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DEVELOPMENT OF DRUG LOADED BIODEGRADABLE POLYMER COMPOSITE MICROPARTICLES

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Table of Contents

Acknowledgement	i
Table of Contents	iii
List of Figures	viii
List of Tables	xiii
List of Symbols	xiv
List of Acronyms and Abbreviations	xvi
List of Publications	xix
Abstract	xxi
Chapter 1	
Introduction.....	1
1.1. Background	1
1.2. Objectives.....	4
1.3. Scope	4
Chapter 2	
Literature review	6
2.1. Hydroxyapatite (HA)	6
2.1.1. Crystal structure of HA	7
2.1.2. Synthesis of HA	9
2.1.3. Synthesis of metal ion doped HA (MHA).....	11
2.1.4. Applications of metal ion doped HA (MHA).....	12
2.1.5. <i>In vitro</i> cytotoxicity test of metal ion doped HA (MHA)	15
2.1.6. <i>In vitro</i> biocompatibility test of metal ion doped HA (MHA)	16
2.2. Poly(lactide-co-glycolide) (PLGA).....	19
2.2.1. Synthesis of PLGA.....	20
2.2.2. Degradation of PLGA	21

2.2.3. Fabrication of microparticles	26
2.2.3.1. Oil-in-water (O/W) single emulsion.....	29
2.2.3.2. Solid-in-oil-in-water (S/O/W) single emulsion	32
2.2.3.3. Water-in-oil-in-water (W/O/W) double emulsion.....	34
2.2.4. Drug release mechanism	36
2.2.5. Mathematical modeling of drug release	39
2.2.5.1. Models based on diffusion.....	39
2.2.5.2. Other models.....	45
2.3. Polymer composite.....	47
2.3.1. Composite materials as scaffold.....	47
2.3.2. Composite materials as drug carrier.....	49
Chapter 3	
Experimental methods and techniques.....	54
3.1. Synthesis of metal ion doped HA (MHA).....	54
3.2. Characterization of metal ion doped HA (MHA)	56
3.2.1. X-ray diffraction (XRD).....	56
3.2.2. Fourier transform infrared (FTIR) spectroscopy.....	57
3.2.3. Field emission scanning electron microscope (FESEM) and energy dispersive X-ray spectroscopy (EDX).....	57
3.2.4. Inductively coupled plasma (ICP).....	57
3.2.5. Electrical conductivity test.....	57
3.2.6. Magnetic property test.....	58
3.2.7. <i>In vitro</i> cell study.....	58
3.2.7.1. Agar diffusion assay	58
3.2.7.2. Extraction assay	59
3.2.7.3. Cell seeding	60
3.2.7.4. Cell fixation	60
3.2.7.5. MTT assay	61
3.2.7.6. ALP assay	61

3.3. Loading cisplatin on MHA nanoparticles	62
3.4. Fabrication of composite microparticles	63
3.4.1. Water-in-oil-in-water (W/O/W) double emulsion.....	63
3.4.2. Solid-in-oil-in-water (S/O/W) single emulsion.....	64
3.5. Drug release and degradation study	65
3.6. Characterization of microparticles	66
3.6.1. Drug loading test	66
3.6.2. Differential scanning calorimetry (DSC)	66
3.6.3. Gel permeation chromatography (GPC)	67
3.7. Statistical analysis	67
Chapter 4	
Characterization of metal ion doped hydroxyapatite	68
4.1. XRD patterns.....	68
4.2. FTIR spectra.....	73
4.3. FESEM and EDX analysis	76
4.4. ICP analysis.....	78
4.5. Electrical conductivity of NdHA and GdHA	81
4.6. Magnetic property of GdHA and FeHA.....	86
4.7. Cytotoxicity of NdHA and GdHA	88
4.8. Cytotoxicity of MnHA and FeHA.....	90
4.9. Cell growth on MnHA and FeHA pellets	92
4.10. Cell morphology analysis.....	98
4.11. Summary	99
Chapter 5	
Cisplatin loaded MHA nanoparticles	102
5.1. As-received cisplatin	102
5.2. Zeta potential of MHA nanoparticles.....	103

5.3. Drug loading conditions	104
5.4. Characterization of cisplatin loaded Fe5 (CDDP-Fe5) nanoparticles..	107
5.5. Summary	110
Chapter 6	
Fabrication of drug loaded composite microparticles.....	111
6.1. W/O/W double emulsion.....	111
6.2. S/O/W single emulsion	114
6.3. Characterization of microparticles produced using S/O/W single emulsion	116
6.4. Summary	120
Chapter 7	
Degradation study on microparticles	121
7.1. Change in molecular weight.....	121
7.2. Change in glass transition temperature (T_g).....	126
7.3. Change in pH of PBS	131
7.4. Morphological changes of microparticles	134
7.5. Summary	145
Chapter 8	
Study of drug release profiles	146
8.1. Cumulative drug release of microparticles	146
8.2. Median effective dose (ED_{50}) of all samples	151
8.3. Study of drug release mechanism	154
8.3.1. Linear fit.....	155
8.3.2. Drug release due to surface diffusion and polymer degradation..	158
8.4. Summary	161
Chapter 9	
Conclusion	162

9.1. Metal ion doped hydroxyapatite (MHA).....	162
9.2. Cisplatin loaded composite microparticles	163
Chapter 10	
Future work.....	166
References.....	168
Appendices.....	185

List of Figures

Fig 1.1. Molecular structure of cisplatin (<i>cis</i> -diamminedichloroplatinum(II), CDDP). .	2
Fig 2.1. XRD pattern of monoclinic HA. Arrows indicate characteristic reflections of monoclinic HA, which are forbidden by the hexagonal symmetry [50].....	7
Fig 2.2. Projection of hexagonal hydroxyapatite onto the (001) plane. Triangles represent PO ₄ tetrahedra.	8
Fig 2.3. Different shaped HA nanoparticles were synthesized following the reaction as shown in Eq 2.1 at (a) 40°C, (b) 80°C, and (c) 100°C [46].....	10
Fig 2.4. FTIR spectra of HA precipitated at reaction temperatures of 40-100°C. (A) indicates the substitution of CO ₃ ²⁻ for OH ⁻ (A-type carbonated HA) and (B) indicates CO ₃ ²⁻ for PO ₄ ³⁻ (B-type carbonated HA) [46].	11
Fig 2.5. (a) Representative control cells stained with Alizarin red-S after 48 h of differentiation; (b) representative deferoxamine (DFO)-treated cells (8 μM DFO) stained with Alizarin red-S after 48 h of treatment and differentiation; and (c) summary of statistical analysis of calcium mineralization in control and treated cells after 48 h of treatment (<i>P</i> < 0.05). The number of nodules in the field was counted by two different individuals [109].	19
Fig 2.6. Molecular structure of (a) PLGA and (b) PLA.	20
Fig 2.7. Stereoisomers of lactic acid and molecular structure of glycolic acid [123]. ..	20
Fig 2.8. Stereoisomers of lactide and molecular structure of glycolide [123].	21
Fig 2.9. Crystallinities determined by X-ray and DSC measurement and water uptake data for PLGA copolymers as a function of composition [35].	24
Fig 2.10. Procedures of making PLGA microparticles with hydrophobic and hydrophilic agents using oil-in-water (O/W) single emulsion and water-in-oil-in-water (W/O/W) double emulsion.	27
Fig 2.11. SEM images of (a) lidocaine-loaded PLGA microparticles produced using O/W single emulsion, top and cross section view [130] and (b) FITC-dextran loaded PLGA microparticles produced using O/W single emulsion with the introduction of DMSO as cosolvent, top and cross section view [29].	31
Fig 2.12. SEM images of cross-sections of 5-FU-loaded PLGA microparticles produced using S/O/W single emulsion, at three different magnifications. The hatched regions correspond to the epon (Epon 812 Fluka) that was used to obtain the cross-sections, the dark grey regions correspond to PLGA, the bright grey regions to 5-FU. A cavity with 5-FU crystals (indicated by an arrow) inside is shown in (c) [112].	33
Fig 2.13. SEM image of 5-FU loaded PLGA microparticles produced using S/O/O single emulsion [32].	33
Fig 2.14. Microparticles with different morphologies produced from different formulations [163]. (A) PLGA, 10% w/v, 1% w/v PVA, 500 rpm, (a) microscope images of the initial W1/O/W2 droplets transformation, (b) SEM image of microparticles morphology; (B) PLGA, 5% w/v, 1% w/v PVA, 8000 rpm, (a) initial W1/O/W2 microdroplets, (b) SEM image of solidified microparticles.	35

Fig 2.15. Drug release mechanism through diffusion (A) (B), chemical reaction (C) (D), matrix swollen (E), pore formed due to osmotic pressure (F), osmotic delivery (G) and enhanced release rate by external magnetic field (H). In all cases, dots represent drug and in (H), larger dots represent magnetic beads [170].	37
Fig 2.16. Fit of cumulative drug release profile of 5-fluorouracil (5-FU) loaded PLGA microparticles in PBS (pH 7.4) at 37°C according to an exact solution of Fick's second law (considering spherical device geometry, constant drug diffusivity, perfect sink conditions, monolithic solution, Eq 2.13: solid line) and Koizumi model (described later, Eq 2.17: dashed curves) [112].	40
Fig 2.17. Cumulative fractional drug release of PLGA microparticles produced using W/O/W double emulsion method. The solid line is a fit of the data according to Eq 2.14 [150].	41
Fig 2.18. Fractional cumulative release of 5-FU from polyacrylamide microparticles containing 10 wt.% of 5-FU and crosslinked with (●) 2% (F-1), (▲) 4% (F-2) and (■) 6% wt.% of NNMBA [174].	42
Fig 2.19. Pseudo steady-state approach applied for the derivation of Higuchi model quantifying drug release from planar systems (monolithic dispersion) [112].	43
Fig 2.20. Higuchi plots of the release profiles of gentamicin from composite microparticles [48].	44
Fig 2.21. Fractional levamisole base release profiles from microspheres containing 19.7% (□), 14.3% (◆) and 2.4% (○) levamisole base in PBS (pH 7.4) fitted according to Eq 2.20 [149].	45
Fig 2.22. Release profiles of salicylic acid (2 wt.%) from D,L-PLA tablets at 37°C [180].	47
Fig 3.1. The schematic diagram of apparatus for MHA nanoparticles production.	55
Fig 3.2. Schematic diagram of sample pellet, agar layer and cells in agar diffusion test.	60
Fig 4.1. The XRD pattern for HA-s, the special sample fabricated similarly to pure HA but without 2-h aging at 98.5°C.	69
Fig 4.2. The XRD patterns of (a) NdHA powder, ▼: Nd(OH) ₃ and (b) GdHA powder, ■: Gd(OH) ₃ .	71
Fig 4.3. The XRD patterns of (a) MnHA powder and (b) FeHA powder.	72
Fig 4.4. The FTIR spectra of NdHA and GdHA.	75
Fig 4.5. The FTIR spectra of MnHA and FeHA.	76
Fig 4.6. The representative FESEM images and the corresponding EDX spectra (insets) for (a) pure HA (b) Nd10 (c) Nd20 (d) Gd20 (e) Mn10 and (f) Fe5. The circles represents Nd(OH) ₃ cylindrical rods.	78
Fig 4.7. Projection of hydroxyapatite onto the (001) plane. Two channels are shown.	83
Fig 4.8. The XRD patterns of (a) NdHA and (b) GdHA after sintered at 800°C for 1 h.	84
Fig 4.9. The FTIR spectra of (a) NdHA and (b) GdHA after sintered at 800°C for 1 h.	85
Fig 4.10. The magnetization curves of GdHA and FeHA powder measured at room temperature.	87
Fig 4.11. Representative light microscope images of cells directly under the sample pellets after one day incubation. (a) Nd1 (b) Nd5 (c) Nd10 (d) Nd20 pellets. The magnification bar is 200 μm.	88

Fig 4.12. Representative light microscope images of cells directly under the pellets of (a) pure HA (b) Gd1 (c) Gd5 (d) Gd10 (e) Gd20 and (f) 1 cm beyond Gd20 pellets after one day incubation. The magnification bar is 200 μm	90
Fig 4.13. (a) Cell number based on MTT test and (b) ALP activity for osteoblast cells cultured in extracted medium for 72 h. Data = mean + standard deviation; n = 4; * $p < 0.05$ as compared to HA; ** $p < 0.05$ as compared to the same metal ion doped HA at higher metal ion concentration, M5 and/or M10.....	91
Fig 4.14. The relative absorbance of MTT test for osteoblast cells growing on all sample pellets for 3 and 7 days (The tests were carried out independently on 3-d and 7-d incubation, so the relative absorbance was calculated based on the absorbance for HA at the same incubation time). Data = mean + standard deviation; n = 4; * $p < 0.05$ as compared to HA at the same incubation time.	93
Fig 4.15. The relative absorbance of MTT test for osteoblast cells growing on FeHA pellets for 3 days. Data = mean + standard deviation; n = 4.	94
Fig 4.16. (a) Cell number based on MTT test and (b) ALP activity for osteoblast cells growing on HA, Mn5 and Fe5 pellets for 3 and 7days. Data = mean + standard deviation; n = 4; * $p < 0.05$ as compared to HA at the same incubation time; ** $p < 0.05$ as compared to 3-day incubation of the same sample; ^^ $p < 0.1$ as compared to 3-day incubation of the same sample.	95
Fig 4.17. Schematic drawing of the interaction between the polarized charge and environmental materials contributing to cell adhesion. Inorganic ions, organic ions, amino acids, and proteins float around the HA ceramics, and these materials are adsorbed to the unpolarized-surface. Cations and positively charged ionic groups are actively adsorbed on the negatively charged surface (N-surface), and Ca^{2+} ion contribute to the formation of the bone-like layer growing on the HA N-surface. Anions and negatively charged species are actively adsorbed on the positively charged surface (P-surface) of HA, which fail to nucleate crystal growth [98].	96
Fig 4.18. Representative FESEM images of cells growing on sample pellets for 3 days. (a) pure HA, (b) Mn5 and (c) Fe5. Scale bar is 10 μm	99
Fig 5.1. FESEM images of as-received cisplatin at magnification (a) $\times 100$ and (b) $\times 500$	103
Fig 5.2. Zeta potential of pure HA, Mn10 and Fe5 powder in PB solution at 6.67 mg/ml. Data = mean + standard deviation; n = 3; * $p < 0.05$ as compared to pure HA at the same temperature.	104
Fig 5.3. FESEM images of (a) cisplatin loaded Fe5 (CDDP-Fe5) and (b) Fe5 nanoparticles at $\times 40,000$ magnification. The scale bar is 300 nm.	108
Fig 5.4. XRD patterns of as-received cisplatin, cisplatin loaded Fe5 (CDDP-Fe5) and Fe5 nanoparticles.	108
Fig 5.5. FTIR spectra of as-received cisplatin, cisplatin loaded Fe5 (CDDP-Fe5) and Fe5 nanoparticles.	109
Fig 6.1. FESEM images of microparticles produced using W/O/W double emulsion method. The insets are images at larger magnification. (a) D-2 (b) D-3 (c) D-4 (d) D-5 (e) D-5-H (f) D-10-H.	114
Fig 6.2. FESEM images of microparticles produced using S/O/W single emulsion method at magnification $\times 100$ and $\times 700$. (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT.....	117

Fig 6.3. XRD patterns of (a) as-received cisplatin (b) blank (c) PT (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) CDDP-Fe5. FTIR spectra of (a) as-received cisplatin (b) blank (c) PT (d) PT-Fe5 and (e) CDDP-Fe5. ↓ absorption bands belonging to CDDP-Fe5, ↓ absorption bands attributed to CDDP.....	119
Fig 7.1. The representative GPC chromatograms of blank sample at different degradation times.....	122
Fig 7.2. The average molecular weight of all samples as a function of degradation time.	123
Fig 7.3. The representative linear regression on $\ln M_n$ of blank sample.....	124
Fig 7.4. The polydispersity index (PI) of all samples as a function of degradation time.	126
Fig 7.5. Schematic of the proposed complexation mechanism of cisplatin and carboxyl groups [4].....	126
Fig 7.6. Representative DSC thermograms from the first and second heating cycle for sample (a) blank, and (b) 2PT-Fe5/PT at different degradation times.	127
Fig 7.7. The representative DSC thermogram of second heating cycle for blank sample after immersed in PBS for 1 day at 37°C. T_g was calculated as the onset temperature of the glass transition by extrapolating the baseline in the glassy state to meet extrapolated line with the slope of the steepest part of the transition curve, where the meet point was T_g	129
Fig 7.8. The change of T_g values of microparticles as a function of degradation time. (a) from the first heating cycle, (b) from the second heating cycle.....	131
Fig 7.9. The pH values of PBS solution for all samples as a function of degradation time.	132
Fig 7.10. The representative linear regression figure for pH values of sample blank as a function of degradation time.....	134
Fig 7.11. FESEM images of microparticles (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT after degradation in 10 mM PBS solution at 37°C for 7 days.....	135
Fig 7.12. FESEM images at magnification $\times 700$ and $\times 5000$ for microparticles (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT after degradation in 10 mM PBS solution at 37°C for 14 days.....	137
Fig 7.13. FESEM images at magnification $\times 700$ and $\times 5000$ for microparticles (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT ($\times 400$ and $\times 5000$) after degradation in 10 mM PBS solution at 37°C for 28 days.....	139
Fig 7.14. FESEM images at magnification $\times 700$ and $\times 5000$ for microparticles (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT after degradation in 10 mM PBS solution at 37°C for 35 days.....	141
Fig 7.15. FESEM images at magnification $\times 700$ for microparticles (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT after degradation in 10 mM PBS solution at 37°C for 42 days.....	142
Fig 7.16. FESEM images at magnification $\times 700$ and $\times 2000$ or higher for microparticles (a) PT-Fe5 (b) PT-Fe5/PT and (c) 2PT-Fe5/PT after degradation in 10 mM PBS solution at 37°C for 49 days.	143
Fig 8.1. Fractional cumulative release of cisplatin from (a) CDDP-Fe5 nanoparticles and Fe5/PLGA microparticles (b) PT-Fe5 (c) 2Fe5/PT (d) 2PT-Fe5/PT (e) PT (f) PT-Fe5/PT in 10 mM PBS (pH 7.4) at 37°C. Data = mean $\pm$ standard deviation; n = 3.....	148

Fig 8.2. Cumulative release of cisplatin ($\mu\text{g-drug/mg-particle}$) from (a) CDDP-Fe5 nanoparticles and Fe5/PLGA microparticles (b) PT-Fe5 (c) 2Fe5/PT (d) 2PT-Fe5/PT (e) PT (f) PT-Fe5/PT in 10 mM PBS (pH 7.4) at 37°C. Data = mean $\pm$ standard deviation; n = 3.	149
Fig 8.3. The representative XRD patterns of (a) as-received cisplatin, (b) PT and (c) PT-Fe5/PT before degradation and after 55-day degradation, (d) CDDP-Fe5.	150
Fig 8.4. Cisplatin concentrations of PT ($\blacktriangle$) and PT-Fe5/PT ($\blacktriangleleft$) at various time intervals. Data = mean $\pm$ standard deviation; n = 3. The dash lines correspond to 0.35 $\mu\text{g/ml}$ and 1.4 $\mu\text{g/ml}$ respectively.	151
Fig 8.5. Cisplatin concentrations of CDDP-Fe5 ($\circ$) and PT-Fe5 ($\bullet$) at various time intervals. Data = mean $\pm$ standard deviation; n = 3. The dash lines are representing 0.35 $\mu\text{g/ml}$ and 1.4 $\mu\text{g/ml}$ respectively.	153
Fig 8.6. Cisplatin concentrations of 2PT-Fe5/PT ($\blacktriangledown$) and 2Fe5/PT ($\blacksquare$) at various time intervals. Data = mean $\pm$ standard deviation; n = 3. The dash lines are representing 0.35 $\mu\text{g/ml}$ and 1.4 $\mu\text{g/ml}$ respectively.	154
Fig 8.7. Curve fit of cumulative release profile of 2PT-Fe5/PT.	156
Fig 8.8. Sectional curve fits of cumulative drug release data for (a) PT and (b) PT-Fe5/PT.	157
Fig 8.9. Curve fits of drug release profiles of sample (a) PT (b) PT-Fe5 (c) PT-Fe5/PT (d) 2PT-Fe5/PT and (e) 2Fe5/PT according to Eq 8.2.	160

List of Tables

Table 2.1 Ion concentrations of simulated body fluid (SBF) and human blood plasma [84]	17
Table 2.2 List of solvents commonly used for microencapsulation by solvent evaporation [161].....	29
Table 2.3 Release kinetics parameters based on Eq 2.15 of different samples as shown in Fig 2.22 [174]	42
Table 3.1 Information of the PLGA used for the fabrication of composite microparticles	54
Table 3.2 Zone Index and Lysis index description in agar diffusion assay	59
Table 3.3 Loading parameters for cisplatin on MHA nanoparticles in the study	62
Table 3.4 The fabrication parameters for microparticles produced using W/O/W double emulsion technique	64
Table 3.5 Solids in 10 ml DCM for the fabrication of drug loaded composite microparticles using S/O/W single emulsion technique	65
Table 4.1 Lattice parameters and crystal size for MHA from XRD patterns.....	73
Table 4.2 The atomic ratios for MHA from ICP results	80
Table 4.3 Electrical conductivity σ and resistivity ρ of NdHA and GdHA pellets (sintered at 800°C for 1 h) measured at room temperature.....	82
Table 4.4 Cytotoxicity of NdHA and GdHA using agar diffusion method	89
Table 5.1 Drug loading (DL) and loading efficiency (LE) of cisplatin on MHA at different loading conditions. The concentration of cisplatin was 0.4 mg/ml in 10 mM phosphate buffer solution (free of Cl ⁻) at pH 7.4	105
Table 6.1 PLGA concentration and volumetric ratio of W1/O/W2 for the fabrication of microparticles and the corresponding particle size.....	112
Table 6.2 Feed ratios of solids in DCM for the fabrication of microparticles and the properties of microparticles after solidification.....	115
Table 7.1 The degradation rate constant k_{Mn} for all samples based on the equation $\ln M_n = -k_{Mn} \times \text{Time} + \text{const}$	124
Table 7.2 The k_{pH} , const and R^2 values for all samples based on the equation $\text{pH} = -k_{pH} \times \text{Time} + \text{const}$	133
Table 8.1 Fractional cumulative release of cisplatin from CDDP-Fe5 nanoparticles and Fe5/PLGA microparticles on the specific incubation day	148
Table 8.2 Release kinetics parameters for all samples according to Eq 8.1.....	156
Table 8.3 Release kinetics parameters for all samples according to Eq 8.2.....	158

List of Symbols

a, b, c, γ	lattice parameters
$B_{1/2}$	full width at half-maximum intensity (FWHM) of peak (Eq 3.1)
C	concentration of drug (Eq 2.11)
C	equilibrium concentration (mg/l) of adsorbate (Eq 5.3)
C_0	the drug concentration, a constant (Eq 2.17)
C_p	polymer concentration (% w/v) (Eq 2.10)
C_s	solubility of the drug (Eq 2.17)
D	diffusion coefficient of drug (Eq 2.11)
d	crystal size (Eq 3.1)
D_0	diffusion coefficient of drug before degradation (Eq 2.19)
d_f	diameter of the final microparticle (Eq 2.10)
d_i	diameter of the initial microdroplet (Eq 2.10)
D_{Mw}	diffusion coefficient of drug at polymer weight M_w (Eq 2.19)
F_B	fraction of drug released during the initial burst release phase (Eq 2.21)
F_b	total burst fraction at time infinity (Eq 2.22)
F_{TOT}	total fraction of drug release at time t (Eq 2.22)
k	shape coefficient (Eq 3.1)
k	constant reflecting adsorption affinity (Eq 5.3)
$k.$	rate constant (Eq 2.20)
k_b	burst release constant (Eq 2.21)
k_D	decreasing constant of D (Eq 2.19)
k_d	rate constant for polymer degradation release (Eq 2.22)
k_{degr}	degradation constant (Eq 2.18)
K_g	polymer specific constant for Fox-Flory Equation (Eq 2.9)
k_{Mn}	rate constant of degradation process
k_{pH}	decreasing constant of pH
m	constant reflecting adsorption capacity (Eq 5.3)
m	mass transfer coefficient (Eq 2.14)

M_{∞}	cumulative drug release at time infinite (Eq 2.13)
M_n	number-average molecular weight of polymer
$M_{n,0}$	number-average molecular weight of polymer before degradation
M_s	saturation magnetization
M_t	cumulative drug release at time t (Eq 2.13)
$M_{w,0}$	molecular weight of polymer at degradation time 0 (Eq 2.18)
$M_{w,t}$	molecular weight of polymer at degradation time t (Eq 2.18)
n	an empirical parameter characterizing the release mechanism (Eq 2.15)
Q	amount adsorbed (mg/g) (Eq 5.3)
r	radial coordinate (Eq 2.12)
R	radius of particles
r	ionic radius
s	a constant characteristic of the drug-polymer system (Eq 2.15)
T_g	glass transition temperature
$T_{g\infty}$	glass transition temperature at an infinitely high number-average molecular weight
$t_{\max}$	time at which maximum rate of drug release achieved (Eq 2.20)
V_p	polymer volume (Eq 2.10)
V_s	solvent volume (Eq 2.10)
W	fraction of drug released at time t (Eq 2.21)
X	fractional cumulative drug release (Eq 2.20)
x, y, z	three Cartesian coordinates (Eq 2.11)
X_M	atomic ratio of added metal ions with added Ca
α	polarizability (section 4.5)
α_i	shrinkage factor (Eq 2.10)
λ	wavelength of Cu $K\alpha$ radiation (Eq 3.1)
ρ	electrical resistivity
ρ_p	polymer density (g/ml) (Eq 2.10)
σ	electrical conductivity
χ_g	magnetic susceptibility

List of Acronyms and Abbreviations

ACP	calcium phosphate
ALP	alkaline phosphatase
ATCC	American Type Culture Collection
BSA	bovine serum albumin
CDDP	cisplatin
DCM	dichloromethane
DFO	deferoxamine
DL	drug loading
DLS	dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DSC	differential scanning calorimeter
EDX	energy dispersive X-ray spectroscopy
FBS	fetal bovine serum
FeHA	Fe ³⁺ doped HA
FESEM	field emission scanning electron microscope
FTIR	Fourier transform infrared spectroscopy
FWHM	full width at half-maximum intensity
GdHA	Gd ³⁺ doped HA
g-HA	surface grafted-HA
GPC	gel permeation chromatography
HA	hydroxyapatite
HCA	hydroxyl carbonate apatite
HDPE	high density polyethylene
hFOB	human fetal osteoblast cell line
HPLC	high performance liquid chromatography
ICP	inductively coupled plasma
IV	inherent viscosity
LE	loading efficiency

LDF	linear driving force
LDH	lactate dehydrogenase
MHA	metal ion substituted hydroxyapatite
MnHA	Mn ²⁺ doped HA
MRI	magnetic resonance imaging
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NdHA	Nd ³⁺ doped HA
O/O	oil-in-oil single emulsion
O/W	oil-in-water single emulsion
OC	osteocalcin
PB	10 mM phosphate-buffered solution (without NaCl)
PBS	phosphate buffer saline solution
PDLLA	poly(D,L-lactide)
PEEK	polyetheretherketone
PEMA	poly(ethyl methacrylate)
PI	polydispersity index
PLA	poly(DL-lactide)
PLGA	poly(lactide- <i>co</i> -glycolide)
PLLA	poly(L-lactide)
PMMA	poly(methyl methacrylate)
pNPP	<i>p</i> -nitrophenyl phosphate
PVA	poly(vinyl alcohol)
S/O/O	solid-in-oil-in-oil single emulsion
S/O/W	solid-in-oil-in-water single emulsion
SBF	simulated body fluid
SSA	specific surface area
THF	tetrahydrofuran
TiHA	Ti ⁴⁺ doped HA
VSM	vibrating sample magnetometer
W/O/W	water-in-oil-in-water double emulsion

XRD X-ray diffraction

List of Publications

Journal Articles

- Yan Li, Jiahui Ho and Chui Ping Ooi. Antibacterial efficacy and cytotoxicity studies of copper(II) and titanium(IV) substituted hydroxyapatite nanoparticles. *Materials Science and Engineering C*, 2010, 30, 1137-1144
- Yan Li, Chai Teck Nam and Chui Ping Ooi. Iron(III) and manganese(II) substituted hydroxyapatites nanoparticles: characterization and cytotoxicity analysis. *Journal of Physics: Conference Series*, 2009, 187, 012024
- Yan Li, Chui Ping Ooi, Philip Hong Ning Cheang and Khiam Aik Khor. Synthesis and characterization of neodymium(III) and gadolinium(III) substituted hydroxyapatite as biomaterial. *International Journal of Applied Ceramic Technology*, 2008, 6, 501-512

Conference Presentations

- Yan Li, Rike Oktavianti Toyip and Sierin Lim. Incorporation of E2 protein cage in PLGA matrix. 2nd International Conference on Cellular and Molecular Bioengineering (ICCMB2), Singapore, Aug 2010.
- Chui Ping Ooi, Yuting Lin and Yan Li. 5-Fluorouracil encapsulation in hydroxyapatite/poly(lactide-*co*-glycolide) composite microspheres for drug delivery. 11th International Congress of the IUPESM - Medical Physics and Biomedical Engineering, Munich, Germany, Sep 2009.
- Wanquan Yong, Yan Li, Chui Ping Ooi and Cheang Hong Ning, Philip. The effect of pH and temperature on the morphology of hydroxyapatite synthesized using wet chemical precipitation. 7th International Conference on Materials Processing for Properties and Performance (MP³), Singapore, Nov 2008.
- Yan Li, Chai Teck Nam and Chui Ping Ooi. Iron(III) and manganese(II) substituted hydroxyapatites nanoparticles: characterization and cytotoxicity analysis. APCTP-ASEAN Workshop on Advanced Materials Science and Nanotechnology (AMSN), Nha Trang, Vietnam, Sep 2008.

- Yan Li, Chui Ping Ooi, Philip Hong Ning Cheang and Michael Khiam Aik Khor. Synthesis and characterization of novel metal ion-substituted hydroxyapatite. The International Conference on Materials for Advanced Technologies (ICMAT), Singapore, June 2007.

Abstract

Calcium hydroxyapatite (HA) is the main inorganic component of natural bones and widely used as coating material on bone implants due to its osteoconductive characteristic. Other metal ions besides Ca can be introduced into HA through ion exchange or coprecipitation method to form metal ion doped HA (MHA), which has been used as catalyst or absorbent. In this investigation, gadolinium(III) (Gd^{3+}), neodymium(III) (Nd^{3+}), manganese(II) (Mn^{2+}) and iron(III) (Fe^{3+}) substituted HA at different molar ratios ($M_{\text{added}}/\text{Ca}_{\text{added}} = X_M$, 0.01, 0.05, 0.1 and 0.2) were synthesized using wet chemical method coupled with ion exchange mechanism. Calcium hydroxide $\text{Ca}(\text{OH})_2$ and phosphoric acid H_3PO_4 were used as reactants. To characterize MHA, X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, field emission scanning electron microscope (FESEM) coupled with energy dispersive X-ray spectroscopy, and inductively coupled plasma (ICP) were used.

All MHA were single phased except when X_{Nd} and $X_{\text{Gd}} = 0.2$ and all powders were well crystallized based on the XRD patterns. When X_{Nd} and $X_{\text{Gd}} = 0.2$, the second phase was $\text{M}(\text{OH})_3$. The FTIR spectra showed that all samples possessed the characteristic phosphate and hydroxyl absorption bands of HA with PO_4^{3-} groups partially replaced by CO_3^{2-} groups (B-type carbonated HA). This was reasonable since wet chemical method usually involved CO_3^{2-} groups from atmosphere or reactants. All MHA nanoparticles were elongated spheroids with small size distribution and of around 70 nm, regardless of metal ion substitution concentrations. When $X_{\text{Nd}} = 0.2$, the cylindrical rods of $\text{Nd}(\text{OH})_3$ were observed in the FESEM image. The presence of metal ions was confirmed by EDX spectra. ICP results revealed all MHA were non-stoichiometric (Ca/P atomic ratio deviated from 1.667).

The electrical conductivity of NdHA and GdHA were much higher than pure HA and increased with metal ion content. GdHA and Fe5 ($X_{\text{Fe}} = 0.05$) nanoparticles were paramagnetic and the magnetic susceptibility increased with

Gd content proportionally. Cytotoxicity test were carried out for all MHA. All samples were non-cytotoxic to osteoblast cells except when $X_{\text{Gd}} = 0.2$. The toxicity was speculated to result from the free Gd^{3+} ions not located in the HA lattice.

All MHA nanoparticles had large surface area where drugs could be adsorbed on the surface. Cisplatin (CDDP) is an effective anticancer drug; however the applications have been limited by its adverse side effects. It is positively charged in aqueous phase and can be adsorbed onto the negatively charged HA nanoparticles for localized release. Based on cytotoxicity and zeta potential results, Mn10 ($X_{\text{Mn}} = 0.1$) and Fe5 ($X_{\text{Fe}} = 0.05$) were selected for drug loading studies. Loading conditions such as shaking speed and temperature were optimized to achieve high drug loading. ICP was used to quantify the drug quantity based on Pt concentration. The drug loading of Mn10 and Fe5 was higher than pure HA whereas Fe5 had the highest loading efficiency (57% higher than pure HA). Cisplatin loaded Fe5 (CDDP-Fe5) nanoparticles were characterized using XRD, FTIR and FESEM. Since cisplatin was surface adsorbed onto Fe5 nanoparticles, the XRD pattern and morphology of CDDP-Fe5 was similar to Fe5, indicating that the loading process did not significantly affect the structure of Fe5. The presence of cisplatin on CDDP-Fe5 was confirmed by ICP and FTIR where the absorption bands belonging to cisplatin were found on the FTIR spectrum of CDDP-Fe5.

CDDP-Fe5 nanoparticles were further encapsulated into poly(lactide-*co*-glycolide) (PLGA) microparticles using solid-in-oil-in-water (S/O/W) single emulsion method. The presence of Fe5 did not affect the microparticle size, which was around 110 μm in diameter. The Fe5 did however decrease drug loading and appear to cause the surface of microparticles to wrinkle. The degradation and drug release profiles of PLGA microparticles were investigated in phosphate buffer saline solution (PBS) (pH 7.4) at 37°C. All samples became porous, then disintegrated and fused to form pellets as degradation proceeded (until 55 days). It was observed that both Fe5 and cisplatin delayed the degradation of the polymer.

The direct release of cisplatin from CDDP-Fe5 nanoparticles in PBS had a high initial burst (42%) and a short release time range (25 days). By encapsulating CDDP-Fe5 into PLGA microparticles, the initial burst was lowered to 23% and the release time was lengthened to 55 days due to the presence of the polymer matrix acting as a diffusion barrier. The typical triphasic release profile of microparticles with only cisplatin was modified to a linear profile after the initial burst for the sample with cisplatin and CDDP-Fe5 at high feed ratio of Fe5/PLGA of 1/4. Our results demonstrated that PLGA microparticles encapsulated cisplatin along with CDDP-Fe5 is a potential chemotherapeutic carrier for treatment of bone cancer.

Chapter 1

Introduction

1.1. Background

Cancer is one of the deadliest killers and responsible for millions of deaths annually worldwide. Among different types of cancers, osteosarcoma (or osteogenic sarcoma) is the most common type of bone cancers [1]. The cancer cells, like bone forming cell osteoblast, produce bone matrix that is not as strong as normal bones and easily fractured. Osteosarcoma can spread into the nearby tissues. The cancer cells can also break away from the main tumor to enter into other bones or internal organs through blood circulation. Osteosarcoma develops in any bone and the most common location is near the ends of long bones, especially around the knees and the proximal humerus (the part of the upper arm bone close to the shoulder). Osteosarcoma happens at any age and it mostly occurs in children and young adults between the ages of 10 and 30.

Osteosarcoma is classified as low grade, intermediate and high grade based on how closely it resembles normal bone. Tumor with many dividing cells is considered high grade. All patients with high-grade osteosarcoma require both chemotherapy and surgery. Chemotherapy is usually given before surgery (neoadjuvant chemotherapy) for 10 weeks to shrink the tumor and produce the sharp edge for easier removal [2, 3]. Neoadjuvant chemotherapy is also a unique tool to assess the *in vivo* activity of anticancer drugs [2, 3]. Chemotherapy is also given to patients after surgery (adjuvant chemotherapy) for several months to approximately one year to prohibit the recurrence of cancer. Chemotherapy is a systemic therapy, which means anticancer drugs enter the blood circulation and destroy the cancer cells throughout the body. Hence it does not only kill cancer cells, but also damages some normal cells and causes many side effects; nausea, vomiting, loss of appetite, loss of hair,

mouth sores and low blood cell counts are the commonly observed side effects among others.

