EFFECT OF MECHANICAL MILLING TIME ON PREPARATION CHARACTERISTIC, MAGNETIC BEHAVIOUR AND *IN-VIVO* STUDY OF HEMATITE-HYDROXYAPATITE NANOCOMPOSITE

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DEDICATION

For my beloved parents and husband

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ABSTRACT

Bovine bone is one of the natural resources of hydroxyapatite (BHAp). It also has been commercialized in the market due to its biocompability and better osseointegration. However, there has been reported of poor mechanical properties of BHAP which lead to premature fracture in implants. The combination of hematite (α-Fe_2O_3) and BHAp were taking into consideration to improve the mechanical properties and enhance its bioactivity. Mechanical milling was employed to fabricate the nanocomposite that occupied dispersion of metallic, generation and migration of defects in the microstructure and plastic deformation of particle. The aim of this project is to explore how milling time effects the structural features as strengthening mechanisms. Hematite substituted BHAp magnetic nanoparticle was prepared by mechanical activation (ball-milling). Synthesizing of BHAp composite showed dispersion of particles and improved strength, as it engaged to nanocrystalline metal. Effect Fe content (5%, 15% and 30%), milling time (3, 6, 9 and 12 h) before and after annealed for 2 h at 900 ^{o}C were investigated. Comprehensive characterization techniques including X-ray diffraction, field emission scanning electron microscopy, and transmission electron microscopy were used to examine effect Fe^{3+} on structural properties such as lattice parameter of BHAp, crystallinity and morphology of the phase. Compression strength and Young's modulus of the nanocomposites were found increased at 36.29% and 26.15% respectively, as the milling was prolonged from 3 to 12 h. Immersion studies in simulated body fluid (SBF) showed better bioactivity but different mechanism on the growth of apatite at different milling time due to surface properties and microstructure. Different magnetic properties exhibited at different milling time in 30% α-Fe_2O_3+BHAp due to the arrangement of Fe ion in BHAp structure. Based on the extraction assay for cytotoxicity test, the presence of higher Fe^{3+} ion substitution in BHAp has enhanced the cell proliferation on fibroblast with better adhesion of osteoblast on the surface until 14 days. The preliminary *in-vivo* studies on rat show better osseointegration for 12 h milling of 30% α-Fe_2O_3+BHAp indicated by callus formation after 30 days. Finally, morphology of 30% α-Fe_2O_3-+HAp particles behaviour strongly influenced by the milling time that regenerated new performance in bone-implant material.

ABSTRAK

Tulang lembu merupakan salah satu sumber semulajadi untuk penghasilan hidroksiapatit (BHAp). BHAp juga telah dikomersialkan di pasaran oleh kerana sifat biokompatibiliti dan osseointegrasi yang baik. Namun, kekurangan dalam ciri mekanikal contohnya keretakan implant pramatang sering dilaporkan. Hematit (α-Fe_2O_3) adalah besi teroksida yang paling stabil di bawah keadaan sekeliling dan merupakan bahan bermagnet untuk aplikasi bioperubatan, penghasilan BHAp dengan pengukuhan α- Fe_2O_3 bersaiz nano telah dipertimbangkan untuk menambahbaik ciri mekanikal dan meningkatkan bioaktiviti. Penggilingan secara mekanikal telah digunakan untuk penghasilan nanokomposit yang melibatkan penyebaran bahan logam, penjanaan dan pergerakan kecacatan di mikrostruktur dan pengubahan bentuk partikel plastik. Oleh itu, kajian ini bertujuan untuk mengenalpasti kesan masa penggilingan terhadap sifat struktur untuk mekanisma penguatan. Nanopartikel BHAp bermagnet disubstitusikan dengan hematit telah disediakan melalui pengaktifan mekanikal (bola penggiling). Sintesis BHAp komposit menunjukkan penyebaran partikel dan meningkatkan kekuatan, atas hubungan nano-penghabluran besi. Kesan peratusan ferum (5%, 15% dan 30%), masa penggilingan (3, 6, 9 dan 12 jam) sebelum dan selepas sepuh lindap selama 2 jam pada suhu 900^{o}C telah dikenalpasti. Teknik karakterisasi secara mendalam termasuk XRD, FESEM dan TEM telah dijalankan untuk mengenalpasti kesan Fe^{3+} terhadap sifat struktur seperti parameter kekisi BHAp, kehabluran dan fasa morfologi. Apabila masa penggilingan dipanjangkan dari 3 sehingga 12 jam, kekuatan mampatan dan modulus Young nanokomposit turut meningkat masing-masing kepada 36.29% dan 26.15%. Kajian rendaman dalam larutan SBF menunjukkan sifat bioaktiviti yang baik namun terdapat perbezaan dalam mekanisma pertumbuhan apatit untuk masa penggilingan yang berbeza disebabkan oleh sifat permukaan dan mikrostruktur. Perbezaan masa penggilingan 30% α-Fe_2O_3+BHAp menunjukkan perbezaan sifat magnetik disebabkan oleh susunan ion Fe dalam struktur BHAp. Berdasarkan asai pengekstrakan untuk ujian ketoksikan sel, kehadiran substitusi ion Fe^{3+} yang tinggi dalam BHAp telah meningkatkan proliferasi sel fibroblas sehingga 14 hari dan memberikan kelekatan sel osteoblas yang baik pada permukaan. Kajian awal *in vivo* terhadap tikus selepas 30 hari menunjukkan osseointegrasi yang baik melalui pembentukan kalus untuk 30% α-Fe_2O_3+BHAp dengan masa penggilingan 12 jam. Akhirnya, morfologi partikel 30% α-Fe_2O_3+BHAp sangat dipengaruhi oleh waktu penggiling, dimana mampu melahirkan bahan implant tulang yang baharu dan berpretasi.

