FISH SCALES HYDROXYAPATITE/COLLAGEN COMPOSITES INCORPORATED WITH SILVER NANOPARTICLES FOR BONE FILLER APPLICATIONS

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ABSTRACT

The aim of this study was to improve antimicrobial properties of fish scales hydroxyapatite/fish collagen (FsHA/FsCol) composites through infiltration of silver nanoparticles into the matrix for antimicrobial bone filller substitute. In this sudy both hydroxyapatite and collagen were extracted from fish scales using simple hydrothermal extraction method. Silver nanoparticles (AgNPs) were synthesized from silver nitrate using microwave–assisted method in which neems leaves extract was used as reducing agent while fish collagen as stabilizer. FsHA/FsCol/AgNPs composites were prepared in a form of powders and beads using two different methods. Chemical and physical properties of AgNPs and FsHA/FsCol/AgNPs composites were characterized using field emmision scanning electron microscope with energy dispersive x-ray (FESEM-EDX), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR) and x-ray diffraction technique (XRD). Biological properties of prepared composites were investigated through antibacterial, cytotoxicity and cell attachment tests. The results shown that four different sizes for AgNPs ranging from 28 nm to 100 nm were obtained by using different concentration of collagen. Their antimicrobial activities against *Escherichia coli* and *Staphylococcus aureus* showed that smaller particles sizes of AgNPs have shown better inhibition of bacteria growth. Analysis using XRD and EDX proved that the pattern of spectra corresponded to silver nanoparticles present in the composites. FESEM-EDX analysis indicated that the silver nanoparticles distributed well in the HA composite matrix. Composites with 80 wt.% of HA and 20 wt.% of collagen demonstrated high stability during degradation test. The addition of 20 wt.% starch in the composite during preparation improved the porosity of composites after sintering, thus improved the infiltration of AgNPs-collagen solution and antibacterial properties. In conclusion, composites of FsHA/FsCol/AgNPs powder and beads have been proved to be biocompatible and good cell attachment. The implication of this study is both powder and beads of FsHA/FsCol/AgNPs composties can be used as antimicrobial bone filler substitute.

KOMPOSIT HIDROKSIAPATIT/KOLAGEN SISI IKAN DIGABUNG DENGAN NANOPARTICLES SILVER UNTUK APLIKASI PENGISI TULANG ANTIMIKROBIAL

ABSTRAK

Tujuan kajian ini adalah untuk meningkatkan sifat antimikrobial komposit hidroksiapatit/kolagen (FsHA/FsCol) sisik ikan melalui penyusupan nanopartikel perak ke dalam matriks sebagai pengganti pengisi tulang antimikrobial. Dalam kajian ini kedua hidroksiapatit dan kolagen diekstrak dari sisik ikan menggunakan kaedah pengekstrakan hidrotermal. Nanopartikel perak (AgNPs) disintesis dari perak nitrat menggunakan kaedah berbantukan gelombang mikro di mana ekstrak daun mambu digunakan sebagai agen penurunan manakala kolagen ikan sebagai agen penstabil. Komposit FsHA/FsCol/AgNPs disediakan dalam bentuk serbuk dan manik menggunakan dua kaedah yang berbeza. Sifat kimia dan fizikal AgNPs dan komposit FsHA/FsCol/AgNP dicirikan menggunakan mikroskop pengimbas elektron pelepasan medan dengan penyebaran tenaga sinar-x (FESEM-EDX), spektroskopi inframerah transformasi Fourier (FTIR), mikroskop transmisi elektron (TEM) dan teknik pembelauan sinar-x (XRD). Sifat biologi komposit yang dihasilkan dikaji melalui ujian antibakteria, sitotoksisiti dan lekatan sel. Hasil kajian menunjukkan empat AgNPs yang berbeza saiz antara 28 nm hingga 100 nm dihasilkan dengan menggunakan kepekatan kolagen yang berbeza. Kegiatan antimikrobial komposit terhadap *Escherichia coli* dan *Staphylococcus aureus* telah menunjukkan bahawa ukuran partikel AgNP yang lebih kecil menunjukkan penghalangan pertumbuhan bakteria yang lebih baik. Analisis menggunakan XRD dan EDX telah membuktikan corak spektra sesuai dengan kehadiran nanopartikel perak yang terdapat di dalam komposit. Analisis FESEM-EDX telah menunjukkan bahawa nanopartikel perak tersebar dengan baik dalam matriks komposit HA. Komposit dengan 80% berat HA dan 20% berat kolagen telah menunjukkan kestabilan yang tinggi semasa ujian degradasi. Penambahan 20% berat tepung ubi dalam komposit semasa penyediaan telah meningkatkan keliangan komposit selepas pensinteran, sehingga meningkatkan penyusupan larutan AgNPs-kolagen dan seterusnya meningkatkan sifat antibakteria. Kesimpulannya, komposit serbuk dan manik FsHA/FsCol/AgNPs terbukti bioserasi dan menunjukkan lekatan sel yang baik. Implikasi dari kajian ini adalah serbuk dan manik dari komposit FsHA/FsCol/AgNPs boleh digunakan sebagai pengganti pengisi tulang antimikrobial.

