# INCORPORATION OF BIOACTIVE COLLAGEN TYPE I AND BIOMINERALISED HYDROXYAPATITE ON POLYDOPAMINE GRAFTED STAINLESS STEEL

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A thesis submitted in fulfillment of the
requirements for the award of the degree of
Master of Philosophy Biomedical Engineering

Faculty of Biosciences and Medical Engineering
Universiti Teknologi Malaysia

JANUARY 2018

To my husband, father, mother, and siblings for their support and encouragement. Thank you for all your supports and motivations.

# ACKNOWLEDGEMENT

In the name of Allah, The Most Gracious and The Most Merciful.
Salawat and Salam to prophet Nabi Muhammad S.A.W.

Firstly, I would like to express my greatest gratitude to Allah S.W.T. to give strengths and faith to complete this master project. A million of appreciation to my outstanding supervisor, Dr. Syafiqah binti Saidin for useful comments, remarks and engagement through the learning process of this master project.

I would like to acknowledge Assoc. Prof. Ir. Dr. Belinda Murphy from Universiti Malaya, for her trustworthy, guidance, motivation and opportunity to me to be a part of Tissue Engineering Laboratory, Department of Biomedical Engineering, Faculty of Engineering, Kuala Lumpur in order to complete my cell studies.

Finally, I would like to thank all staffs and friends from Biomedical Science Laboratory and Tissue Engineering Laboratory whom helping me to accomplish my objectives of this study. I am also gratefully indebted to Malaysian Ministry of Higher Education and Universiti Teknologi Malaysia for FRGS grant R.J130000.7845.4F523.

# ABSTRACT

Hydroxyapatite (HA) and collagen have been coated on metallic implants to accelerate osseointegration. Most methods to coat HA require high sintering temperature, high cost and high-energy power while the methods to coat collagen commonly produce unstable coating. Therefore, in this study, a polydopamine film was used as an intermediate layer to immobilise HA and collagen type I on a medical grade stainless steel (SS316L) due to its versatile, strong and stable properties. The SS316L disks were pre-treated and grafted with a polydopamine film. Then, they were covalently immobilised with collagen fibers at different immersion time (6, 12 and 24 hours). The disks were further biomineralised with HA in 1.5× simulated body fluid (SBF) solution for 7 days. The coated surfaces were characterised using FTIR, FESEM-EDX, XRD and contact angle analyses to investigate its chemical composition, morphology, crystallinity and wettability properties. The characterisation analyses showed that increased in collagen immersion time have induced the formation of amide cross-linkage between collagen and polydopamine. Longer immersion time has also produced less agglomerated carbonate HA with a nano lath-like surface. The disks with longest collagen immersion time were selected and subjected to *in vitro* test with human fetal osteoblasts (hFOB). The cell attachment, viability and differentiation were examined through FESEM, Alamar Blue reduction assay and Alkaline Phosphatase (ALP) assay respectively. The disks immobilised with HA and collagen presented highest proportion of cell adhesion, highest viability percentage with 31.8% of reduction potential and highest production level of ALP activity at 6.4 µIU/L compared to the bare SS316L disks and the disks immobilised with collagen. It can be concluded that, the polydopamine film has acted as an intermediate layer for the immobilisation of bioactive HA and collagen which projects a promising technique in the development of bioactive implant coating.