Cisplatin (Fig 1.1) is one of the four frequently used drugs (cisplatin, doxorubicin, methotrexate and ifosfamide) for osteosarcoma [3]. It inhibits the growth of tumor by binding to DNA after cellular uptake, causing DNA cross linking, and then triggering apoptosis [4]. Although the therapeutic activity of cisplatin appears to increase with the intensity of single and cumulative dose, the dose-dependent nephrotoxicity and neurotoxicity has limited the exploitation of its full therapeutic potential [5]. Nephrotoxicity, generally irreversible, is a major cause of cisplatin-related acute and chronic morbidity and toxicity-associated mortality. Neurotoxicity is dependent on cumulative dose. Only after considerable toxicity has happened, symptoms are detected, which usually progress after the therapy and may continue for months or years [5]. Additionally, *in vitro* studies have shown that cisplatin has a direct effect on growth plate chondrocytes, where it results in decreased growth and final height in animals [6]. Osteosarcoma occurs in teenagers mostly during puberty. The osteosarcoma is unfortunately often high grade, which requires both chemotherapy and surgery. These side effects of cisplatin chemotherapy may give further negative effects on patients' quality of life. Besides the toxic effects of cisplatin, the administration route is usually infusion, which is inconvenient and needs high cost of treatment. Thus, drug carrier systems have been proposed for cisplatin to lower the side effects and improve patients' compliance.

Fig 1.1. Molecular structure of cisplatin (*cis*-diamminedichloroplatinum(II), CDDP).

To date, many carriers have been produced for cisplatin, such as polymeric nano- [7, 8] and micro- [4, 9-16] particles, micelles [17], liposomes [18], calcium phosphate nanoparticles [19-21] and insertable matrix-type device [22]. Among them, microparticles have attracted great interest. They can be locally

administered. This help reduce the systemic toxicity and enhance the local efficacy of chemotherapy [23] which is believed to be beneficial to patients. It has been reported that the anticancer agent must remain at the tumor tissues for a required period of time to kill the tumor cells [24].

Calcium phosphate nanoparticles, like hydroxyapatite (HA) loaded with cisplatin [21], has been suggested for treatment of osteosarcoma due to the biocompatibility of HA, such as non-toxicity, lack of inflammatory and immune responses, and high bio-resorbability. The interaction between HA and cisplatin is believed to be an electrostatic attraction. In aqueous phase, one chloride atom on cisplatin is replaced by one water molecule, giving rise to positive charged species, which can be adsorbed onto the negatively charged HA nanoparticles [19-21]. The surface properties of HA nanoparticles play an important role on the drug loading, which can be modified by introduction of other metal ions into HA using ion exchange reaction. However, the release of loaded cisplatin from the surface of HA nanoparticles usually demonstrated a high initial burst [19-21]. In this research, cisplatin-loaded hydroxyapatite nanoparticles were further encapsulated into polymeric microcarriers to lower the initial burst and give a controllable release profile. Polymeric matrix may offer a diffusion barrier for cisplatin and the presence of HA nanoparticles may affect the degradation of polymer, thus the drug release profile.

Among the available microcarriers, poly(lactide-*co*-glycolide) (PLGA) microparticles are the most widely studied drug carriers [10-16, 25-32] due to the biocompatibility and biodegradability. The release profile of solid drug embedded in PLGA microparticles is usually triphasic, first an initial burst followed by a lag time and lastly a second burst release due to the disintegration of microparticles. The degradation of PLGA is a random chain scission process of ester bonds, which is catalyzed by the acidic degradation byproducts. Although near linear drug release profiles were obtained from the multi-reservoir type microparticles produced from PLGA and poly(D,L-lactide) (PLA) through phase separation [11-13], the acidic degradation byproducts may cause inflammation of surrounding tissues [33, 34]. In this investigation, the presence of HA nanoparticles in PLGA microparticles may potentially

neutralize the acidic degradation byproducts of PLGA since HA is basic in nature. The drug release profile would also be modified due to the complex relationship between the autocatalysis of PLGA and the neutralization of HA on the acidic degradation byproducts which led to the autocatalysis process.

1.2. Objectives

This research aims to develop a composite microparticulate drug carrier system using PLGA and metal ion doped hydroxyapatite nanoparticles with surface adsorbed cisplatin. Since the chemotherapy for osteosarcoma needs a sustained drug release profile, this carrier was designed to release cisplatin at the tumor site for a period of 10 weeks [2, 3] in a controlled manner as cisplatin has a very narrow therapeutic index.

To achieve the objective, the research was divided into two major parts:

1. Synthesis and characterization of metal ion substituted hydroxyapatite (MHA). Here, neodymium(III) (Nd^{3+}), gadolinium(III) (Gd^{3+}) and iron(III) (Fe^{3+}) were chosen as dopants due to their corresponding magnetic properties while manganese(II) (Mn^{2+}) was used due to its potential to increase cell adhesion. These metal ions also have the potential to modify the surface properties of HA nanoparticles which may further influence the drug loading.
2. Fabrication of cisplatin loaded MHA/PLGA composite microparticles, followed by drug release and degradation studies. Here, PLGA with molar ratio of D,L-lactide/glycolide 50/50 and inherent viscosity (IV) 0.61 dl/g was used due to its good hydrophilicity among the PLGA copolymers [35].

1.3. Scope

The objectives were accomplished by the following experimental steps:

1. Synthesis of metal ion substituted hydroxyapatite using ion exchange method in aqueous phase.
2. Characterization of the metal ion substituted hydroxyapatite using the typical characterization techniques for bioceramics, such as X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, field emission scanning electron microscope (FESEM) equipped with energy dispersive X-ray

spectroscopy (EDX) and inductively coupled plasma (ICP). For Nd^{3+} , Gd^{3+} and Fe^{3+} doped HA, magnetic property was quantified. Cytotoxicity test was performed for all metal ion substituted HA.

3. Selection of the best candidate among the metal ion substituted HA as drug carrier for cisplatin based on cytotoxicity, zeta potential and drug loading efficiency. Investigation on loading conditions was carried out to achieve the highest drug loading.

4. Fabrication of cisplatin loaded MHA/PLGA composite microparticles using solid-in-oil-in-water (S/O/W) single emulsion and water-in-oil-in-water (W/O/W) double emulsion methods. The fabrication parameters were modified and microparticles were characterized using XRD, FTIR, FESEM, and differential scanning calorimetry (DSC).

5. The drug release and degradation of the composite systems were studied using ICP, DSC and gel permeation chromatography (GPC).

Chapter 2

Literature review

This research aims to develop composite microparticles as a novel drug carrier. Our drug carrier system contains three components: polymer, inorganic filler and drug. The polymer used was poly(lactide-*co*-glycolide) (PLGA), the inorganic filler was hydroxyapatite nanoparticles and the drug was cisplatin. The following is a review on the properties and applications of these materials.

2.1. Hydroxyapatite (HA)

Calcium hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, is commonly referred to as hydroxyapatite (HA). The carbonated HA doped with other ions is a major inorganic component of hard tissue, such as bones and teeth. Because of its osteoconductivity and biocompatibility, HA is widely used as a coating material on metallic implants and low-load bearing implants.

HA can tolerate high ion substitution without changing its lattice structure. Recently, much attention has been paid to the ions substituted HA in order to modify its properties and also broaden the applications [36-40]. Fluorine substitution increased the electrical conductivity of porous hydroxyapatite [36]. Cu^{2+} , Ni^{2+} and Co^{2+} substituted HA have catalytic effect for specific reactions [37, 41]. Ag^+ substituted HA has antibacterial effect [38] and Ti^{4+} substituted HA can be used as a photocatalyst [40, 42].

To date, HA is also used as a drug carrier in various systems [43-45]. The size of HA is usually in nanometers range with high surface area when produced using wet chemical method [46]. Both hydrophilic [47] and hydrophobic [44] drugs can be adsorbed onto the HA surface. However, the drug release profile is hard to control [48]. The drug release profile of HA particulate system usually has a high initial burst and a short release time range [44]. In order to prolong the drug release time frame, porous hydroxyapatite blocks or granules have

been used with drugs impregnated into the pores [45, 49]. The main disadvantage of these blocks or granules delivery systems is that they are so large and can only be implanted in particular tissues. Hence, composites microparticles, consisting of HA and polymer may overcome these drawbacks.

2.1.1. Crystal structure of HA

Hydroxyapatite (HA) has two kinds of unit cells, hexagonal and monoclinic. Only the stoichiometric HA possesses monoclinic structure with space group $P2_1/b$ and lattice parameters $a = 9.426(3) \text{ \AA}$, $b = 18.856(5) \text{ \AA}$, $c = 6.887(1) \text{ \AA}$, $\gamma = 119.97(1)^\circ$ [50]. Most synthetic HA is hexagonal with space group $P6_3/m$ and lattice parameters $a = 9.424(4) \text{ \AA}$, $b = 2a$, $c = 6.879(4) \text{ \AA}$ and $\gamma = 120^\circ$ [51]. The XRD pattern of monoclinic HA is shown in Fig 2.1 [50].

Fig 2.1. XRD pattern of monoclinic HA. Arrows indicate characteristic reflections of monoclinic HA, which are forbidden by the hexagonal symmetry [50].

In the hexagonal structure, the regular phosphate tetrahedra compose the matrix of the crystal (Fig 2.2). There are two nonequivalent sites for calcium, Ca(I) and Ca(II). For the Ca(I) atoms, each is along the threefold axes and bonded to six oxygen atoms at an essentially equal distance, which form a twisted triangular prism. There is also bonding between these Ca(I) atoms and three O atoms at a longer distance. Thus the Ca(I) atoms are coordinated by nine O atoms located in six different phosphate tetrahedra. The Ca(II) atoms, situated around the

hexagonal screw axes, each have an irregular sevenfold coordination with six O atoms from five phosphate groups along with the hydroxyl ion [52]. The OH columns are parallel to c axis and the main lines of the columns pass through the centers of Ca triangles which are on the mirror plane at $z = 1/4$ and $z = 3/4$. The monoclinic HA exhibits structural similarity to the hexagonal HA [50]. The greatest difference between monoclinic and hexagonal HA is the arrangement of the hydroxyl ions. In the monoclinic structure, the hydroxyapatite is pure enough that the OH columns are arranged in an ordered manner, where all the OH ions are displaced in the same direction from $z = 1/4$. Furthermore, all the OH columns point in the same direction within a plane parallel to the a and c axes, but the direction of the displacements alternated between adjacent planes. This ordered arrangement of OH ions in the monoclinic structure changes the mirror planes in $P6_3/m$ into b glide planes in $P2_1/b$ and double the b axis parameter.

Fig 2.2. Projection of hexagonal hydroxyapatite onto the (001) plane. Triangles represent PO_4 tetrahedra.

HA has quite high tolerance for ion substitution due to its special structure. OH^- ions can be replaced by F^- and Cl^- ions; while PO_4^{3-} ions can also be substituted with CO_3^{2-} and SiO_4^{4-} ions. The most interesting is the metal ions taking the

positions of calcium in HA lattice without changing the structure. With the incorporation of metal ions, the properties of HA can also be modified. For example, Eu^{3+} and Tb^{3+} doped HA have applications as fluorescent probes [39].

2.1.2. Synthesis of HA

Two major routes are used to synthesize HA: solid state reaction and wet chemical methods [53]. Solid state reaction is by mixing stoichiometric quantity of calcium precursor (such as CaCO_3) and phosphorus precursor ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ or $\text{Ca}_2\text{P}_2\text{O}_7$), followed by preheat treatment and shaping into disks and eventually reacted at $\sim 1000^\circ\text{C}$ [54-56]. It usually produces stoichiometric and well crystallized HA. However, relatively high temperature and long heat treatment is needed and the sinterability of the product is low. For wet chemical methods, the reaction takes place in aqueous media. The crystallinity and Ca/P ratio depend strongly on the preparation conditions. Wet methods include precipitation, hydrothermal technique and hydrolysis of other calcium phosphates. Among these methods, the precipitation method usually gives nanocrystalline HA and the shape of the nanoparticles can be controlled [46] (Fig 2.3). Nanometer-sized HA has been reported to have better bioactivity than the micrometer-sized HA [57].

The two most popular ways of precipitating HA are shown in Eqs 2.1 and 2.2. The reactions usually involve adding phosphorous precursor slowly into calcium precursor and/or adjusting the reaction pH using ammonia [53, 58-60]. Eq 2.1 is the reaction between calcium hydroxide and ortho-phosphoric acid [46, 50, 61], and Eq 2.2 is the reaction between calcium nitrate and ammonium dihydrogen phosphate with ammonium hydroxide to maintain the pH value of the reaction solution [53, 62, 63]. During the precipitation process, CO_3^{2-} groups are easily introduced into HA lattice. This is probably due to the presence of CaCO_3 in the reactants and dissolved CO_2 from atmosphere. If CO_3^{2-} groups replace OH^- , it is referred to as A-type carbonated HA and if CO_3^{2-} substitutes PO_4^{3-} , this is known as B-type carbonated HA [46]. The different substitutions can be distinguished based on FTIR spectrum, as shown in Fig 2.4 [46].

Fig 2.3. Different shaped HA nanoparticles were synthesized following the reaction as shown in Eq 2.1 at (a) 40°C, (b) 80°C, and (c) 100°C [46].

In order to prevent the presence of CO_3^{2-} groups in HA, some researchers have used CaO as the starting material which is the dehydration product of CaCO_3 [50, 61], while others have applied CO_2 free atmosphere (N_2 or Ar) during the synthesis process [58, 61].

Fig 2.4. FTIR spectra of HA precipitated at reaction temperatures of 40-100°C. (A) indicates the substitution of CO_3^{2-} for OH^- (A-type carbonated HA) and (B) indicates CO_3^{2-} for PO_4^{3-} (B-type carbonated HA) [46].

In this project, Eq 2.1 was used because it is a simple formulation with the advantage of water being its only byproduct. Since CO_3^{2-} groups are naturally present in bones [64], the presence of CO_3^{2-} groups in HA is not expected to affect cell response. Hence, no special procedures were applied to exclude CO_3^{2-} groups here.

2.1.3. Synthesis of metal ion doped HA (MHA)

To synthesize metal ions doped HA, coprecipitation [40, 42, 65, 66] and ion exchange methods [37, 39, 67, 68] are usually used. The former is by including metal ions in calcium solution while the latter is by adding metal ion solutions after HA nanoparticles have been formed. These two methods modify HA in different manners. The coprecipitation method changes both the surface and bulk concentration of metal ions, while the ion exchange method usually results in higher concentration of metal ions on the surface of HA nanoparticles than the bulk since ion-exchange is limited to the particles surface [68]. In this research, HA was produced through Eq 2.1 and $\text{Ca}(\text{OH})_2$ was used. If coprecipitation method was chosen, $\text{M}(\text{OH})_x$ may also be precipitated as one of

the byproducts due to the corresponding low solubility product of the metal hydroxide (M: Gd, Nd, Mn and Fe). This in turn could affect the precipitation of metal ion doped HA. Hence, ion-exchange method was used to synthesize the metal ion doped HA in this investigation.

2.1.4. Applications of metal ion doped HA (MHA)

Ion exchange reaction of HA or metal ion doped HA have been applied in many fields. HA has been used for the removal of heavy metal ions such as Pb^{2+} or Cd^{2+} in waste water or soils [69, 70], and immobilization of radioactive waste [55, 71]. The adsorption capacity depends on the crystallinity and specific surface area (SSA) of HA. HA powder with low crystallinity and a high SSA showed quantitative removal for wide range of heavy metal ions (Pb, Zn, Be, U, Bi, V, Al, Cu and Ga), while the calcined powder with higher crystallinity and lower SSA showed a quantitative removal only for a few elements (Pb, Bi, Ga) [69]. Studies on natural apatites from the fossil nuclear reactor of Oklo found that these compounds supported high doses of external and internal α irradiation with no significant changes in the crystal structure [55]. Ion removal or immobilization by HA takes place through two mechanisms, as shown in Eqs 2.3 - 2.5 [69]. The first mechanism is the dissolution and precipitation theory (Eqs 2.3 and 2.4), where HA provides phosphates by dissolution which then react with the metal ions to precipitate low soluble metal phosphate according to Eq 2.4. The second mechanism is the ion exchange theory (Eq 2.5), where metal ions adsorbed to the surface of HA particles substitute Ca^{2+} ions in the lattice.

Rare earth metal ions doped HA have the potential to be used as luminescence and laser material [72]. As reported by Chane-Ching *et al.* [73], Eu^{3+} and Tb^{3+} doped HA nanorods displayed green and red emission peaks under excitation in the visible spectrum. These nanorods with surface bonded aminoethyl

phosphate groups were individualized and stable in aqueous dispersion at neutral pH without aggregation or precipitation. These results allowed the application of Eu^{3+} and Tb^{3+} doped HA as fluorescence probe in biological fields.

The partial oxidation of methane to carbon monoxide and hydrogen has been reported to be catalyzed by HA at around 600°C. The formation of oxygen radical species on HA has also been found [37]. Introduction of Cu^{2+} and Ni^{2+} ions into HA matrix creates new redox centers and causes a large increase in activity of dehydrogenation of alcohols with little effect on the dehydration function of HA [41].

HA has been reported to decompose compounds such as methyl mercaptane and dimethyl sulfide effectively under UV (254 nm) irradiation [42]. To modify the photocatalysis effect of HA, Ti^{4+} ions were introduced into HA by coprecipitation method [40, 42, 74]. Ti^{4+} doped HA absorbed UV light at a wavelength less than 380 nm, so that it exhibited a much higher photocatalytic activity in oxidation and decomposition of acetaldehyde under UV irradiation than unmodified HA [74]. As reported by Hu *et al.* [42], Ti^{4+} substituted HA had photocatalytic effect both under visible light and UV irradiation and the photocatalytic activity of HA was excited by Ti substitution. Since HA had much higher affinity with biomaterials than TiO_2 , Ti^{4+} modified HA differed from TiO_2 in both adsorption affinity and photocatalytic activity for organisms, such as *E. coli*. Additionally, Ti^{4+} doped HA even showed bactericidal effect in the dark [40, 74].

To improve the antibacterial property, Ag^+ , Cu^{2+} or Zn^{2+} ions were co-substituted into Ti^{4+} doped HA (TiHA) [75]. TiHA was produced using coprecipitation method and Ag^+ , Cu^{2+} or Zn^{2+} ions were introduced by ion exchange method. Ag^+ , Cu^{2+} co-substituted TiHA coated on porous spumous nickel film showed high efficiency in killing *E. coli* and *S. aureus* in the dark and under weak UV irradiation. It was found that oxygen radical formed on Ag^+ , Cu^{2+} co-substituted TiHA at ambient temperature. Antibacterial effect of Ag^+ , Cu^{2+} or Zn^{2+} substituted HA synthesized using coprecipitation method was

also investigated [38]. Kim *et al.* reported that Ag^+ doped HA showed high antimicrobial property while Cu^{2+} and Zn^{2+} in HA did not show any antimicrobial property against *E. coli* [38]. The strong bactericidal effect of Ag^+ and non-antibacterial effect of Zn^{2+} in HA against *E. coli* was similarly reported by Hu *et al.* [75]. While Kim *et al.* observed that Cu^{2+} substituted HA synthesized using coprecipitation method did not show any antimicrobial property against *E. coli* [38]; Hu *et al.* noted that Cu^{2+} co-substituted TiHA was found to kill *E. coli* and *S. aureus* effectively [75]. Another paper on Cu^{2+} doped HA produced by ion exchange method, had high antibacterial activity on *E. coli* and *S. aureus* [76], which was similar with Hu's results [75]. The different observations on the antibacterial effect of Cu^{2+} doped HA was possibly due to the different ways of introducing Cu^{2+} ions into HA, which further resulted in different concentration distribution of Cu^{2+} ions in HA nanoparticles. Introduction of Cu^{2+} ions into HA by coprecipitation did not show bacterial effect while introduction of Cu^{2+} ions by ion exchange method demonstrated high antibacterial property. The coprecipitation method changes both the surface and bulk concentration of metal ions; while the ion exchange method usually gives rise to higher concentration of metal ions on the surface of HA nanoparticles than the bulk since ion-exchange is limited to the surface [68].

Magnetic property of HA can be modified by introducing other metal ions. Co^{2+} doped HA produced by ion exchange method was paramagnetic [37]. For Fe^{2+} doped HA, different results were obtained. Nakahira *et al.* [77] reported that Fe^{2+} doped HA synthesized using ion exchange method was paramagnetic, while Wu *et al.* [78] produced Fe^{2+} doped HA using coprecipitation method and found it was superparamagnetic. In the case of Fe^{3+} substituted HA synthesized using coprecipitation, it was found to be paramagnetic as reported by Low *et al.* [79].

Biological HA crystals contain lots of other ions, such as K^+ , Mg^{2+} , Na^+ , CO_3^{2-} and F^- , to name a few [80]. As the carbonate content in enamel may be related with the susceptibility to dental caries [81], Na^+ and CO_3^{2-} co-substituted HA was fabricated and the structure was refined using Rietveld refinement [81].

La³⁺ and Al³⁺ doped HA were also studied [65, 67] because of the inhibitory effect of La³⁺ and Al³⁺ on the demineralization of dental enamel. Since Mn²⁺ ions increase the ligand binding affinity of integrin and activate cell adhesion [64], Mn²⁺ doped HA has been synthesized and investigated [62, 64]. It was found that the growth of osteoblast cells on Ti coated with Mn²⁺ doped HA was enhanced as compared to uncoated Ti [64]. However, no published data is of yet available about the improvement of cell behavior on Mn²⁺ doped HA as compared to pure HA.

2.1.5. *In vitro* cytotoxicity test of metal ion doped HA (MHA)

Hydroxyapatite is biocompatible. However the cytotoxicity of metal ion doped HA needs to be confirmed due to the presence of metal ions other than Ca.

According to the ISO standard (10993-5), there are typically three methods to screen the cytotoxicity of biomaterials, including direct contact, indirect contact and extraction assay. After each assay, different methods can be applied to quantify the cytotoxicity of materials based on the viability of cells, including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), neutral red, LDH (lactate dehydrogenase) assay and also cell number.

For the direct contact test [82], the biomaterial is in direct contact with the cell monolayer without any barriers. This test method is not applicable for bulk materials due to the possible mechanical damage imposed on cells. In the case of metal ion doped HA nanoparticles, the powder form can be used. As reported by Wu *et al.* [78], medium containing Fe²⁺ doped HA nanoparticles at a concentration ranging from 0 to 0.5 mg/ml was applied in the *in vitro* biocompatibility test.

In the indirect contact method, there is a diffusion barrier between biomaterials and the cell system [82], such as an agar layer or a Millipore filter. According to ASTM F 895-84, a flat surface of specimen is preferred to be directly in contact with the agar surface. Since the agar diffusion assay was shown to be the most sensitive assay among the three cytotoxicity assessment methods [82], and

metal ion doped HA can be easily pressed into pellets, agar diffusion assay is suitable for the cytotoxicity test of metal ion doped HA.

In the extraction method, the cells are exposed to eluant from the biomaterials being tested. According to ISO standard (10993-12), materials in various forms, such as film, tubing, powder, pellet, foam, and membranes can be used for this cytotoxicity study. The extraction assay has been reported to be applied on HA pellets made from HA nanoparticles of different morphologies. The study was followed by cell seeding on the HA pellets in order to investigate the effect of HA nanoparticles morphology on osteoblast proliferation [57].

The number of viable cells is subsequently quantified using different methods. The frequently used ones include MTT and LDH assay. MTT assay is based on the ability of mitochondria from viable cells to reduce yellow MTT to purple formazan in living cells. The formazan is solubilized by detergents, such as dimethyl sulfoxide (DMSO) and the absorbance is measured using spectrophotometric method at 550-600 nm. Since a linear relationship exists between cell number and absorbance, the number of viable cells can be quantified based on the absorbance.

LDH (lactate dehydrogenase) assay is based on the reduction of NAD^+ to NADH catalyzed by LDH which is a cytoplasmic enzyme. NADH is then released into the culture medium upon cell lysis. The formed NADH converts the tetrazolium salt to a red water-soluble formazan that can be measured spectrophotometrically. Thus, LDH assay can be used to assess dead cells quantity by measuring LDH released into the medium or remaining live cells quantity by performing lysis LDH.

2.1.6. *In vitro* biocompatibility test of metal ion doped HA (MHA)

Bioactive ceramics bond spontaneously to living bone via a hydroxyl carbonate apatite (HCA) (also called bone-like apatite) layer without forming fibrous tissue around them when implanted in the body [83]. Kokubo *et al.* [84, 85] first found that bone-like apatite formation on bioactive materials *in vivo* could

be reproduced even in an acellular simulated body fluid (SBF), which has the equal inorganic ion concentrations similar to body plasma (Table 2.1). Since then, SBF test has been widely used for the assessment of bioactivity of various materials [86-94].

Table 2.1 Ion concentrations of simulated body fluid (SBF) and human blood plasma [84]

	Ion concentration (mM)						
	Na ⁺	K ⁺	Mg ²⁺	Ca ²⁺	Cl ⁻	HCO ₃ ⁻	HPO ₄ ²⁻
SBF	142.0	5.0	1.5	2.5	148.8	4.2	1.0
Blood plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0

For hydroxyapatite, the formation of HCA [89] is initialized by the deposition of Ca-rich amorphous or nanocrystalline calcium phosphate (ACP), followed by Ca-poor ACP, which eventually crystallize into HCA. The negatively charged surface of HA in SBF attracts Ca²⁺ ions and forms Ca-rich ACP, gaining positive charge. The Ca-rich ACP interacts with phosphate ions in SBF and forms Ca-poor ACP. Ca-rich ACP and Ca-poor ACP stabilize as HCA with a low solubility in SBF. The abundance of hydroxyl and phosphate groups on HA surface facilitates the deposition of HCA since both groups are responsible for the surface negativity of HA. Additionally, Yamashita *et al.* [93] found that accelerated HCA formation was observed on negatively charged HA surface which was produced by electric poling.

The biocompatibility and bioactivity of materials can also be investigated based on cell response *in vitro* [57, 64, 80, 95-104]. For pure HA and metal ion doped HA, osteoblast or osteoblast-like cells are usually seeded on the sintered pellets [80, 96, 100, 101]. Osteoblast cells adhered and differentiated earlier on pellets of HA doped with trivalent cations (such as La³⁺, Y³⁺, In³⁺ and Bi³⁺) than either HA doped with divalent cations (Mg²⁺ or Zn²⁺) or pure HA [80]. Cell proliferation was monitored using MTT assay [104] [103] or MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay [64, 97]. Both assays rely on the activity of mitochondrial

reductase that reduces MTT or MTS to purple colored formazan. To obtain cell density on pellet, visual counting method was used. After cells were fixed, the nuclei were stained with Hoechst fluorescence dye and visualized under fluorescence microscope. Cell number was counted with image analysis software [101].

As for cell differentiation behavior, alkaline phosphatase (ALP) and osteocalcin (OC) activity of osteoblast cells are typically assessed. Osteoblast cells in culture go through a programmed differentiation procedure [105]. After reaching confluence, cells express high levels of bone cell phenotypic markers, including ALP, OC, type I collagen, to name a few and ultimately deposit extracellular mineralized matrix. Additionally, OC levels increase in response to 1,25-(OH)₂D₃ treatment. Commercialized kits are available for ALP and OC detection and measurement. ALP activity is usually determined using *p*-nitrophenylphosphate as a substrate [106, 107]. ALP catalyzes the colorless substrate to a yellow product, *p*-nitrophenol and phosphate. The reaction is stopped by NaOH solution and the concentration of *p*-nitrophenol is determined spectrophotometrically by measuring the absorbance at 405 nm. As for OC, many assays are available using both polyclonal and monoclonal antibodies together with different standards. Thus, OC values determined by different assays give discordant results [108]. Alizarin Red-S is used for mineral staining since it binds selectively to calcium salts [97, 109]. The stain is extracted and the absorbance is taken using a spectrophotometer. As shown in Fig 2.5, the number of nodule was proportional with the calcium mineralization of hFOB cells [109]. At different growth stages, the morphology of osteoblast cells on pellets can be observed under SEM after fixation and dehydration.

Fig 2.5. (a) Representative control cells stained with Alizarin red-S after 48 h of differentiation; (b) representative deferoxamine (DFO)-treated cells (8 μ M DFO) stained with Alizarin red-S after 48 h of treatment and differentiation; and (c) summary of statistical analysis of calcium mineralization in control and treated cells after 48 h of treatment ($P < 0.05$). The number of nodules in the field was counted by two different individuals [109].

2.2. Poly(lactide-co-glycolide) (PLGA)

Poly(lactide-co-glycolide) (PLGA) and poly(lactide) (PLA) (Fig 2.6) have been extensively used in drug delivery field due to their biocompatibility, biodegradability and good mechanical strengths [110]. To date, micro- or nanoparticles are still the focus as drug carriers [31, 111-116]. Emulsification process is typically used since the technique is easy to manipulate. Spherical particles are usually proposed as drug carriers for localized delivery. However, macrophages can easily internalize spherical particles with diameters up to 15 μ m while the internalization of spheroids is unachievable. Spheroidal PLGA microparticles can be produced using simple oil-in-water single emulsion method [117].

Drug release profiles of PLGA and PLA microparticles are usually triphasic, where the initial burst and an induction period are followed by a second burst release. Modifications have been proposed to lower the initial burst or to increase drug encapsulation efficiency, such as polymer or microparticles blending [31], double walled microspheres [27], drug combination [26], drug polymer conjugate [111, 118] and polymer composite microparticles with inorganic filler [48, 119]. Among these modifications, polymer composite microparticles have several potentials to be used in the drug release field, like broad applications of drugs [43-45, 47, 120, 121] due to the surface property of the inorganic filler, low initial burst [47, 48] and the combination of advantages

belonging to polymer and inorganic filler. Hence, polymer composite microparticle system is used in this investigation.

There are many factors influencing the release profile, such as the polymer composition, polymer molecular weight, polydispersity [31], drug property [26], particle size [122], release medium and release temperature [114]. To date, drug release systems, produced from polymer with inorganic filler, are new and more investigations are needed to explore the properties.

Fig 2.6. Molecular structure of (a) PLGA and (b) PLA.

2.2.1. Synthesis of PLGA

The monomers of PLGA include lactic acid and glycolic acid, which are metabolized *in vivo*. Lactic acid is one of the smallest optically active molecules. It has L(+) and D(-) stereoisomers (Fig 2.7) [123].

Fig 2.7. Stereoisomers of lactic acid and molecular structure of glycolic acid [123].

PLGA is synthesized using two methods, polycondensation and ring opening polymerization [123, 124]. Polycondensation of diol with dicarboxylic acid has also been applied for the synthesis of other aliphatic polyesters. However direct

polycondensation from lactic acid and glycolic acid is not widely used since formation of water generally limited the molecular weight [125].

Fig 2.8. Stereoisomers of lactide and molecular structure of glycolide [123].

Ring opening polymerization method is commonly studied because of the possibility of an accurate control on the chemistry [123-125]. After polycondensation of lactic acid or glycolic acid, depolymerization of the oligomers into dehydrated cyclic dimer, lactide (3,6-dimethyl-1,4-dioxane-2,5-dione) or glycolide (1,4-dioxane-2,5-dione) (Fig 2.8) is followed. An appropriate catalyst is required to make the ring opening polymerization proceed in reasonable conditions and afford polymers with controlled properties and high molecular mass. For PLA, PGA and PLGA, the most widely used catalyst for industrial preparation is tin(II) bis(2-ethylhexanoate) (tin octanoate, $\text{Sn}(\text{Oct})_2$) [125].

To date, PLGA with different compositions (LA/GA molar ratio), molecular weight and end groups (carboxyl, or ester capped) has been commercialized.

2.2.2. Degradation of PLGA

PLGA is insoluble in water, however it is hydrolytically unstable and degraded by hydrolytic attack of the ester bonds [126]. The degradation mechanism of PLGA has been generalized as a random hydrolytic chain scission process [127, 128]. Through this process, PLGA is degraded into lactic and glycolic acids. A

triphasic mechanism has been suggested by Taghuvanshi *et al.* [129]. During the first stage, the molecular weight decreases significantly but no appreciable weight loss happens or no soluble monomers are formed. In the second stage, molecular weight continues decreasing together with a rapid loss of weight since soluble oligomers and monomers are formed. The final stage is where soluble monomers are formed from soluble oligomers, which is attained when the polymer is completely soluble. The random chain scission process progresses throughout the three stages.

The degradation process can be described as follows [127, 128]. Initially, the polymer matrix is homogeneous. Once placed in an aqueous phase, water penetrates into the specimen, leading to the hydrolytic cleavage of ester bonds, producing degradation byproducts of carboxyl and hydroxyl end groups. The new carboxyl end groups then accelerate the hydrolytic reaction of the remaining ester bonds, which is defined as an autocatalysis effect. The acidic byproducts which are close to the matrix surface are neutralized. However, those located inside the matrix cannot diffuse out easily or hard to be neutralized, thus catalyzing the hydrolysis of more ester bonds and increasing the acidity inside the matrix. The difference in acidity between the bulk and surface makes the degradation heterogeneous. If the specimen is large enough, a hollow structure would eventually be formed. Bimodal distributions of molecular weight are also observable because of the presence of two populations of macromolecules degrading at different rates. The amorphous part of the matrix is degraded first compared with the crystalline regions since water penetrates through the amorphous regions easily. This autocatalysis effect will affect the drug release profile from PLGA microparticles [130].

The hydrolysis of PLGA is shown as follows [126, 128]:

Since the degradation is catalyzed by the acidic degradation product, —COOH end groups, the reaction rate can be described as:

$$\frac{d[COOH]}{dt} = p[ester][H_2O][COOH] = k_{Mn}[COOH] \quad (2.7)$$

where $[COOH]$, $[ester]$ and $[H_2O]$ are the concentrations of carboxyl end groups, ester and water in the polymer matrix. It is assumed that in the early stage of degradation, the concentrations of ester and water are constant and $[COOH] = 1/M_n$, thus the Eq 2.7 is rewritten as:

$$\ln M_n = \ln M_{n,0} - k_{Mn}t \quad (2.8)$$

where $M_{n,0}$ is the number-average molecular weight of polymer before degradation and k_{Mn} is the rate constant of degradation process [126, 128, 131, 132].

Many factors influence the degradation rate of PLGA, such as polymer composition [127, 133], molecular weight [25, 134], glass transition temperature [128], crystallinity [127, 128], dimension of specimen [135, 136], additives [127], the degradation temperature [136] and pH of buffer solution [137, 138]. Compared with lactic acid (LA), glycolic acid (GA) is much more hydrophilic and easily accessible to water, which results in the preferential degradation of GA units in the polymer chains of PLGA [127]. Although GA is hydrophilic, PGA has much higher crystallinity than PLGA polymers [35] (Fig 2.9); thus PGA is not easily penetrated by water molecules, giving rise to a slower degradation rate. Among PLGA polymers of different LA/GA compositions, the polymer with LA/GA ratio of 50/50 has the fastest degradation rate [139, 140].

Polymer with higher molecular weight usually has slower degradation rate [25, 134]. For linear polymers, high molecular weight means long polymer chains. The longer the polymer chains, the higher the entanglement ratio and viscosity, and the lower the diffusivity [122] of small molecules, such as water. This leads to lower degradation rate as it is harder for water to penetrate into the matrix.

Fig 2.9. Crystallinities determined by X-ray and DSC measurement and water uptake data for PLGA copolymers as a function of composition [35].

Glass transition temperature is an indicator of the mobility of polymer chains. Lower T_g suggests higher mobility of polymer chains, and also higher diffusivity of water molecules. The relationship between T_g and molecular weight is expressed by Fox-Flory equation (Eq 2.9) [125, 141]:

$$T_g = T_{g\infty} - \frac{K_g}{M_n} \quad (2.9)$$

where $T_{g\infty}$ is the glass transition temperature at an infinitely high number-average molecular weight, K_g is a polymer specific constant and M_n is number-average molecular weight. For polymers with the same LA/GA composition, the one with higher molecular weight has higher T_g . General ranges for T_g include: PLLA, 60–65°C; PDLA, 55–60°C; PLGA 85/15 and 75/25, 50–55°C; PLGA 65/35 and 50/50, 45–50°C [142]. T_g appears to decrease with the

increase of GA content. The T_g values for all PLA and PLGA polymers are higher than the physiological temperature of 37°C [143].