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LIST OF ABBREVIATIONS

AB assay	Alamar Blue Assay
AFM	Atomic Force Microscopy
Ag	Silver
$AgNO_3$	Silver Nitrate
AgNPs	Silver Nanoparticles
Al	Aluminium
BG	Bioactive Glass
BMP-2	Morphogenetic Protein-2
C	Carbon
Ca	Calcium
Cap	Calcium Phosphate
$CHCl_3$	Chloroform
Cl	Chlorine
CPC	Calcium Phosphate Cement
CS	Chitosan
Cu	Copper
DAPI	4′,6-Diamidino-2-Phenylindole
DCPA	Dicalcium Phosphate Anhydrous
DCPD	Dicalcium Phosphate Dehydrate
DHT	Dehydrothermal

DLS	Dynamic Light Scattering
DMEM	Dulbecco'S Modified Eagle'S Medium
DMF	N, N-Dimethylformamide
DW	Distilled Water
E. coli	Escherichia Coli
EDTA	Ethylenediaminetetraacetic Acid
EDX	Energy Dispersive X-Ray Analysis
FAK	Focal Adhesion Kinase
Fe	Iron
FGF-2	Growth Factor Of Fibroblast
FsCol	Fish Scals Collagen
FsHA	Fish Scals Haydroxyapitite
FTIR	Fourier-Transform Infrared Spectroscopy
FWHM	Full-Width At Half-Maxima
GS	Green Synthesis
HCl	Hydrochloric Acid
HMD	Hyaline Membrane Diseases
LAT	Laser Ablation Technique
MAGS	Microwave-Assisted Green Synthesis
Mg	Magnesium
MSCs	Mesenchymal Stem Cells
N	Nitrogen
NA	Nutrient Agar

Na	Sodium
$NaBH_4$	Sodium Borohydride
NCPs	Non-Collagenous Proteins
NE	Neem Extract
NGF-β	Nerve Growth Factor
NPs	Nanoparticles
O	Oxygen
OC	Osteochondral
P	Phosphorus
PBS	Phosphate Buffer Saline
PCL	Polycaprolactone
PLA	Poly (Lactic Acid)
PLCL	Poly (Lactide-Co-Ecaprolactone)
PLGA	Poly-(Lactide-Co-Glycolide
PLLA	Poly (L-Lactic Acid)
PMMA	Poly (Methyl Methacrylate
Poly-HEMA	Poly-Hydroxyethyl Methacrylate
PVA	Poly (Vinyl Alcohol)
PVP	Polyvinylpyrrolidone
ROS	Reactive Oxygen Species
S. aureus	Staphylococcus Aureus
SBF	Simulated Body Fluid
SEM	Scanning Electron Microscope

Si	Silicon
SPR	Surface Plasmon Resonance
TEM	Transmission Electron Microscopy
TTCP	Tetracalcium Phosphate
UTIs	Urinary Tract Infections
UV-Vis	Ultraviolet-Visible Spectroscopy
XPS	X-Ray Photoelectron Spectroscopy
XRD	X-Ray Diffraction Analysis
β	Beta
β TCP	B-Tricalcium Phosphate
λ	X-Ray Radiation