TABLE OF CONTENTS

	TITLE	PAGE
	DECLARATION	**ii**
	DEDICATION	**iii**
	ACKNOWLEDGEMENT	**iv**
	ABSTRACT	**v**
	ABSTRAK	**vi**
	TABLE OF CONTENTS	**vii**
	LIST OF TABLES	**xii**
	LIST OF FIGURES	**xiv**
	LIST OF ABBREVIATIONS	**xix**
	LIST OF SYMBOLS	**xx**
	LIST OF APPENDICES	**xxi**
CHAPTER 1	**INTRODUCTION**	**1**
1.1	Background	1
1.2	Problem statement	3
1.3	Objective	5
1.4	Scopes	5
1.5	Significances of study	6
1.6	Thesis Structure and Organisation	7
CHAPTER 2	**LITERATURE REVIEW**	**9**
2.1	Introduction	9
2.2	Biomaterials	9
2.3	Bioceramics	10
2.4	Hydroxyapatite	11
	2.4.1 The crystal structures of hydroxyapatite	13
	2.4.2 Natural hydroxyapatite	15
	2.4.2.1 Bovine bone HAp	16

2.4.2.2 Thermal process 17

2.5 Hydroxyapatite composite 19

2.6 Iron and Iron Oxide 21

2.6.1 Iron (III) oxide: Hematite (α-Fe_2O_3) 22

2.6.2 Iron oxide-Hydroxyapatite 23

2.7 Mechanochemical Process 26

2.7.1 Brief history 27

2.7.2 Theory Mechanochemistry 27

2.7.3 Planetary mills 30

2.7.4 Milling time 31

2.8 Magnetic material 33

2.8.1 Types of Magnetism in Materials 34

2.8.2 Magnetic Hysteresis: Variable and properties 36

2.8.3 Application of magnetic materials in Biomedical 38

2.8.3.1 Drug delivery and cell separation 40

2.8.3.2 MRI contrast agents 41

2.8.3.3 Hyperthermia 43

2.9 Bone 44

2.9.1 Mechanism of bone formation 45

2.9.2 Wound healing 46

2.9.3 Bone healing 48

2.10 Summary of literature 51

CHAPTER 3 RESEARCH METHODOLOGY 55

3.1 Introduction 55

3.2 Sample preparation 55

3.2.1 Bovine hydroxyapatite preparation 57

3.2.2 Stage 1: Mixing of iron-BHAp ratio 58

3.2.3 Stage 2: Mixing 30% Fe-BHAp in difference milling time 59

3.3 Samples characterisation 60

3.3.1 Physiochemical properties 60

		3.3.1.1 X-ray diffraction (XRD)	60
	3.3.2	Morphology Analyses	61
		3.3.2.1 Field emission-Scanning electron microscopy (FE-SEM)	61
		3.3.2.2 High ResolutionTransmission electron microscopy (HRTEM)	61
	3.3.3	Surface wettability	62
		3.3.3.1 Contact angle (CA) analysis	62
	3.3.4	Magnetic properties	62
		3.3.4.1 Vibrating sample magnetometer (VSM)	62
3.4	Radiopaque analysis testing		62
3.5	Mechanical testing		63
3.6	*In-vitro* biomineralisation study		63
3.7	Cell culture		64
	3.7.1	MTT assay study	65
	3.7.2	Cell attachment	66
3.8	Preliminary study of in-vivo biocompability		66
	3.8.1	Animal model	66
	3.8.2	Implantation	67
	3.8.3	Samples characterisation	68
		3.8.3.1 X-ray Diffraction (XRD)	68
		3.8.3.2 Fourier transform InfraRed (FTIR)	68
	3.8.4	Radiological images analysis	68
	3.8.5	Peripheral blood profile analysis	69
CHAPTER 4	**RESULTS AND DISCUSSIONS**		**71**
4.1	Introduction		71
4.2	Analysis of Bovine Hydroxyapatite–Iron Composite with different Percentages of Iron.		71
	4.2.1	Phase Determination of Nanocomposite with Various Iron Percentages	72
	4.2.2	Structural and Microstructural Characterizationof Nanocomposite with Various Iron Percentages	73