T_g is also affected by thermal histories and additives. Annealing of PLGA induces higher crystallinity. Due to the loss of amorphous material, T_g decreases with the increase of crystallinity. Meanwhile the hydrolytic degradation is retarded to some extent, beyond which formation of voids through annealing increases degradation rate [128]. For microparticles fabricated using water-in-oil-in-water double emulsion method, the introduction of poly(vinyl alcohol) in the inner water phase demonstrates antiplasticizing [144] (increase of T_g) while inclusion of Triton X-100 in the inner water phase results in plasticizing effect (decrease of T_g) [145].

The dimension of specimen plays an important role as degradation of PLGA is autocatalyzed. The acidic degradation byproducts, carboxyl and hydroxyl end groups close to the surface of specimen are easier to be neutralized than the ones located inside the matrix due to the shorter diffusion path length. The autocatalysis effect results in the heterogeneous degradation of PLGA. Hollow structure and bi-modal distribution of molecular weight of sample after degradation were observed [127]. The autocatalysis affects the degradation rate of PLGA microspheres (condensed and porous microparticles) and thus the drug release rate [112, 122, 130, 146]. It was found that a linear relationship existed between degradation rate and particle size, where particles of larger size degraded faster [147]. Furthermore, aggregated microparticles degraded faster than well dispersed ones as the acidic degradation products were not easily flushed out in the former, which decreased the local pH and enhanced autocatalysis effect [132].

Since the degradation products of PLGA are acidic, the pH of buffer solution also affects the degradation rate. As reported by Zolnik *et al.* [137], PLGA microparticles degraded at pH 2.4 had larger degradation rate constant than at pH 7.4. Most significantly, morphological studies on microparticles suggested that the mechanism of PLGA degradation changed from “outside-in” at pH 2.4 to “inside-out” at pH 7.4. At pH 2.4, microparticles maintained the smooth

surface throughout the degradation process whereas at pH 7.4, surface pitting and pore formation were observed on microparticles, similar to the usual morphological changes. Degradation proceeded in a more homogeneous manner at pH 2.4 than at pH 7.4. This was possibly due to the lower solubility of acidic oligomers at pH 2.4 than at pH 7.4, which resulted in the entire microparticles experiencing a close-to-uniform pH at pH 2.4. At pH 7.4, the microenvironmental pH within the microparticles has been reported to vary considerably.

Another important factor influencing degradation of PLGA is additives. Incorporation of acidic compounds accelerates the degradation process while for basic materials, two effects can intervene simultaneously: base catalysis on cleavage of ester bonds and neutralization of carboxyl end groups which would suppress the autocatalysis effect. The degradation rate is accelerated or slowed down depending on which effect takes the lead [127]. From the application point, the neutralization of acidic degradation products due to basic additives may be beneficial to patients with PLGA implants since it has been found that adverse response to these implants occurred weeks or months postoperatively, which may be related to the biodegradation of these implants [148].

2.2.3. Fabrication of microparticles

Drug-loaded polymeric microparticles are usually fabricated using emulsion [25, 26, 31, 32, 47, 48, 112, 114, 116, 118, 122, 130, 144, 145, 149-156], spray drying [157-159] and phase separation (coacervation) methods [160]. All these methods have to meet the following requirements. First, the stability and biological activity of drugs should not be adversely affected. The size of microparticles should be in the desired range (up to 250 μm , ideally < 125 μm) and microparticles have high drug encapsulation efficiency. The microparticles quality and drug release profiles should be reproducible within specified limits. Lastly, no aggregation or adherence of microparticles happened which should be a free flowing powder.

For emulsion method, depending on the hydrophilicity of the drug, specific method, including oil-in-water (O/W), oil-in-oil (O/O), solid-in-oil-in-water (S/O/W) single emulsion and water-in-oil-in-water (W/O/W) double emulsion are typically applied. All these methods involve dispersing one phase of small quantity into another phase of much larger quantity with the help of homogenizer or sonicator or stirrer. For hydrophilic drugs, O/O, S/O/W single emulsion and W/O/W double emulsion methods are usually used while for hydrophobic drug, O/W and S/O/W single emulsion methods are frequently chosen. The overall O/W and W/O/W procedures are shown in Fig 2.10.

Fig 2.10. Procedures of making PLGA microparticles with hydrophobic and hydrophilic agents using oil-in-water (O/W) single emulsion and water-in-oil-in-water (W/O/W) double emulsion.

Based on the fabrication routine used, drugs will exist as solid particles embedded or molecularly dispersed in the polymer matrix. Most microparticles are spherical in morphology and of controlled size. Recently spheroidal shaped

microparticles were also fabricated using simple O/W single emulsion [117]. This shape is able to facilitate localized drug release since giant cells or macrophages cannot internalize such microparticles. Furthermore, a computational model study suggested that spheroidal particles preferentially drifted towards vascular wall over spherical ones because of the complex hydrodynamic forces and torques acting on them. This indicated that spheroidal microparticles would possess a higher affinity for the vascular wall compared to the spherical ones.

Spray drying is commonly used to preserve dairy products in food industry [159]. It has also been extensively applied to dry heat-sensitive materials, improve flow properties, prepare free-flowing granules for tablet production and increase the solubility of poorly water-soluble substances in pharmaceutical industry. Spray drying can be adapted to fabricate matricial systems of microspherical type, thus it has also been employed as a microencapsulation method [159]. A spray dryer disperses a liquid feed into a hot drying medium and the liquid feed turns to solid particles. This is a continuous process involving several steps, which includes atomization, mixing of spray with the drying gas, evaporation and product separation. The liquid feed is made up of complex mixtures consisting of an active material dissolved, dispersed, or emulsified with a polymer in an aqueous or an organic solvent. Depending on the formulations, products are either microparticles or microcapsules.

As for the phase separation (coacervation) method, the process has some steps similar to the emulsion technique. Drugs are either dissolved or dispersed in polymer solution or dissolved in water which is further dispersed in polymer solution to form water-in-oil (W/O) emulsion. An organic nonsolvent is then added to the polymer-drug-solvent system under stirring. The solvent is gradually extracted and very soft coacervate droplets with entrapped drugs are formed. These soft droplets are then transferred to a large quantity of another organic nonsolvent for being hardened [160]. Since there is no surfactant (used in emulsion method) present to stabilize the microdroplets, agglomeration is a frequent problem.

Among the three fabrication methods above, emulsion method is the most cost effective, easy to manipulate, controllable and of various parameters to adjust so as to achieve drug-loaded microparticles for specific applications.

2.2.3.1. Oil-in-water (O/W) single emulsion

For O/W single emulsion [160-162], polymer and drug are dissolved in a water immiscible oil phase (organic solvent) and dispersed into a water solution containing a surfactant under stirring with the help of a magnetic stirrer, mechanical impeller, or homogenizer. The microdroplets are stabilized due to the surfactant and the organic solvent is slowly diffused into aqueous phase and evaporated eventually (microparticles are solidified). Microparticles are then washed, collected by filtration, sieving or centrifugation and dried in ambient conditions or by lyophilization.

Table 2.2 List of solvents commonly used for microencapsulation by solvent evaporation [161]

Name	A; B; s*	Advantages	Disadvantages
Chloroform	212; 61; 8	Low solubility in water	Higher toxicity than dichloromethane
Dichloromethane (methylene chloride)	453; 39.7; 20	Dissolution of most polymers; almost immiscible in water; high volatility and quite low boiling temperature	High toxicity
Ethyl acetate	100; 77; 90	Low toxicity	Partially soluble in water, very low vapor pressure
Ethyl formate	259; 54; 105	Low toxicity	Partially soluble in water

*A; B; s: Vapor pressure (mbar) at 20°C; boiling point (°C); solubility in water (g/L) at 20°C

The most commonly used solvents are listed in Table 2.2. Among them, dichloromethane (DCM) is widely used due to its high volatility and high immiscibility with water. However, DCM is confirmed carcinogenic according to the Environmental Protection Agency [161]. As some hydrophobic drugs are not soluble in DCM, cosolvent is introduced [149, 153], such as methanol [28], ethanol [161] and dimethyl sulfoxide [29] which are miscible with water. The

presence of cosolvent may affect the elimination rate of organic solvent during the solidification of microparticles.

There are two steps for the O/W single emulsion process [163]. The first is emulsification which mainly determines the microdroplets size distribution and the second step, organic solvent diffusion from the microdroplets, influences the particle morphology, encapsulation efficiency and drug release profile. As reported by Rosca *et al.* [163], spherical microparticles are formed by accelerated solvent elimination resulted from the combination effects of high solvent volatility and polymer precipitation. With the elimination of solvent, the polymer gradually precipitates at the water-droplet interface and tends to form a continuous crust around the droplets. In the meantime, the particles shrink due to the loss of solvent. Hence the polymer crust is continuously fragmented and nanoparticles are generated, which was observed for samples produced from low polymer concentration such of 1% w/v due to the large shrinkage factor. According to Eq 2.10, the shrinkage factor (ratio between the diameter of the emulsion droplet and the diameter of the solvent-free microparticles) decreases with the increase of polymer concentration in organic solvent if the same stirring condition is applied. O/W single emulsion is usually used to encapsulate hydrophobic drugs which agglomerate toward the microparticles surface during solvent elimination. This enriched layer will contribute to the initial burst release.

$$\alpha_i = \frac{d_i}{d_f} = \left(\frac{V_p + V_s}{V_p} \right)^{1/3} = \left(1 + \frac{100\rho_p}{C_p} \right)^{1/3} \quad (2.10)$$

Here, d_i is the diameter of the initial microdroplet; d_f is the diameter of the final microparticle; V_p the is polymer volume; V_s is the solvent volume; ρ_p is the polymer density (g/ml) and C_p is the polymer concentration (% w/v). For PLGA, the density ρ_p is calculated as 1.31 g/ml [163]. The shrinkage factors calculated according to d_i/d_f where d_i and d_f are measured on microscope images, are reasonably close to their corresponding theoretical values calculated based on C_p and ρ_p in Eq 2.10 if the experiment errors considered [163].

For O/W single emulsion technique, both emulsification and solvent elimination influence the size of solidified microparticles. According to Gabor *et al.*, increasing the stirring rate decreased the microparticles size and narrowed the size distribution; while polymer concentration increased, the size of the microparticles increasing proportionally [164]. It is reasonable that higher shear force produce smaller droplets and higher polymer concentration gives higher viscosity which makes it harder to separate to smaller droplets. In addition, according to Eq 2.10, microparticles produced from higher polymer concentration has smaller shrinkage factor.

Microparticles produced using O/W single emulsion are condensed particles with smooth surface. The introduction of cosolvent introduces pores inside microparticles while the surface is still smooth, as shown in Fig 2.11.

Fig 2.11. SEM images of (a) lidocaine-loaded PLGA microparticles produced using O/W single emulsion, top and cross section view [130] and (b) FITC-dextran loaded PLGA microparticles produced using O/W single emulsion with the introduction of DMSO as cosolvent, top and cross section view [29].

Many factors influence the drug loading efficiency. Microparticles of large size usually have higher loading efficiency due to longer diffusion path length. Those produced from polymers with higher molecular weight have higher loading efficiency due to the higher polymer chain entanglement ratio. Higher

drug concentration in the polymer solution usually results in lower loading efficiency since higher gradient exists between polymer solution and surrounding media. Other parameters affecting drug loading, includes composition and end groups of PLGA and the drug/polymer interaction.

In order to increase the loading efficiency of water soluble drugs, oil-in-oil (O/O) single emulsion has been developed [160]. The drug and polymer are first dissolved in a hydrophilic solvent [9, 10, 14], such as acetonitrile, dimethylformamide and the solution is then emulsified in an oil phase such as light mineral oil, liquid paraffin containing a surfactant like Span. The solvent is then eliminated by evaporation or extraction. Microparticles formed are washed using solvents like *n*-hexane.

2.2.3.2. Solid-in-oil-in-water (S/O/W) single emulsion

Some drugs are not soluble in organic solvents, or have very low loading efficiency using O/W or O/O single emulsion, thus solid-in-oil-in-water (S/O/W) single emulsion is usually employed [162]. This method is applicable for both hydrophobic [162, 165] and hydrophilic [25, 112, 114, 122] drugs. The procedures are quite similar to O/W single emulsion. The only difference is that drug particles are suspended in polymer solution for S/O/W single emulsion while in the O/W single emulsion method, drug is dissolved in the organic solvent.

Small-sized drug particles are required for S/O/W single emulsion in order to achieve complete encapsulation [162]. For non-hygroscopic drugs, a smooth mortar and pestle are used to ground the drug particles to smaller size. Most drug particles have a tendency to show sedimentation due to their higher density compared to the suspension medium, which results in low loading efficiency. Different from the molecular homogenous distribution of drug in the matrix of microparticles produced using O/W single emulsion, the drug particles in S/O/W method are still crystals in polymer matrix after the particles have hardened (Fig 2.12). Release profile is affected since the drug crystals are

interconnected inside microparticles and upon released into media, the remaining space is occupied by water and diffusion channels are thus formed.

Fig 2.12. SEM images of cross-sections of 5-FU-loaded PLGA microparticles produced using S/O/W single emulsion, at three different magnifications. The hatched regions correspond to the epon (Epon 812 Fluka) that was used to obtain the cross-sections, the dark grey regions correspond to PLGA, the bright grey regions to 5-FU. A cavity with 5-FU crystals (indicated by an arrow) inside is shown in (c) [112].

Fig 2.13. SEM image of 5-FU loaded PLGA microparticles produced using S/O/O single emulsion [32].

For hydrophilic drugs, S/O/W single emulsion sometimes results in a low loading efficiency due to the continuous loss of drug crystals to water solution.

Hence, some authors have applied solid-in-oil-in-oil (S/O/O) single emulsion [32]. The procedure for S/O/O is similar to O/O single emulsion. However, since the drug is suspended in the polymer solution and not soluble in the continuous oil phase, the microparticles surface is different from the ones produced using O/O method due to the presence of drug crystals (Fig 2.13). In this case, the surface is still smooth with some drug crystals adhering over the periphery of microparticles [32].

2.2.3.3. Water-in-oil-in-water (W/O/W) double emulsion

Hydrophilic drugs are also encapsulated into microparticles by water-in-oil-in-water (W/O/W) double emulsion. Bioactive large compounds, such as proteins are usually entrapped inside microparticles using this method.

The procedure for the fabrication process is shown in Fig 2.10. Drugs are dissolved in aqueous media with or without surfactant, which is the W1 phase. The W1 phase is then emulsified in polymer organic solution with the help of a homogenizer or sonicator at a very high speed, like 10,000 rpm. This makes the W1/O emulsion, which is further emulsified in a large volume aqueous phase containing surfactant (W2) at a much lower stirring speed, forming the second emulsion. Organic solvent is slowly eliminated by evaporation or extraction and microparticles are eventually hardened, washed, collected by centrifugation, filtration or sieving and dried at ambient conditions or by lyophilization.

Microparticles with different morphologies are fabricated dependent on the relative size of inner aqueous droplets and the final emulsion droplets. During solvent elimination and shrinkage of microparticles, the inner droplets have a tendency to gradually coalesce under the pressure of the precipitating polymer. Hence, if the inner droplets are not much smaller than the final emulsion droplets, closed and open microcapsules are easily formed (Fig 2.14B, c1 and c2). Only when the inner W1 droplets are much smaller than the final emulsion droplets, microparticles with honeycomb structure are formed (Fig 2.14A), which is the most suitable structure for efficient entrapment.

Fig 2.14. Microparticles with different morphologies produced from different formulations [163]. (A) PLGA, 10% w/v, 1% w/v PVA, 500 rpm, (a) microscope images of the initial W1/O/W2 droplets transformation, (b) SEM image of microparticles morphology; (B) PLGA, 5% w/v, 1% w/v PVA, 8000 rpm, (a) initial W1/O/W2 microdroplets, (b) SEM image of solidified microparticles.

The size of inner droplets depends on the preparation parameters, such as W1/O emulsification energy, polymer concentration, with or without surfactant in W1 phase, volumetric ratio of W1/O, volume of W2 phase, surfactant concentration in W2 and the stirring speed of the second emulsification. According to Rosca *et al.* [163], W1 droplets in the W1/O emulsion with diameter larger than 10 μm were present even at a very high stirring speed of 20,000 rpm.

Higher homogenizing speed may narrow the size distribution of W1 droplets and thus increase the loading efficiency [29]. Increasing polymer concentration in organic solvent stabilizes the inner W1 droplets against coalesce and usually leads to higher viscosity of W1/O emulsion, which results in larger particle size after the second emulsification. Thus the diffusion path length also increases and the higher viscosity may restrict the diffusion of the drug in the matrix. The loading efficiency increases accordingly [29, 166]. Similarly, the presence of surfactant like PVA in W1 phase also stabilizes the W1/O emulsion, which

gives a more uniform drug distribution and a slower drug release [167]. As with the increase of W1 volume, size of microparticles increases slightly while the loading efficiency decreases significantly [29]. Low volume of W1 phase results in a minimal porosity of the polymer matrix, whereas high W1 volume causes an increase of micropores and channels thereby facilitating the rapid diffusion of drug in W1 phase into the continuous W2 phase during microparticles solidification [29, 166]. Due to the presence of inner droplets, the shrinkage factor (ratio between the diameter of the emulsion droplets and the diameter of the solvent-free microparticles, Eq 2.10) of microparticles produced using W/O/W double emulsion is much smaller than the ones using O/W single emulsion. However, the inner W1 phase is still partly lost during the solvent elimination and shrinkage of microparticles [163]. Increasing the concentration of surfactant like PVA (0.1% to 1%) in the external W2 phase, size of microparticles decreases due to the stabilization effect of surfactant. The higher concentration of PVA also restricts the diffusion of drug from W1 to continuous W2 phase [29, 167, 168]. Thus the loading efficiency also increases.

The hydrophobicity of polymer also affects the inside structure of microparticles. As reported by Ruan *et al.* [169], microparticles produced from PELA (PLA-PEG-PLA) had smaller and more uniform inside pores as compared to the ones made from PLGA. This is because the aqueous phase W1 preferred the hydrophilic chains segment of PEG in PELA and thus the W1/O emulsion is easier to be stabilized. PELA microparticles had higher encapsulation efficiency and more rapid *in vitro* release than PLGA microparticles.

2.2.4. Drug release mechanism

According to Langer, drug release mechanism of polymeric microparticulate systems can be divided into the following typical categories [170]. The most common is diffusion, where drugs migrate from the initial position in the polymeric system to the surrounding media. Diffusion may happen in a reservoir (Fig 2.15A) or a matrix system (Fig 2.15B). For the reservoir system,

the drug core is surrounded by a polymer film while for the matrix system, drug particles are well dispersed or dissolved in the polymer matrix.

Fig 2.15. Drug release mechanism through diffusion (A) (B), chemical reaction (C) (D), matrix swollen (E), pore formed due to osmotic pressure (F), osmotic delivery (G) and enhanced release rate by external magnetic field (H). In all cases, dots represent drug and in (H), larger dots represent magnetic beads [170].

Drugs are also released by chemical reactions such as degradation of polymer (Fig 2.15C) or cleavage of the drug from a polymer chain (Fig 2.15D). Water molecules penetrate into the polymer matrix once the systems are immersed in an aqueous media. Some systems swell in aqueous media and drugs locked inside are then released out (Fig 2.15E). While for some systems, pores are formed due to the osmotic pressure, through which drugs are released (Fig 2.15F). An ideal system using osmotic effect is a pill that has a laser-drilled hole in the surface of a polymer coating, where constant release rate can be achieved (Fig 2.15G). Some polymer systems can be externally activated using

forces like magnetism to release more drug when needed. The magnetic beads in polymer matrix would squeeze drug containing pores and force more drug out of the matrix by applying an external magnetic field (Fig 2.15H). Combinations of the above mechanisms are also possible.

For PLGA microparticles, drug release profile is usually a triphasic pattern. After an initial burst release, a lag time with very slow release rate is observed and followed by a second burst release due to the disintegration of microparticle structure. The drug release is usually diffusion controlled, polymer degradation controlled or a combination of the two [160]. Diffusion is known to play a major role in affecting drug release rate from PLGA microparticles. Hence, lower release rate is expected for larger microparticles due to the longer diffusion pathways. However, it has been reported that the drug release rate was not affected by particles size for microparticles produced using O/W single emulsion with size ranging from 7 μm to 53 μm [130]. This is due to the autocatalysis effect of PLGA. These microparticles are condensed in nature and without pores. The acidic degradation products inside the matrix continue to catalyze the cleavage of ester bonds. The degradation of PLGA is heterogeneous and pores are gradually formed both on the surface and internally while the particles size is not significantly affected. After the initial burst and lag period, the remaining drug in microparticles is released out through the channels formed. Hence the release mechanism is described as a combination of mechanism B and F in Fig 2.15. The only difference is that pores in PLGA are formed due to the polymer degradation and not osmotic pressure as in Fig 2.15F. For larger microparticles, the degradation rate is faster due to the autocatalysis effect. The higher diffusivity resulted may compensate the longer diffusion path length, which led to a comparable release rate from microparticles of different sizes [130]. Different from condensed microparticles of identical composition, the drug release rate from porous microparticles produced using W/O/W double emulsion decreased with increase of particle size [146]. The degradation rate of porous microparticles increased with particle dimensions, indicating that autocatalytic effect still played an important role even in small and highly porous microparticles. This autocatalysis effect was much less pronounced in porous microparticles than in condensed ones [146].

The drug release profile of PLGA microparticles is usually a triphasic pattern. This triphasic pattern was shifted to a continuous release by inclusion of medium chain triglycerides in PLGA microparticles [28]. Leuprolide was chosen as a model drug and O/W single emulsion method was used. The presence of medium chain triglycerides led to the formation of microparticles with irregular surface and highly porous inner structure, which could have contributed to the accelerated leuprolide release in the lag time and reduced release rate in the second burst phase.

2.2.5. Mathematical modeling of drug release

2.2.5.1. Models based on diffusion

Many factors have been reported to affect drug release kinetics of PLGA microparticulate system, including particle size, molecular weight and composition of copolymer, interaction between drug and polymer, to name a few. To simplify the modeling process, it is assumed that the drug release is governed solely by diffusion, a mass transfer process which can be described using Fick's second law [171]:

$$\frac{\partial C}{\partial t} = \frac{\partial}{\partial x} \left(D \frac{\partial C}{\partial x} \right) + \frac{\partial}{\partial y} \left(D \frac{\partial C}{\partial y} \right) + \frac{\partial}{\partial z} \left(D \frac{\partial C}{\partial z} \right) \quad (2.11)$$

where C and D are the concentration and the diffusion coefficient of drug, respectively, t represents time and x, y, z are three Cartesian coordinates. Since microparticles are spherical in geometry, Eq 2.11 can be written as 2.12 using Spherical coordinates.

$$\frac{\partial C}{\partial t} = D \left(\frac{\partial^2 C}{\partial r^2} + \frac{2}{r} \frac{\partial C}{\partial r} \right) \quad (2.12)$$

where r is the radial coordinate. Assuming constant drug diffusion coefficient D, perfect sink condition and a uniform drug concentration which is smaller

than its solubility in microparticles, the following analytical solution can be used to describe the fractional cumulative drug release.

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} e^{-\left(\frac{n^2 \pi^2 D}{R^2} t\right)} \quad (2.13)$$

Here, M_t and M_∞ is the cumulative drug release at time t and infinite, respectively. R is the radius of particles. The model was adapted to fit the drug release data of biodegradable polymer nanoparticles [172] and PLGA condensed [112, 130] and porous [146] microparticles. The representative fitting curves are shown in Fig 2.16 [112].

Fig 2.16. Fit of cumulative drug release profile of 5-fluorouracil (5-FU) loaded PLGA microparticles in PBS (pH 7.4) at 37°C according to an exact solution of Fick's second law (considering spherical device geometry, constant drug diffusivity, perfect sink conditions, monolithic solution, Eq 2.13: solid line) and Koizumi model (described later, Eq 2.17: dashed curves) [112].

When the fractional cumulative drug release is larger than 0.6, Eq 2.13 can be approximated using the linear driving force (LDF) model as Eq 2.14 [150]:

$$\frac{M_t}{M_\infty} = 1 - e^{-mt} \quad (2.14)$$

where m is a mass transfer coefficient. The equation also gives a good fit of data for early diffusion [150], as shown in Fig 2.17.

Fig 2.17. Cumulative fractional drug release of PLGA microparticles produced using W/O/W double emulsion method. The solid line is a fit of the data according to Eq 2.14 [150].

Based on Eq 2.13, a semi-empirical equation has been proposed [173] to fit the drug release data, as shown as Eq 2.15.

$$\frac{M_t}{M_\infty} = st^n \quad (2.15)$$

where s is a constant representative of the drug-polymer system and the exponent n is an empirical parameter characterizing the release mechanism. For spheres, pure Fickian release had the limiting n value of 0.43 and the value for Fickian diffusion depended on the size distribution of polymer microparticles. The model was used to fit the release data of 5-fluorouracil from crosslinked polyacrylamide microparticles [174]. The constant s and n was listed in Table 2.3. The representative drug release profiles are shown in Fig 2.18.

Fig 2.18. Fractional cumulative release of 5-FU from polyacrylamide microparticles containing 10 wt.% of 5-FU and crosslinked with (●) 2% (F-1), (▲) 4% (F-2) and (■) 6% wt.% of NNMBA [174].

Table 2.3 Release kinetics parameters based on Eq 2.15 of different samples as shown in Fig 2.22 [174]

Sample name	s	n	Correlation coefficient, r
F-1	0.1283	0.346	0.806
F-2	0.0682	0.508	0.974
F-3	0.0494	0.660	0.813

One major assumption of the above models is that the drug is molecularly dispersed within the system (monolithic solution). However, for many drug loaded polymer microparticles, the drug is in a crystalline state and dispersed in the polymer matrix as solid particles. Many models have been reported to quantify drug release from the system in which the drug is homogeneously distributed throughout the system with an initial concentration higher than its solubility in the carrier material (monolithic dispersion). The most important theory among the models is developed by Higuchi who had used a pseudo steady-state assumption [112]. A representative diagram using a thin slab exposed in perfect sink condition is shown in Fig 2.19. The solid line is the

concentration of drug in the matrix and for distance farther from h (distance from absorbing surface), the drug concentration is assumed a constant, C_0 , which is much larger than the solubility of the drug C_s ($C_0 \gg C_s$). Upon drug release, the new concentration profile of drug is represented by the broken line. Under these conditions, a simple relationship between the release rate of the drug and the square root of time was derived by Higuchi in the case of planar geometry. Later the theory was extended to spherical devices. The modified Higuchi equation [48] can be shown as Eq 2.16.

$$\frac{M_t}{M_\infty} = \sqrt{\frac{D}{\pi R^2} t} \quad (2.16)$$

To date, many authors have applied this model to fit the drug release data [48, 175-178]. The representative fractional cumulative release of gentamicin from HA/PHBV composite microparticles as a function of square root of time is shown in Fig 2.20 [48]. The curves are almost linear lines after the initial burst.

Fig 2.19. Pseudo steady-state approach applied for the derivation of Higuchi model quantifying drug release from planar systems (monolithic dispersion) [112].

A very similar approach as Higuchi's model was used by Koizumi and Panomsuk [112]. The obtained (approximate) equation is shown as Eq 2.17, which is in an explicit form and is easier to handle than the respective Higuchi equation.

$$M_t = 4\pi R^2 [\sqrt{2(C_0 - C_s)C_s Dt} + \frac{4C_s Dt}{9R} (\frac{C_s}{2C_0 - C_s} - 3)] \quad (2.17)$$

This model has been used to fit the 5-FU release data from PLGA microparticles [112, 114]. The representative fitting curves are shown in Fig 2.16.

Fig 2.20. Higuchi plots of the release profiles of gentamicin from composite microparticles [48].

The Koizumi model is based on the assumption that drug diffusion coefficient D is a constant during drug release. However, for degradable polymer device, the diffusion coefficient increases with the degradation time due to the decrease of molecular weight. For PLGA system, the decrease of molecular weight with degradation time is described in Eq 2.18 while the dependence of D on M_w is expressed in Eq 2.19.

$$M_{w,t} = M_{w,0} e^{-k_{deg} t} \quad (2.18)$$

$$D_{M_w} = D_0 + \frac{k_D}{M_w} \quad (2.19)$$

Where $M_{w,t}$ and $M_{w,0}$ is the molecular weight of polymer at degradation time t and 0, respectively. k_{degr} is the degradation constant. D_{Mw} and D_0 is the diffusion coefficient of drug at polymer weight M_w and before degradation. k_D is the decreasing constant of D . Koizumi's model coupled with Eq 2.18 and 2.19 has been used to fit 5-FU release data of PLGA microparticles system [112, 122].

2.2.5.2. Other models

For PLGA system, drug release rate depended on polymer degradation. Fitzgerald and Forrigan [149] quantified diltiazem release from polylactide-*co*-glycolide carrier using a polymer degradation model. This model, based on copolymer degradation and dissolution, is expressed as Eq 2.20:

$$\ln \frac{X}{1-X} = k.t + q \quad (2.20)$$

where X is the fractional cumulative drug release, k . is a rate constant and $q = -k.t_{\text{max}}$. t_{max} is the time at which maximum rate of drug release achieved. The model fitted well with the release data of levamisole from PLGA microspheres and disks [149] (Fig 2.21).

Fig 2.21. Fractional levamisole base release profiles from microspheres containing 19.7% (□), 14.3% (◆) and 2.4% (○) levamisole base in PBS (pH 7.4) fitted according to Eq 2.20 [149].

Another model is proposed by Gallagher and Corrigan [179], who expressed the fractional cumulative drug release as a sum of drug release resulted from surface diffusion and degradation of polymer matrix. If the surface diffusion release is due to rapid dissolution of the drug at solid-liquid interface, the proportion and size distribution of drug at the interface, and the solubility of the drug influence the magnitude of surface diffusion. As dissolution proceeds, the proportion of surface occupied by drug will decrease. Since the available surface area of drug is assumed to be proportional to the weight of the undissolved drug, the dissolution of drug can be described by a first-order process under sink conditions and declining surface area of drug. Assuming that no drug is dissolved at time zero, the initial surface drug release can be described by Eq 2.21.

$$W = F_B(1 - e^{-k_b t}) \quad (2.21)$$

W is the fraction of drug released at time t, F_B is the fraction of drug released due to surface diffusion and k_b is the burst release constant. Drug release due to polymer degradation is described in Eq 2.20. Rearranging Eq 2.20 and combining with Eq 2.21, the model proposed by Gallagher and Corrigan [179] is shown as Eq 2.22. The model fitted well with the release data of propranolol HCl, diclofenac sodium, salicylic acid and sulfasalazine from poly(D,L-lactic acid) tablets [180] (Fig 2.22).

$$F_{TOT} = F_{b\infty}(1 - e^{-k_b t}) + (1 - F_{b\infty}) \frac{e^{k_d t - k_d t_{\max}}}{1 + e^{k_d t - k_d t_{\max}}} \quad (2.22)$$

Where F_{TOT} is the total fraction of drug release at time t, $F_{b\infty}$ is the total surface diffusion fraction at time infinity and k_d is the rate constant for polymer degradation release. k_d is the same parameter as k. in Eq 2.20.

Fig 2.22. Release profiles of salicylic acid (2 wt.%) from D,L-PLA tablets at 37°C [180].

2.3. Polymer composite

Composite material is a mixture of two or more phases bonded together so as to possess a combination of properties which cannot be achieved by a single phase [181]. In biomedical field, composite materials are normally fiber-reinforced and particle-reinforced composite. Ceramics and glasses are usually brittle and associated with low resistance against fatigue failure, whereas polymers are not as strong as ceramics but have viscoelastic properties, excellent toughness and shock-absorbance ability [182]. Thus composite materials of polymers with ceramics or glasses usually have improved mechanical properties compared to single phase materials.

2.3.1. Composite materials as scaffold

Hydroxyapatite (HA) has been widely used as inorganic filler in polymer scaffolds due to its osteoconductivity. The presence of HA also improve the mechanical properties of the polymer matrix.

HA reinforced high density polyethylene (HDPE) is commercially available as HAPLEX™. By introducing HA, the bonding between bone and the composite graft is enhanced. *In vivo* test have shown that beyond a minimal volume

percentage of about 20% HA, bone apposition and bone bonding was achieved in a HA-HDPE composite implant [183]. Recently, HA/polyetheretherketone (PEEK) composite materials have attracted attention. The Young's modulus and tensile strength of HA/PEEK composite made by injection molding were reported to be in the range of 2-10 GPa and 40-90 MPa, respectively, which were in the regime of cortical bone. Bioactivity study on HA/PEEK composite fabricated by compaction after powder mixing showed that the apatite forming ability increased with the increase of HA volume fraction in the composite [91]. These results indicate that HA/PEEK composites are potential candidate in load-bearing applications.

HA reinforced biodegradable polymer composite, like PLA, PLGA and poly(ϵ -caprolactone) has also been developed. HA/PLLA composite scaffold may have osteoconductivity, biodegradability and high mechanical strength [116, 184-187]. HA/PLGA fibrous scaffold, which was produced by electrospinning polymer solution with well dispersed HA nanoparticles, had enhanced mechanical strength for HA in the range of 1% to 5% by weight [188-190]. This might be due to the uniform distribution of HA nanoparticles in polymer matrix. Increasing HA quantity higher than 5 wt%, defects formed and the storage modulus decreased [188]. In order to create a better osteophilic environment, fibrous scaffold of poly(L-lactic acid-*co*- ϵ -caprolactone) and gelatin was fabricated by combining the electrospinning and electrospraying techniques [97]. HA nanoparticles were no longer inside the polymer matrix as described above, but were electrosprayed on electrospun fibrous scaffold. This kind of scaffold had improved mechanical properties than HA blended scaffold. Additionally, the rough surface of HA electrosprayed on scaffold showed better osteoblast proliferation than HA blended scaffold.

HA/PDLLA scaffold produced by press-molding has been reported to possess much better shape memory ability than pure PDLLA scaffold [191]. In order to well disperse HA nanoparticles in the polymer matrix, the nanoparticles were first suspended in ethanol with homogenizer and the suspension was slowly added into polymer solution under stirring. Some polymer would precipitate due to the ethanol. The organic solvent was slowly evaporated. The floccules

were completely dried and press-molded to a designed shape at 105°C. 68.0°C was chosen as the shape deformed temperature and 70.0°C was the recovered temperature. The shape recovery ratios for all HA/PDLLA composites were higher than 95% and reached a peak value of 99.6% when HA/PDLLA weight ratios were 1:2 and 1:2.5. Pure PDLLA scaffold, on the other hand, had a low shape recovery ratio of 80.6%. This was probably because of the stationary and reversible phases formed in HA/PDLLA composites, the presence of which was a necessary condition to form shape memory effect.

The properties of composite materials depended on the component materials, distribution and the interaction among components. In order to improve the bonding ability between HA nanoparticles and polymer matrix, such as PLLA, the PLLA polymer chains were grafted onto HA nanoparticles surface [192, 193]. The surface grafted-HA (g-HA) nanoparticles were homogeneously suspended in chloroform and the suspension was added to PLLA/chloroform solution. The mixture was precipitated in excess ethanol and after drying, the precipitate was hot-press molded into the required shape. At a low g-HA content (~4 wt%), g-HA/PLLA composite showed higher bending strength and impact energy than PLLA, and at a high g-HA content of 20 wt%, the modulus was remarkably increased. g-HA nanoparticles functioned as inter-tying molecules and improved the adhesive strength between the nanoparticles and the polymer matrix. Additionally, the presence of g-HA facilitated both adhesion and proliferation of chondrocytes on g-HA/PLLA composite film [193]. The mechanical strength was significantly improved and the biocompatibility of the scaffold was also enhanced.

2.3.2. Composite materials as drug carrier

Composite materials have also been developed as drug carriers, either in the form of condensed disks, film coating on scaffold or microparticles, since it can combine the advantage of the constituent materials.

Gentamicin sulphate (antibiotic) loaded composite were fabricated by polymerization after mixing HA, poly(ethyl methacrylate) (PEMA) and

gentamicin sulphate solid with poly(methyl methacrylate) (PMMA) monomer [194]. Gentamicin sulphate was released at a fast rate during the first 10 h, followed by a diminishing rate until 70 days. The release percentage increased proportionally with the quantity of HA in the composite since HA increased the porosity and hydrophilicity of samples.

Drugs can also be loaded onto the inorganic filler. Toremifene citrate was first adsorbed onto the surface of silica xerogel, which was further mixed with poly(ϵ -caprolactone-*co*-D,L-lactide) and compression molded into disk [195]. The release flux of toremifene citrate increased with the increase of caprolactone quantity in the copolymer while silica xerogel had a minor enhancing effect on the release rate of toremifene citrate.

