iJRASET
International Journal For Research in Applied Science and Engineering Technology

INTERNATIONAL JOURNAL FOR RESEARCH

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

Volume: 3 **Issue:** VI **Month of publication:** June 2015

DOI:

www.ijraset.com

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International Journal for Research in Applied Science & Engineering Technology (IJRASET)

Naturally Derived Porous Hydroxyapatite/ Polymer Biocomposite of Cuttlebone and Eggshell for Dental and Orthopedic Applications

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Abstract*--Hydroxyapatite [HAp, $Ca_{10}(PO_4)_6(OH)_2$], a well-known mineral component and chemically similar to inorganic constituent of bone and teeth, possess good osteoconduction and osseointegration properties. Bio inspired lightweight materials such as cuttlebone and eggshell are rich in $CaCO_3$. Since they possess good permeability, porosity and compressive strength much interest are shown in orthodontics and orthopedic applications. Several attempts had been made to synthesize HAp from cuttlebone and eggshell. The aim of the study is to synthesize a biocomposite materials with a blend of cuttlebone and eggshell HAp by a simple wet chemical method. The newly formed composite showed a good crystalline nature. The presence of calcium phosphate in HAp is confirmed by X-ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR) analyses. Mold prepared by solvent/solution casting method showed appreciable compressive strength and porosity (80-85%). Further, invitro biocompatibility studies were done with MG-63 osteoblast cells.
***Keywords: Hydroxyapatite; cuttlebone; eggshell; orthodontics; orthopedic*.**

I. INTRODUCTION

Synthesis of hydroxyapatite (HAp) with porous morphology is a defied work in the field of bone tissue engineering [1, 2]. In recent decade HAp with osteoconduction and osseointegration properties has shown tremendous interest in oral/orthopedic surgery for bone repair or replacement [3]. The chemical composition of hydroxyapatite (HAp) is similar to that of natural bone tissue exhibits non toxicity, non-inflammatory, non-pyrogenic response, no fibrous tissue formation between implant and bone, in addition to that they possess the ability to bond directly to the host bone and hence deliberated as an elite biocompatible material in bone graft substitute and used in bulk form, as a coating and/or cements [4, 5]. Currently, HAp is the material used in various biomedical applications, e.g. as a replacement for bony and periodontal defects, alveolar ridge, middle ear implants, tissue engineering systems, drug delivery agent, dental materials [6-14]. Although synthetic HAp has been widely used in biomedical field, natural materials are most preferred in medical applications. Over last decade, several attempts to synthesize hydroxyapatite from natural sources such as coral [15], bovine bone [16] and sea shells [17] are seen. Utilization of the same resource will lead to extinction of species. So it's crucial to discover a new alternative material with comparable properties, should be easily accessible, renewable and cost effective. Eggshell and cuttlefish bone being an inexpensive, abundance and rich in calcium source can be converted into HAp granules. An eggshell is made up by a three layered structure, viz. the cuticle, the spongeous layer and the lamellar layer [17]. The cuticle layer represents the outermost surface and consist of a number of proteins. Spongeous and lamellar layer form a matrix constituted by protein fibers bonded to calcite (Calcium Carbonate) crystals [18]. The eggshells constitute 11% of the total weight of the egg and are composed of calcium carbonate (94%), calcium phosphate (1%), organic matter (4%) and magnesium carbonate (1%) [19]. According to the Food and Agricultural Organization of the United Nations, the world's egg production was approximately 64 million metric tons in 2010, and India occupies the third position with 3.4 million metric tons. A huge amount of egg shells being produced daily are of no use causing environmental pollution [20]. HAp being derived from egg shell is a low cost material and at the same time reduces environmental pollution related problems. Cuttlebone (CB) is a cheap, easily available natural biomaterial with similar chemistry and crystallography as coral [21, 22]. CB is a rigid structural component consists of a dorsal shield which act as an external wall and an internal lamellae matrix. The lamellar matrix is formed by a parallel sheet structure and is interconnected, which results in highly porous properties and consists primarily of Aragonite, a crystallized form of $CaCO_3$ [23]. Due to this unique property, several studies have explored the transformation of CB into hap. Moreover, cuttlebone Hap as a filler in acrylic bone cements showed enhanced osseointegration and no evidence for secondary infection during *in vivo* testing. Further it shows appreciable mechanical properties when compared to those of commercial bone cement [24].