4.2.3 Phase Determination of Nanocomposite with Various Iron Percentages after Sintering 75
4.2.4 FESEM for Various Iron Percentages 76
4.2.5 Compression Testing 78
4.2.6 Radiodensity with Different Percentages of Hematite 79
4.2.7 MTT Assay for Various Percentages of Iron 80
4.2.8 Summary of findings 82
4.3 Analysis of Bovine Hydroxyapatite–Iron Composite with Different Milling Time 82
4.3.1 Phase Determination of Nanocomposite with Various Milling Time Durations 83
4.3.2 Structural and Microstructural Characterization of 30% Fe-BHAp Nanocomposite with Various Milling Time Durations 85
4.3.3 Phase Determination of Nanocomposite with Various Milling Time Durations after Sintering 88
4.3.4 FESEM for Various Milling Time Durations 92
4.3.5 High Resolution-Transmission Electron Microscopy Study 95
4.3.5.1 Morphology of 30%Fe-BHAp with Various Milling Time Durations 95
4.3.5.2 Morphology of 30% α-Fe_2O_3-BHAp with Various Milling Time Durations 98
4.3.6 Compression Testing 100
4.3.7 Radiopaque Analysis 101
4.3.8 Magnetic Properties Analysis 102
4.3.9 Wettability 106
4.3.10 *In-Vitro* Study by Immersion in SBF 106
4.3.11 Cell Attachment 110
4.3.12 Preliminary *In-Vivo* Study 114
4.3.12.1 Peripheral blood analysis 115
4.3.12.2 Red blood cells analysis 115
4.3.12.3 White blood cells analysis 118

4.3.12.4 X-ray radiography 121

4.3.12.5 Material Characterisation After Implantation 125

4.3.12.6 X-ray Diffraction Analysis 126

4.3.12.7 FTIR Analysis 128

4.3.13 Summary of findings 130

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS 131

5.1 Introduction 131

5.2 Conclusion 131

5.3 Future work 133

REFERENCES 135

LIST OF PUBLICATIONS 180

LIST OF TABLES

TABLE NO.	TITLE	PAGE
Table 2.1	Implants materials classified based on the material types (50).	10
Table 2.2	Types of bioceramic interaction with surrounding (51)	11
Table 2.3	Calcium phosphate and their uses (45,57,58).	12
Table 2.4	Natural sources synthesized to HAp with method, calcinations temperature on phase stability and phase decomposition (79).	16
Table 2.5	Various of hydroxyapatite composite research through many years.	20
Table 2.6	Hydroxyapatite with iron oxide composite studied by other research	52
Table 3.1	Iron containing the prepared BHAp synthesis	58
Table 3.2	Ion concentration of SBF and human blood plasma	64
Table	4.1 Average FHWM, crystal size, and lattice strain of BHAp	74
Table 4.2	The phase fraction, lattice parameter, and crystallite size of BHAp and α-Fe_2O_3 phase in composite phases	91
Table 4.3	Saturation magnetization, remanent magnetization, and coercivity values against milling time.	103
Table 4.4	Saturation, remanent magnetization and coercivity values against milling time.	105
Table 4.5	Contact angle of BHAp composite by Fe reinforcement for different milling time durations.	106
Table 4.6	The number of red blood cells ($10^6/mm^3$) and hemoglobin (g/dL) for rats control group, and 30% α-Fe_2O_3+BHApfor different milling time durations (3, 6, 9, and 12 h) at 0, 7, 14, 30, and 60 days.	116
Table 4.7	List of Rat's Haematologic (268)	118
Table 4.8	The number of white blood cells ($10^3/\mu L$), neutrophils (%WBC), lymphocytes (%WBC), monocytes (%WBC), eosinophils (%WBC), and basophils (%WBC) for rats control group, and 30% α-Fe_2O_3+BHApfor different	

milling time durations (3, 6, 9, and 12 h) at 0, 7, 14, 30, and 60 days. 120

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
Figure 2.1	Different HAp structure: monoclinic (top) and hexagonal (bottom) unit cells. (64)	13
Figure 2.2	Crystal structure of HAp in the c-direction into the plane of the paper. O-red, Ca(I)-light blue, Ca(II)-dark blue, P-yellow, OH—pink. (65)	14
Figure 2.3	Schematic morphology of BHAp during as-received and heat treated at 1250 °C to 900 °C (86).	19
Figure 2.4	The multidisciplinary nature of iron oxide research (102).	21
Figure 2.5	Schematic illustration of (a) hexagonal unit cell and (b) rhombohedral primitive cell of α-Fe_2O_3. Color scheme : Fe = grey and O = red (118).	23
Figure 2.6	a) Microstructure model of hydroxyapatite- iron composite (Fe(II)-HAp) and b) Vibrating Sample Magnetometer (VSM) curve of Fe(II)-Hap (32).	25
Figure 2.7	TEM image of a) cubic shaped pristine of magnetite nanoparticle (MNP), b) MNP-hyroxyapatite (m-HAp) synthesized at 35kHz at 320W and c) comparison VSM curve MNP and m-HAp ((a) (131).	26
Figure 2.8	Magma-plasma model: E-exo-electrons, N-underformed solid, D-highly deformed surface layer, P-plasma (37).	28
Figure 2.9	The change of specific surface are, S in the course of a mechanochemical reaction(37)	28
Figure 2.10	a) Defects created by mechanical activation (MA) (37) and (b) TEM image of bending plane, dislocation and non-ordered structure of HAp grain (139)	29
Figure 2.11	Main stress in mills, R1-compression, R2-shear (attrition), R3-impact (stroke), R4-impact (collision), circle- mass of milling media, square-mass of material charge, rectangle-mass of mill (37).	30
Figure 2.12	(a) Planetary mills (Google image), and (b) Movements of working parts and ball in planetary mill (36).	31