So far, the common way to fabricate polymer microparticles is emulsion solvent evaporation method. The composite microparticles are either fabricated using S/O/W single emulsion [48, 116, 152, 154] or W/O/W double emulsion [196, 197]. Thus the surface property of the inorganic filler, especially the interfacial compatibility between inorganic filler and polymer phase may be a key factor for the properties of composite microparticles [198]. The hydrophilic nature of HA makes the HA nanoparticles inclined to be located in the water phase and the higher density of HA nanoparticles led to sedimentation of HA during the preparation process of S/O/W emulsion. This may result in very small encapsulation amount of HA in composite microparticles. Grafting with PLLA polymer chain was reported to modify the hydrophilic nature of HA nanoparticles [192, 193, 199]. The compatibility between PLLA matrix and HA was significantly improved and the loading percentage of HA in microparticles was as high as 30% by weight [193]. Although the compatibility between HA and PLLA was improved, the surface of HA nanoparticles was modified to be hydrophobic which was not applicable as drug carrier for cisplatin. Hence, grafting with PLLA polymer chain was not used in this investigation.

In microparticulate system, drugs can be deposited onto the inorganic filler and further encapsulated into the polymer matrix [48, 152, 197]. Compared to the bare drug loaded inorganic filler, the initial burst of microparticle system is

much lower and the release time lasts much longer [152]. HA/PLGA microparticles produced by W/O/W double emulsion with bovine serum albumin (BSA) as model protein, demonstrated slower release rate than those free of HA [197]. HA nanoparticles initially located inside microparticles became surface associated ones as release proceeded. It was speculated that HA was dissolved by the acid degradation products of PLGA and reprecipitated on microparticles surface due to the very low solubility product (K_{sp}) of HA at pH 7.4. It indicated that the presence of HA could control the degradation rate of PLGA matrix by affecting the pH of the microenvironment in the microparticles.

Besides encapsulation in microparticles, HA nanoparticles were also coated onto liposomes [200] and PLGA microparticles [151] using constant composition method. Briefly, a stable supersaturated solution of HA was prepared by mixing CaCl_2 and KH_2PO_4 at the molar ratio of 1.67. Both pH and Ca^{2+} activity were monitored. Liposomes or PLGA microparticles were added to the reaction vessel to induce the precipitation of HA. The consequent drop in pH or Ca^{2+} activity triggered the simultaneous addition of titrants from two autoburettes containing CaCl_2 and KH_2PO_4 , KOH respectively. HA coated liposomes reduced the release rate of the model hydrophobic drug, indomethacin over uncoated liposomes. It has been observed that for uncoated liposomes, the drug was released at a greater rate at pH 7.4 than at pH 4 since the drug was more soluble under basic conditions. In the case of HA coated liposomes, release rate at pH 7.4 was slower than at pH 4. This was because HA was more soluble in acidic conditions. This suggested that it was possible to have microenvironmental control on the drug release rate from HA coated liposomes. For PLGA microparticles, in order to induce the precipitation of HA, anionic surfactant was used and a hydrophilic model drug amoxicillin was encapsulated using S/O/W single emulsion. Compared to the uncoated microparticles, drug release profile of HA coated PLGA microparticles did not demonstrate significant difference. However, the presence of coated HA on microparticles showed a statistically significant buffer ability of pH of PBS after 13 days as compared to PLGA microparticles. Even after PLGA was greatly degraded, HA coating was only partially dissolved.

Recently, the possible negative effects caused by acidic degradation byproducts of PLGA polymer have attracted great interest. *In vivo*, occurrences of a sterile sinus discharge or inflammatory discharge related to PLA-PGA devices have been reported in podiatry field. It was speculated that the adverse response was related to the biodegradation of PLA-PGA implants [148]. *In vitro*, cell seeding test showed that osteoblast cells death and detachment was observed after growing on microporous PGA substrate for 36 h [95]. Furthermore, different apatite forming ability was observed on PLGA-siloxane nanohybrid material with different polymer compositions (low degradability 90PLA10PGA, high degradability 50PLA50PGA) [92]. PLGA-siloxane hybrid materials were synthesized by end-capping PLGA (with acidic end groups) with 3-isocyanatopropyl triethoxysilane and following the sol-gel reaction with calcium nitrate tetrahydrate. The bioactivity of the samples was tested using SBF method for 28 days. The low degradable PLGA hybrid showed apatite forming ability within 3 days of incubation while the highly degradable samples had no apatite formed even after 28 days. Although the pH of SBF with the highly degradable hybrid was not low enough to inhibit the apatite crystal formation, it was speculated that the measured pH value was averaged and did not reveal the local pH on the specimen surface. It was believed that the fast degradation of the highly degradable hybrid had an acidic surface and thus prohibited the apatite deposition. Hence, basic materials have been proposed to be incorporated into PLGA materials so as to modify the local pH and improve the biocompatibility. It was reported that composite scaffold containing PGA and amorphous carbonated apatite demonstrated good biocompatibility. *In vitro* long term (4 weeks) osteoblast cell culture revealed good cell attachment, collagen and osteocalcin expression, and mineral deposition. The pH of medium decreased to 6.2 after pure microporous PGA scaffold immersed for 12 h while the pH of medium with composite scaffold remained in the physiological range (pH 7.2 - 7.6) within 4 weeks [95].

To date, basic materials, like HA [95, 148, 201], wollastonite, bioglass 45S5 [201], tri-calcium phosphate [202], calcium carbonate, sodium bicarbonate [148] have been incorporated into PLGA matrix. The effect on degradation rate

was studied. For HA, conflicting results have been reported. HA is slightly basic and would in theory neutralize the acidic degradation products. Li *et al.* [201] reported that incorporation of HA accelerated the degradation of HA/PLGA sponge-like scaffold; while Agrawal *et al.* [148] reported that HA decreased the degradation of HA/PLGA condensed implants. As for microparticles system, the effect of HA on the degradation of PLGA still need to be investigated.

Chapter 3

Experimental methods and techniques

The delivery system contains three major components, cisplatin (CDDP), inorganic filler which was metal ion doped hydroxyapatite (MHA) and polymer, PLGA. Both the drug and polymer were used as received while MHA was synthesized using ion exchange method in aqueous phase. Cisplatin was from Sigma Aldrich Chemical Company and basic information of PLGA used is summarized in Table 3.1. The drug carrier was fabricated using single or double emulsion techniques.

Table 3.1 Information of the PLGA used for the fabrication of composite microparticles

Synonym	Molar ratio of lactide/glycolide	Intrinsic viscosity (dl/g)	Company
P(0.8)	75/25	0.8	Boehringer Ingelheim
P(0.61)	50/50	0.61	Durect Corporation

MHA was produced using ion exchange method and characterized by the following typical techniques, including X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, field emission scanning electron microscopy (FESEM) equipped with energy dispersive X-ray spectroscopy (EDX) and inductively coupled plasma (ICP). Besides the structure and composition information, the potential properties and applications of the products were also explored.

3.1. Synthesis of metal ion doped HA (MHA)

Pure hydroxyapatite (HA) was synthesized as follows. 3.71 g Ca(OH)_2 (Fluka, 96.0 wt% pure with less than 4 wt% CaCO_3) was added to 250 ml deionized water (DI water) in a beaker and the suspension was stirred using a magnetic stirrer at 500 rpm. Ca(OH)_2 suspension was stabilized at 98.5°C (where

spherical HA nanocrystallite would be precipitated [46]) for half an hour before 0.12 M H_3PO_4 (Sigma-Aldrich, A.C.S. reagent, 85.0 wt% in water solution) solution was transferred to the suspension at 4 ml/min using a peristaltic pump. The addition of acid was stopped when the pH of suspension dropped to 7.8 (for Gd^{3+} and Nd^{3+} doped HA), 5.5 (for Mn^{2+} doped HA), and 5.0 (for Fe^{3+} doped HA). The slurry was aged at 98.5°C for another 2 h with stirring at 500 rpm and kept overnight at room temperature (25°C). The supernatant was decanted and the precipitate was washed thrice with DI water. The final product was dried at 100°C in an oven and ground to powder for characterizations.

Metal ion substituted HA (MHA) was produced using ion exchange method. The solution containing Nd^{3+} ($\text{NdCl}_3 \cdot 6\text{H}_2\text{O}$, Aldrich, 99.9%), Gd^{3+} ($\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$, Sigma, 99% titration), Mn^{2+} ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, Riedel-de Haën) or Fe^{3+} ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, Sigma-Aldrich) of 0.0002, 0.001, 0.002 and 0.004 mol was transferred to the HA slurry when the addition of H_3PO_4 solution was stopped. Ammonia hydroxide (Sigma-Aldrich, A.C.S reagent) was then added to adjust the pH of the slurry to the specific values. This was followed by aging for 2 h at 98.5°C at 500 rpm and kept at room temperature (25°C) overnight without stirring. The products were identified as MX (where X was defined as the atomic percentage of added M to added Ca).

Fig 3.1. The schematic diagram of apparatus for MHA nanoparticles production.

To confirm that the mechanism involved for MHA production was ion exchange, the special sample, HA-s, was synthesized following similar procedures as above but without the 2-h aging at 98.5°C. After the addition of phosphorus acid, the precipitate was filtered, dried at room temperature (25°C) and grounded to powder for XRD characterization.

3.2. Characterization of metal ion doped HA (MHA)

All samples were characterized using the following techniques. Since electromagnetic fields have been shown to accelerate healing in bone fractures [203, 204] and ferromagnetic or superparamagnetic nanoparticles have the potential to be used as drug carriers, contrast agents and heat mediators for hyperthermia [78], the electrical conductivity (for Nd³⁺ and Gd³⁺ doped HA) and magnetic property (for Gd³⁺ and Fe³⁺ doped HA) were also tested.

3.2.1. X-ray diffraction (XRD)

The phases present in the powders were identified using X-ray diffraction (XRD). Bruker D8 Advance X-ray diffractometer with Cu K α radiation at 40 kV and 40 mA was used. The step size and scan time at each step were 0.04° and 10 s, respectively. The Rietveld refinement was carried out using program TOPAS 2.1 (Bruker) and the starting structure was a hexagonal model with space group P6₃/m reported by Sudarsanan, K and Young, R.A [51]. The scale factor, zero shift (2θ) and background coefficients were refined first. The lattice parameters a and c were also allowed to vary along with the earlier parameters. The crystal size was tabulated using Scherrer equation [205]:

$$d = \frac{k\lambda}{B_{1/2} \cos \theta} \quad (3.1)$$

where, d is the crystal size, k is the shape coefficient and 0.9 for elongated apatite crystallites, λ is the wavelength of Cu K α radiation (1.540980 Å), and $B_{1/2}$ (rad) is the full width at half-maximum intensity (FWHM) of peak belonging to the (002) plane.

3.2.2. Fourier transform infrared (FTIR) spectroscopy

The functional groups of the powders were examined by Fourier transform infrared (FTIR) spectroscopy (PerkinElmer, Spectrum GX). The scans were averaged over five runs at a resolution of 1 cm^{-1} . The specimens were prepared by mixing 0.5 mg sample powder with 150 mg KBr and compressing the powder into pellets at 8-ton load.

3.2.3. Field emission scanning electron microscope (FESEM) and energy dispersive X-ray spectroscopy (EDX)

The morphology of nanoparticles was observed with a JEOL field emission scanning electron microscope (FESEM) (JSM-6700F) and the elements present were confirmed using energy dispersive X-ray spectroscopy (EDX). To obtain a better image of the nanoparticles under FESEM, samples were slightly compressed into pellet at 0.5-ton load. Before observation, pellets were attached to a holder using double faced carbon tape and coated with platinum at 20 mA for 100 s using a sputter (JEOL JFC-1600).

3.2.4. Inductively coupled plasma (ICP)

The concentrations of the elements in each sample were quantified by ICP (Prodigy-Teledyne Instruments Leeman Lab). ICP was calibrated by standard solutions of the interested element and samples were prepared by dissolving 40 mg powder in 250 ml 1% w/v HNO_3 .

3.2.5. Electrical conductivity test

The electrical conductivity of Nd^{3+} doped HA (NdHA) and Gd^{3+} doped HA (GdHA) was measured at room temperature with a 6'' probe-station (Cascade Microtech SUMMIT 11751B-6) connected to Agilent E5270B semiconductor parameters analyzer. 0.25 g powder was pressed into pellet at 1-ton load and sintered (particles coalescence begins at 700°C [206] and thus make the pellet more compact) at 800°C (non-stoichiometric HA would partially decompose to α -TCP at temperature above 800°C [207]) for 1 h. The heating and cooling rate

were 4°C/min. Before measurement, both surfaces of the pellets were painted with silver to form the electrically conductive layers.

3.2.6. Magnetic property test

For Gd³⁺ doped HA (GdHA) and Fe³⁺ doped HA (FeHA), the magnetic property was examined using vibrating sample magnetometer (VSM) (Lake Shore 7404) at room temperature (25°C) in the range of magnetic field 0-10 kOe.

3.2.7. *In vitro* cell study

Pellets of metal ion substituted HA (MHA) were prepared by pressing 120 mg powder in a 13 mm die (Specac[®], PT. No. 3006 BDH) at 1 ton-load and autoclaved before use. The diameter of pellets was 13 mm and thickness was around 1 mm. Human fetal osteoblast cell line hFOB 1.19 from American Type Culture Collection (ATCC) was cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with 10% v/v fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ in air at 37°C. Among the three frequently used cytotoxicity test methods (agar diffusion assay, extraction assay and direct contact assay), agar diffusion assay was reported as the most sensitive method [82]. Thus, agar diffusion assay was used to quantify the *in vitro* cytotoxicity of NdHA and GdHA, while extraction assay were applied for MnHA and FeHA. As for MnHA and FeHA, osteoblast cells were further seeded onto the pellets so as to study the *in vitro* cell proliferation. The extraction assay was better to support the cell seeding results as the concentration of metal ions in the eluant of extraction assay could be measured.

3.2.7.1. Agar diffusion assay

Agar diffusion method was performed for NdHA and GdHA in accordance with ISO 10993 Part 5 and ASTM F895-84 due to its high sensitivity. Osteoblast cells were seeded in 35 mm Petri dishes at 1.0×10^5 cells/ml (2 ml/dish) for one day. The medium was then discarded and replaced by agar medium. The agar medium was prepared as follows. 2% w/v agar water solution was autoclaved and mixed with two times concentrated DMEM/F12 at volumetric ratio 1/1.

The mixture was cooled to 39°C and then poured into Petri dishes containing the cells. After the agar had solidified, the specimen (sample pellets, negative and positive controls of high density polyethylene (HDPE) and latex rubber respectively) was placed on the agar. The pellets had been presoaked in DMEM/F12 medium for 2 h to wet them. For each composition, triplicates were prepared for the test. The cells were then incubated for another day (24 ± 1 h) according to ASTM F895-84. After one day, neutral red solution was added and the cells were incubated for another hour. Finally the Zone Index and Lysis Index were determined under a light microscope according to the guidelines in Table 3.2. Schematic diagram of pellets, agar layer and cells are shown in Fig 3.2.

Table 3.2 Zone Index and Lysis index description in agar diffusion assay

Index	Description for Zone index	Description for Lysis index
0	No detectable zone around or under specimen	No observable cytotoxicity
1	Zone limited to area under specimen	Less than 20% of zone affected
2	Zone extends less than 0.5 cm beyond specimen	20 to 39% of zone affected
3	Zone extends 0.5 to 1.0 cm beyond specimen	40 to 59% of zone affected
4	Zone extends greater than 1.0 cm beyond specimen but does not involve entire dish	60 to 80% of zone affected
5	Zone involves entire dish	Greater than 80% of zone affected

3.2.7.2. Extraction assay

The cytotoxicity of Mn^{2+} doped HA (MnHA) and Fe^{3+} doped HA (FeHA) was tested using extraction assay. Cells were seeded into 24-well plates at a density of 5×10^4 cell/ml (1 ml/well) and incubated for one day. The medium in each well was then replaced by the extraction medium (4 replicates for each composition, 1 ml/well) and incubated for another 72 h. The extracted medium

was obtained from 2.12 ml DMEM/F12 medium, which was used to incubate one pellet at 37°C for 72 h. The cells cultured with DMEM/F12 medium acted as the control. MTT method was used to measure the proliferation of cells and ALP activity of cells was further tested by *p*-nitrophenyl phosphate (pNPP) liquid substrate system (N7653, Sigma). The concentration of Fe in the extracted medium was measured using colorimetric assay which made use of Fe chelator (orthobathophenanthroline (OBP)). The absorbance of the conjugate was monitored at 538 nm [208]. The detailed protocol is included as Appendix C.

Fig 3.2. Schematic diagram of sample pellet, agar layer and cells in agar diffusion test.

3.2.7.3. Cell seeding

Since FeHA and MnHA have potential to be more bioactive than pure HA, osteoblast cells were seeded on FeHA and MnHA pellets at a density of 5×10^4 cell/ml in 24-well plates (4 replicates for each composition). The wells without pellets were used as the controls. The culture medium was changed every 2 days and MTT method was applied to quantify the cell proliferation. The cell growth profile and cell activity on the pellets were investigated for 3 days and 7 days. ALP test was carried out to assess the cell activity. For the interpretation of cell adhesion result, zeta potential of HA, Mn5 and Fe5 powder in DI water was assessed using Malvern Zetasizer Nano-ZS at 37°C.

3.2.7.4. Cell fixation

Osteoblast cells on HA, Mn5 and Fe5 pellets were fixed after 3-day incubation (duplicates for each sample). The samples were first rinsed with PBS, followed by fixing the cells in 2.5% v/v glutaraldehyde in 0.1 M sodium cacodylate at room temperature (25°C). After 45 min, the samples were washed with 0.1 M

sodium cacodylate. The cells were further fixed in 1% w/v tannic for 30 min at 4°C and rinsed in 0.1 M sodium cacodylate buffer. The samples were then dehydrated in a graded series of ethanol and subsequently another graded series of hexamethyldisilazane. (The detailed protocol is included as Appendix D). The cell morphology was observed under FESEM (JEOL JSM-6700F).

3.2.7.5. MTT assay

MTT assay is based on the transformation of the pale yellow MTT to dark blue formazan crystals by a mitochondrial dehydrogenase enzyme in viable cells. The formed formazan is impermeable to cell membranes and cumulated within healthy cells. At each test point, 100 µl MTT-PBS solution (5 mg/ml) was added to each well and the plate (covered with alumina foil to protect it from light) was incubated at 37°C in an incubator for 2 h. The supernatant was then carefully removed and the dark blue formazan was dissolved by adding 500 µl dimethyl sulfoxide (DMSO) to each well and incubating at 37°C for 2 h. The solution was loaded into another 24-well plate and the absorbance was measured at 490 nm with reference at 630 nm (to minimize the noise influence) using a microplate reader (BioTex ELx 800). A calibration curve between cell number and MTT absorbance value was obtained following the protocol in Appendix E.

3.2.7.6. ALP assay

Alkaline phosphatase (ALP) is one of osteoblast markers. It can catalyze the hydrolysis of a colorless organic phosphate ester substrate, *p*-nitrophenyl phosphate (pNPP), to a yellow product *p*-nitrophenol and phosphate. Here, pNPP liquid substrate system (N7653, Sigma) was used for ALP activity test. The cells were first washed with PBS before 500 µl pNPP solution was added to each well. The 24-well plate was then kept in the dark for 2 h at room temperature (25°C) until the solution changed from colorless to yellow. The reaction was stopped by adding 100 µl 3M NaOH solution, following which the yellow solution was aliquoted into another 24-well plate and the absorbance was measured at 405 nm using a microplate reader (BioTex ELx 800). The ALP

activity was calculated based on the quantity of the yellow product *p*-nitrophenol, as shown in Appendix F.

3.3. Loading cisplatin on MHA nanoparticles

It has been reported that the affinity of cisplatin onto HA nanoparticles depended on loading temperature [20] and shaking speed [19-21]. In this project, two factors - temperature and shaking speed - were varied to obtain maximum cisplatin loading, as summarized in Table 3.3.

Table 3.3 Loading parameters for cisplatin on MHA nanoparticles in the study

Shaking speed (rpm)	Temperature (°C)	
	25	37
0	√	√
200	√	√

Cisplatin ($\geq 99.9\%$ trace metal basis, Aldrich) was first dissolved at 0.4 mg/ml in 10 mM phosphate-buffered solution (PB). NaCl was omitted in the buffer solution since Cl ions have been found to prohibit the binding of cisplatin onto HA nanoparticles [19]. MHA nanoparticles were then suspended in the cisplatin solution at 6.67 mg/ml and sonicated for 10 min. The suspension was incubated at the shaking speed and temperature shown in Table 3.3 for 24 h. It has been reported that the uptake of cisplatin by HA reached maximum at 24 h [21]. After 24 h, samples were centrifuged at 7000 rpm for 10 min. The supernatant was discarded and the precipitate was washed once with DI water, dried in a vacuum oven at room temperature (25°C) and ultimately grounded to powder for characterization, including XRD, FTIR, FESEM and ICP as described in section 3.2. ICP was used to quantify the drug loading and loading efficiency were calculated using Eqs 3.2 and 3.3. The zeta potentials of pure HA, Mn10 and Fe5 powder in 10 mM phosphate-buffer solution (without NaCl) (PB) at 6.67 mg/ml were also assessed using Malvern Zetasizer Nano-ZS at 25°C and 37°C. The specific surface area of pure HA, Mn10 and Fe5 was measured using BET method.

$$\text{Drug loading (\%)} = \frac{\text{weight of CDDP loaded}}{\text{weight of particles}} \times 100\% \quad (3.2)$$

$$\text{Loading efficiency (\%)} = \frac{\text{drug loading}}{\text{theoretical drug loading}} \times 100\% \quad (3.3)$$

3.4. Fabrication of composite microparticles

Emulsification solvent evaporation method was used to fabricate the composite microparticles. In order to obtain high drug loading and controllable drug release profile, water-in-oil-in-water (W/O/W) double emulsion and solid-in-oil-in-water (S/O/W) single emulsion were used.

3.4.1. Water-in-oil-in-water (W/O/W) double emulsion

As described in Section 3.3, cisplatin was adsorbed onto MHA nanoparticles in PB solution, thus water-in-oil-in-water (W/O/W) double emulsion was first proposed to fabricate cisplatin loaded composite microparticles as follows. After the nanoparticles had been suspended in cisplatin solution for 24 h, the suspension was injected into PLGA in dichloromethane (DCM) (VWR, AnalaR, 99.5%) solution to form the first emulsion. This was followed by the second emulsification in poly(vinyl alcohol) (PVA) (Aldrich, 87-89% hydrolyzed, average molecular weight 13,000-23,000) aqueous solution.

The major challenge here was to find the right parameters to produce microparticles with high drug loading and loading efficiency. For this, condensed surface is preferred as porous microparticles usually give rise to low drug loading. To optimize the fabrication parameters for condensed surface, pure HA without any cisplatin was first used instead of CDDP-Fe5 in the initial study.

The details of fabrication parameters are shown in Table 3.4. Pure HA nanoparticles were suspended in 10 mM PB solution and ultrasonic for 30 min to well disperse the nanoparticles. The suspension was then emulsified in PLGA-DCM solution using a homogenizer at 10,000 rpm for 30 s, producing the first emulsion. The first emulsion was further injected into 1% w/v PVA

solution and emulsified at 400 rpm for 1 h using a magnetic stirrer. The stirring speed was lowered to 200 rpm for another 2 h to evaporate the DCM at room temperature (25°C) in a fume hood. The composite microparticles were collected by sieve in the range of 20 µm to 150 µm, washed with DI water and dried. The final product was stored at -20°C.

Table 3.4 The fabrication parameters for microparticles produced using W/O/W double emulsion technique

Sample name	W1 ^a			O ^b			W1/O/W2 ^c (Volumetric ratio)
	HA (g)	W1 (ml)	C of HA ^d (g/ml)	P(0.8) ^e (g)	DCM (ml)	C of P(0.8) ^f (% w/v)	
D-2	0.05	1	0.05	0.4	20	2%	1/20/200
D-3	0.05	1	0.05	0.6	20	3%	1/20/200
D-4	0.05	1	0.05	0.8	20	4%	1/20/200
D-5	0.05	1	0.05	1.0	20	5%	1/20/200
D-5-H	0.10	2	0.05	1.0	20	5%	1/10/100
D-10-H	0.10	2	0.05	2.0	20	10%	1/10/100

^a W1, 10 mM phosphate-buffered solution (without NaCl) (PB)

^b O, oil phase, PLGA-DCM solution

^c W2, 1% w/v PVA solution

^d the concentration of HA in W1, PB solution

^e PLGA with molar ratio of lactic acid/glycolic acid 75/25 and inherent viscosity 0.8 dl/g

^f concentration of P(0.8) in DCM solution

3.4.2. Solid-in-oil-in-water (S/O/W) single emulsion

The solid-in-oil-in-water (S/O/W) emulsion method was applied to prepare MHA/PLGA composite microparticles. Briefly, all solids including PLGA, Fe5 and/or cisplatin shown in Table 3.5 were dissolved or suspended in 10 ml DCM at 400 rpm for 4 h using a magnetic stirrer. The suspension was injected into 200 ml 1% w/v PVA solution to be emulsified at 400 rpm for 1 h using a magnetic stirrer. The stirring speed was then lowered to 200 rpm for another 3 h to completely evaporate the DCM at room temperature (25°C) in a fume hood. Once the microparticles solidified, the pH of the emulsion was adjusted to pH

3.5 by adding 3.7% w/v HCl solution to eliminate the surface adsorbed or unincorporated Fe5 nanoparticles. The microparticles were collected by sieve of size 20 μm to 150 μm , washed with DI water and dried. The final product was stored at -20°C .

3.5. Drug release and degradation study

In the drug release study, 20 mg of microparticles were precisely weighed and dispersed in 2 ml PBS solution (pH 7.4, Sigma) in 2 ml microtubes. At predetermined time points, 1.8 ml PBS was collected and stabilized in 1% w/v HCl solution at volumetric ratio 1/1 for ICP test. Exact amount of fresh PBS was used to replace the extracted PBS each time. The concentration of cisplatin was based on the concentration of Pt and cisplatin release was calculated in terms of cumulative release (% w/w). To verify the repeatability of the drug release, all tests were carried out in triplicates. To explore the drug release mechanism, the fractional cumulative drug release profiles were fitted according to two semi-empirical models: linear fit and surface diffusion along with polymer degradation model.

Table 3.5 Solids in 10 ml DCM for the fabrication of drug loaded composite microparticles using S/O/W single emulsion technique

Sample name	Solid in DCM (g)				Feed ratio	
	CDDP	Fe5	CDDP-Fe5 ^a	P(0.61) ^b	Fe5/P(0.61)	Fe5/CDDP
blank	0	0	0	1.54	--	--
PT	0.17	0	0	1.54	--	--
PT-Fe5	0	0	0.38	1.54	1/4	--
PT-Fe5/PT	0.17	0	0.17	1.54	1/9	1/1
2PT-Fe5/PT	0.17	0	0.38	1.54	1/4	9/4
2Fe5/PT	0.17	0.38	0	1.54	1/4	9/4

^a cisplatin loaded Fe5 nanoparticles, with drug loading 4.01%, fabricated as described in section 3.3

^b PLGA with molar ratio of lactic acid/glycolic acid 50/50 and inherent viscosity 0.61 dl/g

For the degradation study, the same procedure as the drug release study was used. At fixed time intervals, samples were collected, washed with DI water and dried in a vacuum oven at room temperature (25°C) for characterizations. All samples were stored at -20°C.

3.6. Characterization of microparticles

The microparticles morphology and size were observed using FESEM and light microscope. For structural information, XRD and FTIR were used, as described in section 3.2.

3.6.1. Drug loading test

The drug loading and encapsulation efficiency of composite microparticles was determined using extraction method. Briefly, 10 mg microparticles were dissolved in 0.5 ml DCM and 5 ml 1% w/v HCl was added to extract the entrapped cisplatin. The mixture was vortexed for 5 min and centrifuged. The upper aqueous solution was collected for ICP test. For each composition, triplicates were performed. The cisplatin concentration in aqueous solution was based on Pt concentration from ICP result. The drug loading and the encapsulation efficiency was calculated using Eqs 3.2 and 3.3.

3.6.2. Differential scanning calorimetry (DSC)

Glass transition temperature, T_g is an important characteristic of an amorphous polymer in assessing the mobility of polymer chains. DSC was used to study the glass transition temperature. Since cisplatin decomposes at 270°C and release toxic gases such as chlorine, the maximum temperature for the DSC study was kept at 100°C. The following heating program was used:

- equilibrium at -20°C for 5 min,
- heating from -20°C to 100°C at 10°C/min,
- equilibrium at 100°C for 5 min,
- cooling down from 100°C to -20°C at 10°C/min,
- equilibrium at -20°C for 3 min,
- heating from -20°C to 100°C at 10°C/min.

The T_g was calculated as the onset temperature of glass transition based on the first and second heating cycle.

3.6.3. Gel permeation chromatography (GPC)

Average molecular weight and polydispersity index (PI) of polymer matrix were determined using a Shimadzu LC-20AD instrument equipped with a RID-10A refractive index detector and a PLgel GPC column. The mobile phase was tetrahydrofuran (THF) (VWR, HPLC grade) at a flow rate of 1 ml/min at 40°C. Samples were dissolved in THF at 2 mg/ml, filtered and filled into 2 ml vials. The solution was then injected into the column using auto sampling. The system was calibrated using a series of polystyrene standards with weight averaged molecular weight ranging from 500 kDa to 162 Da.

3.7. Statistical analysis

Differences between groups were analyzed using Student's t test and were considered statistically significant at $p \leq 0.05$.

Chapter 4

Characterization of metal ion doped hydroxyapatite

Five types of hydroxyapatite were synthesized: pure hydroxyapatite (HA), Nd^{3+} (NdHA) and Gd^{3+} (GdHA) substituted hydroxyapatite, Mn^{2+} (MnHA) and Fe^{3+} (FeHA) substituted hydroxyapatite. Pure HA, NdHA and GdHA were white. As for MnHA, light pink colored powders were obtained and for FeHA, all powders were brown, where the color intensity varied proportionally with substitution concentrations. All samples were characterized using the typical techniques for ceramics, including XRD, FTIR, FESEM, EDX, and ICP. Since pure HA was reported as biocompatible, cytotoxicity of all samples was also quantified. Depending on the potential applications of MHA, different characterization techniques were further applied for different groups of MHA. For NdHA and GdHA, the electrical conductivity was tested as electromagnetic field has been reported to accelerate healing in bone fractures [209]. For GdHA and FeHA, the magnetic property was quantified since ferromagnetic or superparamagnetic nanoparticles could be used as drug carriers, contrast agents and heat mediators for hyperthermia [78]. Osteoblast cells were seeded onto the MnHA and FeHA pellets and the cell growth profile together with ALP activity were determined to establish their bioactivity which was subsequently compared with pure HA. In this investigation, MHA nanoparticles were further used as drug carrier for anticancer drug cisplatin. Thus, based on their properties, the best candidate was chosen for further drug loading studies.

4.1. XRD patterns

XRD was carried out to study the phase present in each sample. Ion exchange method was used to synthesize metal ion substituted HA (MHA). In this method, HA nanoparticles had formed before the metal ion solution was added to the reaction. In order to confirm this, a special sample, identified as HA-s, was produced as described in the section 3.1. The preparation was similar to that for pure HA without 2 h aging. As soon as the addition of phosphoric acid

was stopped, the precipitate was immediately filtered and dried at room temperature. XRD test was carried out on the special sample, HA-s. As shown in Fig 4.1, the peaks agreed well with hydroxyapatite, ICSD file no. 26204. Hence it can be concluded that HA nanoparticles were formed before the ion exchange reaction took place. The added metal ions should then be limited to the nanoparticles surface [37] and the lattice structure of HA would not be significantly affected by the addition of metal ions.

According to the XRD profiles (Figs 4.2 and 4.3), all powders were well crystallized, as demonstrated by the sharp and well resolved peaks. The peaks were in good agreement with ICSD file no. 26204, indicating that all samples were single phased hexagonal hydroxyapatite, except for Nd20 and Gd20. For Nd20, the extra peaks agreed well with ICSD file no. 399, $\text{Nd}(\text{OH})_3$ and the weight percentage of $\text{Nd}(\text{OH})_3$ was calculated as 18% (from the Rietveld refinement). As for Gd20, the extra peak at 29.48° was assigned to $\text{Gd}(\text{OH})_3$, following ICSD file no. 200093 and the weight percentage was calculated as 2.5% (from the Rietveld refinement). NdCl_3 used in the preparation of NdHA is highly soluble in water. As the pH of the slurry was kept at 7.8 with ammonia hydroxide during the synthesis process, Nd ions that were not substituted into the HA lattice would be precipitated as neodymium hydroxide [210]. Similarly for gadolinium hydroxide, the excess Gd^{3+} would be precipitated.

Fig 4.1. The XRD pattern for HA-s, the special sample fabricated similarly to pure HA but without 2-h aging at 98.5°C .

Compared with pure HA, all MHA showed comparable peak positions without significant shift, regardless of substitution concentrations, which confirmed that the lattice structure of HA were not markedly affected by the ion exchange process. Table 4.1 shows the lattice parameters for MHA. For NdHA and GdHA group, the parameter a (9.4114 Å) of pure HA was slightly smaller while parameter c (6.8989 Å) was larger than the corresponding a (9.424 Å) and c (6.879 Å) values for stoichiometric hydroxyapatite [51]. Partial replacement of Ca^{2+} ions by other cations with smaller ionic radii will result in smaller lattice parameters a and c . While compared with the pure HA, a and c values for NdHA and GdHA did not change significantly with the metal ion content. This may be due to the small ionic radius difference between Gd^{3+} (0.94 Å), Nd^{3+} (0.98 Å) and Ca^{2+} (1.0 Å). The difference was not significant enough to affect the parameters a and c , thus no apparent decrease in the unit cell size was observed for NdHA and GdHA (Table 4.1).

For the MnHA and FeHA samples, the unit cell volume of MnHA was slightly smaller than pure HA while for FeHA, it was larger than pure HA. Here, both Mn^{2+} (0.67 Å) and Fe^{3+} (0.65 Å) ions have smaller ionic radii than Ca^{2+} ions (1.00 Å) [211]. Lattice parameters for MnHA decreased as Mn content in HA increased (Table 4.1). In contrast, FeHA resulted in larger lattice parameters compared to those of pure HA (Table 4.1). This was probably due to Fe hydroxo ions ($\text{Fe}(\text{OH})^{2+}$) partially involved in replacing Ca^{2+} as suggested by Wakamura *et al.* [67].

Since ion exchange method was used for the synthesis of MHA, the size of nanocrystallites would not be greatly modified when compared with pure HA. As shown in Table 4.1, crystal size for MHA calculated based on Scherrer equation (Eq 3.1) was comparable to that of pure HA.

Fig 4.2. The XRD patterns of (a) NdHA powder, ▼: Nd(OH)₃ and (b) GdHA powder, ■: Gd(OH)₃.

Fig 4.3. The XRD patterns of (a) MnHA powder and (b) FeHA powder.

Table 4.1 Lattice parameters and crystal size for MHA from XRD patterns

Sample name	Lattice parameters			Crystal size (nm)
	$a(\text{\AA})$	$c(\text{\AA})$	volume($\text{\AA}^3$)	
HA*	9.424(4)	6.879(4)	529.09	
Pure HA ^a	9.4114(7)	6.8989(6)	529.20(9)	38
Nd1	9.4105(6)	6.9029(5)	529.41(8)	38
Nd5	9.4131(8)	6.9016(6)	529.60(11)	39
Nd10	9.4190(11)	6.8996(8)	530.11(14)	38
Nd20	9.4093(7)	6.9026(7)	529.25(11)	39
Gd1	9.4131(6)	6.9008(4)	529.53(7)	38
Gd5	9.4199(7)	6.8998(5)	530.22(9)	38
Gd10	9.4115(10)	6.9022(7)	529.46(13)	36
Gd20	9.4188(21)	6.9003(16)	530.14(27)	37
Pure HA ^b	9.4233(7)	6.8981(6)	530.48(9)	41
Mn1	9.4219(7)	6.9005(6)	530.50(9)	38
Mn5	9.4221(9)	6.8989(7)	530.40(11)	38
Mn10	9.4209(9)	6.8982(7)	530.22(11)	39
Fe1	9.4246(8)	6.9005(6)	530.80(10)	41
Fe5	9.4240(9)	6.9011(7)	530.78(11)	39

*: stoichiometric hydroxyapatite [51].

^a: when pH decreased to 7.8, the addition of H₃PO₄ solution was stopped during synthesis process.

