J. Myanmar Acad. Arts Sci. 2018 Vol. XVI. No.4

APPLICABILITY OF FISH SCALES NANOBIOMATERIALS IN HARD TISSUE ENGINEERING

Lei Lei Aye

Abstract

Extraction of fish scales biomaterials (from *Notopterus notopterus*) was carried out by burning the fish scales at different temperatures. These biomaterials were proved to be nano-sized hydroxyapatite by using X.R.D.(x-ray diffraction), F.T.I.R.(fourier transform infrared spectroscopy) and S.E.M.(scanning electron microscope). Purity of the sample was confirmed by T.G.A.(thermo-gravimetric analysis), protein content and Ca/P weight ratio. Porosity in the sample was calculated by image J software. Toxic heavy metal was not detected by mineral analysis. Evaluation of hardness of F.S.HAp-Glass Powder-GIC Composite was done by Mitutoyo micro-hardness testing machine. Hardness of pure Glass Ionomer (Japan) was found to be 3.5 Mohs while that of F.S.HAp-Glass Powder-GIC Composite was 4 Mohs. As the hardness is very near to teeth and skeletal tissue, it is suitable for substitution in tooth defect area.

Keywords: hydroxyapatite, *Notopterus notopterus*, Mohs, F.S.HAp-Glass Powder-GIC Composite

Introduction

Background of the Study

Nowadays, fishery by-products are subject to strict environmental regulations due to limited land and increased environmental concerns such as groundwater contamination and foul odor. Compliance with environmental standards and a better understanding of the potential values of processing by-products for a variety of applications have resulted in technological innovations for seafood wastes as nutraceuticals and functional foods. However, lack of adequate utilization of technology to convert such wastes into value-added products must be seriously addressed. Currently there is an growing interest in natural ingredients available from animal by-products to fulfill the needs of human being. Biomaterials are widely used in human body for tissue repair and substitution still expand to date. Biomaterials have recently been extracted from various calcium rich biowastes including corals, shells and vertebrate bones and the extracted biomaterials can be used in reconstruction of hard tissues.

Assistant Lecturer, Dr., Department of Zoology, University of Mandalay

The aquatic biowastes, fish scale are biocomposites of highly ordered type I collagen fibers and hydroxyapatite (Fengxiang Zhang, anning wang,2011). Genetically, the same genes involved in tooth and hair development in mammals are also involved in scale development (Scale Anatomy,Fish scales, Wikipedia, the free encyclopedia).

Li Li, Haihua Pan, Jinhui Tao, Xurong Xu'(2008) stated that the hydroxyapatite is a "natural building blocks of enamel and 20nm-40nm sized hydroxyapatite particle can effectively remineralize the enamel.Their significant finding is poor adhesive ability of hydroxyapatite synthesized from Orthophosphoric acid solution. It suggests that the better biomaterials require a perfect biocompatibility to reduce the interface between inplanted biomaterials and natural materials.

Structure of Tooth

As with bone, different components of tooth (dentin, enamel, cementum) are distinguished. The periodontal ligament connects the tooth (via the cementum) to the underlying jawbone. The outer coating of the tooth as far as the gum line is enamel, a very hard material with little or no protein. Below the enamel is dentin, the major component of teeth. Separating the dentin from the surrounding jawbone is a bone-dentin composite material, cementum, and a periodontal membrane. The dentin surrounds a pulp cavity that holds the nerves and blood vessels necessary for tooth function.(Figure 1)

In tooth, collagen is the major organic constituent of dentin and cementum, but there is no collagen in enamel. Collagen is the same protein that gives flexibility to ligaments and tendons, but the addition of mineral to the collagen matrix makes it rigid and gives bones and teeth their greater load-bearing capacity. The mineral that reinforces bone and dentin matrices and the major constituent of enamel is an analogue of the mineral hydroxyapatite.