# ABSTRAK

Hidroksiapatit (HA) dan kolagen telah disalut ke atas implan logam untuk mempercepatkan integrasi osseo. Kebanyakan teknik untuk menyalut HA memerlukan suhu pembakaran, kos dan tenaga yang tinggi, manakala, teknik untuk menyalur kolagen selalunya menghasilkan salutan yang tidak stabil. Oleh itu, dalam kajian ini, lapisan polidopamin telah digunakan sebagai lapisan perantara untuk penyahgerakkan HA dan kolagen jenis I di atas besi tahan karat SS316L untuk mengatasi kekurangan-kekurangan tersebut. Cakera SS316L telah diberi rawatan awal dan dicantumkan dengan lapisan polidopamin. Kemudian, permukaannya disekatgerak dengan serat kolagen secara kovalen pada masa rendaman yang berbeza (6, 12 and 24 jam). Cakera tersebut pula melalui pemineralan biologi dengan HA dalam larutan simulasi cecair badan (SBF) 1.5× selama 7 hari. Permukaan salutan tersebut dicirikan dengan analisis FTIR, FESEM-EDX, XRD dan sudut sentuhan air untuk menyiasat komposisi kimia, morfologi, tahap kristal dan sifat kebolehjerapan. Analisis pencirian menunjukkan peningkatan masa rendaman kolagen telah menggalakkan pembentukan amid antara kolagen dan polidopamin. Rendaman yang lebih lama, menghasilkan karbonat HA yang kurang aglomerasi dengan permukaan bilahan bersaiz nano. Cakera yang mempunyai masa rendaman kolagen yang lebih lama telah dipilih untuk ujian *in vitro* menggunakan sel osteoblas janin manusia. Kelekatan sel, kebolehhidupan dan pembezaan sel telah diuji melalui FESEM, asai pengurangan Alamar Biru dan aktiviti alkali fosfat. Cakera yang disekatgerak dengan HA dan kolagen menunjukkan kadar kelekatan, kebolehhidupan sel yang tertinggi iaitu 31.8% dan kadar penghasilan aktiviti ALP yang tertinggi sebanyak 6.4 µIU/L berbanding dengan cakera SS316L dan cakera yang disekatgerak dengan kolagen. Ia boleh disimpulkan bahawa, lapisan polidopamin bertindak sebagai lapisan perantara untuk penyahgerakkan bioaktif HA dan kolagen di mana teknik ini mempunyai potensi untuk pembangunan salutan implant bioaktif.

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| | | |
|---|---|---|
| ALP | - | Alkaline Phosphatase |
| ANOVA | - | One way analysis of variance |
| APS | - | Aminopropylsilane |
| ATR | - | Attenuated total reflectance |
| C | - | Carbon |
| CA | - | Contact Angle |
| Ca | - | Calcium |
| $CaCl_2$ | - | Calcium chloride |
| $CO_2$ | - | Carbon dioxide |
| $CO_3^{2-}$ | - | Carbonate |
| Cr | - | Chromium |
| DI | - | Deionised |
| DMEM | - | Dulbecco's modified eagle's medium |
| ECM | - | Extracellular matrix |
| EDC | - | 1-ethyl-3-(3-dimethylaminoproply) carbodiimide |
| EDTA | - | Ethylenediaminetetraaceticacid |
| EDX | - | Energy dispersive x-ray spectroscopy |
| EPD | - | Electrophoretic deposition |
| FBS | - | Foetal bovine serum |
| FESEM | - | Field emission scanning electron microscope |
| FTIR | - | Fourier transform infra-red spectroscopy |

| | | |
|---|---|---|
| $H_3PO_4$ | - | Phosphoric acid |
| HA | - | Hydroxyapatite |
| HCL | - | Hydrochloric acid |
| HF | - | Hydrofluoric acid |
| hFOB | - | Human foetal osteoblast cells |
| HIP | - | Hot isostatic pressing |
| $HNO_3$ | - | Nitric acid |
| HSD | - | Honestly significant different |
| HUVEC | - | Human umbilical vein endothelial cells |
| KCl | - | Potassium chloride |
| $MgCl_2 \cdot 6H_2O$ | - | Magnesium chloride hexahydrate |
| $Na_2SO_4$ | - | Sodium sulphate |
| NaCl | - | Sodium chloride |
| $NaHCO_3$ | - | Sodium hydrogen carbonate |
| NaOH | - | Sodium hydoxide |
| NHS | - | N-hydroxysuccinimide |
| Ni | - | Nickel |
| $OH^-$ | - | Hydroxyl |
| P | - | Phosphorus |
| PBS | - | Phosphate buffered saline |
| pHAF | - | Polydopamine assisted hydroxyapatite |
| $PO_4^{3-}$ | - | Phosphate |
| REDOX | - | Oxidation-reduction |
| S | - | Sulphur |
| SBF | - | Simulated body fluid |
| SD | - | Standard deviation |
| SEM | - | Standard error mean |
| Si | - | Silicon |
| SS316L | - | Medical grade stainless steel |
| Cr | - | Chromium |

