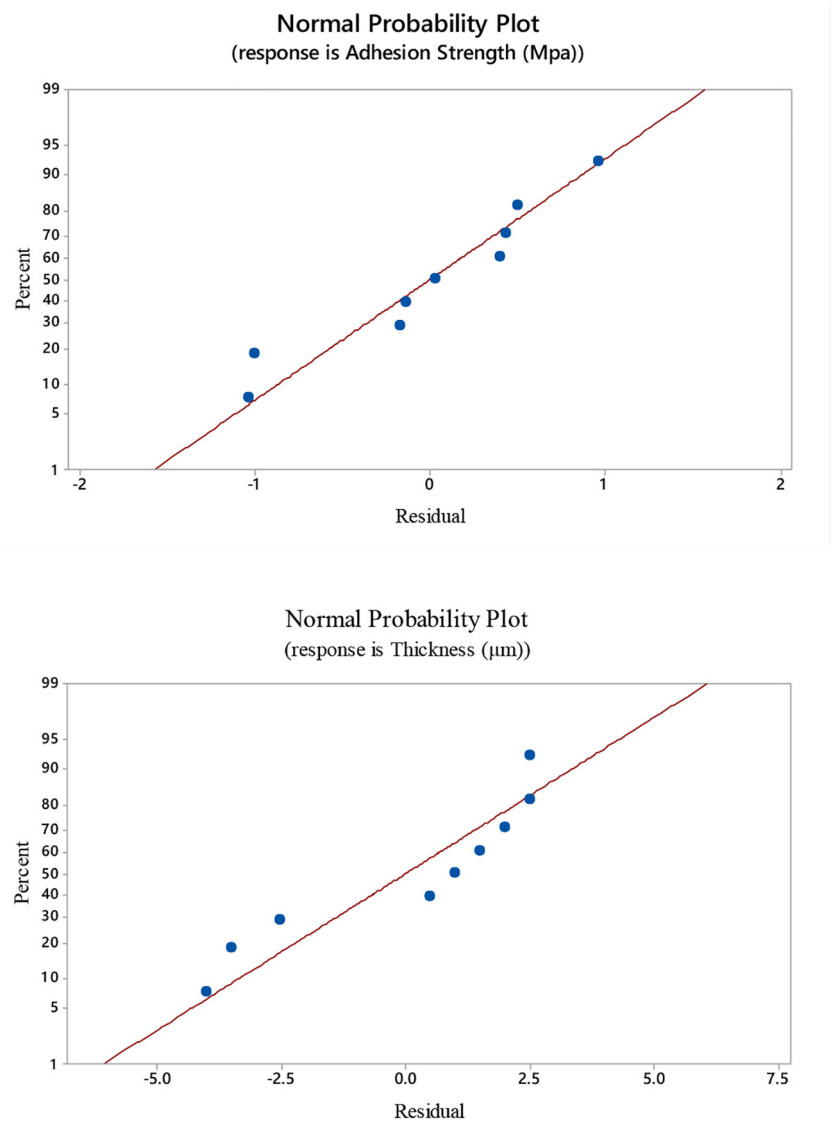


**Fig. 4.** Normal probability plot of residuals for adhesion strength & coating thickness.

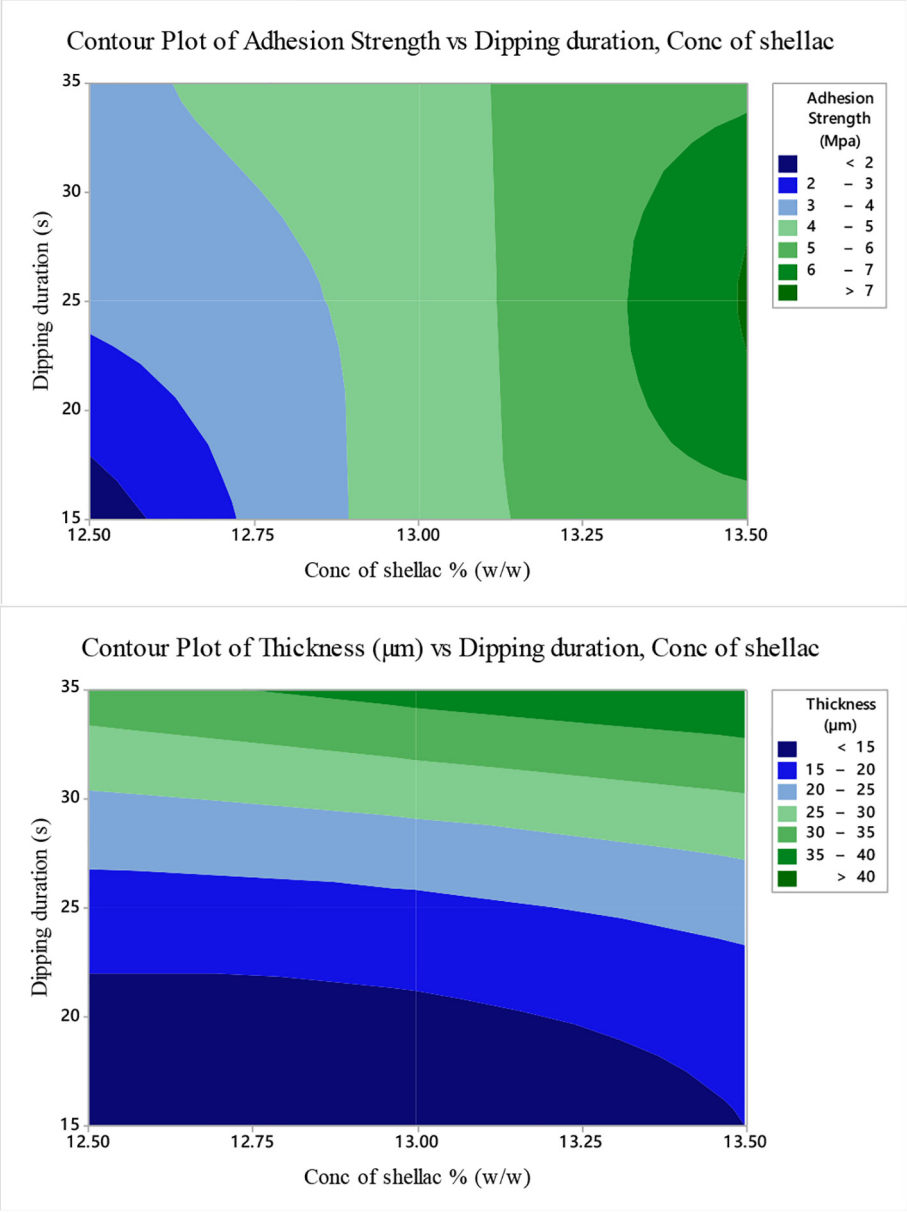


**Fig. 5.** Contour plot for Adhesion strength and thickness v/s Dipping duration & Concentration of Shellac.

observed for 24 hrs, 48 hrs, and 72 hrs and were found good. The cell attachments on the extract prepared from the chitosan-coated sample mixed with different proportions of culture medium are shown in Fig. 7. A steady increase in the number of cells was observed after 72 hrs time period which indicated good cell proliferation.

The samples were also subjected to corrosion studies in the prepared SBF. The SBF was found to have almost the same ion concentration as that of human blood plasma. The potentiodynamic polarization curve of uncoated and chitosan-coated samples are shown in Figs. 8 & 9. The $I_{corr}$value of the uncoated sample is $7.78 \times 10^{-6}$ and that of chitosan-coated is $5.38 \times 10^{-7}$. Lower the value of $I_{corr}$, better the corrosion resistance. Here the chitosan-coated samples are showing better corrosion resistance and hence it can also be inferred that the coating is stable and uniform. The thin layer of coating acts as a protective layer for the sample surface [22].