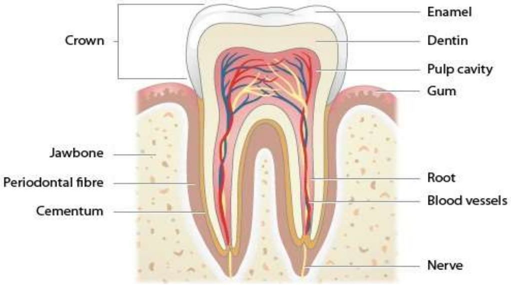


Figure1. Structure of Tooth(Source-Solanki Dental Centre)

Morphology and Component of Fish Scales

Fish scales exhibit large variations in shape, size and arrangement. Teleost scales are composed of collagen fibril type – I, and are partially mineralized with hydroxyapatite (16-59%) mineral content in weight. The outer layer of the scale is significantly more mineralized and often referred to as 'bony layer' . Whereas the inner layer 'basal' or 'collagen' layer is mineralized mostly near the bony layer, but with mineralization pockets proceeding well into the collagen layer. As hydroxyapatite of fish scales is similar to main component of teeth and bone, mineral component of fish scales can be applied in the hard tissues(teeth and skeletal tissues) engineering application of the human body.

The Properties of Glass Ionomer Cement

Glass Ionomer Cement (GIC) was invented by Wilson et. al at the Laboratory of the Government Chemist in early 1970. They are water-based cements, known as polyalkenoate cements. They possess restorative and adhesive properties- adhesion to moist tooth surface and base metals, anticariogenic propertites due to release of fluoride, thermal compatibility with tooth enamel, biocompatibility and low toxicity.(Maya Lyapina[1], Mariana Tzekova[2] *et al*., 2016). Galss ceramics are non-toxic and chemically bond to bone. Glass ceramic elicits osteoinductive property. Bioglass and glass ceramic are embedded in a biomaterial support to form prosthetics for hard tissues.(Thamaraiselvi and Rajeswari, *et al*., 2004)

The mechanical property of hydroxyapatite is very important in hard tissue engineering application. Combination of nano-sized hydroxyapatite with glass ionomer cement has been reported in 2011.The documented effects of these nano-sized particles on the chemistry of these materials include increased biocompatibility and mechanical strength(Nidhi kantharia *et al*,.2011). This study aims to highlight the efficacy of Fish scales HAp in repair of tooth defect area without regarding race and regilion.

Materials and Methods

Fish scales were collected from Mingaladon market.Characterization of fish scales HAp was done at University Research Centre and National Laboratory, Department of Research and Innovation. Extracted teeth were collected from Dental Hospital,University of Dental Medicine, Yangon. This study was taken from 2013 to 2016.

Materials

Fish scales, 1N HCl,1N NaOH, 10% formalin, Glass Ionomer Package and extracted teeth were used in this study.(Figure 2, 3, 4, 5, 6)

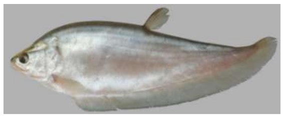


Figure 2.*Notopterus notopterus* (Pallas,1769**)**

Figure 3.*Notopterus notopterus* scales

Figure 4.*Notopterus notopterus* biomaterial powders of different temperatures

Figure 5. GC Glass Ionomer

Figure 6. Extracted Tooth

Method

Extraction of Fish Scales Boimaterials

The following procedure(Figure 7) is used to extract fish scales biomaterials:

Fish(*Notopterus notopterus*) scales are washed thoroughly with tap water

↓

Cleaned fish scales are soaked in 1N HCl solution for 24 hours(Deproteinization)

↓

Washed thoroughly several times with distilled water

↓

Soaked again in 1N NaOH solution for 24 hours(Deproteinization)

↓

Filtered and washed thoroughly with distilled water

↓

Dried in oven

Deproteinized, and dried fish scales are heated at different temperatures in furnace

Fish scale biomaterials powders of different temperatures

Figure 7. Extraction of fish scale biomaterials

Characterization Of Fish Scale Biomaterials Powders

All powders of different temperatures were characterized by XRD and FTIR. Functional groups of all fish scales powders were proved as hydroxyapatite (F.S.HAp) by maching with light absorbant wave numbers of standard hydroxyapatite and those extracted from other biowastes. Purity of F.S.HAp. was confirmed by TGA analysis, protein content and Ca/P weight ratio. Toxic heavy metal content was examined by mineral analysis. Ca/P weight ratio of F.S.HAp. were calculated at laboratory services, AMTT company and matched with that of enamel and dentine. Particle size was observed by SEM. Porosity in the F.S.HAp. was calculated by Image J software.

Evaluation Of Hardness of Pure GC Ionomer and F.S.HAp.-Glass Powder-GIC Composite By Mitutoyo Micro-Hardness Testing Machine

GC glass ionomer (Japan) was available at University of Dental Medicine, Yangon.To make a block of pure GC glass ionomer composite, as described in direction for use, two drops of GIC was added to one scoop of glass powder.(1:2). Procedure for formation of F.S.HAp-Glass Powder-GIC Composite is stated in Figure 8.