^b: when pH decreased to 5.5, the addition of H₃PO₄ solution was stopped during synthesis process.

4.2. FTIR spectra

The FTIR spectra of all samples showed vibrational modes characteristic of PO₄³⁻ and OH⁻ groups in HA (Figs 4.4 and 4.5) [70] [212]. The bands at 570 and 600 cm⁻¹ were assigned to the PO₄³⁻ bending mode (ν_4), while the antisymmetric (ν_3) and symmetric (ν_1) stretching mode of PO₄³⁻ appeared at 1040/1090 and 960 cm⁻¹ respectively. The CO₃²⁻ stretching and bending bands were observed at 875 and 1425 cm⁻¹, respectively. This was due to PO₄³⁻ groups being partially replaced by CO₃²⁻ groups, forming B-type carbonated HA. This would explain the Rietveld refinement results where the lattice parameter a of

pure HA was slightly smaller while c was larger than that of stoichiometric hydroxyapatite [51, 213] (Table 4.1). On the other hand, changes in lattice parameters suggest a possible deviation from stoichiometry and it is well known that precipitation method usually produces non-stoichiometric HA [46]. It is easy to synthesize B-type carbonated HA using precipitation reactions [212] [214, 215] due to the presence of calcium carbonate in the reactant and CO_2 from the atmosphere. The presence of carbonate is not expected to affect cell response since it is naturally present in bones [64]. In addition, a sharp band at 3568 cm^{-1} was observed in all samples, which corresponded to the stretching mode of OH^- . Absorption bands of hydrogen phosphate, such as HPO_4^{2-} and H_2PO_4^- , were not present in all spectra.

For Nd20, the sharp band at 3609 cm^{-1} was due to OH^- stretching of $\text{Nd}(\text{OH})_3$ and the band at 680 cm^{-1} belonged to the libration of OH^- from $\text{Nd}(\text{OH})_3$. In the case of Gd20, a small shoulder at 720 cm^{-1} was observed, which could be due to the OH^- stretching band of $\text{Gd}(\text{OH})_3$.

The intensity of all the characteristic bands did not change significantly with M content, except for the OH^- stretching band at 3568 cm^{-1} of NdHA and GdHA which decreased when $X_M = 0.1$ and 0.2 .

Fig 4.4. The FTIR spectra of NdHA and GdHA.

Fig 4.5. The FTIR spectra of MnHA and FeHA.

4.3. FESEM and EDX analysis

Nanoparticles morphology was observed under FESEM and elements in nanoparticles were studied using EDX. Fig 4.6 shows the representative

FESEM images and EDX spectra of MHA powders. For pure HA, the particles were elongated-spheroidal in shape and with a small size distribution. With the incorporation of metal ions, the morphology and particle size did not change significantly, where the particles were elongated spheroids of around 70 nm. This was because HA nanoparticles had formed before the ion exchange process took place. As demonstrated by the EDX spectra, all elements belonging to the metal ion doped hydroxyapatite phase, including carbon, oxygen, phosphor, calcium and neodymium, gadolinium, manganese or iron, were present and no other element (such as chloride) was detected. In this research, chlorides were used as the source of metal ions, as shown in section 3.1. If ion exchange had not taken place, chloride ions would be detected by EDX due to surface adsorption along with the metal ions.

For Nd20, there were two different shaped particles in the FESEM image (Fig 4.6(c)), elongated spheroids and cylindrical rods. Referring to the sharp peaks and bands in the XRD pattern and FTIR spectrum for Nd20 (Figs 4.2 and 4.4), it can be concluded that the cylindrical rods found in the FESEM image of Nd20 (Fig 4.6(c), as circled) was neodymium hydroxide, which was around 300 nm. The shape of $\text{Nd}(\text{OH})_3$ observed here was similar to that reported by Wang *et al.* in 2006 [210]. The elongated-spheroidal particles of around 70 nm would be NdHA. In the case of Gd20 (Fig 4.6(d)), it was not obvious from the FESEM image that there were particles of different morphologies. However, peaks of $\text{Gd}(\text{OH})_3$ were observed in the XRD (Fig 4.2) and FTIR (Fig 4.4) patterns. Chen *et al.* [216] reported that spherical-like $\text{Gd}(\text{OH})_3$ nanoparticles (size of around 50 nm) were precipitated when $\text{Gd}(\text{NO})_3$ reacted with NH_4OH in solution during stirring. In this research, it was difficult to differentiate the $\text{Gd}(\text{OH})_3$ nanoparticles from GdHA since the shape of both nanoparticles were rather similar to one other. Furthermore, the weight percentage of $\text{Gd}(\text{OH})_3$ was very low, 2.5%, as calculated using Rietveld method. Hence, $\text{Gd}(\text{OH})_3$ was not easily to be differentiated from GdHA in the FESEM image of Gd20.

Fig 4.6. The representative FESEM images and the corresponding EDX spectra (insets) for (a) pure HA (b) Nd10 (c) Nd20 (d) Gd20 (e) Mn10 and (f) Fe5. The circles represents Nd(OH)_3 cylindrical rods.

4.4. ICP analysis

Table 4.2 shows the atomic ratios for all samples calculated from ICP result. For pure HA (reaction stopped at pH 7.8 and pH 5.5), the Ca/P ratio was higher than the stoichiometric HA, 1.667 [96]. This was due to partial replacement of PO_4^{3-} ions with CO_3^{2-} ions in the HA lattice. The Ca/P ratio in MHA is an indicator of how much Ca was replaced by M. This ratio decreased with the increase of X_M except for Nd20, where the Ca/P ratio of Nd20 was higher than Nd10. For Nd20, the presence of second phase Nd(OH)_3 would explain this

smaller Ca/P ratio. This is possibly because ion exchange and precipitation as hydroxide are two competitive reactions. For Nd20, Nd^{3+} ions were concurrently precipitated as $\text{Nd}(\text{OH})_3$ and not involved in the ion exchange reaction with Ca^{2+} ions in HA lattice, which would have led to a smaller Ca/P ratio than Nd10.

The M/Ca ratio increased with X_M proportionally, further supporting that more Ca ions in the lattice structure were replaced by the metal ions. M/Ca was also larger than X_M since X_M was the ratio of initial reaction chemicals. After MHA formed, Ca content decreased due to the ion exchange with M. However, for Mn5 and Mn10, M/Ca was smaller than X_M . That was probably because some Mn^{2+} ions added as reactant were not involved in the ion exchange process and remained in the supernatant which was eventually discarded. When compared with Ca/P^* , the theoretical value assuming that Ca had exchanged with all M added at the corresponding ratio, the detected Ca/P ratio was larger for Nd20, Gd10 and Gd20, MnHA and FeHA. This is reasonable since the ion exchange efficiency was lower than 100%, especially at higher feed concentration of metal ions. For Nd20, the larger Ca/P ratio compared to the theoretical Ca/P^* ratio was also due to the presence of $\text{Nd}(\text{OH})_3$ as a second phase. For NdHA and GdHA, the theoretical Ca/P^* ratio was calculated based on the starting value of 1.743, which was the Ca/P ratio for pure HA at pH 7.8 obtained from the ICP result. For NdHA and GdHA, ion exchange reaction might accompany the production of HCl, which would dissolve the surfaced adsorbed $\text{Ca}(\text{OH})_2$ on HA nanoparticles. Hence, it was reasonable to find the detected Ca/P ratio was lower than the theoretical values in the case of Nd5, Nd10 and Gd5. If the starting value was changed to 1.667 (Ca/P ratio for pure stoichiometric HA), the theoretical Ca/P ratio would be 1.584, 1.500 for $X_M = 0.05, 0.1$ respectively, which were lower than the detected values for M5 and M10 as shown in Table 4.2.

Compared with Mn^{2+} ions, Gd^{3+} and Fe^{3+} ions were more active in replacing Ca^{2+} in HA lattice. As shown in Table 4.2, Mn/Ca atomic ratio was smaller than Gd/Ca and Fe/Ca atomic ratio for X_M of 0.05 and above. The Ca/P atomic ratio for Mn5 was larger than Gd5 and Fe5 while the value for Mn10 was also larger

than Gd10 when the same molar quantity of metal ions was added during the fabrication process. This may be due to the different electron density (atomic valence per volume of ions) between Mn^{2+} and Gd^{3+} , Fe^{3+} ions.

Table 4.2 The atomic ratios for MHA from ICP results

Product name	Atomic ratios				
	X_M	M/Ca	Ca/P	Ca/P*	(M+Ca)/P
HA ^a	--	--	1.743	1.667	--
Nd1	0.010	--	1.735	1.726	--
Nd5	0.050	--	1.612	1.656	--
Nd10	0.100	--	1.518	1.569	--
Nd20	0.200	--	1.551	1.394	--
Gd1	0.010	0.010	1.667	1.726	1.683
Gd5	0.050	0.053	1.582	1.656	1.665
Gd10	0.100	0.112	1.574	1.569	1.751
Gd20	0.200	0.218	1.464	1.394	1.783
HA ^b			1.681	--	
Mn1	0.010	0.010	1.660	1.664	1.675
Mn5	0.050	0.041	1.620	1.597	1.681
Mn10	0.100	0.053	1.599	1.513	1.683
Fe1	0.010	0.010	1.661	1.656	1.678
Fe5	0.050	0.055	1.589	1.555	1.677

^a: when pH decreased to 7.8, the addition of H_3PO_4 solution was stopped during synthesis process.

^b: when pH decreased to 5.5, the addition of H_3PO_4 solution was stopped during synthesis process.

: theoretical atomic ratio of Ca to P assuming Ca was replaced by all the M ions added at the corresponding ratio. For NdHA, GdHA and MnHA, the assumed exchange ratio of M/Ca was 1/1. For FeHA, the assumed exchange ratio of Fe/Ca was 3/2 to maintain the charge balance. The initial value of Ca/P was derived from pure HA ICP result of 1.743 for NdHA and GdHA and 1.681 for MnHA and FeHA.

The missing M/Ca ratios for NdHA were due to the unavailability of Nd standard solution for ICP quantification.

As for (M+Ca)/P ratio, the value was much higher than 1.667 for Gd10 and Gd20 due to surface adsorption of amorphous $\text{Gd}(\text{OH})_3$ and/or $\text{Gd}(\text{OH})_3$ as the

second phase. For MnHA, the value was comparable with the Ca/P ratio for pure HA (pH 5.5). It suggested that Mn^{2+} ions successfully replaced Ca^{2+} in HA lattice, thus resulting in decreased lattice parameters for MnHA as Mn content in HA increased (Table 4.1). The decreased lattice parameters for MnHA were because of the smaller ionic radius of Mn^{2+} (0.67 Å) than Ca^{2+} (1.00 Å). In contrast, FeHA resulted in larger lattice parameters compared to those of pure HA (Table 4.1). This was probably due to Fe hydroxo ions ($\text{Fe}(\text{OH})^{2+}$) partially involved in replacing Ca^{2+} as suggested by Wakamura *et al.* [67]. In our calculation, the exchange ratio of Fe/Ca was assumed 1.5 to maintain the charge balance. However, the measured Ca/P ratios were higher than the theoretical values (Table 4.2), which suggested that the exchange ratio of Fe/Ca was less than 1.5. If the exchange ratio of Fe/Ca was 1, Ca/P ratios from ICP result were less than the theoretical values which should be 1.664 and 1.597 for Fe1 and Fe5 respectively. This suggested that the exchange ratio of Fe/Ca was higher than 1. Thus it can be concluded the exchange ratio of Fe/Ca here was larger than 1 but smaller than 1.5. This observation was consistent with results from Wakamura *et al.* who estimated the exchange ratio of Fe/Ca as 1.3. However, limited by the sample number, the exact exchange ratio was not calculated here.

4.5. Electrical conductivity of NdHA and GdHA

The electrical property of HA is important since electromagnetic fields have been shown to accelerate healing in bone fractures [203, 204]. The electrical conductivity, σ , and resistivity, ρ , of the samples at voltages 30 V and 50 V are shown in Table 4.3. With increase of M ions, the electrical conductivity, σ increased significantly. For GdHA, the maximum σ at 30 V was $3.64 \times 10^{-7} \Omega^{-1} \cdot \text{m}^{-1}$ for Gd20, which was 13481% higher than that of pure HA. As for NdHA, the maximum σ of $2.03 \times 10^{-7} \Omega^{-1} \cdot \text{m}^{-1}$ was obtained for Nd10. The smaller σ in Nd20 compared to Nd10 might be due to the presence of the second phase in the pellet. The resistivity of all pellets was smaller when a higher voltage (50 V) was applied for each composition.

Table 4.3 Electrical conductivity σ and resistivity ρ of NdHA and GdHA pellets (sintered at 800°C for 1 h) measured at room temperature

Product name	σ $10^{-9}(\Omega^{-1}\cdot\text{m}^{-1})$ (30 V)	ρ $10^6(\Omega\cdot\text{m})$ (30 V)*	σ $10^{-9}(\Omega^{-1}\cdot\text{m}^{-1})$ (50 V)	ρ $10^6(\Omega\cdot\text{m})$ (50 V)*
HA	2.7	372.8	3.4	298.7
Nd1	7.1	140.9	10.7	93.9
Nd5	12.2	81.9	19.1	52.3
Nd10	203.5	4.9	293.4	3.4
Nd20	164.2	6.1	261.5	3.8
Gd1	9.1	109.6	14.0	71.3
Gd5	49.6	20.2	96.6	10.4
Gd10	158.9	6.3	422.5	2.4
Gd20	364.0	2.8	634.6	1.6

(30 V)*: The value was measured at voltage 30 V.

(50 V)*: The value was measured at voltage 50 V.

The electrical conductivity, σ , of NdHA and GdHA was significantly higher than that of pure HA. In hydroxyapatite lattice, the compact assemblage of PO_4^{3-} and Ca^{2+} defines two types of zeolite-like channels [37] (Fig 4.7). The first type of channel, with diameter 2.5 Å, is surrounded by six oxygen atoms and contains Ca(I) ions. The second channel, with diameter 3.5 Å, consists of Ca(II) ions, which hosts hydroxyl groups and plays an important role in the electrical properties. It has been reported that the charge carrier of HA is proton [217] and H^+ migrates between the electroattractive ion O^{2-} to give molecular H_2O in the second channels. The high polarizability and the expansion of tunnels with the introduction of big cations favor the ionic conduction [217]. Here, both ions, Gd^{3+} and Nd^{3+} , have smaller ionic radius (r) and higher polarizability (α) than Ca^{2+} ($r(\text{Gd}^{3+}) = 0.94$ Å, $r(\text{Nd}^{3+}) = 0.98$ Å, $r(\text{Ca}^{2+}) = 1.0$ Å; $\alpha(\text{Gd}^{3+}) = 1.01$ Å³, $\alpha(\text{Nd}^{3+}) = 1.23$ Å³ [218], $\alpha(\text{Ca}^{2+}) = 0.6$ Å³ [217]), and OH^- was partially replaced by O^{2-} in the structure of NdHA and GdHA. As rare earth metal ions preferentially occupy Ca(II) sites [219], the higher polarizability may compensate for the smaller channels (due to smaller ionic radii of M(III) ions). The O^{2-} ion can be better stabilized compared to the pure

HA due to higher electrical density produced by M(III). For oxyapatite, O^{2-} ions were proposed to diffuse along the c axis. If O^{2-} was the charge carrier, the ionic transport number would be higher than 1 for the system. Hence these two factors may together influence the electrical property of NdHA and GdHA.

Fig 4.7. Projection of hydroxyapatite onto the (001) plane. Two channels are shown.

The structure of samples was investigated after the pellets were sintered at 800°C for 1 h (Fig 4.8 and 4.9). All samples were still of hexagonal hydroxyapatite phase and B-type carbonated hydroxyapatite. For pure HA, a small peak at 37.28° (as indicated by ■ in Fig 4.8) was found, which belongs to CaO. This was due to the dehydration of surface adsorbed $Ca(OH)_2$, which could have also contributed to the higher Ca/P atomic ratio for pure HA than the theoretical value of 1.667 (Table 4.2). For NdHA, only Nd20 has the second phase of Nd_2O_3 , while the others were still single phased. For GdHA (Gd5, Gd10 and Gd20), a small peak at 24.94°, which was assigned to Gd_2O_3 , appeared after the sintering process. In the XRD pattern and FTIR spectrum for Gd20 before sintering, the extra peak at 29.48° and shoulder band at 720 cm^{-1} , which belonged to $Gd(OH)_3$ (Fig 4.2 (b) and Fig 4.4 (b)) were not observed in the corresponding XRD and FTIR patterns for Gd20 after sintering (Fig 4.8 (b))

and 4.9 (b)). This was because $\text{Gd}(\text{OH})_3$ had dehydrated to Gd_2O_3 during the thermal process. Furthermore, the sintering process greatly improved the crystallinity of the product. The OH^- librational band at 631 cm^{-1} [55] was clearly shown in FTIR spectra for products after sintering (Fig 4.9). This band was absent in the corresponding FTIR spectra before sintering (Fig 4.4). The intensity of OH^- librational band at 631 cm^{-1} and OH^- stretching band at 3568 cm^{-1} both decreased with increase of metal ion content.

Fig 4.8. The XRD patterns of (a) NdHA and (b) GdHA after sintered at 800°C for 1 h.

Fig 4.9. The FTIR spectra of (a) NdHA and (b) GdHA after sintered at 800°C for 1 h.

The decrease of OH⁻ stretching and libration bands intensities with Gd and Nd content (especially evident after sintering) (Figs 4.4 and 4.9) suggested that the following ion exchange mechanism might have taken place [220]:

The mechanism demonstrated that the ion exchange ratio of Ca²⁺ to M³⁺ was 1/1 and with Ca²⁺ exchanging with M³⁺, OH⁻ was simultaneously replaced by

O²⁻ at the same quantity in order to keep the charge balanced. From the mechanism, the atomic ratio (M+Ca)/P should be comparable with the theoretical value, 1.667. However, only Gd1 and Gd5 had ratios in the range (Table 4.2). In the case of Gd10 and Gd20, the (M+Ca)/P ratios were much higher than 1.667 (Table 4.2). This could be due to Gd(OH)₃, either adsorbed on the Gd10 nanoparticles surface or as second phase (for Gd20). After sintering, both Gd10 and Gd20 showed the second phase of Gd₂O₃ in the XRD patterns, a product of dehydration of Gd(OH)₃ (Fig 4.8). From Eq 4.1, it was observed that HCl is a byproduct of the ion exchange process. The presence of HCl could dissolve the surface adsorbed Ca(OH)₂ and resulted in the lower Ca/P ratio compared to the theoretical values in the case of Nd5, Nd10 and Gd5 as shown in Table 4.2.

4.6. Magnetic property of GdHA and FeHA

The magnetic property of GdHA and FeHA was characterized using VSM. Gadolinium is a paramagnetic element and gadolinium chelates can be used as MRI contrast agents to increase the brightness in various parts of the body where the agents reside. It has been reported that the ligand structure had no influence on the magnetic property of Gd chelates and the covalent attachment of Gd-DTPA to a cross-linked starch particle had no impact on the intrinsic magnetic properties [221]. The magnetization of GdHA powders versus external magnetic field is shown in Fig 4.10. The magnetization increased linearly with the external field, indicating that GdHA was paramagnetic. The slope, which represents the magnetic susceptibility (χ_g), increased with Gd concentration. (Magnetic susceptibility is the degree of magnetization of a material in response to an applied magnetic field). Here, for Gd5, Gd10 and Gd20, the χ_g was 9×10^{-6} , 2×10^{-5} , 3×10^{-5} emu·g⁻¹·Oe⁻¹, respectively. The saturation magnetization (Ms) at 10 kOe was 9×10^{-2} , 2×10^{-1} , 3×10^{-1} emu·g⁻¹ respectively.

Fig 4.10. The magnetization curves of GdHA and FeHA powder measured at room temperature.

FeHA was characterized as diamagnetic or paramagnetic depending on Fe content. Fe5 was paramagnetic and the magnetic susceptibility (χ_g) was $9.25 \times 10^{-7} \text{ emu} \cdot \text{g}^{-1} \cdot \text{Oe}^{-1}$ (Ms at 10 kOe was $9.25 \times 10^{-3} \text{ emu} \cdot \text{g}^{-1}$), which was smaller than Gd5 (χ_g was $9 \times 10^{-6} \text{ emu} \cdot \text{g}^{-1} \cdot \text{Oe}^{-1}$). Wu *et al.* [78] reported that Fe^{2+}

substituted HA nanoparticles were superparamagnetic and the highest M_s observed was $20.92 \text{ emu} \cdot \text{g}^{-1}$. The significant difference was probably due to the fabrication techniques and valance of Fe ions used. Here, Fe^{3+} was chosen and the ion exchange method was applied, while Wu *et al.* introduced Fe^{2+} ions into HA nanoparticles by coprecipitation method [78].

Since nanoparticles which are ferromagnetic or superparamagnetic are usually preferred for biomedical applications such as drug delivery, contrast agents for MRI and heat mediators for hyperthermia [78], our results suggested that GdHA and FeHA nanoparticles produced here are not suitable for such applications.

Fig 4.11. Representative light microscope images of cells directly under the sample pellets after one day incubation. (a) Nd1 (b) Nd5 (c) Nd10 (d) Nd20 pellets. The magnification bar is 200 μm .

4.7. Cytotoxicity of NdHA and GdHA

All NdHA and GdHA were tested as non-cytotoxic, except Gd20. The agar diffusion results are listed in Table 4.4. Representative images of the cells under the specimen after one day incubation followed by addition of neutral red

solution are shown in Figs 4.11 and 4.12. The live cells absorbed neutral red while the dead ones did not.

Table 4.4 Cytotoxicity of NdHA and GdHA using agar diffusion method

Product name	Zone index*	Lysis index*	Cytotoxicity
HA	0	0	Non-cytotoxic
Nd1	0	0	Non-cytotoxic
Nd5	0	0	Non-cytotoxic
Nd10	0	0	Non-cytotoxic
Nd20	0	0	Non-cytotoxic
Gd1	0	0	Non-cytotoxic
Gd5	0	0	Non-cytotoxic
Gd10	0	0	Non-cytotoxic
Gd20	2	5	Cytotoxic

Zone index*: Measurement of the clear zone in which cells do not stain with neutral red. Here, “0” corresponds to “No detectable zone around or under specimen” and “2” represents “Zone extends less than 0.5 cm beyond specimen”.

Lysis index*: Measurement of the number of cells affected within the zone of toxicity. Here, “0” corresponds to “No observable cytotoxicity” and “5” represents “Greater than 80% of zone affected”.

As shown in Table 4.4, all NdHA and GdHA were tested as non-cytotoxic, except for Gd20. Free Gd^{3+} ions are extremely toxic, but after coordination to organic ligand, the Gd complexes become very stable and thus exhibit much less toxicity [222]. Several gadolinium-based contrast agents have been approved by FDA, e.g. gadopentetate dimeglumine (Gd-DTPA, Magnevist[®]) and gadoterate meglumine (Gd-DOTA, Dotarem[®]) [223]. GdHA (Gd1, Gd5 and Gd10) was found as non-cytotoxic since Gd ions were bonded in the HA lattice. The toxicity of Gd20 was probably due to the free Gd^{3+} ions not substituted into the HA lattice and were instead adsorbed onto nanoparticle surface or present as $Gd(OH)_3$. As for NdHA, it has been reported that Nd inorganic salts showed a very weak cytotoxic effect on cells [224]. Here, all NdHA powders were characterized as non-cytotoxic, including Nd20 which had two phases, Nd^{3+} substituted HA and $Nd(OH)_3$.

Fig 4.12. Representative light microscope images of cells directly under the pellets of (a) pure HA (b) Gd1 (c) Gd5 (d) Gd10 (e) Gd20 and (f) 1 cm beyond Gd20 pellets after one day incubation. The magnification bar is 200 μm .

4.8. Cytotoxicity of MnHA and FeHA

For MnHA and FeHA, extraction assay was applied to quantify the cytotoxicity. In order to examine the cell viability and activity upon culturing in extracted medium, MTT and ALP tests were conducted after 72 h incubation (Fig 4.13). The colorimetric value from MTT test presented the quantity of vital osteoblast cells. Compared with pure HA, all of the MnHA and FeHA samples had more living cells (Fig 4.13(a)) with Mn5, Mn10 and Fe5 having significantly more ($p < 0.05$). As shown in Fig 4.13(b), the ALP activity for

samples Mn5, Mn10 and Fe5 was significantly higher ($p < 0.05$) than pure HA and the activity increased proportionally to the metal ion concentration ($p < 0.05$).

The concentrations of Fe in the extracted medium were quantified using colorimetric assay. It was 0.182 ± 0.071 ppm, 0.197 ± 0.000 ppm, 0.291 ± 0.047 ppm, 0.478 ± 0.047 ppm for DMEM/F12, HA, Fe1 and Fe5 extracted medium respectively. The concentration of Fe in the extracted medium increased with Fe content in HA, where the concentration of Fe in Fe5 extracted medium was significantly higher than in Fe1 and pure HA extracted medium.

Fig 4.13. (a) Cell number based on MTT test and (b) ALP activity for osteoblast cells cultured in extracted medium for 72 h. Data = mean + standard deviation; $n = 4$; * $p < 0.05$ as compared to HA; ** $p < 0.05$ as compared to the same metal ion doped HA at higher metal ion concentration, M5 and/or M10.

The presence of Mn and Fe in HA demonstrated significant positive effect on osteoblast cells as compared to pure HA. The extraction assay was to quantify the toxicity of water soluble species from samples [57]. For single phased MnHA or FeHA, the possible water soluble components included Mn^{2+} or Fe^{3+} ions in addition to the dissolution products of pure HA such as Ca^{2+} , PO_4^{3-} and OH^- ions. Compared with pure HA, the presence of Mn^{2+} and Fe^{3+} in extracted medium for Mn5, Mn10 and Fe5 promoted cell growth and expression of ALP (Fig 4.13, $p < 0.05$). As for Mn1 and Fe1, both cell number and ALP activity were comparable with pure HA. This could be due to the smaller concentrations of metal ions in the metal ion substituted HA, which gave rise to lower concentrations of metal ions in the extracted medium.

Contradictory results have been reported about the effects of Fe ions on bone properties [107, 109, 225]. From Parelman's [109] *in vivo* results, female rats fed by marginal iron restriction diet had lower whole-body bone mineral content while those fed by marginal calcium restriction diet had lower bone mineral density. Only female rats fed by iron and calcium adequate diet had healthy bones. Additionally, the *in vitro* tests showed that cells cultured in medium which the iron was chelated by deferoxamine (DFO) deposited less mineral as compared to cells in control group [109]. However, the inhibitory effect of Fe ions on bone formation was observed when Fe ions were added to the culturing medium [107, 225]. This indicated a complex relationship between calcium, iron, and bone. In our experiment, the extracted medium contained not only Fe but also Ca ions due to the dissolution of FeHA. Similar to the female rats fed by calcium and iron adequate diet, the combined presence of both iron and calcium resulted in enhanced osteoblast cells growth.

4.9. Cell growth on MnHA and FeHA pellets

Since adhesion of osteoblast to matrix is an important step for osteointegration which prevents motion-induced damage [80], osteoblast attachment to sample pellets was assessed. In the preliminary cell seeding result shown in Fig 4.14 (since different cell densities were used for 3-day and 7-day incubation in two

batches of tests, only relative Abs based on pure HA was shown), no significant difference was found between cell numbers on Mn5 and Mn10 pellets after incubation for 7 days. Mn5 had 27% more cells than Mn1 on day 7. For FeHA, cell proliferation showed Fe concentration dependence as suggested by our results where the cell number on Fe1 pellets was comparable with that on pure HA pellets but there were twice that number of cells found on Fe5 pellets (Fig 4.14). To verify the supposition, samples with higher Fe concentration (Fe10 and Fe15) were produced and cell proliferation was tested as shown in Fig 4.15. Since two batches of tests were performed, only relative Abs was shown. On day 3, the number of cells on these FeHA samples were significantly higher than on pure HA ($p < 0.05$) and Fe5 gave the highest cell proliferation. Thus, Mn5 and Fe5 were chosen for further osteoblast growth test.

Fig 4.14. The relative absorbance of MTT test for osteoblast cells growing on all sample pellets for 3 and 7 days (The tests were carried out independently on 3-d and 7-d incubation, so the relative absorbance was calculated based on the absorbance for HA at the same incubation time). Data = mean + standard deviation; $n = 4$; * $p < 0.05$ as compared to HA at the same incubation time.

The cell growth profile for HA, Mn5 and Fe5 was obtained, as shown in Fig 4.16(a). Only Mn5 demonstrated marked cell proliferation from day 3 to day 7 ($p < 0.1$) while the cell number on both HA and Fe5 pellets did not increase significantly ($p > 0.05$) from day 3 to day 7. Compared with HA, Fe5 pellets had significantly more cells (around 180% higher) both on day 3 and day 7 ($p < 0.05$). Although cell number did not increase significantly from day 3 to day 7

for HA and Fe5 pellets, the ALP activity of cells increased significantly ($p < 0.5$) from day 3 to day 7, as shown in Fig 4.16(b). On the other hand, the increase in ALP activity on Mn5 was not significant ($p > 0.5$) from day 3 to day 7.

Fig 4.15. The relative absorbance of MTT test for osteoblast cells growing on FeHA pellets for 3 days. Data = mean + standard deviation; $n = 4$.

Ohgaki *et al.* reported that negatively charged surface of electrically polarized HA promoted osteoblast-like cells growth *in vitro* [98]. To assess the surface charge, the zeta potential of HA, Mn5 and Fe5 in DI water at 37°C were measured. The zeta potential were determined to be -0.91 ± 0.26 mV, -4.49 ± 0.30 mV and -11.65 ± 3.17 mV, respectively. Fe5 had the largest negatively charged surface among the samples investigated, which could explain the highest cell number compared with the other samples.

Here, ion exchange mechanism was used to fabricate the metal ion doped HA. This technique did not affect the size and morphology of MHA nanoparticles [67]. The size and morphology of MnHA and FeHA nanoparticles did not change significantly compared to pure HA, as confirmed by FESEM images (Fig 4.6). Furthermore, the pellets were made using cold press without sintering for all the cell studies. Thus, the morphology of all pellets' surfaces was similar to each other. Mn^{2+} ions had been reported to increase binding affinity of integrin and activate cell adhesion [64]. However, only ~ 17% more cells grew

on Mn5 pellets while ~140% more cells were observed on Fe5 pellets as compared to pure HA (Figs 4.14 and 4.16(a)).

Fig 4.16. (a) Cell number based on MTT test and (b) ALP activity for osteoblast cells growing on HA, Mn5 and Fe5 pellets for 3 and 7 days. Data = mean + standard deviation; n = 4; * $p < 0.05$ as compared to HA at the same incubation time; ** $p < 0.05$ as compared to 3-day incubation of the same sample; ^^ $p < 0.1$ as compared to 3-day incubation of the same sample.

Fig 4.17. Schematic drawing of the interaction between the polarized charge and environmental materials contributing to cell adhesion. Inorganic ions, organic ions, amino acids, and proteins float around the HA ceramics, and these materials are adsorbed to the unpolarized-surface. Cations and positively charged ionic groups are actively adsorbed on the negatively charged surface (N-surface), and Ca^{2+} ion contribute to the formation of the bone-like layer growing on the HA N-surface. Anions and negatively charged species are actively adsorbed on the positively charged surface (P-surface) of HA, which fail to nucleate crystal growth [98].

Cell number increased at a low rate on all samples pellets from day 3 to day 7 (Fig 4.16(a)). This could be due to the poor mechanical property of cold pressed pellets without sintering. In other work, cell seeding was usually carried out on sintered HA pellets [80, 96, 100-102]. However, metal ion doped HA pellets were reported to show different grain size after sintering compared to pure HA [80, 226] and grain size affected osteoblast proliferation [101]. As reported by

Webster *et al.* [101], osteoblast proliferation was significantly greater on nanophased HA (grain size 67 nm) than conventional HA (grain size 179 nm) after 3 and 5 days. Compared to conventional HA, synthesis of alkaline phosphatase and deposition of calcium-containing mineral was significantly greater by osteoblast cells growing on nanophased HA after 21 and 28 days [101]. Besides the change in grain size, sintering would oxidize Mn^{2+} to Mn^{5+} at 600°C [62], which was no longer the ion of interest in this investigation. Hence, cold pressed pellets were used to keep the surface morphology of pellets comparable to each other and the valence of doped metal ion unchanged even though the pellets formed were brittle in nature. The differentiation of osteoblast cells in culture was reported to go through an early proliferative stage and later postconfluent stages. During the early proliferative stage, osteoblast cells are relatively undifferentiated. When reaching postconfluent stages, osteoblast cells express bone cell phenotypic markers, such as ALP and ultimately deposit calcium containing mineral [105]. ALP activity of postconfluent cells was dramatically higher than subconfluent cells in differentiation media [105]. Here, the cells on pellets had not reached confluence during the culturing time (Fig 4.16(a)) and no differentiation media was used. Hence there was no significant difference in the ALP activity between cells on different pellets (Fig 4.16(b)). However, the higher cell number on Fe5 pellets indicated that the cells on Fe5 may reach confluence and differentiate earlier than the other samples.

Around 170% more cells grew on Fe5 than pure HA and Mn5 on day 3 and day 7 (Fig 4.16(a)). A bonelike apatite layer is formed on HA after soaking the HA samples in simulated body fluid (SBF). The process is initiated by the formation of Ca-rich amorphous or nano-crystalline calcium phosphate due to the negatively charged HA surface after exposure to SBF together with the higher bonding affinity of Ca^{2+} ions than other metal ions [89]. Yamashita *et al.* [93] showed that accelerated apatite growth was observed on negatively charged surface while no crystal grew on positively charged surface of HA, which suggested that negatively charged surface attracted the positively charged Ca^{2+} ions. Furthermore, Ohgaki and Kobayashi *et al.* reported that negatively charged surface of electrically polarized HA promoted colony formation of

osteoblast-like cells *in vitro* [98] and the negatively charged HA conducted formation of bone layer much faster than conventional HA *in vivo* [227]. The schematic drawing of the interaction between the polarized charge and environmental materials contributing to cell adhesion is shown in Fig 4.17. In our investigation, the zeta potential for Fe5 in DI water at 37°C (-11.65 ± 3.17 mV) was more negative than Mn5 (-4.49 ± 0.30 mV) and pure HA (-0.91 ± 0.26 mV). The more negatively charged surface of Fe5 pellets would adsorb more Ca^{2+} ions and eventually more osteoblast cells. The osteoblast cell adhesion initiated by Ca^{2+} adsorption, would further attract cell adhesion proteins, like vitronectin which promoted cell adhesion. Vitronectin is negatively charged in cell culture medium since its isoelectric point is 4.75-5.25 [228]. Based on the results, Fe5 is a potential candidate for implants or coating materials.

4.10. Cell morphology analysis

Filopodia are important characteristic of osteoblast cells and their presence provide further confirmation of osteoblast attachment to sample pellets. Fig 4.18 shows the FESEM images of osteoblast cells growing on HA, Mn5 and Fe5 pellets for 3 days. On all surfaces, osteoblast cells spread well and grew actively. Compared with pure HA, the cells on Mn5 pellets were more flattened and the cellular membrane was closer to the pellet surface, as if they were almost fused together. The osteoblast cells on Fe5 pellets showed more mini-filopodia (indicated by arrow on Fig 4.18(c)) than the cells on both HA and Mn5 pellets. This observation further supported the fact that cells preferred Fe5 pellets over pure HA pellets since the filopodia is regarded as one of the most important cellular sensors [229, 230] and with which epithelium cells would migrate [231]

Fig 4.18. Representative FESEM images of cells growing on sample pellets for 3 days. (a) pure HA, (b) Mn5 and (c) Fe5. Scale bar is 10 μm .

4.11. Summary

Hydroxyapatite (HA) substituted with Nd^{3+} , Gd^{3+} , Mn^{2+} and Fe^{3+} was successfully synthesized by wet chemical method coupled with ion exchange mechanism. All samples were single phased (excluding Nd20 and Gd20) without significant structure deviation and were B-type carbonated HA as carbonate groups partially replaced phosphate groups in the lattice structure. Since ion exchange mechanism was applied, the morphology and crystal size of MHA nanoparticles were not significantly affected as compared to pure HA. All MHA nanoparticles were elongated spheroids with size of around 70 nm. For Nd20, the second phase of $\text{Nd}(\text{OH})_3$ was observed as cylindrical rods of ~ 300 nm, which corresponded to the extra sharp peaks in the XRD pattern and absorption bands in the FTIR spectrum that were not attributed to the NdHA phase. As for Gd20, peaks of $\text{Gd}(\text{OH})_3$ were found in XRD pattern but no particles of the different morphologies were observed in the FESEM image. With the increase of X_M , the quantity of metal ion in HA lattice increased

correspondingly. As suggested by ICP data, Gd^{3+} and Fe^{3+} ions were more active than Mn^{2+} ions at exchanging with Ca^{2+} ions in the HA lattice.