CHAPTER 1

INTRODUCTION

1.1 Background of study

Bone is considered the core unit that forms the human body's skeleton, consisting primarily of hydroxyapatite (HA) and collagen (Bandyopadhyay, 2008). Bone consists of four main components: HA minerals, type-I collagen, water, and non-collagenous proteins (NCPs). By weight, bone is ~70 wt % minerals, ~20 wt % organics, and ~10 wt % water. The structure of natural bone is shown in figure 1.1. Natural bone is a composite material comprised of organic and inorganic elements (Andric et al., 2011). The organic materials are mainly collagen fibers containing tropocollagen, which make up most of the organic constituent of bone, and provide strength to the bone (Basha et al., 2015). The inorganic materials are mainly calcium (Ca) and phosphorus (P) in the form of hydroxyapatite (HA). Kamitakahara et al., (2007), howver reported bone is a composite of 70 mass% inorganic component, i.e., hydroxyapatite, and 30 mass% organic components, i.e., collagen (Kamitakahara et al., 2007).

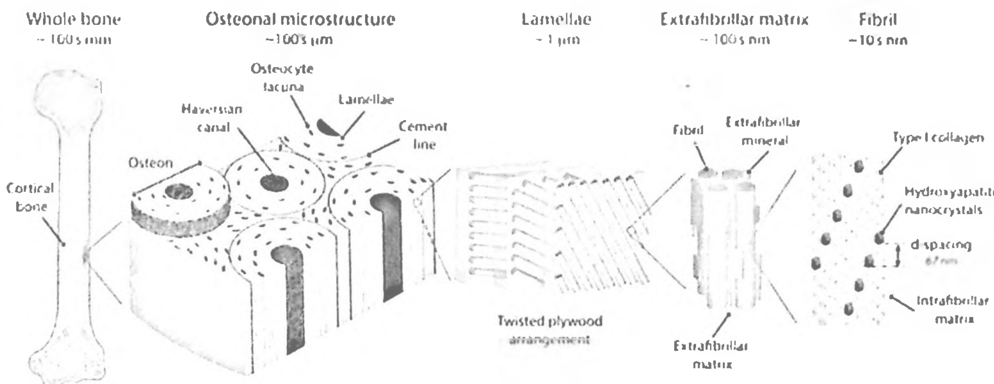

Figure 1.1. The Components of Bone (https://www.nature.com/articles/boneres201759)

Bone defects may occur for several reasons, such as injuries, illnesses, surgical interventions, and accidents, some of which may heal on their own. However, bone defects greater than 1/3 an inch (≈ 8 mm) cannot be healed on their own (Gupta, Thussbas, Koch, & Seebauer, 2018). Therefore, bone substitutes were used to fill up and enhance bone defects to allow for the rapid healing process (Qian et al., 2018). These substitutes provide structural and mechanical support to enhance bone tissue formation or fill gaps to facilitate the healing of bone tissue. Bone substitutes have been widely used in plastic surgery, oral, maxillofacial, dental, and orthopedic surgery, making it one of the most implanted tissues in the medical field (Fliefel, Ehrenfeld, & Otto, 2018).

HA is an inorganic compound with the chemical structure $Ca_{10}(PO4)_6 (OH)_2$. It is a type of biomaterial widely used for orthopedics and dental due to its bioactive properties and osteoconductive properties (Habibah & Salisbury, 2018). The crystal structure of HA is shown in Figure 1.2. HA has been used in many composites to enhance the healing of bone defects, such as HA-poly(lactic acid) as an artificial bone filler (Higashi et al., 1986), HA-polyamide as an osteoconductive agent (Lu et al., 2016), nHA (Nano-HA)-chitosan for orthopedic bone (Zhang et al., 2012), nHA/gelatin for pharmaceutical and cosmetic purposes (Mohamed, Beherei, & El-Rashidy, 2014).