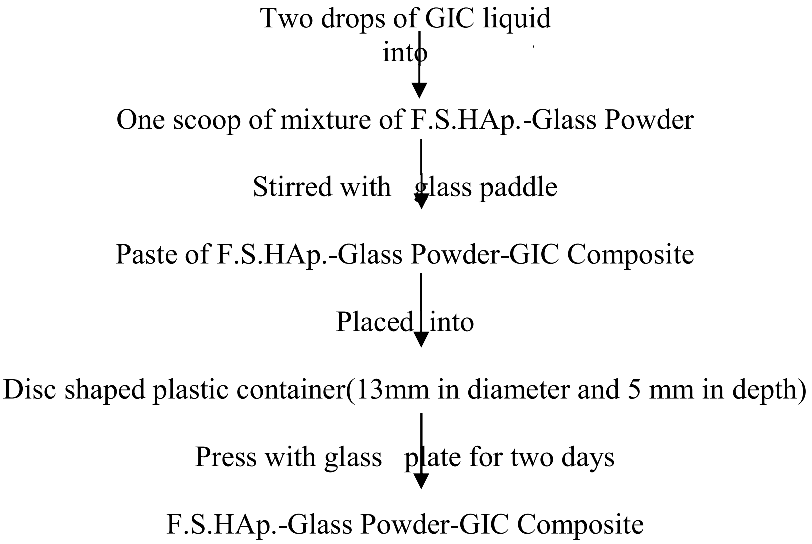


Figure 8. Formation of F.S.HAp.-Glass Powder-GIC Composite

Figure 9. Disc shaped plastic container (13mm in diameter and 5 mm in depth)

Figure 10.F.S.HAp.-Glass Powder -GIC Composite

Hardness measuring of F.S.HAp.-Glass Powder-GIC Composite in different ratios was done by Mitutoyo Micro-hardness testing machine at Meik-Hti-Lar(Figure 11). According to results, the sample composite with hardness very near to that of teeth and skeletal tissue stated in references was selected to repair the defect area in the hard tissues (Teeth and skeleton).

Figure 11 .Hardness measuring of F.S.HAp-Glass Powder-GIC Composite with Mistutoyo Micro-hardness testing machine

I have made four kinds composites in different ratios in order to know which ratio is inline with hardness of teeth(Table 1).

Table 1.The ratios of four samples

Composite	Sample no.	Ratio
Ratio of F.S.HAp.-Glass Powder-GIC composite	1	3:1:8
	2	3:2:10
	3	3:3:12
Pure GC Glass Ionomer (powder& liquid)	4	1:2(as direction for use)

Repair Of Tooth Defect Area

Extracted teeth were collected from Dental Hospital. Defect area of extracted teeth were repaired by following procedure shown in Figure 12.

Extracted tooth
↓
Treated with 10% formalin for 1 day
↓
Clean with purified water
↓
Fill up freshly prepared fish scale HAp-Glass Powder-GIC Composite (3:1:8) in tooth defect area

Figure 12.Procedure for repair of tooth defect area with F.S.HAp-Glass Powder-GIC composite

Results and Discussion

X-ray Diffraction Analysis

Presence of HAp in *Notopterus notopterus* fish scales powder (800°C) was confirmed by a strong diffraction peak at 2θ value of 31.808°(211plane) with 100% intensity (Table 2)

Table 2.Comparison of X.R.D. result datas in I%, 2θ value, hkl of F.S.HAp. and references

Datas	Milenko (2004)	X.R.D. JPCDS-09-0432	*Notopterus notopterus*(800°C)
hkl	211	211	211
I%	100	100	100
2θvalue	31.77	31.77	31.808

Thermal Analysis

The removal of the organic portion was observed at different temperatures with changes in the color of powders. The color of raw fish scale was observed as white, which gradually changed into grey, white, white and white tint with blue green respectively.No significant weight loss was observed at 800˚C,900˚C and 1000˚C(Figure 13).This indicates that fish scale powders at 800˚C is lacking of organic moieties and water.