Figure 2.13	TEM image of FAp-20% wtTiO_2 nanocomposite after (a) 5h, (b) 10h, (c) 15h and (d) 20h milling (143).	32
Figure 2.14	Magnetic moments associated with (a) orbital and (b) spin angular momenta of an electron (148).	33
Figure 2.15	Magnetization curve of LMSO powder with different milling time at 300 K (146)	34
Figure 2.16	Different types of magnetic behaviour (149).	35
Figure 2.17	Hysteresis loop (*www.nde-ed.org*)	37
Figure 2.18	Schematic illustration of the coercivity-size relations of small particles (152)	38
Figure 2.19	Schematic representation of magnetically driven transport of drugs to specific region (155).	39
Figure 2.20	Magnetic carrier for drug targeting and drug delivery system under the influence of external magnetic field (153).	40
Figure 2.21	Illustration of multifunctional imaging/therapeutic MNPs anatomy and potential mechanisms of action at the cellular level. (A) A multifunctional MNP modified with targeting and (B) Four possible modes actions for various therapeutic agents (159).	41
Figure 2.22	Animal trial data on hyperthermia treatments in rabbits showing different time of tumour infused ferromagnetic microspheres; (■) tumour edge, (♦) tumour centre, (▲) normal liver 1-2cm, (×) alternative lobe, and (◊) core body temperature (151).	43
Figure 2.23	The hierarchical structure of bone (Murugan and Ramakrishna, 2005).	45
Figure 2.24	Stages of wound healing (Google image)	46
Figure 2.25	Schematic representation of the three stages of fracture repair (189)	50
Figure 3.1	Flowchart of experiment a procedure. (a) Stage 1: Different percentage of iron (Fe), and (b) Stage 2: 30%Fe_2O_3+BHAp in different milling time.	56
Figure 3.2	Bovine hydroxyapatite procedure	58
Figure 3.3	Schematic view of the mechanosynthesis method	59
Figure 3.4	A male Sprague Dawley rat (Google's image)	67

Figure 4.1 XRD pattern of 5%, 15%, and 30% Fe-BHAp milled nanocomposite powder. (♦: hydroxyapatite (#09-0432), ●: iron (#6-0696)) 72

Figure 4.2 Magnified XRD pattern of BHAp (hkl: 002) peaks. 74

Figure 4.3 (a) XRD pattern of 5%, 15%, and 30% Fe-BHAp milled nanocomposite powder after sintering at 900^{o}C for 2 h, and (b) samples of 5%, 15%, and 30% Fe-BHAp milled. 76

Figure 4.4 FESEM of surface morphologies for different iron percentages Fe-BHAp. (a) 5%, (b) 15% and (c) 30% as milled for 12 h, and histogram of particles distribution for (d) 5%, (e)15% and (f) 30%-Fe-BHAp 77

Figure 4.5 FESEM of surface morphologies for different iron percentages α-Fe_2O_3-BHAp. (a) 5%, (b) 15% and (c) 30% after sintering, and histogram of particles distribution for (d) 5%, (e)15% and (f) 30%- α-Fe_2O_3-BHAp. 78

Figure 4.6 The mechanical properties of 5, 15 and 30% α-Fe_2O_3-BHAp specimens: (a) Young's Modulus and (b) compression strength. 79

Figure 4.7 Statistical analysis of grey scale for radiopacity values of 5%, 15%, and 30% α-Fe_2O_3-BHAp. 80

Figure 4.8 Cell cytotoxicity of the diluted extract of 5%, 15%, and 30% α-Fe_2O_3+BHAp against fibroblast cell. 81

Figure 4.9 XRD spectra of 30%Fe-BHAp samples with various milling time durations. 83

Figure 4.10 Magnified XRD region between (a) hkl: 002 for BHAp (2θ=25^{o}–27^{o}) and (b) hkl: 110 for Fe (2θ=43^{o}–46^{o}) 86

Figure 4.11 (a) Full width at half maximum (FWHM), (b) crystallite size, and (c) lattice strain of BHAp (hkl:002) and Fe (hkl: 110). 87