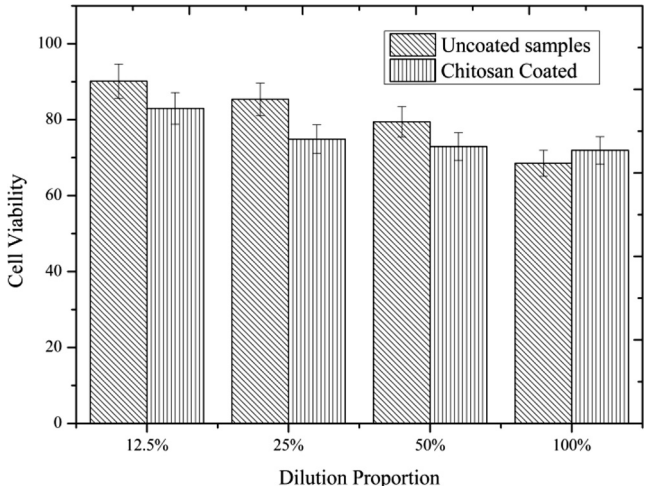


**Fig. 6.** Cell Viability for different dilution ratios.

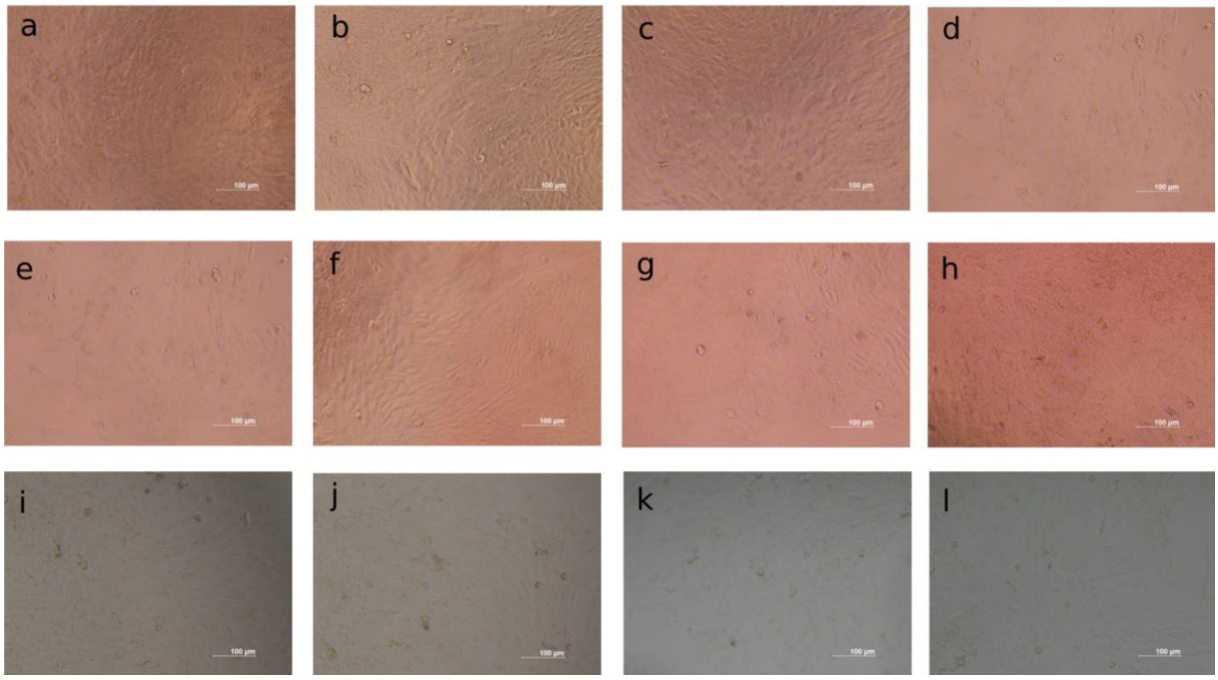


**Fig. 7.** Microscopic images of MG-63 cells exposed to different proportions of extract medium a) 24hrs-12.5% b) 24hrs-25% c) 24hrs-50% d) 24hrs-100% e) 48hrs-12.5% f) 48hrs-25% g) 48hrs-50% h) 48hrs-100% i) 72hrs-12.5% j) 72hrs-25% k) 72hrs-50% l)72hrs-100%