Functional Groups Study

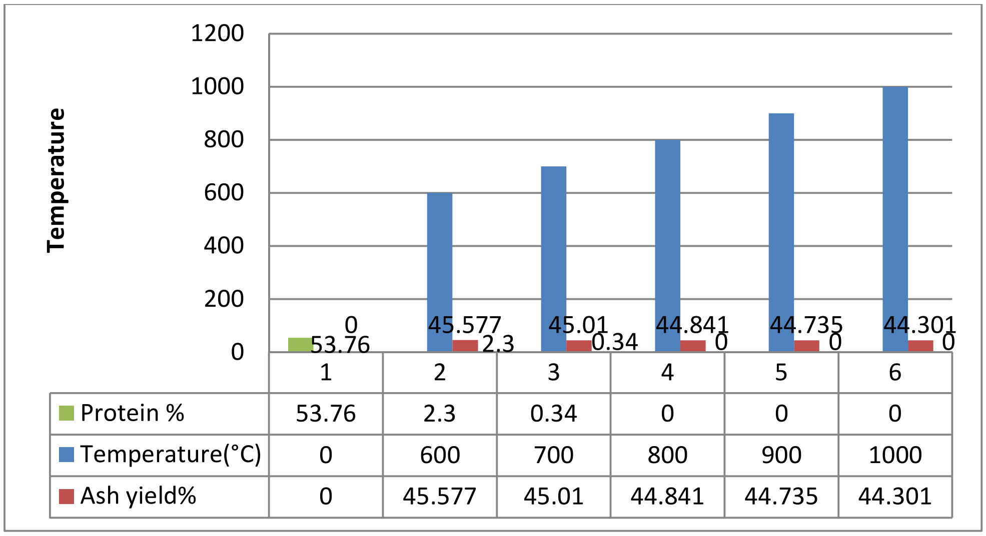
Functional groups of HAp (PO_4^{3-} , CO_3^{2-} , OH^- and HPO_4^{2-}) extracted from fish scale were ascertained by Fourier Transform Infrared spectroscopy. Spectrums of different powders were obtained over the region of 4000 – 400 cm^{-1} . The most characteristic chemical groups in the FTIR spectrum of synthesized HAp are PO_4^{3-}, OH^-, CO_3^{2-} , as well as HPO_4^{2-} that characterize non-stoichiometric HAp. OH^- ions prove presence of HAp.(LigaBerzina-Simdina,2012). Functional groups of fish scales powder at 800°C were also found very close to those of HAp references(Table 3).

Table 3.Comparative study of F.T.I.R. wave numbers Of fish scale HAp. and HAp. references

Kinds Of HAp	Functional Groups Spectrum(cm^{-1})						
Standard Spectrum Of HAp (Lu Xiaoying, 2007)	OH^-	H_2O	CO_3^{2-}	PO_4^{3-}	HPO_4^{2-}	OH^-	PO_4^{3-}
	3570	no	no	1090, 1040,960	no	634	603, 568
Research Of Calcium Phosphate Using FTIR Spectroscopy,\ (LigaBerzina-Simdina,2012)	3420, 3500, 3540, 3570		870-880, 873, 1460-1530, 1450, 1650	1000-1100, 960, 1020-1120, 1094-1090, 1032-1046		630	460, 560-600, 603, 601 565-571
Animal Hard Tissues (LuXiaoying, 2007)	3569, 3571, 3574	1623, 1626, 1631, 1633	1400, 1456, 1460, 1470	960,962, 965,1045, 1047,1049, 1088,1089, 1091,1094	874	632, 634, 636, 642	566, 567, 568, 601, 603, 604, 609
Notopterus notopterus (800°C)	3504		1413.87, 1462.09	962.51, 1037.74, 1089.82		632.67	570.95, 601.81

Ash yield % and Protein content % of F.S.HAp

According to Figure 13 results, ash yield % was about 44%. Protein content was not observed right from at 800˚C. White color appearance of powder at 800˚C was suitable for hard tissue engineering. It is stated that fish scales are biocmposites of highly ordered type I collagen fibers and hydroxyapatite $\{ca_{10}(PO_4)_6(OH)_2\}$(Feng xiang zhang, Anning wang, 2011). As the collagen is a kind of protein, to get only hydroxyapatite from fish scale I have to choose the powder free from protein.