# LIST OF SYMBOLS

| | | |
|---|---|---|
| % | - | Percentage |
| ˚ | - | Degree |
| ˚C | - | Degree celcius |
| < | - | Less than |
| = | - | Equal |
| µL | - | Microliter |
| µm | - | Micrometer |
| $cm^{-1}$ | - | Per centimeter |
| $cm^2$ | - | Square centimeter |
| h | - | Hour |
| mg | - | Milligram |
| min | - | Minutes |
| mL | - | Milliliter |
| mm | - | Millimeter |
| nm | - | Nanometer |
| rpm | - | Rotation per minute |
| α | - | Alpha |
| B | - | Beta |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Research Background

Osseointegration is one of criteria to determine the success of bioactive implant based on close interaction between bones and implant surface without the formation of fibrous connective tissues [1, 2]. The interaction between bone and implant are dependent on the rate and quality of implant surface [2, 3]. Surface modifications on metallic implants are necessary to achieve fast and good osseointegration [4]. Many studies reported that, good bonding and fast osseointegration are important to accelerate bone healing and to increase bone anchorage between bone and implant surface [5, 6], thus, reducing the probability of implant loosening and failure.

Hydroxyapatite (HA) or calcium hydroxide phosphate is a bioactive ceramic that composed of two main components of mineral bone (calcium and phosphorus) [7]. It has been coated on metallic implants to promote osseointegration [7]. Most techniques and technologies to deposit and coat HA on an implant such as plasma spray, electrophoretic deposition, and hot isostatic pressing require high sintering

temperature which lead to crack formation due to mismatch of thermal expansion [8, 9]. Crack formation will further contributes to coating instability and practically interrupt the bone-implant fixation [8, 9].

Biomimetic technique is one of the methods to coat HA on metallic implants [10]. It does not require high energy power and high processing temperature which is beneficial to prevent crack formation and coating instability [9]. The HA coating film produced by this method has a tendency to integrate with bone rapidly as it is comprised of carbonate group [11]. The carbonated HA is beneficial for bioactive coating, bone graft and bone filler due to its biocompatibility and osseoconductivity which can actively provides strong bonding interface between bone and implant [12].

Recently, a polydopamine film is utilised to form biomimetic HA on medical grade stainless steel (SS316L) through a functionalisation process known as polydopamine assisted HA formation (pHAF) [13]. The mechanism of functionalisation process is based on the existence of amine and thiol/catechol functional groups. [14, 15]. The application of polydopamine as an intermediate layer to functionalise biomolecules is adopted from the work of Lee *et al.* [14]. This technique could produce strong and stable anchorage properties [15, 16]. Besides, the biomimetic HA grafted on polydopamine film mimics the natural properties of bone [14-16]. Those properties cause the polydopamine film to be a favourable way to modify the surface of metallic implants [17].

Collagen fibres are also one of main organic components of bone extracellular matrix (ECM) [11]. It is commonly used to improve the biocompatibility of implant surfaces [12]. These fibres act as a building template for bone formation and provide a mechanical strength to bone [13]. The immobilisation of collagen on material surfaces through a physical absorption technique shows simplicity and flexibility, but generally this method produces instability of coating film [14]. Meanwhile, a covalent immobilisation technique compromises better control of coating parameters such as coating thickness, ligand density and molecular

orientation [15]. There are various strategies to covalently immobilise collagen onto metallic surfaces that usually involve complex chemistry procedures [16].