Figure 4.12 XRD spectra of 30%Fe-BHAp samples with various milling time durations after sintering at 900^{o}C for 2 h and (b) samples of 30% Fe-BHAp milling time (3, 6, 9 and 12 h) 90

Figure 4.13 FESEM of surface morphologies of milled 30% Fe-BHAp for different milling time durations. (a) 3 h, (b) 6 h, (c) 9 h, and (d) 12 h. (e-h) histogram of particle size. 93

Figure 4.14 FESEM micrographs showing morphology of sintered 30% α-Fe_2O_3-BHAp cross section pellets for (a) 3 h, (b) 6h, (c) 9h, and (d) 12h of milling time. (e-h) histogram of particle size. 94

Figure 4.15 TEM images of 30%Fe-BHAp after a) 3 h, b) 6 h, c) 9 h, and d) 12 h of milling (magnification at 40k). 96

Figure 4.16 Higher magnification of TEM images of 30%Fe-BHAp after milling for a) 3 h, b) 6 h, c) 9 h, and 12 h (magnification at 500k). (Blue arrow: amorphous region). 97

Figure 4.17 TEM images of 30% α-Fe_2O_3-BHAp sintered pellets after (a) 3 h, (b) 6 h, (c) 9 h, and (d) 12 h of milling. Low magnification images inset show the size of particles. Yellow marks indicate the distribution of Fe_2O_3, white arrows indicate the amorphous region, and red arrows indicate moiré fringes. 98

Figure 4.18 TEM images of 30% α-Fe_2O_3-BHAp sintered pellets after milling for (a) 3 h, (b) 6 h, (c) 9 h, and (d) 12 h. Higher magnification images show the formation of mixed moiré fringes (red mark and white line) and dislocations (inverse 'T'). 99

Figure 4.19 The mechanical properties of 30% α-Fe_2O_3-BHAp with different milling time durations: (a) Young's modulus and (b) compressive strength. 101

Figure 4.20 Statistical analysis of grey scale for radiopacity values of 30% α- Fe_2O_3-BHAp milled for 3 h, 6h, 9h, and 12h. 102

Figure 4.21 Hysteresis loops' dependence on milling time for 30% Fe+BHAp powders. 103

Figure 4.22 Hysteresis loops dependence on milling time of 30% α-Fe_2O_3+BHAp powders. 104

Figure 4.23 SEM and images of BHAp composite (30% α-Fe_2O_3-BHAp) after 3 days of immersion in SBF. (a-b) 3 h, (c-d) 6 h, (e-f) 9 h, and (g-h) 12 h. 108

Figure 4.24 SEM and images of BHAp composite (30% α-Fe_2O_3-BHAp) after 7 days of immersion in SBF. (a-b) 3 h, (c-d) 6 h, (e-f) 9 h, and (g-h) 12 h milling time. 110

Figure 4.25 FESEM images of osteoblast attached on specimen pellet for 1 day dependence on milling time of 30% α-Fe_2O_3+BHAp for (a) 3h, (b) 6h, (c) 9h and (d) 12h. (e-h) higher magnification. (White circle: area magnification) 112

Figure 4.26 FESEM images of osteoblast attached on specimen pellet for 7 days dependence on milling time of 30%-Fe_2O_3+BHAp for (a) 3h, (b) 6h, (c) 9h and (d) 12h. (e-h) higher magnification. (White circle: area magnification) 113

Figure 4.27 AAS 30% α-Fe_2O_3+BHApfor different milling time durations (3, 6, 9, and 12 h) at 0, 7, 14, 30, and 60 days. (a) Fe ions, (b) Ca ions, and (c) P ions. 117

Figure 4.28 Radiograms of 3h, 6h, 9h, and 12 h (30%Fe_2O_3+BHAp) specimens at 7 days, 14 days, 30 days, and 60 days after implantation. (*: area of interest (specimen), B: bone and S: specimen). 122

Figure 4.29 Statistical analysis of grey scale for radiopacity values of 30% α-Fe_2O_3+BHAp against milling time during implantation at 7, 14, 30, and 60 days. 122

Figure 4.30 Radiogram of 9h and 12 h (30%-Fe_2O_3+BHAp) specimens at 30 and 60 days after implantation. (red arrow: area of interest (specimen)) 124

Figure 4.31 Specimen removed from rat's femur at 60 days after implantation. 125

Figure 4.32 XRD spectra of 30%Fe_2O_3+BHAp samples dependence on milling time 60 days after implantation 127

Figure 4.33 FTIR spectra of 30% α-Fe_2O_3+BHAp samples dependence on milling time 60 days after implantation 128