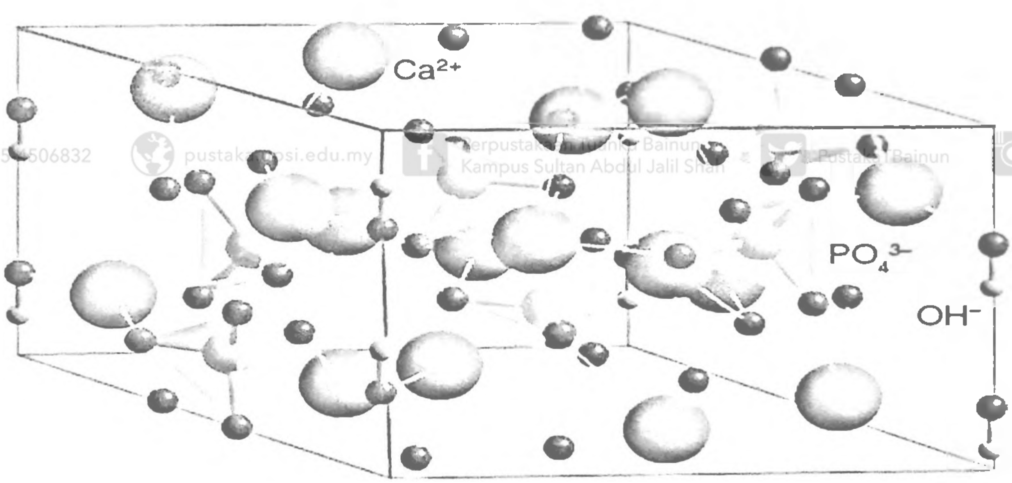

Figure 1.2. Crystal Structure of Hydroxyapatite (https:/ /www.c hemtube3d.com /ss hydroxyapatite /)

Most of the HA used in commercial products are derived through chemical synthesis. However, the high price of chemically-synthesized HA has motivated researchers to find a solution for other sources of HA. One of the alternatives is natural HA from animal bones (Sobczak-Kupiec & Wzorek, 2012). However, the recent problem of animal diseases such as hyaline membrane disease (HMD) and mad cow disease renders animal-based HA not a good alternative of HA. As an alternative, HA from fish scale was studied and found to be the best substitute compared to synthetic HA or animal-based HA (Zainol et al., 2012).

Collagen, a natural polymer that can be isolated from different animal parts such as ligaments, tendons, skin, and bone, is used as a biomaterial due to its biocompatibility (Jia et al., 2013). Collagen has been proven to enhance cell growth and has excellent biological properties (Takitoh et al., 2015; Maslennikova et al., 2015). The structure of collagen is shown in Figure 1.3. Many studies reported the development of highly-modified collagen through the incorporation of different materials, and its use to enhance the healing of bone defects, such as collagen-β-tricalcium phosphate to improve osteoconductivity (Yunoki et al., 2006), collagen-calcium phosphate to improve the biodegradability materials (Du et al., 2000), collagen-poly(lactic-co-glycolic acid) for the healing of cartilage defect (Dean et al., 2003), and collagen-chitosan-glycerin as biostability and biocompatibility materials (Ullah, Zainol, Chowdhury, & Fauzi, 2018).

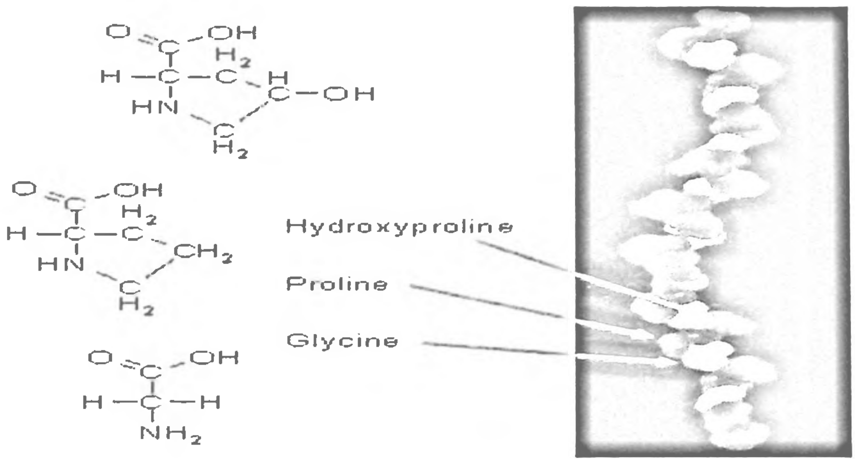

Figure 1.3. Structure of collagen (Silvipriya et al., 2015)

Among several biomaterials investigated to treat bone defects, the composites material exhibited the best effect and can be utilized clinically. HA/collagen composites have been prepared using various preparation techniques and have been used in many bone defects as a biomaterial for bone tissue engineering. Various sizes (nano-size and micro-size) of the Col (collagen)/HA (hydroxyapatite) composites were incorporated with different materials such as poly (l-lactic acid; PLLA) (Liao & Cui, 2004), poly(vinyl alcohol) (PVA); (Song et al., 2012), chitosan (Zhang, Tang, Zhang, Xu, & Wang, 2009), and metals such as Fe (Tampieri et al., 2014), to develop the properties of the scaffolds that exhibit high compatibility and bio-degradable properties for bone repair. The collagen/HA (Col/HA) composites used in many medical applications were fabricated from various sources. However, there was no report on the application of fish scales collagen and fish scales hydroxyapatite as Col/HA composites.