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Several methods have been reported for preparing hydroxyapatite such as chemical precipitation [25], sol-gel process [26], hydrothermal method [27] etc. However, among these methods wet chemical method is more promising owing to its ease of production, low working temperature, inexpensive equipment and also maximum purity [28]. Aragonite and Calcite can be easily sintered to hydroxyapatite by wet precipitation at a temperature of >900˚c showed highest crsytallinity [29]. Brittleness and poor mechanical stability of pure HAp limits its usage in the regeneration of non-load-bearing bone defects and tissue engineering applications [30]. Composite biomaterials like metal and polymer matrix are used to improve the mechanical compatibility of Nano HAp. Further, polymer coating enhances mechanical properties such as porosity and compressive strength of HAp fillers [31]. PEG is one of the widely used polymer and its unique structural feature plays a crucial role in tailoring the applications such as tissue scaffolding and biodegradable scaffolds [32]. The aim of the study is to synthesize a biocomposite materials with a blend of cuttlebone and eggshell HA by a simple wet chemical method. The newly formed composite showed a good crystalline nature. The presence of calcium phosphate in HAp are confirmed by X-ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR) analyses. Mold prepared by solvent/solution casting method showed an appreciable compressive strength and porosity. (80-85%). Further, invitro biocompatibility studies were done with MG-63 osteoblast cells.

II. MATERIALS AND METHODS

A. Synthesis Of Hydroxyapatite From Eggshell

Eggshells boiled for 15 minutes and the protein membrane was removed manually. Eggshells were washed with ethanol to remove the organic residues and dried at 60 ˚C. Dried eggshells were grinded in the agate mortar into a fine powder. By weighing it was found that 41.8 grams of powder obtained from 15 eggshells. Hydroxyapatite is synthesized as follows: Eggshell powder was dissolved in conc. nitric acid. The reaction is given below

$$\mathbf{CaCO_3 + 2HNO_3 = Ca(NO_3)_2 + CO_2 + H_2O}$$

Then 0.6 M ammonium di-hydrogen phosphate was added drop wise to achieve Ca/P ratio and stirred for overnight to form white colored HAp precipitate. The pH of the reaction was adjusted to 9 by adding dil. ammonium hydroxide solution. The precipitate was thoroughly washed and filtered. The residue was dried in an oven at 90˚C for 15 hours. Then the final powder was sintered at 1000˚C for 3 hours [17].

B. Synthesis Of Hydroxyapatite From Cuttlefish Bone

Cuttlebones as a whole (4 no's) were collected from local fish market at Madhavaram in North Chennai. Small pieces of sample were cut from whole cuttlebone using a sharp knife. Aragonite ($CaCO_3$) was obtained from the lamellae of cuttlefish bone powder by using mortar and pestle and heated in a furnace at 350˚c for 3 hours [2, 29]. Wet precipitation method was carried out by mixing 1M $CaCO_3$ and 0.6 M of H_3PO_4 solution with a magnetic stirrer for 30 minutes to achieve molar ratio of Ca/P=1.67. The prepared solution was heated at a temperature of 150˚c for 15 hrs. The resultant powder was cooled at room temperature and then washed with distilled water using a magnetic stirrer. Washing was performed repeatedly in distilled water till it remains at the neutral pH value. This is done to eliminate acidic byproducts. The last wash was performed with methanol to limit agglomeration of HAp during drying. Next, the sample was filtered and dried in oven at 70˚C. Hydroxyapatite formed was further sintered at a temperature of 900˚c for 1 hour and the final product was white in color [33, 34].