LIST OF ABBREVIATIONS

BSE	-	Back Scatter Electron
CA	-	Contact Angle
DMEM	-	Dulbecco’s Modified Eagle Medium
DMSO	-	Dimethyl sulfoxide
EDTA	-	Ethylenediaminetetraacetic
FBS	-	Fetal Bovine Serum
FESEM	-	Field-Emission Secondary Electron microscopy
HAp	-	Hydroxyapatite
Hb	-	Hemoglobin
HRTEM	-	High-Resolution Transmission Electron Microscopy
HSF	-	Primary Human Skin Fibroblast
JSPDS	-	Joint Committee on Powder Diffraction Standards
MM	-	Mechanical Milling
MNP	-	Magnetic Nano-particle
MRI	-	Magnetic Resonance Imaging
MTT	-	Methyl Thiszole Tetrazolium
NHost	-	Normal Human Osteoblast
NLR	-	Neutrophil-to-Lymphocyte Ratio
OD	-	Optical Density
OGM	-	Osteoblast Growth Medium
OBM	-	Osteoblast Basal Medium
PCV	-	Hematocrit
RBC	-	Red Blood Cells
SBF	-	Simulated body fluid
SEM	-	Scanning Electron Microscopy
SPD	-	Severe Plastic Deformation
SPION	-	Superparamagnetic iron-oxide nanoparticle
TEM	-	Transmission Electron Microscopy
VSM	-	Vibrating Sample Magnetometer
WBC	-	White Blood Cells

LIST OF SYMBOLS

rpm	-	Rotation per minute
%wt	-	Percentage of weight
h	-	hour
Å	-	Angstrom
℃	-	Celsius
O^{2-}	-	Oxygen ions
nm	-	nanometer
K	-	Kelvin
T_m	-	Melting Temperature
M_s	-	Saturation Magnetization
H_c	-	Coercivity
χ_m	-	susceptibilities
M_r	-	Remanence Magnetization
M	-	Magnetic Flux Density
H	-	Magnetizing Force
μm	-	Micrometer
cm/s	-	Centimetre per second
∅	-	Diameter
kg/cm^2	-	Kilogram per square centimetre
ml	-	mililiter
kN	-	Kilo Newton
D	-	Crystallite Size
K	-	Broadening constant
λ	-	Wavelength of X-ray Beam
θ	-	Diffraction angle
ε	-	Lattice Strain
kV	-	Kilo Volt
emu/g	-	Mass Magnetization
KOe	-	Magnetic Field Strength
g/dl	-	Gram per Diciliter

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
APPENDIX A	In-vivo Procedure	167
APPENDIX B	EDAX and XRD of bovine hydroxyapatite (BHAp) powder	172
APPENDIX C	XRD spectra of different percentage of iron and	175
APPENDIX D	Contact angle image	178

CHAPTER 1

INTRODUCTION

1.1 Background

Malaysian population is estimated to increase throughout years, from 29.6 million in 2013 to 42.9 million in 2050 with one-third of the total population are over 50 years old (1). Framework of diagnosis and management of osteoporosis provided by the Malaysian Osteoporosis Society (MOS) in Clinical Practice Guideline in 2001, 2006, and 2012 assists doctors to reduce fracture and its accompanying morbidity and mortality (2). Wang et al. (2014) reported that nearly $200 billion per year were spent on osteoporosis-related bone fracture in the United States (3) and orthopaedic product sales also reached $46.6 billion in 2015 worldwide mainly due to product prising and research and development (R&D). Thus, R&D for bone implant materials are still in progressive upon the degree of osseointegration in a sufficient and healthy bone.

Implant biomaterial is necessary in replacing bone fracture by substituting or repairing any damages. Biomaterial is defined by American National Institute of Health as *"any substance or combination of substances, other than drugs, synthetic or natural in origin, which can be used for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual"* (4). The most common materials in biomedical are of metal, polymer, and ceramic categories. They are used as composites to improve material properties. However, the most frequent and widely material used in biomedical implant is ceramic due to biocompability especially hydroxyapatite (HAp) and other calcium phosphates. Researchers like Akoa (1980), Jarcho (1981), and de Groot (1984) pioneered the contributions in orthopaedic and dental fields. Meanwhile, using advanced X-ray powder diffraction technique, Dalconi *et al*. clarified that at the early stages of ossification, structural bioapatite closely resembled the hydroxyapatite ($Ca_{10}(PO_4)_6(OH)$) (5).

Hydroxyapatite (HAp) is bioactive with excellent biocompatibility (6). It is termed as osseointegration (7–9) through its direct bonding responses to surrounding tissues and due to chemical and biological similarities to hard tissues. However, there are limitations in HAp as a result of poor mechanical properties like brittleness and low strength (10,11). Consequently, HAp-based ceramic composites were developed to overcome insufficient mechanical reliability and improved the abilities of living tissues. Composite materials can be composed by ceramic and metallic or polymer matrices reinforced with various components like particulate, short or long fibres, microfillers, and nanofillers (12).

Among all those methods, only modification HAp by doping a small amount of ion substitutions might induce modification in lattice parameter, crystal morphology, crystallinity, solubility, and thermal stability (13,14). In an attempt to improve the mechanical properties, Gautam *et al*. reported that the 5% addition of MgO in HAp was successful to enhance load-bearing capability of pure HAp. Moreover, the increased released amount of zinc ions from the ceramic had increased the growth rate of osteoblast due to nutritional cells (15). The synthesis of substituted hydroxyapatite is one of the options to satisfy clinically improved mechanical properties and bone growth.