Col/HA composites were formulated in different forms such as powder, paste, gel, sponge, and beads. Currently, Morra et al., (2015), reported the preparation of hydroxyapatite (HA)/β TCP (β-tricalcium phosphate)/collagen beads as bone fillers. They placed the HA beads in the collagen solution to allow even distribution of collagen in the porous HA beads. The HA/collagen mixture was then characterized using XPS and FTIR to confirm the structure of the beads. The HA was derived from chemical synthesis, whereas the collagen was extracted from the animal.

The incorporation of HA and collagen is possible due to the biocompatibility of collagen and high mechanical properties of the HA, and it is widely utilized as the biomaterial to enhance the healing of bone defect, as well as a replacement material for bone defects. Nonetheless, despite its advantages, several issues of collagen/HA composite concern the public. Tampieri et al. (2014) reported the successful development of collagen/HA composite that showed excellent bioactivity properties, which lead to its use as a bone filler for the defect bones. However, most of the collagen used in the market is extracted from animals. Thus, halal issues became a significant concern for Muslim users.

Another issue of concern for the consumers is the failure of the implant or foreign body used in orthopedic surgery due to bacterial infections (Ahmed et al., 2016). The adhesion of bacteria to an implant occurs in the form of a biofilm, rendering the bacteria extremely resistant to the host's defense mechanisms and antimicrobials (Dastgheyb et al., 2015). Nonetheless, despite the recognized need for implant-related infection containment and the demonstrated efficacy of some antibacterial coatings, only a few technologies are currently available in orthopedics and trauma (Berend et al., 2013). While some potentially effective solutions have been identified as not suitable for orthopedic implants due to their cytotoxicity, immunoreactivity, or interference with bone healing and osteointegration, those successfully tested in vitro and in vivo may still not be able to achieve large-scale clinical application due to the biotechnological, economy, and regulatory issues (Kurtz et al., 2007). Silver nanoparticles used as agents to inhibit bacterial growth (Chaloupka, Malam, & Seifalian, 2010) have also shown their ability to function as an antibacterial agent when incorporated with composite scaffolds for bone defects (Saravanan et al., 2011). The use of silver nanoparticles (AgNPs) synthesized by chemical, physical, and biological methods helped minimize bacterial infections after bone defect filling, as many unsuccessful orthopedic surgeries were due to bacterial infections (Ahmed et al., 2016). Due to the environmental risks of nanomaterials, which were created by chemical and physical processes, several techniques were developed to solve the problem and to create environmentally friendly, non-toxic materials (Ahmed et al., 2016).

The green synthesis of AgNPs have been conducted by utilizing several plants extract such as *Coffea arabica* (Dhand et al., 2016), *Euphorbia tirucalli* (Kalaiselvi, Mohankumar Shanmugam, Nivitha, & Sundararaj, 2019), *Annona squamosal* (Ruddaraju, Pallela, Pammi, Padavala, & Kolapalli, 2019), *Prosopis juliflora* (Arya et al., 2018). Nanoparticles have been investigated as an excellent antimicrobial agent and applied for many applications such as arthroplasty (Marin et al., 2015), catheters (Rafique, Sadaf, Rafique, & Tahir, 2017), stainless steel materials (Mochochoko, Oluwafemi, Jumbam, & Songca, 2013), dental materials (Emmanuel et al., 2015), and human skin (AlSalhi et al., 2016). Neem is one of the Melia genera belonging to the Meliaceae family, which is widely distributed in India and Malaysia. The Neem has been extracted and used to produce nanosilver, which has shown excellent results by using silver nitrate for anticancer activity (Kathiravan et al., 2014).

In this study, the microwave-assisted green synthesis of silver nanoparticles was conducted using neem extract and collagen as a reducing and stabilizing agent, respectively. The Ag-stabilized collagen was used as an enhancement to infiltrate into the HA beads to form FsHA/FsCol/AgNPs composite. The beads of FsHA/FsCol/AgNPs have been formulated and characterized for bone fillers applications.