	1	2	3	4	5	6
🟩 Protein %	53.76	2.3	0.34	0	0	0
🟦 Temperature(°C)	0	600	700	800	900	1000
🟥 Ash yield%	0	45.577	45.01	44.841	44.735	44.301

Figure 13. Protein % and Ash yield % of *Notopterus notopterus* scales

Mineral Analysis

There is no heavy toxic metals like mercury,arsenic,cadmium and lead at ppm level shown in Table 4.The Ca/P ratio of *Notopterus notopterus* was 1.63. It is very close to the theoretical value of hydroxyapatite (1.67) and found within the range of hydroxyapatite with calcium deficient(1.5-1.67) (LigaBerzina-Simdina, 2012).(Table 4)

Table 4. Mineral analysis Of F.S.HAp

Analyte	***Notopterus notopterus* (%)**	**Ca/P**		
		Notopterus notopterus	Hydroxyapatite with Calcium deficient	Hydroxyapatite
Ca	**5.093(50930ppm)**	1.63	1.5-1.67	1.67
P	**3.062(30620ppm)**			
K	**0.098(980ppm)**			
S	**-**			
Sr	**0.016(160ppm)**			
Fe	**0.007(70ppm)**			
Zn	**0.003(30ppm)**			
Mn	**-**			
Bi	**-**			
Cu	**-**			

Ca/P Weight Ratio

Ca/P weight ratios of Fish scales were calculated at Laboratory Services , AMTT company. 1.94 Ca/P weight ratio of fish scale hydroxyapatite is identical to that of enamel, dentine and tuna bone (Table 5).

Table 5. Ca/P weight ratio of enamel, dentine, tuna bone and F.S.Hap

Ref:	Au. HwaYen liu (2013)		Jayachandran (2010)	In this study
Sample	Enamel	Dentine	Tuna bone	*Notopterus notopterus* scale
Ca/P ratio	1.94-1.83	1.91-1.78	1.94	1.94

Calculation Of Crystallite Size

For tissue engineering work, particle size is important to penetrate into the host tissue. According to X.R.D results, the biomaterial powders extracted from fish scales were proved to be HAp. Average crystallite size of *Notopterus notopterus* at 800°C was 75.8nm.The crystallite size of HAp of different powders were calculated by following equation:

$$\text{Crystallite Size} = \frac{0.9\lambda}{\beta \text{Cos}\theta}$$

SEM Analysis

Measurements of particle size and pore diameter were done by scanning electron microscope at the National Laboratory of the Department of Research and Innovation. Particle sizes are gradually increasing with the rise of temperature upto 900°C but at 1000°C some particles break up into smaller size. Average particle size of F.S.HAp at 800°C was found within the range of 151nm to 265nm (Table 6). All hexagonal shaped particles were fragile in texture.

Table 6. Comparative study of particle size of F.S.HAp at different temperatures

Temperature(°C)	*Notopterus notopterus*
600	45.45 nm to 219.7 nm
700	73.4 nm to 333.6 nm
800	**151.6 nm to265.4 nm**
900	303.3 nm to 925 nm
1000	128.9 nm to 746 nm

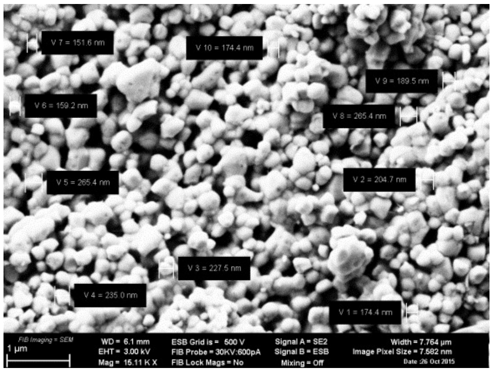


Figure 14.*Notopterus notopterus* scale (800˚C) biomaterial powder

Porosity of the F.S.HAp.

Porisity in the HAp is very important for tissue engineering work. Porosity in the HAp extracted from scales measured by image J software, was found to be 15.86% at 800˚C. (Table 7)

Table 7. Pore % of F.S.HAp.

Notopterus notopterus Scales HAp		Pore %
Total Area	41448400.44 nm^2	15.86
Pore Area	6575553 nm^2	

Toxicity Assessment of F.S.HAp.

As *Notopterus notopterus* is globally recognized as non toxic aquatic fauna, the HAp derived from it is also a non toxic biomaterial. In addition Glass powder and Glass Ionomer Cement are currently used in daily clinical practice and dental therapy. Therefore the composite applied in the present work is needless to say non toxic to human beings. Toxic heavy metals like Mercury, Arsenic, Lead and Cadmium were not detected in the fish scale powders at ppm level (Table 4). Therefore, application of fish scale HAp in hard tissue engineering,I suggest that they are not toxic to host tissue.