Interestingly, substituting iron into HAp lattice contributes to magnetic properties, which is a potential application for magnetic resonance imaging, drug delivery, and hyperthermia. However, cooperating irons into HAp properties are less reported and still in the developed area of research. Iron (Fe) is an essential element in blood production (16–18) and in trace amount in hard tissues (0.003 wt% in enamel, 0.01-0.1 wt% in bone) (18–21). Next, cell study of osteoblast on Fe-HAp reported that the number of cells increased twice in the presence of Fe^{3+} ions in HAp compared to pure HAp. This also showed more mini-filopodia attachment on Fe-HAp pellets, hence indicating a good osseointegration (22). In addition, magnetic-HAp (Fe-HAp) showed a good potential for anti-cancer based hyperthermia due to intrinsic magnetisation that affects heating properties (23). The development of iron oxide incorporation into apatite lattice contributes to biomedical application.

There are various methods reported on the synthesis of Fe incorporated in HAp such as co-precipitation (21,24–30), hydrothermal (31), wet chemical (16,22,32), ion-exchanged (20), pseudo-body (19), microwave (17,31), and milling (33,34). In general, co-precipitation, hydrothermal, wet chemical, ion-exchanged, pseudo-body, and microwave were established using 'trial and error' approaches by controlling the pH, concentration, and temperature during syntheses for chemistry reaction system. Mechanical milling (MM) is the mixing of powder in the right portion and synthesis occurred by perturbation of surface-bonded by the samples, as well as ball and wall relationship. It is a well-known method because of its simple approach, easy to control, and economical (35). The main advantage of MM processing is to produce nano-sized particle during direct process.

The properties of material could be altered by MM through their structures such as crystal structure, unit cell parameter, and lattice volume and density (36,37). The MM accumulates energy in solid form and increases potential energy that is manifested to form lattice defect and other structural imperfections. Consequently, the local stress concentration occurs due to MM which plays an important role in the heat production, new surface formation, plastic deformation, and amorphisation (36,38). Therefore, in order to improve the mechanical, magnetic, and biological properties of natural HAp composite, dispersion toughening of iron oxide and structural transformation by the milling time of process were employed.

1.2 Problem statement

Hydroxyapatite is widely used in biomaterial and tissues engineering due to its similarity with bone matrix. It has low density of HAp effect to mechanical properties and contrast image for early monitoring. Additives were used as radiopacifier like $BaSO_4$ and ZrO_2 (39). Wimhurst *et al.* used rats to study the effect of BaSO4 particulate (radiopacifier) in bone cement and discovered that $BaSO_4$ promotes osteolysis at the bone implant (40). This works to obtain non-inflammatory radiopacity material in enhancing the opacity property of HAp. Therefore, HAp leads to implications like higher resolution contrast with surrounding tissues and improve the mechanical strength.

There were many efforts done in the past focusing on the amount of iron substitution to understand the effects of Fe ions in HAp structure using wet chemical method. However, the occurrence of microstructural modification using mechanical milling (MM) that involves a reduction of particle size has been given a great interest. Milling time is an important parameter to understand the structure-property correlation in the material (41). However, less attention was given in MM and time of milling investigation for the synthesis of iron-hydroxyapatite composite. The development of preparation on Fe-HAp composite using MM to bring multifunctional nanostructured material is a promising opportunity for clinical treatment to enhance bone regenerative engineering.

The fabrication of HAp composite enhances the mechanical properties of HAp-based biomaterials. The reinforcement of 33 wt% Fe in HAp matrix has improved the mechanical properties due to good interfacial bonding with the matrix (42). Ajeesh *et al.* found 40 wt% iron oxide HAp composite enhance X-ray opacity and good biocompatibility (30). An addition of Fe into HAp also produces magnetic stimulation or guidance in bone regeneration by magnetic field. However, the iron overloading causes a Ca and P deficiency, hence decreases the bone's mechanical strength (43). There is no report published the effect of Fe reinforcement of less than 33%. Therefore, the mechanical properties Fe-HAp reinforcement below 33% were unknown.

An antiferromagnetic material such as hematite (α-Fe_2O_3) has been researched to improve bone regeneration by combining the properties of iron oxide and hydroxyapatite. For instance, Ajeesh *et al*. reported that cell cytotoxicity of HAp-hematite composite was higher than that of pure HAp (30). However, they did not investigate the magnetic behaviour of Fe-BHAp partly because they studied the application for drug delivery. Similarly, thus far, in-vivo studies have also not been reported elsewhere for Fe-HAp composite.