For NdHA and GdHA, following the decrease of OH^- concentration with the increase of M content, the proposed ion exchange mechanism is $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + \text{MCl}_3 \rightarrow \text{Ca}_9\text{M}(\text{PO}_4)_6(\text{OH})(\text{O}) + \text{CaCl}_2 + \text{HCl}$. The electrical conductivity increased significantly with M content. This could be due to the higher polarizability of Nd or Gd ions that preferentially occupied Ca(II) sites and the higher ionic transport number, where both H^+ and O^{2-} might be charge carriers for the system.

GdHA and FeHA (high Fe content) were characterized as paramagnetic and the magnetic susceptibility increased with the increase of Gd concentration. However, limited by the low M_s values, these nanoparticles are not suitable for biomedical applications such as MRI contrast agent.

Based on the agar diffusion and extraction assays, it was found that all MHA samples were non-toxic on osteoblast, except for Gd20. The toxicity of Gd20 was probably due to the free Gd^{3+} ions adsorbed on the particle surface. Cell seeding test was further carried out for MnHA and FeHA as both have potential to be more bioactive than pure HA. Compared to pure HA, limited osteoblast proliferation enhancement was observed on MnHA pellets, while the Fe^{3+} ions in HA demonstrated significant positive effect on osteoblast cells proliferation. Although differentiation of osteoblast was not accelerated during the study time range, Fe5 attracted much more cells than pure HA and MnHA samples. Since osteoblast underwent differentiation upon confluence, the higher cell number on Fe5 showed its potential to promote osteoblast differentiation earlier than pure HA. Among all MHA samples investigated, Fe5 is the best candidate as implants or coating materials for bone implants.

In this investigation, MHA nanoparticles were used as drug carriers. These nanoparticles needed to be non-cytotoxic and with high drug affinity. Due to cytotoxicity concern, GdHA was excluded from the investigation while Nd10, Mn10 and Fe5 were chosen for further studies since it was believed that these

single-phased samples with high metal ion content would demonstrate significant different drug loading behavior from pure HA.

Chapter 5

Cisplatin loaded MHA nanoparticles

Cisplatin is a potent anticancer drug and frequently used for osteosarcoma [3]. Its therapeutic activity appears to be proportional to the intensity of single and cumulative dose, however the exploitation of its full therapeutic potential has been limited by dose-dependent nephrological and neurological toxicities [5]. To lower the acute toxic effect, cisplatin was proposed to be loaded onto metal ion doped HA nanoparticles.

As-received cisplatin is a crystalline yellow powder. It is water-soluble and positively charged in aqueous phase due to the exchange of Cl^- ions with water molecules (Eqs 5.1 and 5.2). In this investigation, the drug cisplatin was first adsorbed onto the surface of MHA nanoparticles through electrostatic attraction and the drug loading was quantified using ICP based on Pt concentration. The cisplatin loaded MHA nanoparticles were then characterized using FESEM, XRD and FTIR. These nanoparticles were further encapsulated into PLGA microparticles using solid-in-oil-in-water (S/O/W) single emulsion method. The drug release profiles and polymer degradation were monitored and the results are presented in Chapters 7 and 8.

5.1. As-received cisplatin

Cisplatin is well crystallized. As shown in Fig 5.1, the powder had a polycrystalline structure. The particle size ranged from 10 μm to 100 μm . Cisplatin is slightly soluble in water and the solubility is 2.53 mg/ml at room temperature (25°C) [232]. Here, the concentration used for drug loading was 0.4 mg/ml and the solution was colorless.

Fig 5.1. FESEM images of as-received cisplatin at magnification (a) $\times 100$ and (b) $\times 500$.

5.2. Zeta potential of MHA nanoparticles

Since the interaction between cisplatin and MHA nanoparticles is by electrostatic attraction, zeta potential of selected MHA samples which were characterized as non-cytotoxic was temporarily measured at 10 mg/ml in PBS using Malvern Zetasizer Nano-ZS. The value for pure HA, Nd10, Mn10 and Fe5 in 10 mM PBS was -23.3 ± 1.35 mV, -21.8 ± 0.96 mV, -28.3 ± 0.91 mV and -28.3 ± 1.1 mV, respectively. When these nanoparticles were suspended in DI water, the zeta potential was 0.39 ± 0.38 mV, 13.8 ± 0.93 mV, -6.03 ± 0.66 mV and -10.7 ± 4.01 mV respectively. Negatively charged surface and large magnitude of zeta potential of nanoparticles were needed to attract positively charged drug molecules for high loading efficiency. Hence, Nd10 was excluded from further studies.

As described in section 3.3, MHA nanoparticles were suspended in phosphate-buffered solution (free of NaCl, PB) at 6.67 mg/ml at 25°C or 37°C for drug loading studies. It is believed that these factors, such as the suspended media, particle concentration and measurement temperature affected the zeta potential. Thus the same conditions as drug loading studies were adapted for zeta potential measurement. As shown in Fig 5.2, the magnitude of zeta potential for Mn10 and Fe5 was significantly larger than pure HA while the magnitude of Fe5 was close to Mn10, regardless of the temperature. For each sample, zeta potential at 37°C was slightly smaller than at 25°C ($p > 0.05$). Mn10 and Fe5 were synthesized using ion exchange method which had not markedly affected

the size and morphology of nanoparticles, which were comparable with pure HA (Fig 4.6). The ion exchange method usually gives rise to the distribution of added metal ions on particle surface [37], such as Mn^{2+} and Fe^{3+} in this case, thus modifying the surface properties. This would explain the different zeta potential values observed for pure HA, Mn10 and Fe5, respectively.

Fig 5.2. Zeta potential of pure HA, Mn10 and Fe5 powder in PB solution at 6.67 mg/ml. Data = mean + standard deviation; n = 3; * $p < 0.05$ as compared to pure HA at the same temperature.

5.3. Drug loading conditions

Cisplatin was loaded onto the surface of MHA nanoparticles by electrostatic attraction. In aqueous phase, cisplatin is hydrolyzed as positively charged species, which have been reported to be adsorbed onto negatively charged HA nanoparticles [19]. The hydrolysis processes of cisplatin are shown below [232, 233]:

Since the interaction between cisplatin and MHA nanoparticles was electrostatic attraction, the drug loading (DL) and loading efficiency (LE) values could be affected by the following parameters, such as the concentration

of charged drug species, the surface area of nanoparticles exposed to the drug solution, and the affinity between drug and nanoparticles.

As shown in Table 5.1, the DL and LE values for all samples at shaking speed 200 rpm were higher than those without agitation at the same loading temperature. At fixed shaking speed, the DL and LE values at 37°C were higher than at 25°C for all samples. Under the same loading conditions, the DL and LE values for Mn10 and Fe5 were always higher than pure HA with Fe5 having the highest DL and LE values. Thus, cisplatin loaded onto Fe5 (CDDP-Fe5) under the following conditions - temperature of 37°C and shaking speed of 200 rpm - was further encapsulated into PLGA microparticles for controllable drug release. Under these conditions, DL of CDDP-Fe5 was 4.01%, which was 57% higher than cisplatin loaded HA.

Table 5.1 Drug loading (DL) and loading efficiency (LE) of cisplatin on MHA at different loading conditions. The concentration of cisplatin was 0.4 mg/ml in 10 mM phosphate buffer solution (free of Cl⁻) at pH 7.4

Shaking speed (rpm)	T (°C)	HA		Mn10		Fe5	
		DL (%)	LE (%)	DL (%)	LE (%)	DL (%)	LE (%)
200	25	1.37	22.8	1.93	32.2	2.85	47.5
0	25	1.05	17.5	1.25	20.8	2.54	42.3
200	37	2.55	42.5	2.83	47.2	4.01	66.8
0	37	1.89	31.5	2.22	37.0	3.36	56.0

The adsorption process is well described mathematically by the Freundlich isotherm [19, 20], as shown in Eq 5.3:

$$Q = kC^m \quad (5.3)$$

where Q is the amount adsorbed (mg/g), C is the equilibrium concentration (mg/l) of adsorbate [234, 235] which is charged cisplatin species in this

investigation, k and m are constants reflecting adsorption affinity and capacity. As reported by Barroug *et al.*[19], the increase in ionic strength of the equilibrium solution reduced the adsorption affinity but did not affect the capacity constant. Meanwhile, the presence of phosphate ions and absence of chloride ions in buffer solution enhanced cisplatin uptake. Here, the suspended media used for all samples was 10 mM phosphate-buffered solution free of NaCl. Hence the DL and LE values of various nanoparticles were not influenced by the media in our investigation.

As shown in Eqs 5.1 and 5.2, the charged species are produced by these equilibrated reactions. In the presence of Cl^- ions, the equilibrium shifts to the left side, resulting in less charged species and small DL values. As shown by Yotsuyanagi *et al.*, cisplatin was stabilized in normal saline solution (0.9% w/v), where the fraction of $(\text{NH}_3)_2\text{PtCl}(\text{H}_2\text{O})^+$ (3%) remained unchanged regardless of cisplatin concentration by simulation. The simulation was relied on the hydrolysis of cisplatin and further dissociation of the hydrated species [232]. These hydrolysis reactions (Eqs 5.1 and 5.2) are endothermic [236]. At higher temperature, more charged species will be produced, giving rise to higher equilibrium concentration, C , in the Freundlich isotherm (Eq 5.3). Hence, all samples had higher drug loading at 37°C (Table 5.1), even though the zeta potential values were slightly smaller at 37°C than at 25°C (Fig 5.2). The loading temperature 37°C was used for drug loading in this investigation.

It has been reported that lower crystallinity of HA nanoparticles gives rise to higher loading of cisplatin [20]. This is because HA with lower crystallinity had more defects which functioned as the active sites for cisplatin adsorption. In this investigation, MHA nanoparticles were produced using ion exchange method. As shown in sections 4.1 - 4.3, all MHA nanoparticles had similar crystallinity, morphology and particle size as pure HA. Thus, the theoretical active sites representing adsorption capacity for all samples should be similar to one another. In order to confirm this, specific surface area, which reflects the theoretical adsorption capacity, was measured using BET method. The values obtained for pure HA, Mn10 and Fe5 were similar within experimental error at $90 \pm 18 \text{ m}^2/\text{g}$, $94 \pm 13 \text{ m}^2/\text{g}$, and $92 \pm 15 \text{ m}^2/\text{g}$ respectively.

Here, all samples displayed higher drug loading at 200 rpm compared to those without agitation. This was probably due to the available adsorption sites. The available adsorption sites for a same sample would be different between non-agitation and agitation. Agitation dispersed all samples into smaller aggregates and more active sites were exposed to the equilibrium solution for surface adsorption. This increased the available adsorption sites compared to samples prepared without agitation. Hence, to maximize drug adsorption, shaking at 200 rpm was applied.

Both Mn10 and Fe5 had higher DL values than pure HA. This was probably due to the smaller magnitude of zeta potential for pure HA, which can be reflected as smaller adsorption affinity in the Freundlich isotherm. When compared to Mn10, the higher drug loading of Fe5 could be due to the relatively higher P content on the surface of Fe5 nanoparticles. It has been reported that higher P content on the surface of HA nanoparticles (based on XPS result) would result in higher drug loading of cisplatin [21]. Here, the exchange ratio of Fe with Ca was found to be above 1/1 but less than 3/2, as described in section 4.4, while Mn/Ca was 1/1. Hence the surface of Fe5 would have relatively higher P content compared to Mn10 due to the exchange of Ca with Fe. It was thus reasonable that Fe5 had higher drug loading than Mn10 even though both had close zeta potential.

From the DL and LE results obtained, cisplatin loaded onto Fe5 (CDDP-Fe5) nanoparticles at 37°C under agitation (200 rpm) were chosen for further investigation as potential drug carrier for cisplatin. These nanoparticles were dried in a vacuum oven at room temperature (25°C) and characterized using FESEM, XRD and FTIR.

5.4. Characterization of cisplatin loaded Fe5 (CDDP-Fe5) nanoparticles

Since cisplatin was loaded onto Fe5 nanoparticles by surface adsorption, the morphology and structure of Fe5 nanoparticles were not be markedly changed.

The FESEM images of CDDP-Fe5 and Fe5 are shown in Fig 5.3. CDDP-Fe5 nanoparticles were elongated spheroids of ~70 nm. The particle morphology and size were similar to Fe5. There were no large crystallites of similar morphology to as-received cisplatin (Fig 5.1) observed in the FESEM image of CDDP-Fe5, indicating the cisplatin on Fe5 was not crystallized.

Fig 5.3. FESEM images of (a) cisplatin loaded Fe5 (CDDP-Fe5) and (b) Fe5 nanoparticles at $\times 40,000$ magnification. The scale bar is 300 nm.

Fig 5.4. XRD patterns of as-received cisplatin, cisplatin loaded Fe5 (CDDP-Fe5) and Fe5 nanoparticles.

As shown in Fig 5.4, the XRD peaks of as-received cisplatin were sharp and narrow, confirming that as-received cisplatin was well crystallized. For CDDP-Fe5, only peaks belonging to hydroxyapatite phase were present. Since cisplatin was loaded onto Fe5 nanoparticles through surface adsorption, cisplatin was no longer in a crystalline state and thus did not demonstrate the corresponding peaks in the XRD pattern of CDDP-Fe5. Compared to Fe5, no broadening or shifting of peaks was observed for CDDP-Fe5, indicating that the lattice structure of Fe5 was not affected by the loading process.

Fig 5.5. FTIR spectra of as-received cisplatin, cisplatin loaded Fe5 (CDDP-Fe5) and Fe5 nanoparticles.

Fig 5.5 shows the FTIR spectra of as-received cisplatin, CDDP-Fe5 and Fe5. For cisplatin, the characteristic NH_3 adsorption bands [237] were observed, which included the stretching bands ($3274, 3188 \text{ cm}^{-1}$), asymmetric deformation modes ($1618, 1534 \text{ cm}^{-1}$), skeletal deformation modes ($1304, 1298 \text{ cm}^{-1}$) and phase rocking mode (797 cm^{-1}). When compared to Fe5, only the

NH₃ stretching bands at around 3300 cm⁻¹ and skeletal deformation bands at around 1300 cm⁻¹ were observed in CDDP-Fe5 nanoparticles (as indicated by arrows). This was probably due to the low cisplatin quantity in CDDP-Fe5 (4.01% w/w) and/or the overlap of absorption bands.

Although cisplatin was not shown in FESEM image and XRD pattern, the absorption bands belonging to NH₃ were observed in FTIR spectrum of CDDP-Fe5. The presence of cisplatin on CDDP-Fe5 was also confirmed by ICP data which was used to quantify the DL and LE values.

5.5. Summary

Cisplatin was successfully loaded onto hydroxyapatite nanoparticles. Due to the larger magnitude of zeta potential and higher affinity with cisplatin, Mn10 and Fe5 had higher drug loading (DL) and loading efficiency (LE) than pure HA with Fe5 giving the highest DL and LE values (57% higher than pure HA). This was probably also due to the relatively higher P content on Fe5 nanoparticles surface than Mn10. Since cisplatin was surface adsorbed on Fe5 and not in crystalline state, no peaks belonging to cisplatin in the XRD pattern and no cisplatin crystallites in the FESEM image of cisplatin loaded Fe5 (CDDP-Fe5) were observed. The lattice structure of Fe5 was not affected while the morphology and size of CDDP-Fe5 nanoparticles was similar to Fe5 as observed under FESEM. Although limited by the resolution of FTIR and concentration of cisplatin, the absorption bands of NH₃ were still observed in the FTIR spectrum of CDDP-Fe5. The presence of cisplatin on CDDP-Fe5 was also confirmed from the ICP results since the drug loading of CDDP-Fe5 was quantified using ICP based on Pt concentration.

Chapter 6

Fabrication of drug loaded composite microparticles

In this research, water-in-oil-in-water (W/O/W) double emulsion and solid-in-oil-in-water (S/O/W) single emulsion methods were used to produce the composite microparticles. In order to obtain high drug loading (DL) and loading efficiency (LE), W/O/W double emulsion technique was first studied to fabricate microparticles with condensed surface by varying polymer concentration in DCM. Cisplatin loaded PLGA microparticles were fabricated using S/O/W single emulsion. The morphology of microparticles was observed under FESEM and the structure information was obtained using XRD and FTIR. Polymer degradation and drug release were further examined where the results are shown in Chapters 7 and 8.

6.1. W/O/W double emulsion

Water-in-oil-in-water (W/O/W) double emulsion was first studied as cisplatin was loaded onto Fe5 nanoparticles in PB solution as described in section 3.3. The major challenge was to produce microparticles with high drug loading and loading efficiency. For this, condensed surface is preferred since porous microparticles usually give rise to low drug loading. To obtain condensed surface, the fabrication parameters were optimized. Pure HA without any cisplatin was first used instead of CDDP-Fe5 in the initial study and PLGA concentration was varied from 2% to 10% (w/v) (Table 6.1).

With the increase in polymer concentration (2% to 5% w/v), the particle size did not increase significantly (Table 6.1) while the surface of microparticles became less porous and more condensed, as shown in Fig 6.1(a-d). For D-5 (Fig 6.1(d)), no open pores were observed on the surface of the particles. When the volume of W1 was increased to increase the theoretical drug loading, the surface of sample D-5-H became porous (Fig 6.1(e)). The surface porosity was minimized by doubling the polymer concentration, where D-10-H had

condensed surface but much larger particle size due to the increase in polymer concentration.

Table 6.1 PLGA concentration and volumetric ratio of W1/O/W2 for the fabrication of microparticles and the corresponding particle size

Sample name	Fabrication parameters		Particle size ^c (μm)
	C ^a	W1/O/W2 ^b	
D-2	2%	1/20/200	55 $\pm$ 10
D-3	3%	1/20/200	67 $\pm$ 12
D-4	4%	1/20/200	65 $\pm$ 12
D-5	5%	1/20/200	72 $\pm$ 10
D-5-H	5%	1/10/100	66 $\pm$ 15
D-10-H	10%	1/10/100	129 $\pm$ 33

^a concentration of P(0.8) in DCM, % w/v

^b volumetric ratio of inner aqueous phase W1 with oil phase O and outer aqueous phase W2

^c averaged over 200 particles based on light microscope images

The size of the microparticles at low polymer concentrations was similar to those prepared at high polymer concentrations. This was surprising since larger particles were expected at higher polymer concentration due to the higher viscosity if the same stirring rate was applied. The similarity in the size of microparticles at different polymer concentrations was possibly due to the larger swelling of W1 phase at lower polymer concentrations resulting from mass transfer. The W1 phase is HA suspension in 10 mM phosphate buffer solution (free of NaCl), which was emulsified in the oil (O) phase, PLGA-DCM solution to form the W1/O emulsion. The W1/O emulsion was further dispersed into W2 phase (large volume of 1% w/v PVA solution) and microdroplets were formed. During the second emulsification, the organic solvent, DCM slowly diffused into the surrounding W2 phase and evaporated off. Besides the elimination of organic solvent, there was mass transfer between the W1 and W2 phases through the organic layer [30, 162] due to concentration gradient or osmotic pressure. Water may diffuse from the outer W2 phase into the inner W1 phase through the oil layer and cause the final volume of W1 to increase.

Here, only PLGA with LA/GA molar ratio of 50/50 at IV of 0.61 dl/g was used. The viscosity of the polymer solution increased with polymer concentration. At lower polymer concentrations, such as 2% w/v, water diffusion through the oil layer was easier than at higher polymer concentrations. This was due to the higher permeability of the oil layer at lower polymer concentrations. The increase in volume of W1 phase may lead to the swelling of microdroplets. This would explain the similar particle size of samples prepared at different polymer concentrations (2% to 5% w/v, Table 6.1).

Surface porosity of microparticles decreased with the increase of polymer concentration. During the evaporation of DCM and solidification of polymer, a gel-like layer would be formed on the surface of microdroplets. At low polymer concentrations, the swelling of microdroplets caused by the increase in W1 volume through mass transfer could result in a thin gel-like layer on the surface of microdroplets. This thin gel-like layer was easily distorted, thus pores were easily formed on the surface of the microparticles. At high polymer concentrations, such as 5% and 10% w/v, the high viscosity of the polymer layer restricted water diffusion. The swelling of W1 phase was limited and the gel-like layer on microparticles surface was thick, thus surface porosity was minimized (Fig 6.1(d) (f)).

Only microparticles produced at polymer concentration as high as 5% w/v had condensed surface. If assuming there was saturated cisplatin (2.53 mg/ml [232]) and CDDP-Fe5 (drug loading of 4.01%) at a concentration of 50 mg/ml in W1 phase, the theoretical drug loading of fabricated samples following the parameters of D-5 and D-10-H as shown in Table 3.4 was 0.45% according to Eq 3.2. Although the value was twice of cisplatin loaded microparticles without CDDP-Fe5 nanoparticles, the theoretical drug loading was still low. Additionally, the actual DL value would be lower since drug loss happened during the fabrication process of microparticles [197]. Hence, the W/O/W method was discarded.

Fig 6.1. FESEM images of microparticles produced using W/O/W double emulsion method. The insets are images at larger magnification. (a) D-2 (b) D-3 (c) D-4 (d) D-5 (e) D-5-H (f) D-10-H.

6.2. *S/O/W single emulsion*

The solid-in-oil-in-water (S/O/W) single emulsion method was used to fabricate the drug loaded microparticles. It was initially proposed to entrap only cisplatin loaded Fe5 (CDDP-Fe5) nanoparticles into PLGA microparticles. However, due to the low drug loading values, as-received cisplatin was also added in DCM phase to increase the drug loading of microparticles.

In order to study the effect of hydroxyapatite phase on the degradation of PLGA, the quantity of CDDP-Fe5 was varied in samples PT-Fe5/PT and 2PT-Fe5/PT. As shown in Table 6.2, blank and PT (PLGA microparticles encapsulating only cisplatin) samples, were made as controls. For comparison purposes, microparticles with a mixture of unloaded Fe5 nanoparticles and cisplatin, 2Fe5/PT was also fabricated.

Table 6.2 Feed ratios of solids in DCM for the fabrication of microparticles and the properties of microparticles after solidification

Sample name	Feed ratio of solid		Property of microparticles		
	Fe5 /CDDP	Fe5/ P(0.61)	DL ^a (%)	LE ^b (%)	Particle size ^c (μm)
blank	--	--	0	0	100 ± 24
PT	--	--	6.83 ± 0.04	68.7 ± 0.4	122 ± 34
PT-Fe5	--	1/4	0.56 ± 0.01	70.2 ± 1.5	122 ± 38
PT-Fe5/PT	1/1	1/9	3.59 ± 0.06	39.7 ± 0.6	134 ± 30
2PT-Fe5/PT	9/4	1/4	2.72 ± 0.04	33.5 ± 0.4	100 ± 34
2Fe5/PT	9/4	1/4	2.23 ± 0.11	27.4 ± 1.4	120 ± 40

^a: drug loading, $W_{\text{drug}}/W_{\text{particle}} \times 100\%$, W_{drug} was determined as described in section 3.6.1.

^b: loading efficiency, actual drug loading/theoretical drug loading $\times 100\%$.

^c: averaged over 200 particles based on light microscope images.

The particle size was similar for all samples within experimental error. The size was around 110 μm regardless of the quantity of solids in DCM phase. Sample PT, without Fe5, had the highest drug loading (DL) 6.83% and sample PT-Fe5, with only CDDP-Fe5 nanoparticles, had the lowest drug loading 0.56%. The presence of Fe5 nanoparticles appeared to have a negative effect on the DL and loading efficiency (LE) values. As shown in Table 6.2, for samples with the as-received cisplatin, PT, PT-Fe5/PT and 2PT-Fe5/PT, the DL and LE values decreased with the increase of CDDP-Fe5. For sample 2Fe5/PT, a mixture of Fe5 nanoparticles and cisplatin in the microparticles, the DL and LE values were also much smaller than PT. Hence, it can be concluded that the presence of Fe5 nanoparticles decreased the DL and LE values. The negative effect of

Fe5 on drug loading of microparticles may be due to its hydrophilicity which facilitated the diffusion of drug into the surrounding aqueous phase during the solidification of microparticles.

6.3. Characterization of microparticles produced using S/O/W single emulsion

The morphology of microparticles was observed under FESEM while the structure information was obtained using XRD and FTIR.

Fig 6.2. FESEM images of microparticles produced using S/O/W single emulsion method at magnification $\times 100$ and $\times 700$. (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT.

As shown in Fig 6.2, all microparticles were spherical but with different surface morphologies. The blank and PT samples, which were free of Fe5 nanoparticles, had smooth surfaces with no pores or defects on the surfaces. For those with Fe5 nanoparticles, the surface was wrinkled and some pore defects were observed. This was probably due to the insolubility of Fe5 nanoparticles in aqueous phase, which affected the polymer precipitation during DCM elimination. HCl solution was added to remove the unincorporated or surface adsorbed Fe5 nanoparticles after the microparticles had solidified. This was to

narrow down the parameters influencing drug release and degradation of polymer. The elimination of surface adsorbed Fe5 nanoparticles probably resulted in these defective pores.

The presence of Fe5 nanoparticles appeared to cause the surface of microparticles to wrinkle. This could be due to the water insolubility and hydrophilicity of Fe5. In the preparation of cisplatin loaded PLGA microparticles, all solids (cisplatin, and/or CDDP-Fe5 or Fe5) were first suspended in PLGA-DCM solution because of the polymer viscosity and agitation. When the polymer solution was emulsified in PVA solution to form the O/W emulsion, the organic phase including the solids was separated into small droplets. As DCM slowly diffused into the aqueous phase and gradually evaporated, PLGA droplets with their solid contents gradually solidified to form microparticles. Compared to the oil droplets in the PVA solution, these solidified microparticles were shrunken in nature due to the loss of organic solvent [163]. Meanwhile, due to the shrinking of microdroplets, the encapsulated substances were partially expelled, decreasing the loading efficiency. As reported by Nagata *et al.* [238], hydroxyapatite nanoparticles precipitated from calcium nitrate and ammonium hydrogen phosphate solution at the oil/water interface of PLA microdroplets contributed as a stabilizer for microparticles formation. Here, Fe5 nanoparticles were present inside the oil droplets. Some of the Fe5 nanoparticles would stabilize in the interfacial layer of microdroplets, affecting the shrinkage process of these oil droplets. This would account for the wrinkled texture observed on the surface of microparticles. Since acid solution was used to remove the surface adsorbed Fe5, no Fe5 nanoparticles were observed in the FESEM images (Fig 6.2). However, some defects like pores were observed instead.

The presence of Fe5 nanoparticles decreased the drug loading and loading efficiency of PLGA microparticles. During the elimination of DCM, a gel-like layer would be formed on the surface of microdroplets. The presence of Fe5 nanoparticles in the gel-like layer created micro-channels for drug to diffuse to the surrounding PVA solution, thus decreasing the drug loading. Here the

solubility of cisplatin in water at room temperature (25°C) was 2.53 mg/ml [232].

Fig 6.3. XRD patterns of (a) as-received cisplatin (b) blank (c) PT (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) CDDP-Fe5. FTIR spectra of (a) as-received cisplatin (b) blank (c) PT (d) PT-Fe5 and (e) CDDP-Fe5. ↓ absorption bands belonging to CDDP-Fe5, ↓↓ absorption bands attributed to CDDP.

The as-received cisplatin was a crystalline powder and remained crystallized once encapsulated into microparticles. As shown in Fig 6.3, the XRD patterns of microparticles had the sharp peaks belonging to cisplatin. When compared to the as-received drug, the peaks of cisplatin in microparticles were slightly broader, indicating that small drug crystals were successfully entrapped in the microparticles. This is reasonable since the large drug crystals would be more difficult to entrap inside the microdroplets during solvent elimination. The XRD peak intensity was proportional to the quantity of crystals in samples. For samples with higher drug loading, the peak intensity of cisplatin was relatively higher. Similarly, those with higher Fe5 quantity also had higher peak intensity. The XRD pattern of Fe5 peaks did not change significantly (including positions

and width) after the nanoparticles were entrapped in the microparticles, suggesting the structure of the nanoparticles not influenced by the fabrication process. In addition, the broad peak of polymer PLGA at around 20° was observed in all microparticles.

Limited by the resolution of FTIR spectra, only absorption bands at $\sim 3200\text{ cm}^{-1}$ (indicated by $\downarrow$ in Fig 6.3) belonging to NH_3 stretching bands [237] was observed in sample PT. Meanwhile, the water stretching band at $\sim 3300\text{ cm}^{-1}$ and PO_4^{3-} bending mode at 570 and 600 cm^{-1} belonging to CDDP-Fe5 [70, 212] (indicated by $\downarrow$ in Fig 6.3) were observed for sample PT-Fe5.

6.4. Summary

Porous microparticles were produced using water-in-oil-in-water (W/O/W) double emulsion method. The surface became more condensed with the increase in polymer concentration. No open pores were observed on the surface of microparticles of samples D-5 and D-10-H, which was fabricated at PLGA concentrations 5% and 10% w/v respectively. The highest theoretical drug loading (DL) was calculated as 0.45%. Since drug loss happened during the fabrication of microparticles, W/O/W double emulsion method was not used to encapsulate cisplatin due to the low drug loading.

Cisplatin loaded composite microparticles with high DL values were successfully fabricated using solid-in-oil-in-water (S/O/W) single emulsion. All samples were spherical in morphology and the presence of Fe5 demonstrated minor effect on the surface morphology of the microparticles as compared to samples free of Fe5. In the samples with Fe5, the wrinkled surface with defective pores may further contribute to the different drug release behaviors from the sample without Fe5 nanoparticles. The presence of cisplatin and Fe5 nanoparticles were confirmed by XRD and FTIR. Both were in crystalline state and the encapsulation process did not greatly modify the structures of cisplatin and Fe5. The Fe5 nanoparticles decreased DL values of microparticles.

Chapter 7

Degradation study on microparticles

To examine the morphological changes of microparticles and changes in molecular weight of polymer during drug release, degradation study was conducted on drug loaded Fe5/PLGA composite microparticles. Microparticles were incubated in 10 mM PBS at 37°C. Two sets of samples were prepared. At predetermined time points, 1.8 ml of total 2 ml PBS was collected for pH measurement and refilled with fresh PBS for further degradation at 37°C. Samples of the other set were removed, washed with DI water and dried for further characterizations, including FESEM, DSC and GPC.

7.1. Change in molecular weight

The change in molecular weight was monitored using GPC. For the same type of polymer, those with small molecular weight have small dynamic volume in solution and a short retention time through the GPC column. The representative chromatograms of blank sample at different degradation times are shown in Fig 7.1. The peak shifted to longer retention time and broadened after 21-d incubation and narrowed again after degradation for 42 days. Although PLGA matrix degraded through a heterogeneous reaction, only single peaks which indicated the single distribution of polymer molecular weight were observed in the GPC chromatograms.

As shown in Fig 7.2, the number-average molecular weight (M_n) and weight-average molecular weight (M_w) decreased upon immersion to the endpoint of study, 55th day. Upon immersion into the aqueous phase, water molecules penetrated into the polymer matrix and cleavage of ester bonds began. The M_n and M_w profiles for all samples demonstrated a rapid decrease until 28 days and a slower decrease rate afterwards. In the case of PT, M_n profile had high values until 14th day and then decreased fast afterwards.

Fig 7.1. The representative GPC chromatograms of blank sample at different degradation times.

As described in section 2.2.2, the hydrolysis of PLGA is shown as follows:

The reaction rate is given by:

$$\frac{d[\text{COOH}]}{dt} = p[\text{ester}][\text{H}_2\text{O}][\text{COOH}] = k_{Mn}[\text{COOH}] \quad (7.2)$$

where $[\text{COOH}]$, $[\text{ester}]$ and $[\text{H}_2\text{O}]$ are the concentrations of carboxyl end groups, ester and water in the polymer matrix. It is assumed that in the early stage of degradation, the concentrations of ester and water are constant and $[\text{COOH}] = 1/M_n$, thus Eq 7.2 can be rewritten as:

$$\ln M_n = \ln M_{n,0} - k_{Mn} t \quad (7.3)$$

where $M_{n,0}$ is the number-average molecular weight of polymer before degradation and k_{Mn} is the rate constant of the degradation process.

Fig 7.2. The average molecular weight of all samples as a function of degradation time.

Based on Eq 7.3, the degradation rate constants for all samples were calculated from the slope of the linear regression fit of logarithm of M_n (between 0 and 49 days of degradation) versus degradation time. The curve of blank sample is shown in Fig 7.3, which is representative of the rest samples.

Fig 7.3. The representative linear regression on $\ln M_n$ of blank sample.

Table 7.1 The degradation rate constant k_{Mn} for all samples based on the equation $\ln M_n = -k_{Mn} \times \text{Time} + \text{const}$

sample	Feed ratio		Linear regression		
	Fe5 /CDDP	Fe5/ P(0.61)	k_{Mn}	<i>const</i>	R^2
blank	--	--	0.0477	10.163	0.9786
PT	--	--	0.0475	10.240	0.9434
PT-Fe5	--	1/4	0.0367	10.148	0.9707
PT-Fe5/PT	1/1	1/9	0.0428	10.156	0.9566
2PT-Fe5/PT	9/4	1/4	0.0346	10.118	0.9687
2Fe5/PT	9/4	1/4	0.0438	10.071	0.9661

The degradation rate constant k_{Mn} of all samples are listed in Table 7.1. Samples sorted according to k_{Mn} values from largest to smallest, were blank > PT > 2Fe5/PT > PT-Fe5/PT > PT-Fe5 > 2PT-Fe5/PT. Based on this trend and compositions of all samples, it was found that the presence of cisplatin and/or Fe5 decreased the degradation rate of PLGA.

The polydispersity index (PI) represents the distribution of molecular weight. The PI values increased up to 28 days of degradation and decreased thereafter

until the end of study, 55th day (Fig 7.4). The pattern was similar to Chen's results [113], where PI values increased initially (up to 13th week of degradation) and eventually decreased at longer degradation time (22 weeks). The distribution of molecular weight first broadened and then narrowed as degradation continued. The initial increment of PI values was due to the random cleavage of ester bonds which could decrease the molecular weight of the polymer and broaden its distribution. As degradation continued, the degradation byproducts were small enough to be released into the surrounding solution thus decreasing the PI values. The reported critical molecular weight of oligomers to be soluble in water is around 1100 [239]. The PI values of blank sample reached the maximum value earliest on the 21st day while PT-Fe5 and 2PT-Fe5/PT reached the peak value much later on the 35th day (Fig 7.4). The results obtained further supported the observation that the presence of Fe5 nanoparticles delayed the degradation of PLGA.

As described in section 2.2.2, the hydrolysis of PLGA was catalyzed by the acidic degradation byproducts, giving rise to autocatalysis effect. For large devices, the autocatalysis effect was distinctive and hollow structures were finally formed [111]. As reported by Siepmann *et al.* [130] and Klose *et al.* [146], even very small-sized microparticles (< 20 μm) were affected by the autocatalysis effect. Here, the microparticles investigated were around 110 μm , thus autocatalysis was believed to influence the degradation of PLGA. Fe5 (Fe^{3+} doped hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$)) is basic in nature. For basic material, it has two opposing effects on PLGA degradation [240]. The basic nature can catalyze the cleavage of ester bonds, thus enhancing the degradation. At the same time, it can also neutralize the acidic degradation products and suppress the autocatalysis effect, resulting in lower degradation rate. Whether the degradation of PLGA is enhanced or delayed depends on which effect takes the lead. Here all samples with Fe5 had smaller degradation rate constant k_{Mn} than the blank sample (Table 7.1). This was probably because that the catalysis effect of Fe5 on the cleavage of ester bonds was not as dominant as the suppressing effect of Fe5 on the autocatalysis of PLGA due to the neutralization of Fe5 on the acidic degradation byproducts.

Fig 7.4. The polydispersity index (PI) of all samples as a function of degradation time.

Besides Fe5, it was also observed that cisplatin decreased the degradation rate of PLGA. The interaction between cisplatin and the acidic degradation byproducts of PLGA was probably due to complexation effect as shown in Fig 7.5. It has been reported that the ionic complexation was used to entrap cisplatin in micelles [8, 241] or formed cisplatin conjugate [242]. The acidic end groups were thus shielded and the autocatalysis effect was inhibited.

Fig 7.5. Schematic of the proposed complexation mechanism of cisplatin and carboxyl groups [4].