Evaluation of Hardness of Pure GC Glass Ionomer and F.S.HAp-Glass Powder-GIC Composite by Mitutoyo Micro-hardness Testing Machine

Hardness of all samples were measured by Mitutoyo Micro-hardness testing machine. According to results(Table.8), the hardness value of pure GC Glass Ionomer was found to be 3.5 Mohs. Ismail(2013) stated that adding of glass ionomer cement into hydroxyapatite-silica nanopowder composite makes enhancement of hardness. So many efforts were done in order to increase hardness. Incorporation of Glass Powder-GIC into F.S.HAp. gave increasement in hardness of 50HV compared to the pure GC Ionomer. Among the samples of F.S.HAp.-Glass Powder-GIC composite, according to test results(Table 8), the most hardest value of 272 (average HV) was found in the sample of 3 : 1 : 8 ratio. It was selected to use in the hard tissue engineering application.

Table 8. Hardness measurement by Mitutoyo Micro Hardness Testing Machine

Kind of Sample	Ratio	Hardness(HV)	Hardness (Mohs)
Pure GC Glass Ionomer (Powder : Liquid)	1 : 2	222(average)	3.5
F.S.HAp-Glass Powder-Glass Ionomer Cement Composite	3 : 1 : 8	272(average)	4
	3 : 2 : 10	261(average)	4
	3 : 3 : 12	268(average)	4
JK Chun, HH Choi and JY Lee (2014)	Enamel	274.8	
	Dentine	65.6	

SEM Analysis Of F.S.HAp– Glass Powder-GIC Composite

According to literature (Jayachandran, 2010) saying, the very important factor in the tissue engineering application is to have quite enough pore diameter in the substituted tissue for nutrient inflow and cell to cell connection. Therefore SEM analysis of the composite sample that is to be used in hard tissue engineering, was done at the Department of Research and Innovation. With Scanning Electron Microscope, pore diameter in the FS HAp-Glass Powder- GIC composite was found within the range of 26 nm – 1402 nm. Majority of pores in the composite was nano-sized.(Figure.15)

Meenakhi Mour and Debarun *et.al.*(2010) stated that minimal necessary for bone ingrowth is considered to be approximately 100 µm. The minimum pore size require to generate mineralized bone is generally considered to be 100 µm due to cell size, migration requirement and nutrient transport. Large pores(100-150 and 150-200µm) showed substantial bone growth. Small pores (75-100µm) resulted in ingrowth of unmineralized osteroid tissue. Smaller pores (10-44 and 44-75 µm) were penetrated only by fibrous tissue.

According to evidence of literature(Capillaries-Histology guide; University of Leeds), the diameter of the smallest capillary is 3 to 4 µm. I found that the significant difference in diameter of pore and capillary may lead to a barrier for nutrient supplementation and cell to cell connection into the substituted tissue.

Since enamel is mainly composed of HAp and without supplying of blood vessels and nerves, it is a structure of non living materials. So once eroded, there is no regeneration of HAp in the enamel defect area. Therefore I have done the substitution of F.S. HAp in the enamel defect area with the aid of Glass Ionomer Cement.

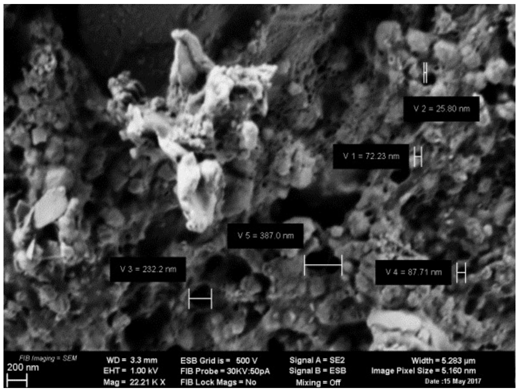


Figure 15.Pore diameter measurement of F.S.HAp-Glass Powder-GIC composite by SEM

Substitution Of F.S.HAp-Glass Powder-GIC Composite In Tooth Depfect Area(In vitro Study)

Since enamel being itself mainly composed of 20-40 nm sized HAp particles and without supplying of blood vessels and nerves, it is a structure made of non-living minerals. Once eroded the enamel, there is no regeneration of HAp in the enamel defect area. Apart from this, particle size difference between F.S.HAp and enamel is a barrier for supplementation of mineral requirement of enamel by diffusion.