1.3 Objective

This research focuses to produce multifunctional HAp composite with several integrated properties that improves mechanical properties of HAp, imaging capabilities for early stage monitoring, and better biocompatibility. The preparation of HAp-Fe involves the mechanical milling. The effect of milling time during milled process is the key point of this research. Hence, the research objectives are as follows:

1. To analyse structural relationship of Fe-BHAp prepared from mechanical milling at various milling time;

2. To evaluate the effect of deformation mechanism on mechanical properties and magnetic behaviour from solid solution to the strengthening of material; and

3. To evaluate the biocompatibility of composites through in-vitro and in-vivo studied.

1.4 Scopes

Scope of study is crucial in order to provide a clear direction towards achieving the research objectives. In the development of multifunctional composite, Bovine hydroxyapatite (BHAp) was prepared using thermal decomposition method reinforced with (44) and pure iron (particle size 450 µm, Goodfellow, UK). The materials were ball milled using planetary milling with Teflon vial and zirconia balls. The treatment was conducted at 1500 rpm and ball/powder ratio was 10:1 with a total powder mass of 5 g.

The investigation on the properties of Fe-substituted BHAp was attended to two stages: one, Iron concentration of 5, 15, and 30%wt iron at 12 h milling and two, 30%wt iron at different milling times: 3, 6, 9, and 12 h. The powder was compacted to a pellet and sintered at 900 ^{o}C for 2 h. The samples before and after sintered were then characterised using various techniques, including X-ray diffraction (XRD), Field emission scanning microscopy (SEM), Transmission electron microscopy (TEM), Vibrating sample magnetometer (VSM), contact angle measurement, Fourier transform infrared spectroscopy (FTIR), and Atomic absorption spectroscopy (AAS).

The compression strength was investigated through compression test using universal testing machine (Instron) containing load cell 10kN at across head speed of 1 mm/min for various milling time of 30% α-Fe_2O_3-BHAp (sintered samples). The biocompatibilities study was conducted using immersion test in Kokubo's solution with pH 7.4 for 3 and 7 days for various milling time of 30% α-Fe_2O_3-BHAp. The cytotoxicity test was performed using skin fibroblast (HSF 1,184) using the extraction assay of hematite (Fe_2O_3) concentration (5, 15, and 30%wt) and cell attachment using osteoblast (NHost) on various milling time of 30% α-Fe_2O_3-BHAp.

Furthermore, preliminary in-vivo study under the ethical clearance of Animal Care and Use Ethics Committee of Bogor Agricultural University, Indonesia (Ethical number: 024/KEH/SKE/III/2015) was conducted using 21 male Sprague Dawley rats (weight 147 ± 10g, age ± 3 months). The rats were implanted with various milling times of 30% α-Fe_2O_3-BHAp at the right leg of their femur bone. The observation and analysis were conducted on radiological image analysis and peripheral blood profile of the rats for 0, 7, 14, 30, and 60 days.

1.5 Significances of study

This is a comprehensive study on a material from preparation to in-vivo evaluation. Previous studies mostly focused on the content ratio of proposed material while this study focuses on milling time. Milling time is an important parameter in material preparation as it controls over material structure, hence contributes towards the enhancement of many material properties such as mechanical properties and biocompatibility. Hematite and hydroxyapatite material are excellent prospects in nanomedicine with multifunctional biomaterial.

1.6 Thesis Structure and Organisation

This thesis consists of five chapters which are organised as follows.

Chapter 1 provides a content overview of the research presented via problem statement and research objectives. It gives knowledge description about HAp composite materials and introduces the concept of MM process.

Chapter 2 discusses a comprehensive literature review with overviews on biomaterial, bone properties, summary of the properties of HAp, Fe, and magnetic material application in biomedical field. Special attention is paid to mechanochemical process and background theory. The present knowledge about bone mechanism and healing process are also included in this chapter.

Chapter 3 gives a detailed description of the experimental procedures used in this research, including (i) the materials used in the preparation of 30% iron-BHAp, (ii) the procedures of cell culture experiment and animal testing, and (iii) the outline of various material characterisations and testing procedures.

Chapter 4 presents the finding and results of analyses in the experiments. First, the new composite is explained in terms of synthesis production along with analysis of the changes. Then, the test results which include *in-vitro* and *in-vivo* works are discussed and analysed.

Finally, the conclusion of research and recommendations for future research are presented in **Chapter 5**.

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LIST OF PUBLICATIONS

Journal with Impact Factor

1. Nordin, J. A., Prajitno, D.H., Saidin, S., Nur, H., & Hermawan, H (2015). Structure-properties relationship of iron-hydroxyapatite ceramic matrix nanocomposite fabrication using mechanosynthesis method. Materials Science and Engineering C, 51, 294-299. http://doi.org/10.1016/j.msec.2015.03.019 **(Q1, IF: 3.420)**

Indexed Journal

1. Nordin, J. A., Nasution, A. K., & Hermawan, H. (2013). Can the Current Stent Manufacturing Process be Used for Making Metallic Biodegradable Stents. *Advanced Materials Research*, *746*, 416–421. https://doi.org/10.4028/www.scientific.net/AMR.746.416. **(Indexed by SCOPUS)**