7.2. Change in glass transition temperature (T_g)

Glass transition temperature (T_g) of a polymer is an important parameter to determine the mobility of polymer chains. If the environmental temperature is

higher than its T_g , the polymer is in rubber state and the mobility of polymer chain is relatively high; otherwise the polymer is in glassy state and the mobility of polymer chain is lower than at rubber state.

Fig 7.6. Representative DSC thermograms from the first and second heating cycle for sample (a) blank, and (b) 2PT-Fe5/PT at different degradation times.

The T_g value can be measured using DSC. On DSC thermogram, glass transition appears as a step in the baseline due to sample undergoing a change in heat capacity. Glass transition is a non-equilibrium process. Many factors affect the measurement of T_g value, such as thermal history of samples, heating rate, to name a few. The DSC thermograms of blank sample and 2PT-Fe5/PT at different degradation times are shown in Fig 7.6. On the thermograms from the first heating cycle, a transition step from ~40°C to ~52°C with an endothermic peak (due to structural relaxation) superimposed on the transition was observed. On the thermograms of the second heating cycle which cleared the thermal

history of samples, only a glass transition step from $\sim 43^{\circ}\text{C}$ to $\sim 53^{\circ}\text{C}$ was observed (Fig 7.6). No crystallization or melting peak was observed in all samples.

Here, the PLGA used had a T_g value (41.4°C , onset temperature of glass transition on the DSC thermogram of second heating cycle) much higher than the experimental temperature (37°C) and storage temperature (-20°C). Hence all samples were in a glassy state at both temperatures throughout the experiments. Glassy polymers are in general non-equilibrium [243]. They are characterized by excess structural energy, which drives the glassy polymer to relax towards equilibrium state. The relaxation process is referred to as structural relaxation. PLGA, like other amorphous polymers, relaxes via molecular rearrangements at temperature below T_g . The molecular motions involve side chains, which is β relaxation, and represents energy minimization of the non-equilibrium nature of glassy state [144]. The loss of enthalpy during structural relaxation increases polymer stability through a reduction in free volume and thus an increase in density. This process is also known as physical aging [244]. On DSC thermograms, the structural relaxation is observed as an endothermic peak superimposed on the glass transition [133, 144, 244] as shown in Fig 7.6 and the area of the peak is the enthalpy relaxation. Here, T_g was calculated as the onset temperature of glass transition as shown in Fig 7.7 to minimize the effect of relaxation peak on the calculation of T_g values.

Fig 7.7. The representative DSC thermogram of second heating cycle for blank sample after immersed in PBS for 1 day at 37°C. T_g was calculated as the onset temperature of the glass transition by extrapolating the baseline in the glassy state to meet extrapolated line with the slope of the steepest part of the transition curve, where the meet point was T_g .

Factors influencing T_g values from the first heating cycle were complicated. The presence of HA nanoparticles in PLGA fibrous scaffold had been reported to result in higher T_g than those without HA [188, 190]. In this investigation, some samples had Fe5 nanoparticles which would increase the T_g of polymer. Microparticles were immersed in PBS at 37°C, which was below the T_g of PLGA and the samples underwent structural relaxation. It was reported that the structural relaxation rate depended on temperature and excess structural energy [243] while the enthalpy relaxation increased with the relaxation time [144, 244]. T_g values increased with enthalpy relaxation due to the concomitant decrease in the molecular mobility of polymer chains [144, 244]. In order to minimize the effect of the relaxation peak, T_g was calculated based on the onset temperature of glass transition [144] as shown in Fig 7.7. Hence, if without other factors such as presence of HA and degradation, T_g of microparticles should increase with the incubation time due to structural relaxation. Upon immersion of microparticles into PBS solution, water penetrated into the polymer matrix, acting as a plasticizer. This would lead to a decrease in the T_g [245] and a change in the kinetics of relaxation. Furthermore, polymer degradation began once water penetrated into polymer matrix (observed from

the decrease in M_n and M_w profiles, Fig 7.2) and resulted in a decrease in the molecular weight. According to Fox-Flory equation (Eq 7.4), T_g decreased with molecular weight decreasing [131]. Thus, T_g values should decrease due to the polymer degradation. Here, DSC test was carried out after microparticles had been dried overnight at room temperature in a vacuum oven. The drying process eliminated the plasticizing effect of water and introduced further structural relaxation of polymer, which might result in higher T_g values than samples before drying. Based on the above complicated effects, T_g from the first heating cycle increased initially and then decreased at longer degradation time (Fig 7.8(a)).

After thermal history was cleared, only a transition step belonging to the polymer glass transition was observed on thermograms of the second heating cycle. The glass transition step gradually shifted to lower temperatures with respect to degradation time (Fig 7.6). This agreed well with the Fox-Flory equation (Eq 7.4) where T_g decreased as molecular weight decreased:

$$T_g = T_{g\infty} - \frac{K_g}{M_n} \quad (7.4)$$

where $T_{g\infty}$ is the glass transition temperature at an infinitely high number-average molar mass, K_g is a polymer specific constant and M_n is number-average molecular weight.

Fig 7.8. The change of T_g values of microparticles as a function of degradation time. (a) from the first heating cycle, (b) from the second heating cycle.

7.3. Change in pH of PBS

The degradation of PLGA was catalyzed by the acidic degradation byproducts, thus the pH values of PBS solution were monitored during the degradation process. At predetermined time points, 1.8 ml of 2 ml PBS from degradation group was removed for pH measurement and fresh PBS of the same volume was refilled. It has been reported that lactic acid and glycolic acid were detected in the incubation media after 1-day degradation of PLGA microspheres [246]. This is believed to be due to the random chain scission of the PLGA chains

which occurred once microparticles were immersed in aqueous media. This was supported by the M_n and M_w profiles in Fig 7.2, where the molecular weight of polymer decreased right from the beginning of degradation study. Due to the buffer ability of PBS solution, the pH value remained around 7.4 for the first 20 days in all samples, followed by a rapid decrease with further degradation and eventually stabilized after 40 days of degradation, as shown in Fig 7.9. For samples with Fe5, the pH values at around 50th day were higher than those samples without Fe5. This was possible due to the delay effect of Fe5 nanoparticles on polymer degradation or neutralization of the acidic degradation products by Fe5. Additionally, pH of 2Fe5/PT began to drop earlier than the other samples. This was in accordance with the largest degradation rate constant, k_{Mn} among samples at feed ratio of Fe5/PLGA of 1/4 (Table 7.1), indicating 2Fe5/PT degraded faster than other samples at feed ratio of Fe5/PLGA of 1/4.

Fig 7.9. The pH values of PBS solution for all samples as a function of degradation time.

Table 7.2 The k_{pH} , $const$ and R^2 values for all samples based on the equation $pH = -k_{pH} \times \text{Time} + const$

sample	Feed ratio		Linear regression		
	Fe5 /CDDP	Fe5/ P(0.61)	k_{pH}	$const$	R^2
blank	--	--	0.1121	10.1310	0.9939
PT	--	--	0.0905	9.5003	0.9773
PT-Fe5	--	1/4	0.0348	8.0470	0.9843
PT-Fe5/PT	1/1	1/9	0.0660	8.8430	0.9848
2PT-Fe5/PT	9/4	1/4	0.0350	8.0531	0.9845
2Fe5/PT	9/4	1/4	0.0494	8.0491	0.9821

The sharp decrease in the pH values were fitted using linear regression ($pH = -k_{pH} \times \text{Time} + const$). The curve fit for the blank sample is shown in Fig 7.10. The slope and R^2 values for all samples are listed in Table 7.2. The R^2 values for all samples were larger than 0.97, indicating that a strong linear correlation existed between the pH value and degradation time. Samples sorted based on k_{pH} value from largest to smallest were blank, PT, PT-Fe5/PT, 2Fe5/PT, 2PT-Fe5/PT and PT-Fe5. The trend was similar to sample sequence based on the degradation rate constant k_{Mn} . The only difference was that the degradation rate constant k_{Mn} of 2Fe5/PT was larger than that of PT-Fe5/PT while the slope k_{pH} of 2Fe5/PT was smaller than the value of PT-Fe5/PT. This was probably due to the neutralization of Fe5 nanoparticles on the acidic degradation byproducts since Fe5 is basic in nature. 2Fe5/PT was fabricated at feed ratio of Fe5/PLGA as 1/4 while PT-Fe5/PT at 1/9, thus 2Fe5/PT had more Fe5 than PT-Fe5/PT and pH values of 2Fe5/PT decreased slower although 2Fe5/PT degraded faster than PT-Fe5/PT. Sample PT-Fe5 had larger degradation rate constant k_{Mn} than 2PT-Fe5/PT while their k_{pH} values were similar, which was also probably due to the neutralization effect of Fe5 nanoparticles where both samples were fabricated at feed ratio of Fe5/PLGA as 1/4. Here, the results were consistent with published data [95, 148, 201] showing that hydroxyapatite neutralized the acidic degradation byproducts of PLGA or PGA and led to higher pH values than samples without HA.

Fig 7.10. The representative linear regression figure for pH values of sample blank as a function of degradation time.

7.4. Morphological changes of microparticles

Although molecular weight of polymer decreased on the 7th day of degradation due to random chain scission (Fig 7.2), there was almost no change in the morphology of microparticles after 7-day incubation in PBS at 37°C, as shown in Fig 7.11. All samples were spherical and the surface of microparticles was similar to samples before degradation (Fig 6.2).

Fig 7.11. FESEM images of microparticles (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT after degradation in 10 mM PBS solution at 37°C for 7 days.

After 14 days in PBS at 37°C, small pores of around 100 nm were observed on the surface of microparticles for all samples, as shown in Fig 7.12. The surface of blank and PT microparticles was no longer smooth as compared to the surface on the 7th day of degradation (Fig 7.11(a) (b)). As for the other samples, no such changes on the surface were observed.

Fig 7.12. FESEM images at magnification $\times 700$ and $\times 5000$ for microparticles (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT after degradation in 10 mM PBS solution at 37°C for 14 days.

After 21 days (FESEM images not included), the pores on the surface of microparticles were enlarged and the quantity increased. The surface of all microparticles appeared to be rougher when compared to samples after 14 days of degradation. All samples were still spherical in morphology. Sample 2Fe5/PT was observed to be swollen as some particles were much larger than $150\text{ }\mu\text{m}$. The initial maximum size of the microparticles was $150\text{ }\mu\text{m}$ since all samples were sieved in the range of $20\text{ }\mu\text{m}$ to $150\text{ }\mu\text{m}$ for the drug release and degradation study as described in section 3.4.2.

The change in the morphology of the microparticles was obvious after 28 days of degradation (Fig 7.13). For samples without Fe5 nanoparticles, blank and PT, the surface of microparticles became wrinkled and the images at larger magnification demonstrated sponge-like microstructure (Fig 7.13(a) (b)). As for samples with Fe5 nanoparticles, the surface of microparticles was much smoother and the surface pores were much smaller (Fig 7.13(c)-(f)) than samples without Fe5, blank and PT. Some particles of 2Fe5/PT had disintegrated by 28 days of degradation and porous interior was observed, while some particles were still swollen after on the 28th days of degradation.

×700

×5000

Fig 7.13. FESEM images at magnification $\times 700$ and $\times 5000$ for microparticles (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT ($\times 400$ and $\times 5000$) after degradation in 10 mM PBS solution at 37°C for 28 days.

All samples had some particles disintegrating by 35 days of degradation. For sample without Fe5, blank and PT, microparticles still had wrinkled surfaces (Fig 7.14(a) (b)) while for those with Fe5, the surface of microparticles were rougher (Fig 7.14(c)-(e)) as compared to samples on the 28th day of degradation (Fig 7.13(c)-(e)). Size of surface pores on all samples appeared to decrease as compared to the 28th day. All particles for 2Fe5/PT had disintegrated and porous structure was observed (Fig 7.14(f)).

Fig 7.14. FESEM images at magnification $\times 700$ and $\times 5000$ for microparticles (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT after degradation in 10 mM PBS solution at 37°C for 35 days.

After 42 days of degradation, the microparticles for all samples, except for PT-Fe5 and 2PT-Fe5/PT, had completely disintegrated (Fig 7.15(a) (b) (d) (f)). For blank sample, the polymer had fused to form pellets (Fig 7.15(a)) while there were still some particles observed for samples PT-Fe5 and 2PT-Fe5/PT (Fig 7.15(c) (e)).

Fig 7.15. FESEM images at magnification $\times 700$ for microparticles (a) blank (b) PT (c) PT-Fe5 (d) PT-Fe5/PT (e) 2PT-Fe5/PT and (f) 2Fe5/PT after degradation in 10 mM PBS solution at 37°C for 42 days.

After 49 days of degradation (Fig 7.16), for samples free of Fe5, blank and PT, the polymer had fused after drying and the morphology was similar to blank sample on the 42nd day of degradation. There were still some particles observed for sample PT-Fe5 while 2PT-Fe5/PT microparticles were completely disintegrated and in powder form. At larger magnification, porous structure and presence of Fe5 nanoparticles was observed (as indicated by arrows).

At the end of degradation study, 55th day, all samples had disintegrated and the pieces were fused together. The morphology was similar to blank sample on the 42nd day of degradation (Fig 7.15(a)).

Fig 7.16. FESEM images at magnification $\times 700$ and $\times 2000$ or higher for microparticles (a) PT-Fe5 (b) PT-Fe5/PT and (c) 2PT-Fe5/PT after degradation in 10 mM PBS solution at 37°C for 49 days.

The morphological changes of microparticles with degradation time were similar among different samples. In summary, pores were formed on particles surface on the 14th day of degradation, which gradually enlarged and increased with further degradation until 28 days, then decreased after 35 days. Concurrently, the surface of microparticles roughened with degradation time, followed by the disintegration of microparticles structure at long degradation time.

A relationship existed between the change in morphology and the change in glass transition temperature T_g . Small pores were observed on the microparticles surface on the 14th day (Fig 7.12) while T_g began to decrease around 14 days of degradation as shown in Fig 7.8(a). This could be due to the formation of small voids inside the microparticles which enabled the polymer to move easily, resulting in lower T_g values. After 35 days of degradation, T_g was smaller than 37°C (Fig 7.8(a)), the degradation temperature. During this time, the pores on microparticle surface were observed to be smaller (Fig 7.14) than

on the 28th day (Fig 7.13). This was probably due to the high mobility of polymer chains in rubbery state. Furthermore, during incubation in PBS, the water molecules in the polymer matrix functioned as a plasticizer, further enhancing the molecular motion of polymer and resulted in decreasing pore size. This was similar to Bouissou's results [145] where microparticles containing model protein FIII9'-10 and surfactant Triton X-100 stored at relative humidity 75% for 24 h at 20°C demonstrated reduction of surface pore depth due to the high mobility of polymer chains in rubber state. The smaller pore size after 35 days of degradation may also reduce the release rate of the water soluble acidic degradation byproducts. This would explain the stabilizing of pH values of the PBS solution at longer degradation time (Fig 7.9).

The presence of Fe5 nanoparticles delayed the polymer degradation as some particles were observed for samples PT-Fe5 and 2PT-Fe5/PT after 42 days of degradation when samples free of Fe5, blank and PT were already completely disintegrated (Fig 7.15). This was consistent with the GPC results that PT-Fe5 and 2PT-Fe5/PT had smallest degradation rate constants k_{Mn} among all samples (Table 7.1). The roughness of microparticles surface was lowered due to the presence of Fe5 nanoparticles on the 28th and 35th day of degradation as compared to samples without Fe5 (Figs 7.13 and 7.14). This might be due to an increase in the mechanical properties of PLGA matrix by Fe5, just as Nie *et al.* [190] and Jose *et al.* [188] reported that encapsulation of hydroxyapatite in PLGA fibrous scaffold gave higher tensile strength or storage modulus when compared to those samples without hydroxyapatite.

The different degradation behavior between 2PT-Fe5/PT and 2Fe5/PT was possibly because of the weak bonding of different substances inside 2Fe5/PT microparticles. 2PT-Fe5/PT contained polymer, cisplatin, cisplatin loaded Fe5 while 2Fe5/PT had polymer, cisplatin and Fe5. Among all samples, 2Fe5/PT was the first having disintegrated particles during the degradation study (28 days of degradation). As for 2PT-Fe5/PT, some particles were still observed after 42 days of degradation (Fig 7.15). It was possible that water molecules diffused easily through the polymer matrix of 2Fe5/PT, thus both autocatalysis effect and inhibitory effect of Fe5 on autocatalysis were weakened. As

compared to samples free of Fe5, blank and PT, the presence of Fe5 in 2Fe5/PT still delayed the polymer degradation (Table 7.1).

7.5. Summary

The presence of Fe5 nanoparticles and/or cisplatin decreased the degradation rate of PLGA. The average molecular weight of PLGA decreased gradually with degradation time. Compared with samples without Fe5, those with Fe5 had lower degradation rate constant k_{Mn} . Fe5 nanoparticles were basic in nature, which simultaneously catalyzed the cleavage of ester bonds and neutralized the acidic degradation byproducts. Here the catalysis effect on chain scission was less pronounced as compared to the neutralization effect on the acidic degradation byproducts which induced autocatalysis of PLGA. Thus the degradation rate was lowered as compared with samples without Fe5. As Fe5 neutralized the acidic degradation byproducts, the pH values of PBS solutions decreased at a slower rate with degradation time compared to the sample with smaller degradation rate constant k_{Mn} but smaller Fe5 quantity (2Fe5/PT compared with PT-Fe5/PT). Cisplatin would form complex structure with the degradation byproducts such as carboxyl end groups and thus diminish the autocatalysis effect. When compared to Fe5, the suppressing effect of cisplatin on the degradation rate of PLGA was weaker. As degradation proceeded, the condensed microparticles became porous (sponge-like), disintegrated and ultimately formed gel-like structures. Samples with Fe5 appeared to demonstrate higher mechanical strength according to the facts that these samples had smoother surface than samples without Fe5 and the spherical morphology of samples with Fe5 lasted longer.

Chapter 8

Study of drug release profiles

Cisplatin release was tested in multiplicity ($n = 3$) for all samples in 10 mM PBS at 37°C. At predetermined time points, supernatant was collected for ICP test to quantify cisplatin concentration based on Pt concentration. In order to explore the drug release mechanism, two semi-empirical models, linear model and surface diffusion along with polymer degradation model were applied to fit the fractional cumulative drug release profile.

8.1. Cumulative drug release of microparticles

Fractional cumulative cisplatin release is shown in Fig 8.1 for all samples. For CDDP-Fe5 nanoparticles where cisplatin was loaded directly on Fe5 nanoparticles, a high initial burst (41.8% on the 1st day, Table 8.1) followed by a continuous release was observed (Fig 8.1(a)). The profile was similar to Palazzo's results [21]. All cisplatin on CDDP-Fe5 was released with the fractional cumulative release reaching 100% on the 25th day (Table 8.1).

Upon encapsulation of CDDP-Fe5 in PLGA microparticles (PT-Fe5), the initial burst was much smaller (Table 8.1) and the release time lasted much longer (55 days) when compared with CDDP-Fe5 (25 days) (Fig 8.1(b)). Due to the introduction of polymer matrix as a diffusion barrier, the drug release rate from PT-Fe5 was much slower than from CDDP-Fe5 and the release rate was also more controllable than CDDP-Fe5.

For the sample with only cisplatin, PT, the drug release followed a typical triphasic manner. The initial burst release and induction period lasted for 23 days, followed by a final burst release (1.58 %/day), as observed in Fig 8.1(e).

For the sample with cisplatin and CDDP-Fe5 nanoparticles at low feed ratio (Fe5/PLGA = 1/9), PT-Fe5/PT, the release profile (Fig 8.1(f)) was similar to PT

(Fig 8.1(e)) before 25 days. After 25 days, the release profile of PT-Fe5/PT appeared to be linear.

For the sample with cisplatin and CDDP-Fe5 nanoparticles at high feed ratio ($\text{Fe5/PLGA} = 1/4$), 2PT-Fe5/PT, the release profile was nearly linear after the initial burst (Fig 8.1(d)). The release rate of 2PT-Fe5/PT was slower than PT-Fe5 which had only CDDP-Fe5 at the same feed ratio ($\text{Fe5/PLGA} = 1/4$).

For the sample with cisplatin and Fe5 nanoparticles at a feed ratio of Fe5/PLGA as 1/4, 2Fe5/PT, the release profile (Fig 8.1(c)) was similar to PT-Fe5 after 15 days. Before 15 days, the release rate of 2Fe5/PT was slower than PT-Fe5.

For these samples at high Fe5 feed ratio ($\text{Fe5/PLGA} = 1/4$), PT-Fe5, 2PT-Fe5/PT and 2Fe5/PT, the initial burst was $\sim 23\%$, which was higher than samples with lower and no Fe5, PT-Fe5/PT and PT ($\sim 10\%$, Table 8.1). This might be due to the hydrophilicity of Fe5 nanoparticles which attracted water and facilitated drug diffusion. As compared with CDDP-Fe5 nanoparticles, the initial burst from the CDDP-Fe5 loaded microparticles was much smaller (Table 8.1). This was due to the presence of a diffusion barrier in the form of polymer matrix, which cisplatin had to diffuse through before it was released into the surrounding media.

At the end of study, only 3 samples had cumulative drug release reaching higher than 90% while the other 3 samples had a cumulative release of $\sim 75\%$ (Table 8.1). As described in section 7.4, all microparticles had disintegrated after 55 days of degradation. This discrepancy was possibly due to the interaction between cisplatin and the acidic degradation byproducts of PLGA, like carboxyl acid end groups. The interaction was probably complexation effect as shown in Fig 7.5. Hence, cisplatin was entrapped in the polymer matrix and the cumulative release was less than 100%.

Fig 8.1. Fractional cumulative release of cisplatin from (a) CDDP-Fe5 nanoparticles and Fe5/PLGA microparticles (b) PT-Fe5 (c) 2Fe5/PT (d) 2PT-Fe5/PT (e) PT (f) PT-Fe5/PT in 10 mM PBS (pH 7.4) at 37°C. Data = mean $\pm$ standard deviation; n = 3.

Table 8.1 Fractional cumulative release of cisplatin from CDDP-Fe5 nanoparticles and Fe5/PLGA microparticles on the specific incubation day

Sample name	Feed ratio of solid		Day		
	Fe5 /CDDP	Fe5/ P(0.61)	1	25	55
CDDP-Fe5	--	--	41.8 $\pm$ 1.4	100.2 $\pm$ 1.1	
PT	--	--	9.1 $\pm$ 0.1	20.4 $\pm$ 0.4	67.8 $\pm$ 2.5
PT-Fe5	--	1/4	22.5 $\pm$ 0.1	77.0 $\pm$ 0.2	97.3 $\pm$ 0.5
PT-Fe5/PT	1/1	1/9	10.6 $\pm$ 0.5	29.5 $\pm$ 0.2	65.3 $\pm$ 2.2
2PT-Fe5/PT	9/4	1/4	22.1 $\pm$ 0.4	55.4 $\pm$ 0.6	75.2 $\pm$ 0.4
2Fe5/PT	9/4	1/4	24.4 $\pm$ 0.9	75.6 $\pm$ 0.1	93.0 $\pm$ 0.6

Fig 8.2. Cumulative release of cisplatin ($\mu\text{g-drug/mg-particle}$) from (a) CDDP-Fe5 nanoparticles and Fe5/PLGA microparticles (b) PT-Fe5 (c) 2Fe5/PT (d) 2PT-Fe5/PT (e) PT (f) PT-Fe5/PT in 10 mM PBS (pH 7.4) at 37°C. Data = mean $\pm$ standard deviation; n = 3.

The cumulative drug release of all samples in $\mu\text{g-drug/mg-particle}$ is shown in Fig 8.2. With drug loading taken into account, the profiles were slightly different from the fractional ones. The patterns were still triphasic for samples with no or little Fe5, PT and PT-Fe5/PT. The release was nearly linear for samples with high Fe5 content (feed ratio of Fe5/PLGA as 1/4), PT-Fe5, 2PT-Fe5/PT and 2Fe5/PT. For CDDP-Fe5 nanoparticles, the release rate of cisplatin was much faster than the PLGA microparticles since there was no diffusion barrier for CDDP-Fe5 nanoparticles.

Not all the drug in microparticles had been released during the time range investigated (55 days), hence XRD analysis was carried out to confirm the presence of cisplatin and also Fe5 in samples after drug release study. Fe5 nanoparticles could be dissolved in acidic microenvironment resulted from the degradation of PLGA. The representative XRD patterns of samples PT and PT-

Fe5/PT before degradation and after 55 days of drug release are shown in Fig 8.3, where the peaks of cisplatin and Fe5 were all present. For cisplatin, peaks were still sharp after 55 days of degradation as compared to 0 day samples, indicating the drug was still crystallized in samples. As for Fe5 in PT-Fe5/PT, the relative intensity of peak belonging to plane (211) was much higher than the peak belonging to plane (112) and the other peaks (as marked by arrows in Fig 8.3), suggesting that the orientation of Fe5 nanoparticles have changed after being embedded in PLGA matrix for 55 days. Fe5 nanoparticles were elongated spheroids (Fig 4.6(f)). The random distribution of these nanoparticles in the polymer microparticles before degradation gave similar XRD patterns of Fe5 phase in microparticles as CDDP-Fe5 powder. After the microparticles were degraded for 55 days in PBS at 37°C, the distribution of Fe5 nanoparticles was probably no longer random and may have a preferred direction, which resulted in the much higher intensity of peak belonging to plane (211) than plane (112) and others planes.

Fig 8.3. The representative XRD patterns of (a) as-received cisplatin, (b) PT and (c) PT-Fe5/PT before degradation and after 55-day degradation, (d) CDDP-Fe5.

Microparticles of sample PT were ultimately disintegrated and fused together after 55 days of degradation as described in section 7.4. However the cumulative drug release was less than 100% (Table 8.1) and the presence of cisplatin was confirmed by XRD (Fig 8.3). Hence, it was highly possible that the acidic degradation byproducts of polymer delayed the release of cisplatin through a complexation effect as described in section 7.1 (Fig 7.5).

8.2. Median effective dose (ED_{50}) of all samples

The median effective dose (ED_{50}) of cisplatin has been reported to be 0.35-1.4 $\mu\text{g/ml}$ *in vitro* [247]. Since different samples had different drug loading and all samples were incubated in 2 ml PBS for drug release study, different initial concentrations of microparticles are required to ensure that the cisplatin concentrations fall within the therapeutic range, as shown in Figs 8.4-8.6.

Fig 8.4. Cisplatin concentrations of PT (▲) and PT-Fe5/PT (▼) at various time intervals. Data = mean $\pm$ standard deviation; $n = 3$. The dash lines correspond to 0.35 $\mu\text{g/ml}$ and 1.4 $\mu\text{g/ml}$ respectively.

For sample PT (microparticles with only cisplatin), the cisplatin concentrations from the 3rd to 19th day were in the ED_{50} range of cisplatin (Fig 8.4), which were 0.35-1.19 $\mu\text{g/ml}$ and could be achieved at PT concentration of 2.32 mg/ml.

During this period, diffusion dominated the drug release. Beyond this time frame, the concentrations of cisplatin were much higher than the therapeutic range. After the diffusion release, the concentration reached maximum value on the 31st day and gradually decreased afterwards. In the case of PT-Fe5/PT (microparticles with cisplatin and CDDP-Fe5 at feed ratio of Fe5/PLGA of 1/9), the concentrations from the 28th to 55th day fell within the range of 0.35-0.78 µg/ml and were in the therapeutic range of cisplatin. This could be obtained at PT-Fe5/PT concentration of 0.79 mg/ml. The concentrations of diffusion release from the 3rd to 19th day were below the therapeutic range. After diffusion stage, the concentration reached maximum value on the 25th day and maintained ~0.50 µg/ml with further drug release time. This period was within ED₅₀ range of cisplatin and lasted for 27 days. For PT, a shorter time range (17 days) was applicable during the drug release study. As for PT-Fe5/PT, the period within therapeutic range was much longer.

For CDDP-Fe5 nanoparticles, cisplatin was released directly from the nanoparticles surface and demonstrated a high initial burst (Fig 8.1). As shown in Fig 8.5, the concentrations of cisplatin fell within the ED₅₀ range between the 12th and 25th day, which were 0.35-0.95 µg/ml and lasted for 14 days. This range could be achieved at CDDP-Fe5 concentration of 1.35 mg/ml. Once CDDP-Fe5 nanoparticles entrapped inside polymer microparticles (PT-Fe5), the release rate became more controllable (Fig 8.5). The concentrations in the ED₅₀ range were from the 9th to 55th day with the exception of 19th to 25th day. The values were between 0.35 and 0.89 µg/ml and could be obtained at PT-Fe5 concentration of 8.77 mg/ml. The cisplatin concentrations within the ED₅₀ range lasted for 37 days, which was much longer than 14 days for CDDP-Fe5 nanoparticles.

Fig 8.5. Cisplatin concentrations of CDDP-Fe5 (○) and PT-Fe5 (●) at various time intervals. Data = mean $\pm$ standard deviation; n = 3. The dash lines are representing 0.35 $\mu\text{g/ml}$ and 1.4 $\mu\text{g/ml}$ respectively.

For 2PT-Fe5/PT, the one with cisplatin and CDDP-Fe5 at feed ratio of Fe5/PLGA of 1/4, the concentrations at various time intervals in the ED₅₀ range were 0.35-1.09 $\mu\text{g/ml}$ from the 6th to 19th day and 0.35-0.75 $\mu\text{g/ml}$ from the 28th to 55th day. This would be obtained at 2PT-Fe5/PT concentration of 1.89 mg/ml. The cisplatin concentrations within ED₅₀ range nearly lasted the entire drug release period, 55 days except for the initial burst release and the 2nd burst release period during which the concentration reached maximum value. The time range in which cisplatin concentrations fell within the therapeutic range (~48 days) was longer than PT-Fe5. For 2Fe5/PT, the one with cisplatin and Fe5 at feed ratio of Fe5/PLGA of 1/4, the cisplatin concentrations fell within ED₅₀ range during the later stage of drug release, from the 25th to 55th day. The values were between 0.35 $\mu\text{g/ml}$ and 1.03 $\mu\text{g/ml}$ and could be achieved at 2Fe5/PT concentration of 2.81 mg/ml. The period within ED₅₀ range was shorter than 2PT-Fe5/PT as shown in Fig 8.6.

Fig 8.6. Cisplatin concentrations of 2PT-Fe5/PT (▼) and 2Fe5/PT (■) at various time intervals. Data = mean $\pm$ standard deviation; $n = 3$. The dash lines are representing 0.35 $\mu\text{g/ml}$ and 1.4 $\mu\text{g/ml}$ respectively.

Based on the comparison, it was found the microparticles at feed ratio of Fe5/PLGA of 1/4 had more controllable cisplatin concentrations at various time intervals than microparticles with less or no Fe5 and also free CDDP-Fe5 nanoparticles without polymer encapsulation. Among these three samples at feed ratio of Fe5/PLGA of 1/4, 2PT-Fe5/PT had the longest release time with drug concentrations in the ED_{50} range of cisplatin. This was ideal for the treatment of osteosarcoma as the chemotherapy for osteosarcoma usually lasts 10 weeks to a year [2, 3]. A preincubation step to decrease the initial burst release would be necessary in the actual drug formulation.

8.3. Study of drug release mechanism

As mentioned earlier under literature review (section 2.2.5), many mathematical models have been used to describe the drug release profiles from PLGA microparticles [48, 149, 150, 172, 173, 180]. Among them, many models were built based on the diffusion mechanism while drug release from PLGA microparticles was believed to be a result of the combination effects of

diffusion and polymer degradation. Hence, a semi-empirical model, surface diffusion along with polymer degradation model was selected to fit the fractional cumulative drug release profiles. Since linear release was the ideal result, linear model was also applied for the release profiles. Among all models, these two showed the best fitting results.

8.3.1. Linear fit

To prove whether the release was zero order, the linear model was used. The fractional cumulative release, M_t/M_∞ increased linearly with time t in the linear fit model.

$$\frac{M_t}{M_\infty} = K't + const \quad (8.1)$$

K' is the kinetic constant and *const* is the value close to the initial burst.

As shown in Fig 8.1, the release profile of CDDP-Fe5 nanoparticles had a high initial burst and a short release time. Once CDDP-Fe5 nanoparticles encapsulated into PLGA microparticles, the initial burst of PT-Fe5 microparticles was much lowered and the release time was lengthened as compared to CDDP-Fe5 nanoparticles. The release profile of microparticles with only cisplatin, sample PT was a typical triphasic model. After the initial burst release and a lag time, a second burst release was observed. The triphasic profile changed to linear with the increase of CDDP-Fe5 nanoparticles in the system. The release profile of PT-Fe5/PT (feed ratio of Fe5/PLGA as 1/9) was similar to sample PT. The release profiles of 2PT-Fe5/PT and 2Fe5/PT (feed ratio of Fe5/PLGA as 1/4) were almost linear. As shown in Table 8.2, the cumulative release profile of 2PT-Fe5/PT had the best linear fit among all samples with R^2 value as 0.9700. The curve fit of 2PT-Fe5/PT cumulative release data is shown in Fig 8.7.

Since the release profiles of PT and PT-Fe5/PT was triphasic, the profiles were further fitted sectionally (Fig 8.8).

Fig 8.7. Curve fit of cumulative release profile of 2PT-Fe5/PT.

Table 8.2 Release kinetics parameters for all samples according to Eq 8.1

Sample	K'	<i>const</i>	R ²
CDDP-Fe5	0.0191	0.6123	0.7114
PT	0.0125	0.0185	0.9313
PT-Fe5	0.0120	0.3805	0.9210
PT-Fe5/PT	0.0112	0.0291	0.9620
2PT-Fe5/PT	0.0096	0.2669	0.9700
2Fe5/PT	0.0126	0.3446	0.9077

As shown in Fig 8.8, the slow diffusion and the second burst release of PT and PT-Fe5/PT fitted well with the linear model. For both samples, the second burst release rate was much higher than the diffusion rate. The diffusion rate of PT (0.0029 %/day) was similar to PT-Fe5/PT (0.0028 %/day) while the burst release rate of PT (0.0164 %/day) was larger than PT-Fe5/PT (0.0117 %/day). The presence of Fe5 nanoparticles did not significantly affect the diffusion release rate but lowered the second burst release rate. This was probably due to the larger pores formed on microparticles surface of PT as compared to PT-

Fe5/PT after the samples had been degraded for 28 days (Fig 7.13). The second burst release began around 28th day of drug release.

Fig 8.8. Sectional curve fits of cumulative drug release data for (a) PT and (b) PT-Fe5/PT.

8.3.2. Drug release due to surface diffusion and polymer degradation

Gallagher and Corrigan expressed the fractional cumulative drug release as a sum of drug release by surface diffusion and degradation of polymer matrix, as shown in Eq 8.2 [180]:

$$F_{TOT} = F_{b\infty}(1 - e^{-k_b t}) + (1 - F_{b\infty}) \frac{e^{k_d t - k_d t_{\max}}}{1 + e^{k_d t - k_d t_{\max}}} \quad (8.2)$$

where F_{TOT} is the total fraction of drug release at time t , $F_{b\infty}$ is the total surface diffusion fraction at time infinity, k_b is the surface diffusion rate constant, $t_{\max}$ is the time for maximum release rate and k_d is the release rate constant due to polymer degradation. The drug release contributed by surface diffusion and polymer degradation was both taken into account in this model.

The fractional cumulative release profiles were fitted according to Eq 8.2 using software Origin Pro 8 (Origin Lab Corporation). As no polymer was involved in CDDP-Fe5 nanoparticles, the release profile of CDDP-Fe5 was excluded. As shown in Fig 8.9, the model fitted well with all the drug release profiles of microparticles. The release kinetics parameters obtained from the curve fit are listed in Table 8.3.

Table 8.3 Release kinetics parameters for all samples according to Eq 8.2

Sample	$F_{b\infty}$	k_b	k_d	$t_{\max}$	R^2
PT	0.1663 ± 0.2479	0.0000 ± 0.0230	0.0849 ± 0.0093	35.7 ± 2.8	0.9799
PT-Fe5	0.4047 ± 0.0451	0.4483 ± 0.0837	0.0932 ± 0.0084	23.8 ± 2.0	0.9879
PT-Fe5/PT	0.2224 ± 0.2227	0.0000 ± 0.0166	0.0745 ± 0.0086	32.5 ± 3.0	0.9775
2PT-Fe5/PT	0.2607 ± 0.0513	0.5016 ± 0.1856	0.0554 ± 0.0067	37.7 ± 3.0	0.9624
2Fe5/PT	0.4852 ± 0.1531	0.1034 ± 0.0527	0.0630 ± 0.0177	21.9 ± 3.8	0.9751

For PT and PT-Fe5/PT, the total surface diffusion fraction at time infinity, $F_{b\infty}$ values were comparable and the diffusion rate constant, k_b values were both negligible. The k_b values for these two samples were much smaller than the release rate constant due to polymer degradation, k_d . The k_d value of PT was larger than PT-Fe5/PT. This was consistent with the results of sectional fitting using linear model, where the diffusion rate of these two samples were comparable and the burst release rate of PT was larger than PT-Fe5/PT (Fig 8.8). This was possibly because of the larger pores formed on microparticles surface of PT than PT-Fe5/PT after degraded for 28 days (Fig 7.13). This phenomena was similar to Bouissou's results [145] where the initial burst release of microparticles containing model protein FIII9'-10 and surfactant Triton X-100 was attenuated due to the reduction of surface pore depth. The time for maximum release rate, t_{max} , for both samples were similar within experimental error as shown in Table 8.3.