C. Preparation Of Hap Scaffold With Peg

To analyze the compressive strength and porosity HAp powder were molded into different shape by solvent/solution casting method [35, 36]. Polymer solution was prepared by dissolving Polyethylene glycol-600 in the distilled water and stirred for 30 minutes. Then HAp powder from different sources were dissolved in polymer solution and stirred for 1 hour. The solution was dried in a Hot air oven at 70 ˚C and dried for 24 hours. Then the mold was cut into a desired shape.

D. Characterization Of Hap And Hap/Peg

Synthesized HAp powder were mixed in different proportion (50CB HAp: 50ES HAp; 70CB HAp: 30ES HAp) and characterized by scanning electron microscopy, X-ray diffraction and Fourier transform infrared spectroscopy. Finally MTT assay has been performed on MG 63 Osteoblast cells.

1) *X-Ray Diffraction:* XRD analysis was performed using a Rigaku Miniflex ii C (Japan) diffractometer with Cu-Kα incident radiation (30 kV, 10 Ma) (λ=1.5405 Å). The diffraction patterns were collected at room temperature over the 2θ range of 10–80 ° and at step size angle of 0.8˚.
2) *Fourier Transform Infrared Spectroscopy:* Perkin-Elmer Spectrum RS I spectrophotometer was used for the identification of functional groups in the HAp. The samples were first ground in a mortar, and then mixed with pure moisture-free KBr

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powder in a ratio of 1:100, in a form of thin pellet. Infrared spectra were recorded in the region 4000–400 cm^{-1}, with 3600 scans performed at a resolution of 1 cm^{-1}.

3) *Bulk Density And Porosity Values:* The bulk density and porosity values were determined using a liquid displacement method. Scaffold initial dry weight (W) was recorded and immersed in a known volume of ethanol (V1). Test tube is tightly capped to drive ethanol into the porous structure of scaffold. After 30 minutes, the total volume of ethanol and ethanol infiltrated scaffold were noted as (V2). Scaffold was removed and the residual volume is noted (V3). The bulk density of the scaffold can be calculated by W/(V2-V3) and the porosity of the scaffold by (V1-V3)/(V2-V3) ×100% [3].
4) *Mechanical Properties:* Porous scaffold (3mm breadth×12mm width) was subjected to a compression test using an Instron test machine by applying a load via 1000N load cell at a cross head speed of 0.001 mm/min under ambient conditions. Load was applied on the top of the scaffold until it cracks. The results were interrupted from the stress vs. strain curve.
5) *Scanning Electron Microscopy:* The surface topography and microstructure of the HAp samples were observed using scanning electron microscopy (VEGA 3 TESCAN) by coating gold on the surface to reduce charging of the samples.
6) *Cell Viability/Cytotoxicity:* Human osteoblastic cells (MG 63) cell line was procured from the National Center for Cell Sciences (NCCS) Pune, India. The cells were grown in culture flasks containing DMEM supplemented with 10% FBS in a 95% air and 5% CO_2 in a humidified atmosphere at 37˚C. Indirect MTT assay was carried out to test the cytotoxicity. Cell viability was assessed by MTT (3-(4, 5-dimethylthiazolyl-2)-2,5diphenyl-tetra-zolium bromide) method. Human osteoblastic cells (MG63) at a concentration of 10,000 cells were seeded to the 96 well cell culture plate. Briefly, 50 mg of hydroxyapatite (HAp) scaffold was weighed and soaked in 500 µl of the DMEM medium for 24 h. The supernatant termed as conditioned medium was taken at different volumes (10µl, 20µl, 50µl and 100µl) and made up to 1 ml with medium and added to the wells containing cells and incubated for 24 h. The media were removed from the wells and 100µl of 0.5% MTT solution was added to each well and incubated for another 4 h at 37˚C. DMSO was used to dissolve the formed formazan crystals, and the optical densities (OD) were determined using the spectrophotometer (BioTEk micro plate reader, USA) at 570 nm. The intensity of the colors obtained (red and blue respectively) is directly proportional to the viability and metabolic activity of the cell population and inversely proportional to the toxicity of the material.