In this study, the crystal size of *Notopterus notopterus* was 75.8nm. Particle size difference of HAp between tooth and fish scales was found to be a barrier for adsorption of particles to the host tissue. In order to overcome the problem of particle size difference in repair of tooth defect area, 4 drops of liquid of GC Glass Ionomer was added into the mixture of fish scale HAp-Glass Powder (1:1). Having no interference from the time of commencement of filling upto this time of presentation, the substituted HAp-Glass powder-GIC composite is still attached to the area of filling in the defect area. (Figure.16, 17, 18)

Figure 16. Before filling

Figure 17. After filling

Figure 18. Binding of vertically divided tooth

Conclusions

In this study, nano-sized hydroxyapatite biomaterial can be produced start right from *Notopterus notopterus* scales at 800°C. Significant finding is that without aid of cement, it is impossible to repair the enamel by using 75.8nm sized F.S.HAp. The application of F.S.HAp.-Glass Powder-Glass Ionomer Cement in repair of enamel defect area is possible only by substitution. It can be used in the hard tissues engineering applications for all people without regarding race and religion.

Acknowledgements

I really thank Professor Dr. Myinzu Minn (Pro-rector, Yadanarbon University) and Professor Dr.Thida Lay Thwe (Head, Department of Zoology, University of Yangon) for their invaluable advices given to me during my research work concerning with nanobiomaterial in the field of Zoology.I also extend my thanks to Dr. Nyein Nyein Hlaing (Associate Professor, Department of Zoology, University of Yangon) and to all those who help me whole-heartedly and enthusiastically in doing this research work. Lastly, I do wish to express my great appreciation and special thanks to Myanmar Academy of Arts and science for giving permission me to read my thesis work in this auspicious occasion.

References

Antonio Zanotto[1*],Maria Luisa Saladino[2], Delia Chillura Martino[2], Eugenio Caponetti[1,2](2012), Centro Grandi Apparecchiature, "Influence of Temperature on Calcium Hydroxyapatite Nanopowders", Uni Net Lab, Universita degli Studi di Palermo, Italy, *Advances in Nanoparticles*, 2012,1,21-28.

Dasgupta Adak[1], M., and Purohit[2], K.M.(2011), "Synthesis of nanocrystalline Hydroxyapatite from dead snail shells for Biological Implantation", Department of Life Sciences, National Institute of Technology, Rourkela, 769008, *Trends Biomater.Artif.Organs,*25(3),101-106(2011).

Dersot, J.M., Septier,D., Llorens, A. and Saffar, J.L.* (1993)," Multinucleated Giant Cells-Hydroxyapatite Interactions: A time-related Quantitative Study in a Rat Skull defect", Laboratoire de Biologie et Biomateriaux du Milieu Buccal et Osseux, Faculte de Chirurgie dentaire, Universite Paris-V, 1 Rue Maurice, Arnoux,92120 Montrouge,France, *Cells and Materials*, Vol.3,No.4, 1993(pages 395-405).

Fengxiang Zhang1[1*], Anning Wang[2] ,Zhihua Li[1],Shengwen He[1], Lijun Shao[1](2011), "Preparation and characterization of collagen from freshwater fish scales", Weifang Medical University, Weifang, China, *Food and Nutrition Sciences*,2011,2,818-823.

Hedzelek[a], W., Wachowiak[b].R.,Marcinkowska[a,*]and Domka[c],L.(2008), "Infrared Spectroscopic Identification of Chosen Dental Materials and Natural

Teeth",University of Medical Sciences, Poland, *Acta Physica Polonica A*,Vol.114, Issue 2, p.471.

Hinedi Z.R., Goldberg S., Chang A.C., and Yesinowski. J.P. (1992), " A^{31}P and ^{1}H MAS NMR Study of Phosphate Sorption onto Calcium Carbonate", Department of Soils and Environmental Sciences, University of California, USA, *Journal of Colloid and Interface Science,* Vol.152, No.1, August 1992.

Hwa-Yen liu[1*], Jiunn-Hsing Chao[2],Chun-Yu Chuang[1],Hung-Lin Chiu[3], Chung-Wei Yang[1], Yuh-Chang Sun[1](2013),"Study of P,Ca,Sr, Ba and Pb level in enamel and dentine of human third molar for environmental and archaeological research", Au. Institute of Anthropology", College of Humanities and social Sciences, National Tsing Hua University, Taiwan, *Advances in Anthropology*, 2013.Vol.3,No.2,71-77.