1.2 Problem statements

The need for the grafts of bones or substitutes increased with the increasing need for suitable materials to be used as substitutes in many cases, such as the treatment of fractures revision hip arthroplasty, spine fusion, and tumors in addition to reconstructive surgery. This situation renders researchers look for vital materials that can be utilized to perform this purpose (Pedersen et al., 2005). Several requirements apply to bone fillers and any other implantable biomaterials, i.e., they must be non-toxic and biocompatible, do not initiate an adverse inflammatory reaction, and must be commercially viable to manufacture and cost-effective for the surgeon to use.

The high cost of chemicals used in the synthesis of HA has motivated researchers to find a solution for another source of HA. One of the alternatives is natural HA from animal bones (Sobczak-Kupiec & Wzorek, 2012). However, animal diseases such as hyaline membrane disease (HMD) and mad cow disease poses a threat to the production of HA from animal bones, rendering animal-based HA not a good alternative of HA. Thus, the fish scale HA was studied and was found to be the best alternative compared to synthetic HA or animal-based HA (Zainol et al., 2012). Fish scales consist of 50 wt% collagen and 50 wt% HA and could be extracted from fish scales using a simple extraction method (Zainol et al., 2012). FsHA has been used in many biological and medical applications due to osteoinductive and osteoconductive properties. Other characteristics that make HA attractive for use as bone replacement are that it is a significant component of bones and hard tissues, is non-toxic and nonimmunogenic, has the mechanical strength and surface properties that are suitable for bone tissue regeneration (Pon-On et al., 2016). However, the report regarding the potential use of fish scales as HA/collagen composites as bone filler is lacking.

Despite the increasing utilization of implanted biomaterials, their long-term durability is not guaranteed, besides the risk of infection that dictates for early failure in orthopedics and trauma. The economic and social costs of implant-related infections are significant. Silver nanoparticles can reduce the potential of bacterial infections from implant materials (Moriarty et al., 2016). The antibacterial activity of silver nanoparticles is well known, mostly dependent on dissolved cations' ability to interfere with bacterial cell membrane permeability and cellular metabolism (Schmidt-Braekling et al., 2017). However, a few implant designs are incorporated with silver due to the cost of the technology. Therefore, to solve this problem, the current study utilized low-cost silver nanoparticles prepared from natural materials.

Based on previous studies, no research was reported on the incorporation of silver nanoparticles in HA/collagen composites. Thus, a novel fish scales HA/collagen/silver composite was developed for cheaper and safer bone filler materials in this study.

1.3 Research objectives

1. To synthesis nanosilver particles using microwave-assisted green synthesis with neem (*M. dubia*) as a reducing agent and collagen as a stabilizer.
2. To formulate and characterize fish scales HA/collagen/silver nanoparticles (FsHA/FsCol/AgNPs) composites for bone filler applications.
3. To investigate the antibacterial activities of FsHA/FsCol/AgNPs composites against pathogenic *Escherichia coli* and *Staphylococcus aureus*.
4. To evaluate the biostability and biological properties of FsHA/FsCol/AgNPs composites.

1.4 Significant of the study

The use of biomaterials to fabricate a suitable substance for an implant in many applications such as bone graft, drug delivery, bone fillers, and tissue engineering of the bone has been one of the significant challenges for many years. HA is one of the materials that have been reported to have excellent properties as a biomaterial, such as biocompatibility, non-inflammatory, non-toxic, osteoconductivity, and none-immunogenicity. The fabrication of collagen with HA is suitable as bone fillers, as well as bone graft and other biomaterials field due to its excellent mechanical properties, biological activity, and others. This study aimed to improve the biological properties of fish scales HA/fish collagen through incorporation with silver nanoparticles, which exhibits antimicrobial activity that allow the beads to be successfully used in bone graft and bone fillers surgery. This preparation could overcome the problems due to bacteria infection.

This study successfully produced FsHA/FsCol/AgNPs composite that possesses biological properties and mechanical properties suitable for bone fillers. The product is relatively cheap and affordable for the public. Due to low cost of raw materials i.e fish scales. Furthermore, the material used is halal and suitable for Muslims around the world.