III. RESULTS AND DISCUSSION

A. XRD Analysis

Fig 1 shows the X-ray diffraction patterns of the synthesized HAp and of the two different sources. As shown in this figure, HA samples synthesized had similar XRD patterns and no other crystalline phase was observed other than HA. Identification of the phases was realized by comparing the experimental XRD pattern to standards compiled by the International Centre for Diffraction Pattern (ICDD) using the JCPDS Card No 09-432 for hexagonal HAp structure. Well-resolved characteristic peak of highest intensity was obtained at 2theta value of 31.77˚ corresponding to 211 plane of HAp [37]. Complete crystallization of the powders has been confirmed due to sharp peak intensity and well-resolved peaks in XRD patterns of the powders [17, 33].

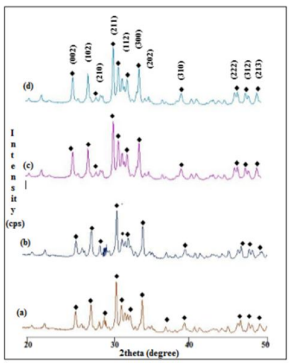

Fig 1 XRD of a) Eggshell HAp b) Cuttlebone HAp c) 50:50 HAp of CB and ES d) 70:30 HAp of CB and ES.

The crystallite sizes of HAp particles from both sources were calculated using Debye Scherer's equation $D = 0.9\ k/B\ \cos\theta$, where D represents mean grain size, B stands for full width at half maximum of the peak, k is the diffraction wavelength(0.154059 nm) and θ is the diffraction angle. The Bragg reflection at 002 planes of HAp was considered to calculate the crystallite size [28]. This indicates that the various proportions of HAp from CB and ES (50:50 HAp of CB and ES; 70:30HAp of CB and ES) has not hindered the formation of HA phases resembles the same peaks of eggshell and cuttlebone synthesized HAp. These characteristic feature resemble those of naturally occurring bone apatite. The crystallite sizes are found

to be 68nm, 67nm, 69nm and 67nm for eggshell, cuttle bone, 50:50 Hap and 70:30 Hap respectively.Due to their crystallite dimension in nanometer the HAp particle can be reinforced with fibers in a better way to improve the mechanical strength and hence favours the bone cell attachment and growth [38].

B. FTIR Analysis

FT-IR spectrum of HAp produced by wet precipitation method of the two different composition from cuttlebone and eggshell are shown in Fig 2 which shows all the characteristic bands for hydroxyapatite. In the HAp derived from cuttlebone, the major peaks are identified at approximately 560 – 630 cm^{-1}, 1030 – 1090 cm^{-1} due to the asymmetric stretching mode of PO_4 group indicates the presence of $(PO_4)^{3-}$ and and OH^{-1} group at 3568 cm^{-1} indicates the hydroxyl group and also contain broad peaks at approximately 1410 –1570 cm-1 in the spectra suggesting the presence of CO_3^{2-} substituted in the HAp. HAp from Eggshell shows the characteristic bands where $(PO_4)^{3-}$ modes were detected at 1079cm^{-1},1033cm^{-1}, 560 cm^{-1} in the spectra and OH^{-1} mode was at 3447 cm^{-1}. The bands at 3751.7 $cm^{-1}$and 1637.2 cm^{-1} corresponds to absorbed H_2O [39-41]. Both the composition showed the same functional groups in cuttlebone and eggshell HAp.

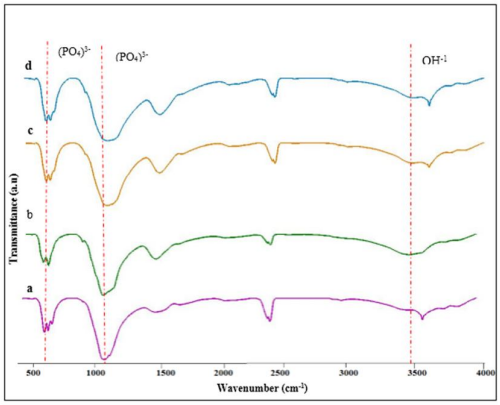

Fig 2 FTIR of a) Eggshell HAp b) Cuttlebone HAp c) 50:50 HAp of CB and ES d) 70:30 HAp of CB and ES.