Ismail Ab Rahman[*],Sam'am Malik Masudi, Norhayati Luddin and Rayees Ahmad Shiekh (2013), "One-pot synthesis of Hydroxyapatite-silica nanopowder composite for hardness enhancement of glass ionomer cement", School of Dental Sciences, Universiti Sains Malaysia,*Bull.Mater.Sci., Vol.37,No.2, ©Indian Academy of Sciences.* April2014,pp.213-219.

Jayachandran Venkatesan[1] and Se Kwon Kim[1,2,*](2010), "Effect of Temperature on Isolation and Characterization of Hydroxyapatite from Tuna bone", Department of Chemistry, Pukyong National University,Busan, Korea, *Materials* 2010,3(10),4761-4772;doi:10.3390/ma3104761.

Liga Berzina-Cimdina and Natalija Borodajenko,(2012). "Research of calcium phosphate using Fourier Transform Infrared Spectroscopy", Riga Technical University, Institute of General Chemical Engineering, Lavia, *Infrared Spectroscopy-Materials Science, Enginering and Technology*.

LiLi[a], Haihua Pan[a], Jinhui Tao[a], Xurong Xu[a],Caiyun Mao[b], Xinhua Gu[b], and Ruikang Tang[*a](2008). "Repair of enamel by using hydroxyapatite nanoparticles as the building blocks", Department of Chemistry, Zhejiang University, Hangzhou, Zhejiang,China, www.rsc.org/materials| *Journal of Materials Chemistry*, DOI:10.1039/b806090h.

Lu Xiaoying,Fan Yongbin, Gu Dachun and Cui Wei, (2007). "Preparation and characterization of Hydroxyapatite from Animal Hard Tissues", State key laboratory of Biotechnics, Dept. of Biomedical Engineering, Southeast University, China, *Key Enginnering Materials* Vols.342-343(2007)pp213-216,©(2007)*TransTech Publications,Switzerland.*

Maya, G., Lyapina[1], Mariana Tzekova[2], Maria Dencheva[2], Assya Krasteva[2], Mariela Yaneva-Deliverska[3], Angelina kisselova[2](2016) "Nano-Glass Ionomer Cement In Modern Restorative Dentistry",Department Of Hygiene, Medical Ecology and Nutrition, Medical Faculty, Medical University- Sofia, Bulgaria, *Journal of IMAB-Annual Proceeding Scientific Paper*, vol. 22, issue2.

Meenakshi Mour[1], Debarun Das[1], Thomas Winkler[2], Elisa Hoenig[2], Gabriela Mieke[2], Michael M. Morlock[2] and Arndt F.Schilling[2*],(2010). "Advances in Porous Biomaterials for Dental and Orthopaedic Applications”,Department of Mechanical engineering, National Institute of Technology, Durgapur 713209, India, *Materials* 2010,3,2947-2974;10.3390/ma3052947.

Milenko Markovic, Bruce O. Fowler and Ming S. Tung, (2004). “Preparation and comprehensive characterization of a calcium hydroxyapatite reference material”, *Journal of Research of the National Institute of Standards and Technology*, volume109,No.6, *J.Res.Natl.Inst.Stand.Technol.* 109,553-568(2004).

Nihi Kantharia, Sonali Naik[1], Sanjay Apte[1], Mohit Kheur, Supriya Kheur[2], Bharat kale[1] (2011). “Nanohydroxyapatite and its comtemporary application”, Department of Prosthodontics, M.A. Rangoonwala Dental College, India, *Journal of Dental Research and Scientific Development*, 2014,vol1,issue1.

Sunho oh[1], Namsik Oh[2], Mark Appleford[1], Joo L.Ong[1](2006). “Bioceramics for Tissue Engineering Application, A Review”, The University of Tennessee Health Science Center,U.S.A., Inha University, Incheon, South Korea, *American Journal of Biochemistry and Biotechnology* 2(2):49-56,2006,ISSN 1553-3468.©,2005 *Science Publications.*

Thamaraiselvi, T.V. and.Rajeswari. S. (2004). “Biological Evaluation of Bioceramic Materials-A Review”, Department of Analytical Chemistry, University of Madras, Guindy Campus, Chennai 6000125, *Trends Biomater. Artif.Organs,* Vol18(1),pp9-17(2004).

Wikipedia, the free encyclopedia.Scale anatomy.*https://en.wikipedia.org*>*wiki*>*Scale*.