Therefore, in this study, a polydopamine film was used to covalently immobilise collagen type I and biomineralised HA on medical grade SS316L. The immobilisation of both elements using an intermediate polydopamine film will prevent production of toxic compound and accelerate osseointegration on the surface of metallic implant to avoid coating and implant failures

## 1.2 Problem Statement

One of the problems arise during early stage of implantation, is coating failure and low rate of osseointegration [18, 19]. Most techniques and technologies to deposit and coat HA on an implant such as plasma spray, electrophoretic deposition, dip coating and hot isostatic pressing require high sintering temperatures which lead to HA decomposition that will increase the percentage of coating inhomogeneity and coating failure [9]. Furthermore, these techniques are expensive and require high energy power to operate those instruments [20].

Other than that, the procedures to immobilise collagen on metallic implants usually experience complex chemistry process which normally will introduce and produce extra toxic factors on the targeted surface [21, 22]. Therefore, in this study, a polydopamine film was utilised as an intermediate layer for collagen immobilisation and HA biomineralisation through three approaching steps: grafting polydopamine, covalent immobilisation of collagen type 1 and HA biomineralisation.

There are several factors have contributed to low osseointegration such as implant materials, mechanical load and surgical technique [23]. The low rate of osseointegration occurs when the osteoblasts are unable to attach and fil interface between metal implant and bone in order to form new tissues generation for cells proliferation, differentiation and maturation [24]. This incident will further reduce bone quantity and quality, slower bone healing and interrupt bone regeneration [2].

## 1.3 Objectives

This study has three main objectives which are:

i. To identify method to immobilise collagen fibre type I and biomimetic HA on polydopamine grafted SS316L.
ii. To characterise the chemical composition, morphology, crystallinity and wettability properties of the grafted films.
iii. To investigate the biocompatibility of the grafted films with human foetal osteoblasts.

## 1.4 Scope of the Study

The SS316L disks were pre-treated and grafted with a polydopamine film. It was covalently immobilised with collagen fibres at different immersion time (6, 12 and 24 hours). The disks were further biomineralised with HA in 1.5× simulated body fluid (SBF) solution for 7 days. The chemical composition of the grafted film

was characterised using Fourier transform infrared spectroscopy (FTIR) and energy dispersive X-ray spectroscopy (EDX). The crystallinity and morphology of the grafted film were investigated by X-ray diffractometer (XRD) and field emission scanning electron microscope (FESEM), respectively. The contact angle (CA) analyses were performed to determine the wettability property of the grafted film. Several samples were subjected to *in vitro* test with human foetal osteoblast cells through Alamar Blue assay and Alkaline Phosphatase (ALP) activity tests. Cell attachment, proliferation and differentiation on the grafted films were viewed under FESEM.

## 1.5 Significance of the Study

This study is significance in the field of orthopaedic implant applications. It involves greater research area on the development of bioactive implant coating. The incorporation of HA within the collagen type I matrix assisting by a polydopamine film has improved the bioactivity properties of metallic implant. The characterisation and cell culture analyses on this coating may promote further modifications and ideas on bioactive implant coating.

Besides, the incidence of implant coating failure could be reduced due to the stable covalent linkage between the implant surface and the coating. It has shown that covalent immobilisation has actively promoted osseointegration without implementing complex chemistry and without producing toxic residues. The HA-collagen grafted polydopamine film also is forecasting to accelerate bone healing and bone growth on metallic implant.

Furthermore, the Orthopaedic Industry Annual Report has stated that, the orthopaedic revenues have reached $48.1 billion worldwide in 2016 and grew at 3.2%, over 2015 [25]. This report presented the needs of orthopaedic products and the implantation is increasing year by year due to infection, old age, obesity, accident and demands to replace dysfunctional/old implant [26]. Therefore, the development of implant coating in orthopaedic field is necessary to fulfil the market needs to prevent second surgery for implant replacement due to implant failure.

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