C. Bulk Density, Porosity And Compressive Strength Analysis

Hap scaffold prepared by solution/solvent casting method is analyzed for porosity and bulk density by liquid displacement method. Fig 3 shows the stress vs. strain curve obtained for the HAp/PEG scaffold from compressive strength analysis. From the curve, the values are interpreted and listed in the Table 1. The compressive strength obtained at the maximum force of 101.1 N is 2.84 MPa. The porosity and compressive strength value obtained is appreciable for the applications of cortical bone [42].

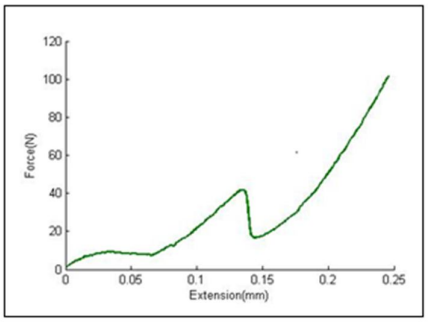

Fig 3 Stress Vs. strain Curve

TABLE I

VALUES FROM STRESS VS STRAIN CURVE

Compressive strength (MPa)	2.824
Max Force (N)	101.1
Yield strength (Mpa)	0.2598
Yield Force (N)	9.35

D. SEM Analysis

Microstructures of the Hap derived from cuttlebone and eggshell were studied by Scanning Electron Microscopy (SEM). Images were shown in Fig 4 (a,b,c &d) which clearly shows that the synthesized HAp consisted of agglomerates. Individual fine particles with spherical and semi-spherical shapes were observed in Fig 4a & 4b. Images showed that biocompatible composites consisted of a highly porous network structure in the range between 1μm and 5μm [43]. The agglomerates (Fig 4c and 4d) were of irregular shape like oval shape and spherical shape [44]. Agglomerates as big as 5µm could also be seen, however, the predominant sizes ranges between 1 and 3µm. Crystallites of nano-sized particle with a tendency to create porous formation

Volume 3 Issue VI, June 2015
ISSN: 2321-9653
International Journal for Research in Applied Science & Engineering Technology (IJRASET)

which are more beneficial and permits the circulation of body fluid throughout the coating when it is used as biomaterial [40].