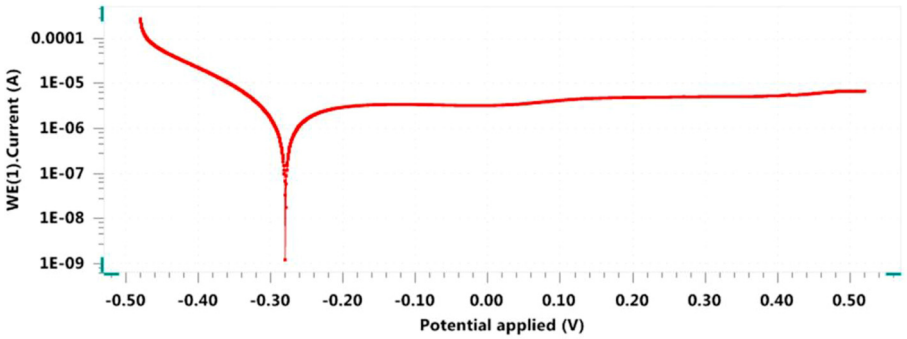


**Fig. 8.** Potentiodynamic polarization curve of uncoated samples.

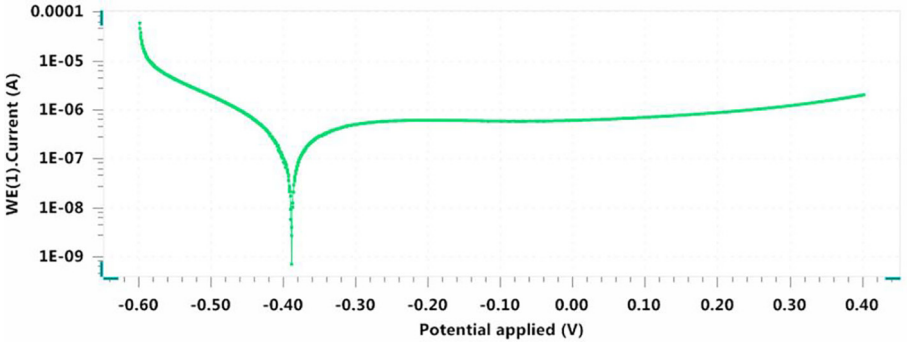


**Fig. 9.** Potentiodynamic polarization curve of Chitosan samples.

## 4. Conclusion

Chitosan was extracted from crustacean shells and it was found crystalline. A uniform thin layer of chitosan coating was developed on Ti6Al4V implant materials. An intermediate layer of shellac was applied to obtain better adhesion between the implant material and chitosan. Shellac concentration and dipping duration were considered as the coating parameters for this study. Shellac with concentrations of 12.5, 13.0, and 13.5% w/w and the dipping duration of 15 s, 25 s, and 35 s was considered in this study. Taguchi

optimization was used to find the optimal coating parameters and a mathematical model was developed to predict optimal values within the coating parameter range. The sample developed with optimal coating parameter was found to be non-toxic and also could promote cell attachment and proliferation. The corrosion studies revealed that chitosan-shellac coated implant material has much better corrosion resistance than uncoated samples.

## CRediT authorship contribution statement

**A. Ritwik:** Conceptualization, Investigations, Writing - original draft. **K.K. Saju:** Writing - review & editing. **P.K. Binsi:** Supervision. **A.R. Reghuraj:** .

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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