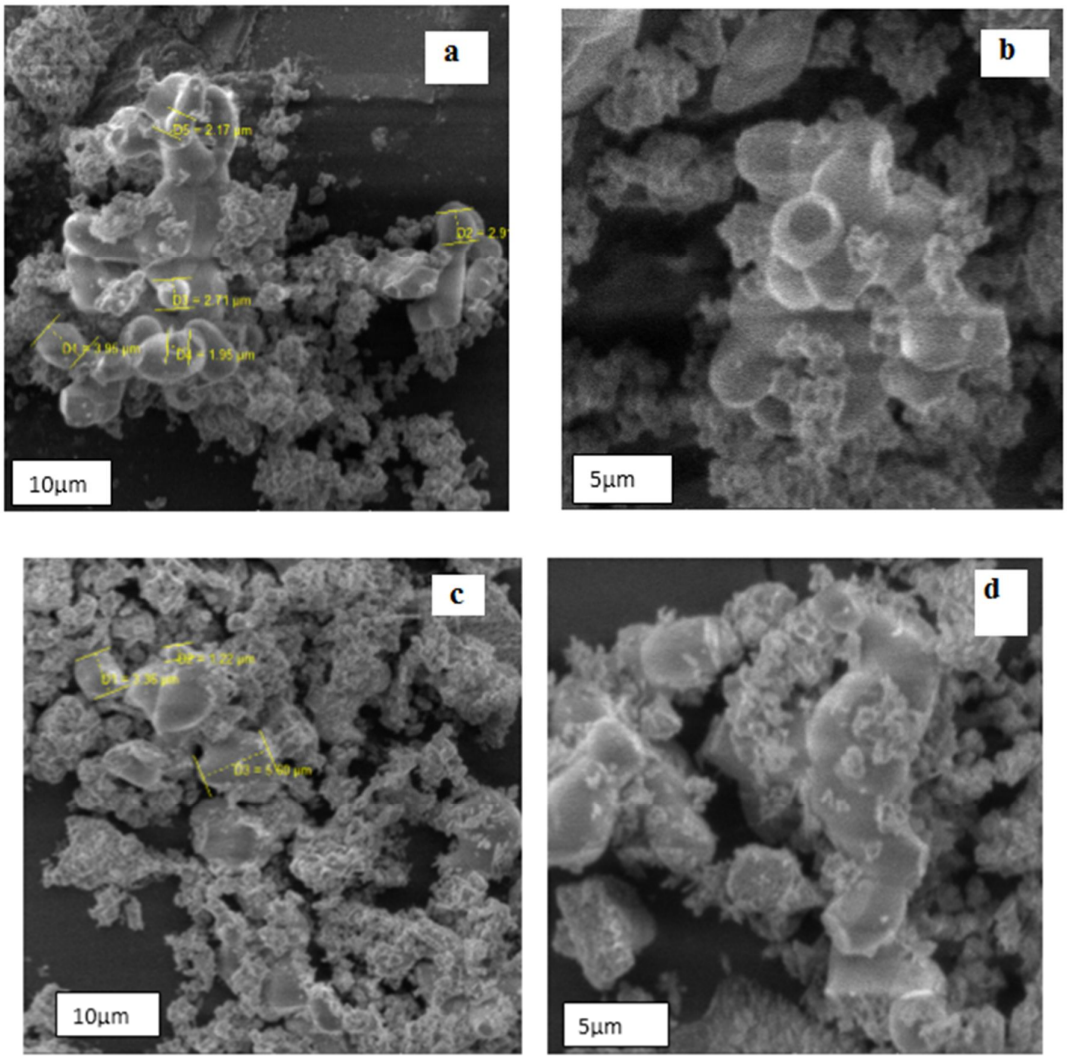

Fig 4 SEM images of a & b 50:50 HAp of CB and ES; c &d 70:30 HAp of CB and ES.

E. MTT Assay

All data were analyzed using GraphPad Prism 5.0 Software (GraphPad Software, La Jolla, USA) and expressed as mean ± standard error of mean (SEM). From the graph it was observed that for 24 hours the cell viability test shows a constant OD value which is similar to the positive control value [1,23].

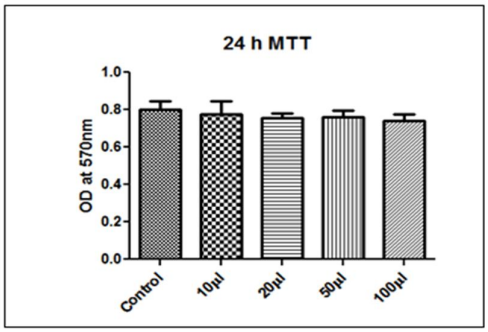

Fig 5. Mean ± SEM of observations denotes at $p<0.05$ compared with control

IV. CONCLUSIONS

The present study reveals that the phase pure HAp were prepared in two different proportions from the cuttlebone and eggshell by wet precipitation method in a cost effective manner. Two composite material revealed their crystalline nature of nano size by XRD analysis. FTIR analysis showed the presence of functional groups such as calcium and phosphate in HAp. Porous scaffold showed 85% of porosity with compressive strength 2.84 MPa which is beneficial to be used as filler and load bearing in biomedical applications. SEM images showed that the scaffold had pore diameters in the range 2~5 µm and pores were interconnected which promotes cellular vascularization. This was supported by invitro assay. The experimental results revealed that the HAp granules had fine biocompatibility. Altogether, our findings clearly suggest that the HAp granules obtained from two different sources may be used as a bone substitute. However, further invivo research studies has to be carried out to explore the benefits of HAp granules in tissue engineering.

V. ACKNOWLEDGEMENT

The authors would like to thank the heads of the various departments for utilizing the facilities at Crystal Growth Centre, Chemistry Department and Printing Technology, Anna University, Chennai-25. We extend our gratitude to the head of the department of Biotechnology, TANUVAS, Veppery, Chennai-07.

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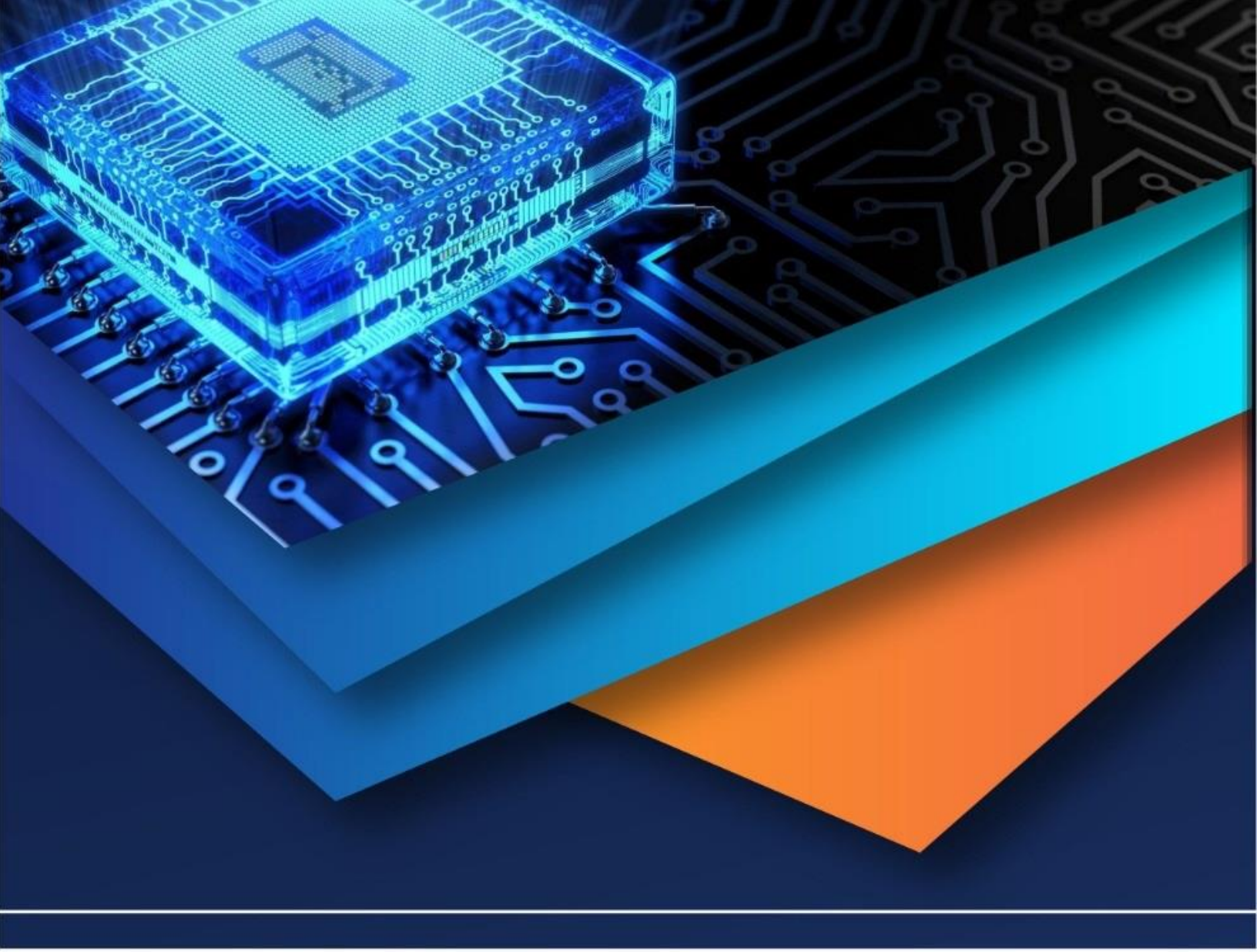

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