Among samples at the same feed ratio of Fe5/PLGA of 1/4, the total surface diffusion fraction $F_{b\infty}$ was similar for PT-Fe5 and 2Fe5/PT, and the values of these two samples were higher than 2PT-Fe5/PT. PT-Fe5 had larger k_b and k_d values than 2Fe5/PT, which might be due to the different degradation rates. PT-Fe5 degraded slower than 2Fe5/PT (smaller degradation rate constant k_{Mn} of PT-Fe5, Table 7.1); hence PT-Fe5 had less carboxyl end groups than 2Fe5/PT. Carboxyl end groups were speculated to form complexation structures with cisplatin (Fig 7.5). Thus cisplatin was easier released out from PT-Fe5 with less acidic end groups than 2Fe5/PT due to surface diffusion. As for the larger k_d value in PT-Fe5, it was also probably due to the larger pores on the microparticle surface of PT-Fe5 than 2Fe5/PT (Fig 7.13). In the case of 2PT-Fe5/PT, k_b was largest but k_d were smallest among these three samples (PT-Fe5, 2PT-Fe5/PT and 2Fe5/PT). It was reasonable that 2PT-Fe5/PT had the largest k_b value as 2PT-Fe5/PT degraded slowest among these three samples (Table 7.1). As for the k_d value of 2PT-Fe5/PT, besides the small pores on microparticles surface, it was also possibly influenced by the smallest cumulative release percentage of 2PT-Fe5/PT among these three samples (Table 8.1).

Fig 8.9. Curve fits of drug release profiles of sample (a) PT (b) PT-Fe5 (c) PT-Fe5/PT (d) 2PT-Fe5/PT and (e) 2Fe5/PT according to Eq 8.2.

The k_b values of those samples at feed ratio of Fe5/PLGA of 1/4 were higher than their k_d values. The trend was different from PT and PT-Fe5/PT, where the k_b values of these two samples were smaller than the k_d values. This might be due to the hydrophilicity of Fe5 nanoparticles, which facilitated the absorption of water and thus drug diffusion.

Based on the fitting results, it could be concluded that the drug release of PT and PT-Fe5/PT was mainly dominated by polymer degradation while the drug

release from samples at feed ratio of Fe5/PLGA of 1/4 was influenced by both surface diffusion and polymer degradation.

Although Fe5 decreased the DL values of cisplatin in microparticles, its effect on drug release was significantly positive. The release profile changed from triphasic to near linear after the initial burst stage. This was made possible by the combination effects of hydrophilicity of Fe5 nanoparticles (attracting water absorption and increasing drug diffusion rate), the suppressing effect of Fe5 on autocatalysis of polymer, and also the complexation of cisplatin with the acidic degradation byproducts (carboxyl end groups).

8.4. Summary

The drug release profiles of cisplatin-loaded microparticles were significantly modified by the presence of Fe5 nanoparticles. The typical triphasic pattern of microparticles with only cisplatin, sample PT changed to linear release profile when CDDP-Fe5 nanoparticles along with cisplatin were encapsulated into microparticles at feed ratio of Fe5/PLGA of 1/4 (sample 2PT-Fe5/PT). The drug release profile of 2PT-Fe5/PT fitted better with the linear model than the other samples. According to the fitting results using surface diffusion along with polymer degradation model, it was found that the drug release from microparticles with higher Fe5 content was controlled by both surface diffusion and polymer degradation while the drug release from samples with lower or no Fe5 was mainly dominated by polymer degradation. As compared with free CDDP-Fe5 nanoparticles, the entrapment of CDDP-Fe5 in microparticles significantly lowered the initial burst and prolonged the release time. Among all samples investigated, 2PT-Fe5/PT had the longest time range with drug concentrations within the ED₅₀ range of cisplatin. Steps such as preincubation might be required to remove the initial burst release.

Chapter 9

Conclusion

9.1. Metal ion doped hydroxyapatite (MHA)

Hydroxyapatite (HA) was synthesized by reacting calcium hydroxide and phosphorous acid in aqueous solution. HA nanoparticles were precipitated before the reactants were consumed and HA substituted with Nd^{3+} , Gd^{3+} , Mn^{2+} and Fe^{3+} was successfully produced using ion exchange reaction. Since ion exchange mechanism was applied, the structure and morphology of MHA were not greatly modified as compared to pure HA; while the properties of MHA were significantly improved due to the metal ions added.

All MHA were single phased (excluding Nd20 and Gd20) without significant structure deviation and were B-type carbonated HA. The nanoparticles were all elongated spheroids with size of around 70 nm. For Nd20, the second phase of $\text{Nd}(\text{OH})_3$ was cylindrical rods of around 300 nm while for Gd20, peaks of $\text{Gd}(\text{OH})_3$ were shown in the XRD pattern. However, no $\text{Gd}(\text{OH})_3$ particles of different morphology were observed in the FESEM image. With the increase of X_M , the quantity of metal ion in HA lattice also increased. As suggested by the ICP data, Gd^{3+} and Fe^{3+} ions were more active than Mn^{2+} ions at exchanging with Ca^{2+} ions in the HA lattice.

For NdHA and GdHA, the electrical conductivity increased significantly with M content. This could be due to the higher polarizability of M ions that preferentially occupied Ca(II) sites and the higher ionic transport number, where both H^+ and O^{2-} might be the charge carriers for the system. The presence of O^{2-} was indicated by the ion exchange mechanism of NdHA and GdHA ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + \text{MCl}_3 \rightarrow \text{Ca}_9\text{M}(\text{PO}_4)_6(\text{OH})(\text{O}) + \text{CaCl}_2 + \text{HCl}$). GdHA and Fe5 were characterized as paramagnetic and the magnetic susceptibility increased with the increase of Gd concentration. Limited by the

low Ms (saturation magnetization) values, biomedical applications such as MRI contrast agents or targeted drug delivery are not recommended.

All MHA, except for Gd20 were tested as non-toxic on osteoblast using agar diffusion or extraction assay. The toxicity of Gd20 was probably due to the free Gd^{3+} ions which were not located in the HA lattice. Hence NdHA was a better candidate as implants or coating materials due to its much higher electrical conductivity ($203.5 \times 10^{-9} \Omega^{-1} \cdot m^{-1}$ at 30 V) than pure HA ($2.7 \times 10^{-9} \Omega^{-1} \cdot m^{-1}$ at 30 V) and also its non-cytotoxicity as compared to GdHA. For MnHA and FeHA, cell seeding test was carried out to investigate their bioactivity as compared to pure HA. Limited osteoblast proliferation enhancement was observed on MnHA pellets as compared with pure HA while Fe^{3+} ions in HA demonstrated significant positive effect on osteoblast cells proliferation. Although differentiation of osteoblast was not accelerated during the study time range (7 days), Fe5 had the potential to promote osteoblast differentiation much earlier than pure HA since osteoblast begin differentiation after reaching confluence and much more cells (~180% more) were growing on Fe5 pellets than pure HA.

9.2. Cisplatin loaded composite microparticles

Mn10 and Fe5 were chosen as cisplatin carrier because of their non-cytotoxicity and higher zeta potential values than pure HA. Due to the larger magnitude of zeta potential and possible higher affinity with cisplatin, Fe5 had much higher drug loading than pure HA (57% higher) and Mn10 (42% higher). Since cisplatin was surface adsorbed onto Fe5 nanoparticles, no cisplatin peaks were observed in the XRD pattern and no crystallites of cisplatin were observed in the FESEM image of cisplatin loaded Fe5 (CDDP-Fe5). The morphology and size of CDDP-Fe5 nanoparticles were similar to Fe5 nanoparticles and the lattice structure of Fe5 was not modified by the loading process. Although limited by the resolution of FTIR and quantity of cisplatin adsorbed, the absorption bands of NH_3 were still observed in the FTIR spectrum of CDDP-Fe5. The presence of cisplatin on CDDP-Fe5 was also confirmed by ICP where the drug loading was quantified based on Pt concentration.

The CDDP-Fe5 nanoparticles were further encapsulated into poly(lactide-co-glycolide) (PLGA) microparticles. Due to the very low theoretical drug loading of microparticles fabricated using water-in-oil-in-water (W/O/W) double emulsion method, this method was not used for the further tests. Cisplatin loaded composite microparticles with high drug loading were successfully produced using solid-in-oil-in-water (S/O/W) single emulsion. All samples were spherical in morphology. As confirmed by XRD, cisplatin and CDDP-Fe5 were in crystalline state. The presence of Fe5 decreased the drug loading and appeared to cause the surface of microparticles to wrinkle. This was probably due to its water insolubility and hydrophilicity, which affected the shrinkage of oil droplets during the fabrication process and thus drug loading and the surface morphology.

The presence of Fe5 and cisplatin decreased the degradation rate of PLGA. When compared with samples without Fe5, those with Fe5 had smaller degradation rate constant k_{Mn} . Since Fe5 was basic in nature, it was able to catalyze the cleavage of ester bonds and neutralize the acidic degradation products which led to autocatalysis of PLGA. Here the catalysis on chain scission was less pronounced than the neutralization of the acidic degradation byproducts. Hence the autocatalysis of PLGA was inhibited, and the degradation rate of PLGA was lowered. Due to the alkaline property of Fe5, the rate in which the pH values of PBS solution decreased with degradation time was also lowered (smaller slope, k_{pH}). Cisplatin would form complex structures with the degradation byproducts such as carboxyl end groups, thus diminishing the autocatalysis effect. As compared to Fe5, the suppressing effect of cisplatin on polymer degradation was weaker.

For all samples, the condensed microparticles became porous (sponge-like), disintegrated and ultimately fused to form pellets as degradation proceeded. Samples with Fe5 appeared to demonstrate higher mechanical strength since these samples had smoother surface than samples without Fe5 throughout the degradation study. Furthermore, particles were still observed for samples with Fe5 while samples without Fe5 had disintegrated and fused.

The entrapment of CDDP-Fe5 inside PLGA microparticles significantly lowered the initial burst release as compared to the free CDDP-Fe5 nanoparticles. The drug release manner of microparticles was markedly modified by the presence of Fe5 nanoparticles from typical triphasic to linear pattern. The cumulative drug release of 2PT-Fe5/PT fitted well with the linear model. Based on the fitting results using surface diffusion along with polymer degradation model, it was found that drug release of microparticles with Fe5 at higher quantity ($\text{Fe5/PLGA} = 1/4$) was controlled by both surface diffusion and polymer degradation while drug release from samples with no or lower Fe5 was mainly dominated by polymer degradation.

Among all samples, 2PT-Fe5/PT was the most suitable candidate carrier for cisplatin as it was able to provide a sustained release of cisplatin within the therapeutic range ($0.35\text{-}1.4\text{ }\mu\text{g/ml}$) for around 48 days. This was ideal for the treatment of bone cancer as the chemotherapy for bone cancer usually lasts 10 weeks to a year. It must be noted that initial pre-incubation step would be required to remove the initial burst stage before microparticles were injected to the tumor site.

Chapter 10

Future work

Metal ion doped HA and drug loaded composite microparticles were successfully fabricated. Among all MHA, Fe5 was found to be more bioactive than pure HA according to the completed cell studies. For composite microparticles, *in vitro* drug release and degradation in PBS were carried out. Based on the results obtained, further related investigations were proposed as follows.

10.1 Application of Fe5 as coating materials

In this project, Fe5 demonstrated higher bioactivity than pure HA according to the preliminary cell seeding tests and also had much higher drug loading of cisplatin than pure HA. As the bone matrix produced by bone cancer cells is easily fractured and metallic implants might be needed, Fe5 could be used as drug-loaded coating material on these metallic implants for localized drug release. Heat treatment is usually involved in coating process. Whether CDDP-Fe5 decomposed after coating is however unknown. Thus, the stability of CDDP-Fe5 after being coated on implants needs to be studied. Additionally, the biocompatibility of CDDP-Fe5 after coating is also of interest to be investigated. Here, cold-pressed pellets were used to keep the surface morphology of pellets similar among samples. Limited by the mechanical strength, cell proliferation was slow in the time range studied (7 d). After heat treatment, mechanical property of Fe5 pellets would improve, while the surface of Fe5 pellets may have different grain size, zeta potential values and thus different cell behavior from cold-pressed Fe5 pellets. The possible application of CDDP-Fe5 needs further exploration.

10.2 In vitro and in vivo studies of Fe5/PLGA composite microparticles

In this project, *in vitro* drug release and degradation of Fe5/PLGA composite microparticles in PBS have been studied. The *in vitro* cytotoxicity of these

microparticles needs to be investigated and compared with free CDDP or naked CDDP-Fe5 nanoparticles. For this, osteosarcoma cell lines, such as MG-63, Saos-2, and U-2 OS can be used. Subsequently, *in vivo* toxicity of the composite microparticles can be done in animal model. As osteosarcoma also occurs frequently in dogs, canine osteosarcoma model can be studied in future work. Injection of microparticles to the tumor (osteosarcoma model) is adapted. The antitumor activity of microparticles based on the growth rate of tumor will be monitored. Meanwhile, the histopathological studies of tumor will be performed. As Fe5 was entrapped to neutralize the acidic degradation products so as to improve the outcome of the interaction between microparticles implants and surrounding tissues, the long-term of animal studies will be conducted.

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Appendices

Appendix A - Fabrication and release characteristic of E2 protein loaded PLGA microparticles:

Besides MHA nanoparticles, E2 protein was considered as a potential drug carrier. The E2 protein is derived from *Bacillus stearothermophilus* pyruvate dehydrogenase complex with a hollow dodecahedral-cage structure of approximately 24 nm in diameter [1, 2]. The cage structure is formed by the self-assembly of sixty subunits of E2 protein and maintains its integrity when foreign molecules are introduced to either internal or external surface [1]. E2 protein is being engineered to carry both hydrophilic and hydrophobic molecules. Modification of E2 protein by engineering a pH sensitive switch may facilitate the release of molecular contents in acidic environment of endosomes after endocytosis process [3, 4].

E2 protein has 12 openings of 5 nm each on the surface [1, 2]. These openings result in E2 protein porous nanosphere appearance. It is speculated that these openings contribute to the escape of molecular contents and significantly affect the encapsulation efficiency of the contents in E2 protein [5]. Additionally, as most proteins in general have short *in vivo* half lives [6-8], it is presumed that the release rate of molecular contents from the E2 protein is fast and uncontrollable.

To achieve a controllable release profile, E2 protein was encapsulated into PLGA microparticles to form a composite structure in this investigation. The encapsulated substances in PLGA microparticles have been reported to be released at a controlled rate over a prolonged period of time, allowing less frequent administrations [9-11]. It is also an advantage that localized drug release could be achieved for PLGA microparticles [11, 12]. Since the E2 protein can be engineered to deliver drug within endosomes, the composite microparticles have the potential to demonstrate an efficient treatment outcome.

In this project, water-in-oil-in-water (W/O/W) double emulsion method was used to fabricate the PLGA microparticles. Bovine serum albumin (BSA) was chosen as model protein to optimize the fabrication parameters. The influence of fabrication parameters on the morphology of microparticles was extensively studied using light microscope and FESEM. The integrity of E2 protein after encapsulation process was examined based on the size measurement using dynamic light scattering (DLS). The *in vitro* E2 protein release study was carried out in PBS at 37°C and morphology of microparticles after protein release was observed under FESEM.

Materials and methods

Materials

PLGA was from Boehringer Ingelheim with LA:GA molar ratio of 75:25 and inherent viscosity of 0.68 dl/g. PVA (87-89% hydrolyzed, molecular weight of 13,000-23,000) and PBS were from Sigma-Aldrich. DCM was from Analar. GF buffer solution (phosphate buffer solution: 50 mM potassium phosphate, 150 mM NaCl, 5 mM EDTA, 0.02% w/v NaN₃, pH 7.4) was used to dissolve the protein. BSA, supplied by Calbiochem, was used as a model protein to achieve the best fabrication parameters. E2 protein was produced in *E. coli* strains BL21(DE3) following the published method [1]. Pierce BCATM Protein Assay Kit was purchased from Thermo Scientific.

Optimization of fabrication parameters

The fabrication process of W/O/W double emulsion was first optimized using BSA as the model protein. The fabrication parameters were varied, including the stirring speed of the second emulsification, volume of the external aqueous phase (W2), type of solutions as W2, concentration of surfactant in W2, polymer concentration in the oil phase (O) and protein concentration in the inner aqueous phase (W1).

Throughout the optimization process, stirring speed for the first emulsification was kept constant at 10,000 rpm for 30 s.

Preparation of E2-loaded PLGA microparticles

E2-loaded PLGA microparticles were fabricated as follows. 1 ml GF buffer solution containing E2 protein (6.2 mg/ml, the internal aqueous phase (W1)), was emulsified in 10 ml PLGA-DCM solution (5% w/v, the oil phase (O)) using a high-speed homogenizer (Heidolph Silent Crusher M) at 10,000 rpm for 30 s. The first emulsion (W1/O) was then injected into 200 ml GF buffer solution containing 0.1% or 1% w/v PVA (W2) at 400 rpm using a magnetic stirrer for 1 h. The emulsion was continuously stirred at 200 rpm for another 3 h in a fume hood to allow DCM evaporation and microparticles solidification. The E2-loaded microparticles were then filtered, washed with DI water, and dried overnight at room temperature (25°C). The microparticles were stored at -20°C.

Characterization of microparticles

Microparticles size analysis

Microparticle shape and size were examined using a light microscope (Olympus BX51). After microparticles solidified, small amount of the final emulsion was placed onto a glass slide, and observed under the light microscope. Pictures were digitally taken and analyzed using the microscope software to calculate microparticle size (averaged over 200 particles).

Surface morphology observation

The surface morphology of microparticles was examined using JEOL FESEM (JSM-6700F). Small amount of microparticles were mounted onto a metal stub using double-sided carbon adhesive tape. Samples were coated with a thin layer of platinum at 10 mA for 200 s using a sputter (JEOL JFC-1600).

Confirmation of E2 protein integrity

The integrity of E2 protein after W/O/W double emulsion process was examined using DLS after extracting E2 protein from PLGA microparticles. The microparticles (20 mg) were first dissolved in 1.2 ml of DCM. GF buffer (2.4 ml) was added and the mixture was shaken for 7 h using a New Brunswick Scientific shaker at 200 rpm at 37°C. The mixture was then centrifuged at 2000 rpm (using Hettich Zentrifugen Universal 32) for 5 min to separate the DCM

from the GF buffer containing the extracted E2 protein. The size of E2 protein in GF buffer (1 ml) was determined using DLS (Malvern Zetasizer Nano ZS90).

Determination of protein encapsulation efficiency

The amount of protein encapsulated in PLGA microparticles was determined by hydrolyzing PLGA microparticles to water soluble monomers and measuring the protein quantity. Protein-loaded microparticles (20 mg) were hydrolyzed in 1.2 ml of 0.3 M NaOH. The solution was diluted five times with GF buffer, and analyzed using Pierce BCATM Protein Assay Kit. A calibration curve between protein concentrations and absorbance values of BCA assay was obtained as shown in Appendix G. Each sample was studied in triplicate.

The protein loading and encapsulation efficiency of protein in PLGA microparticles were calculated using the following equations:

$$\text{Theoretical protein loading} = \frac{W_{\text{added protein}}}{W_{\text{added polymer}} + W_{\text{added protein}}} \times 100\% \quad (\text{A.1})$$

$$\text{Actual protein loading} = \frac{W_{\text{protein}}}{W_{\text{microparticles}}} \times 100\% \quad (\text{A.2})$$

$$\text{Encapsulation efficiency} = \frac{\text{actual protein loading}}{\text{theoretical protein loading}} \times 100\% \quad (\text{A.3})$$

In vitro E2 protein release study

In vitro E2 protein release was carried out in triplicate. PLGA microparticles (20 mg) was suspended in 2 ml PBS (pH 7.4) and incubated in water bath at 37°C without agitation to simulate the *in vivo* conditions. At predetermined time points, 1 ml of the supernatant was taken for protein concentration analysis using Pierce BCATM Protein Assay Kit and replaced by the same volume of fresh PBS. On the 14th day, the release was stopped. Samples were washed with DI water and dried in a vacuum oven overnight at room temperature (25°C). The sample was then observed under FESEM to examine the morphological changes of the microparticles.

Results and discussion

Investigation on fabrication parameters

Stirring speed of the second emulsification

Stirring speed of the second emulsion is important in determining microparticle size because it provides shear energy to disperse the first emulsion (W1/O) into the external aqueous phase (W2) [13, 14]. In this investigation, 8000 rpm for 30 s, 1000 rpm for 30 min and 400 rpm for 60 min were respectively applied to produce the second emulsion.

Fig A.1 shows the light microscope and FESEM images of microparticles fabricated at the second stirring speed of 8000 rpm. The light microscope image shows that most of microparticles did not encapsulate the internal aqueous phase (W1) within the microparticle matrix. The stirring rate for the second emulsification was close to the first emulsification (8000 rpm to 10000 rpm), resulting in the corresponding diameter of final emulsion droplets to be close to the inner aqueous droplets; hence the inner aqueous phase was not encapsulated completely [14]. On the FESEM image, open pores were observed on the surface of microparticles. The microparticles size was determined to be $11 \pm 3 \mu\text{m}$.

Decreasing the stirring speed from 8000 rpm to 1000 rpm resulted in the increase of microparticle size from $11 \pm 3 \mu\text{m}$ to $101 \pm 28 \mu\text{m}$. However, some of microparticles were not spherical but tablet in shape. Shape deviation of microparticles indicated that during microparticle solidification, the emulsion droplets were exposed to excessive shear energy thus microparticles shape was distorted [13]. The microparticles had open pores on the surface.

At the stirring speed of 400 rpm, the microparticles size was close to that at 1000 rpm, which was $108 \pm 33 \mu\text{m}$ to $101 \pm 28 \mu\text{m}$ (at 1000 rpm). While the microparticles were more uniform and less distorted in shape when compared with those produced at 1000 rpm. The microparticles were also porous.

Fig A.1. Microparticles prepared at stirring speed 8000 rpm for 30 s, 200 ml of DI water with PVA (1% w/v) as external phase and polymer concentration of 5% w/v. (a) light microscope image (b) FESEM image.

At the condition of 400 rpm for 60 min, the inner droplets were much smaller than the final emulsion droplets, thus the inner droplet could be successfully encapsulated and the solidified microparticle shape was well defined.

Volume of external aqueous phase (W2)

The effect of W2 volume on the size of the microparticles was investigated by keeping other conditions constant. When W2 volume decreased from 400 ml to 200 ml, the microparticles size decreased from $108 \pm 33 \mu\text{m}$ to $83 \pm 26 \mu\text{m}$ (Table A.1). This was possibly due to the increase in shear energy density.

The surface of microparticles at W2 of 200 ml and 400 ml showed little difference (Fig A.2), where both demonstrated open pores. However, the morphology of particles produced at W2 of 200 ml appeared to be better defined. As microparticles with well defined morphology were preferred and small volume of W2 phase is easy to manipulate, W2 volume of 200 ml was subsequently used to prepare the microparticles.

Type of solution used as external aqueous phase (W2)

In this work, two types of solutions were used as the external aqueous phase, DI water with PVA and GF buffer with PVA. Microparticles fabricated using DI water with PVA as external aqueous phase were more porous than those prepared using GF buffer with PVA (Fig A.3).

Table A.1 Fabrication parameters for PLGA microparticles using W/O/W double emulsion method and the size of the solidified microparticles

Parameters		Microparticle size (μm)
Stirring speed of second emulsification (rpm)	400	108 ± 33
	1000	101 ± 28
	8000	10 ± 3
Volume of external aqueous phase (ml)	200	83 ± 26
	400	108 ± 33
Types of solution used as external aqueous phase	DI water	83 ± 26
	GF buffer	67 ± 13
Polymer concentration in organic phase (DCM) (w/v)	1%	45 ± 11
	2.5%	53 ± 10
	5%	73 ± 15
PVA concentration in external phase (w/v)	0.1%	71 ± 16
	1%	62 ± 13

Fig A.2. Surface morphology of microparticles fabricated at external aqueous phase of (a) 200 ml and (b) 400 ml. Stirring speed: 400 rpm for 1 h, external phase: DI water with PVA (1% w/v), polymer concentration: 5% w/v.

When DI water with PVA was used as W2, there was a large concentration gradient between the inner droplets (protein-GF buffer solution as W1 phase) and the external aqueous phase W2 [12]. Due to this concentration gradient, water may diffuse into the internal phase (W1) from external aqueous phase through the organic layer during the elimination of organic solvent (DCM). Water diffusion and the swelling of W1 phase would lead to the formation of

pores on microparticles surface. This highly porous condition was minimized when GF buffer was used as the external aqueous phase W2, where microparticles with closed pores on surface were formed (Fig A.3). Surface pores might reduce the encapsulation efficiency of protein in microparticles since these pores act as channels for protein to diffuse into W2 phase during the solidification of microparticles. Furthermore, pores might also influence the release kinetics of microparticle system [15, 16].

The microparticle size was smaller when GF buffer was used as W2 phase, $66 \pm 13 \mu\text{m}$ compared to $83 \pm 26 \mu\text{m}$ when DI water was used as W2 phase. This also supported the speculation that W1 phase was swollen due to water diffusion driven by large concentration gradient when DI water was used as external phase W2. In this work, GF buffer with PVA as W2 was used for the fabrication of protein-loaded microparticles.

Fig A.3. Surface morphology of microparticles fabricated with (a) DI water with PVA (1% w/v) and (b) GF buffer with PVA (1% w/v) as external aqueous phase. Stirring speed: 400 rpm for 1 h, external phase: 200 ml, polymer concentration: 5% w/v.

Polymer concentration in DCM

Viscosity of organic phase is one of the important factors influencing the properties and release profiles of microparticles [17, 18]. In this investigation, the viscosity was determined by polymer concentration in organic solvent. Three concentrations of PLGA in DCM (w/v), 1%, 2.5%, and 5% were studied. The sizes of microparticles formed at these polymer concentrations were $45 \pm 11 \mu\text{m}$, $53 \pm 10 \mu\text{m}$, and $65 \pm 16 \mu\text{m}$, respectively. This was consistent with

published data that the size of microparticles increased as the polymer concentration increased [12, 19]. Keeping other fabrication parameters constant, polymer solution with higher viscosity was dispersed into larger droplets when the same stirring speed was applied during the second emulsification step; hence the size of the resulting microparticles was larger.

Fig A.4. Surface morphology of microparticles fabricated at different PLGA concentrations (w/v) (a) 1%, (b) 2.5% and (c) 5%. Stirring speed: 400 rpm for 1 h, external phase: 200 ml of GF buffer with PVA (1% w/v).

The surface porosity of microparticles decreased when polymer concentration increased. Fig A.4 shows that microparticle produced at polymer concentration of 5% w/v had condensed surface. During the elimination of organic solvent, polymer precipitated as a gel-like layer on the surface of the microdroplets [13], which would inhibit the diffusion of water. In viscous solution, the permeability of this layer was lower than less viscous solution, leading to less swelling of W1 phase. Additionally, viscous solution had higher polymer concentration, which resulted in thicker gel-like layer on microparticle surface than less viscous solution. Thus fewer pores were formed on the microparticles surface produced at higher polymer concentration.

Protein concentration in internal aqueous phase (W1)

To investigate the effect of protein concentration in the W1, fabrication parameters determined in previous experiments were kept constant while the protein concentration in the W1 was varied. Similar surface morphology was observed for microparticles produced at two protein concentrations, 25 mg/ml and 40 mg/ml (Fig A.5). On the other hand, higher protein concentration reduced the encapsulation efficiency (Table A.2). High protein concentration in W1, led to the increase of protein concentration gradient between the inner and external aqueous phases. As the concentration gradient was higher, protein was easier to diffuse out of the droplets towards the external aqueous phase, causing more protein loss during the solidification of microparticles. Thus, the encapsulation efficiency was reduced at higher protein concentration in W1.

Table A.2 Comparison of encapsulation efficiency of microparticles produced at different protein concentrations in the W1

	BSA-loaded microparticles	
Protein concentration (mg/ml)	25	40
Theoretical protein loading (%)	5	8
Actual protein loading (%)	1.72 ± 0.02	2.36 ± 0.03
Encapsulation efficiency (%)	34.5 ± 0.3	29.5 ± 0.4

PVA concentration in external aqueous phase (W2)

At higher PVA concentration, smaller microparticles were produced in this work since PVA stabilized the emulsion droplets against coalescence. When the PVA concentration increased from 0.1% w/v to 1% w/v, microparticle size decreased slightly from $71 \pm 16 \mu\text{m}$ to $62 \pm 13 \mu\text{m}$. The morphologies of microparticles produced at different PVA concentrations were almost identical (Fig A.6). All microparticles were spherical in shape and had closed pores on the surface.

Fig A.5. Surface morphology of microparticles fabricated at protein concentration in the W1 of (a) 25 mg/ml and (b) 40 mg/ml. Stirring speed: 400 rpm for 1 h, external phase: 200 ml of GF buffer with PVA (1% w/v), polymer concentration: 5% w/v.

Fabrication of E2-loaded PLGA microparticles

Characterization of E2-loaded PLGA microparticles

E2-loaded PLGA microparticles were fabricated using W/O/W double emulsion method with different PVA concentrations in the external aqueous phase (W2). The size of microparticles was in the range of 50 to 80 µm, regardless of the PVA concentration. The microparticles had closed pores distributed on the surface (Fig A.7).

Fig A.6. Surface morphology of microparticles fabricated in 200 ml GF buffer with (a) 0.1% w/v PVA and (b) 1% w/v PVA. Stirring speed: 400 rpm for 1 h, polymer concentration: 5% w/v.

Fig A.7. Surface morphology of microparticles fabricated at different PVA concentrations (w/v) (a) 0.1% and (b) 1% in GF buffer. Stirring speed: 400 rpm for 1 h, external phase: 200 ml, polymer concentration: 5% w/v, E2 protein concentration in W1: 3.3 mg/ml.

E2 protein integrity in PLGA microparticles

Integrity of the E2 protein upon encapsulation in polymeric matrix is important for sustained release of molecules from the E2 protein. To examine the integrity of E2 protein after encapsulated in PLGA microparticles, E2 protein was extracted from microparticles for DLS analysis. The size was determined to be 27 ± 6 nm, indicating that the E2 protein was stable during the microparticles fabrication process (Fig A.8) since the size of intact E2 protein has been reported to be in the range of 24-28 nm [1]. This suggested that the specified conditions were suitable for embedding E2 protein in the microparticle matrix.

Fig A.8. Size distribution of extracted E2 protein measured using DLS. A peak at 28 nm is shown on the graph.

In vitro E2 protein release study

The release profiles of microparticles produced at different PVA concentrations of 0.1% w/v and 1% w/v were studied (Fig A.9). After the initial burst release,

a sustainable protein release was followed up to 14 days for both samples. The initial burst release was possibly resulted from the E2 protein located near the surface of microparticles that was expelled to PBS at the early stage of incubation [6, 12, 13]. Larger amount of protein was released for the 0.1% w/v PVA microparticles compared to the 1% w/v PVA microparticles during the burst period.

The release rate of E2 protein appeared to be faster from 0.1% w/v PVA sample than from 1% w/v PVA sample. The 0.1% w/v PVA microparticles had completed release on the 14th day of incubation, while the 1% w/v PVA microparticles released 95% of the total protein encapsulated. These results suggested that higher PVA concentration in W2 during the fabrication of microparticles not only reduced the initial burst but also the release rate. A high PVA concentration hindered the transfer of E2 protein from microparticles to the surrounding [6, 12, 13]. Hence, E2 protein in 0.1% w/v PVA microparticles was easier to diffuse out compared to that in 1% w/v PVA microparticles.

Fig A.9. The influence of PVA concentration in W2 (w/v) during the fabrication process on the in vitro release of E2 protein from PLGA microparticles (protein loading 0.44%). (●) 0.1% PVA, (▲) 1% PVA.

Morphology change of microparticles after protein release

After 14-day incubation, the microparticles had smoother surfaces compared to before incubation for both 0.1% and 1% w/v PVA microparticles (Fig A.10). This might arise from the presence of water molecules as plasticizer during the protein release study in PBS at 37°C. As a plasticizing agent, water molecules diffuse into polymer matrix, and increase the flexibility of polymer chains. During the incubation in PBS at 37°C, polymer chains were relatively more mobile than during storage (at -20°C), resulting in the reduction of surface roughness. This observation was consistent with the published data where the size of external pores on microparticles reduced after incubation [20, 21].

Fig A.10. Change in surface morphology of microparticles fabricated at different PVA concentrations in W2 (w/v) of (a) 0.1% and (b) 1% before incubation and after 14-d incubation.

Summary

The E2-loaded PLGA microparticles were spherical and had closed pores on the surface. Increase in the volume of external aqueous phase decreased the microparticles size. In contrast, increase in polymer concentration in the oil phase increased the microparticle size and reduced the surface porosity. When GF buffer with PVA was used as the external aqueous phase, the surface porosity of the microparticles was minimized compared to those prepared with

DI water with PVA. This was due to the reduction of the concentration gradient between inner and external aqueous phase when GF buffer with PVA was used. Moreover, increase in protein concentration in inner aqueous phase enlarged the concentration gradient, thus decreased the encapsulation efficiency. PVA concentration in the external phase during the fabrication of microparticles influenced the microparticle size and the release rate of E2-loaded PLGA microparticles. Increase in PVA concentration decreased the size of microparticles. Higher PVA concentration also resulted in lower burst release and slower release rate of E2 protein from microparticles. The sustained E2 protein release lasted for 14 days. The surface roughness of microparticles was reduced upon incubation in PBS due to the presence of water as plasticizer. As confirmed by size measurement, the integrity of E2 protein was not compromised by the encapsulation process.

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Appendix B - Protocol for osteoblast cell culture:

Cell recovery

- Thaw cells in 37°C water bath as soon as possible and put in 15 ml centrifuge tube.
- Mix with about 5 ml of medium.
- Centrifuge the mixture at 1000 rpm for 5 min. The cell will be at the bottom of the centrifuge tube.
- Take out the liquid above the cell using pipette. The liquid in the frozen cells contains DMSO which is poisonous to the cells. DMSO needs to be removed.
- Put in about 5 ml medium, and make the cells homogeneous in the medium.
- Transfer all the mixture into the flask.
- Add more medium depending on the flask size (75 cm² flask need 15 ml medium).

Changing the medium

- Take out the medium from the flask using pipette.
- Add about 6-7 ml of PBS to the flask and gently shake the flask, and decant the PBS.
- Add the fresh medium to the flask.

Subculture cells

- Take out the medium using pipette.
- Rinse the flask with 10 ml of PBS, pour away the PBS.
- Add 4 ml of trypsin to each flask (75 cm²).
- Incubate the flask for 2 min (time is a critical factor; appropriate time for cell detachment should be chosen). Transfer all the trypsin with the cells into a centrifuge tube. Add about 8 ml of medium to neutralize the trypsin.
- Centrifuge at 1000 rpm for 5 min.

- Pour out the upper solution. The cell pellet is at the bottom of centrifuge tube.
- Add 8-10 ml medium (DMEM/F12).
- Make the cells homogeneous in the medium using pipette.
- Transfer all the mixture into the flask.
- Add more medium depending on the flask size (75 cm² flask need 15 ml medium. 25 cm² flask need about 5 ml medium).
- Incubate the cells and change the medium every 2 or 3 days.

Cell counting

- Make the cells homogeneous in the medium after cells treated with trypsin and resuspended in medium.
- Mix 10 µL cell suspension with 10 µl Trypan Blue solution.
- Fill in the haemocytometer (Improved Neubauer) with 10 µl of the mixed cell suspension.
- Count live cells under light microscope.

The cell depth on it is 0.100 mm $\pm$ 2% and its rulings cover 9 mm² (Fig A.1).

- The number of cells per ml is: Number of cells counted per square millimeter $\times$ dilution factor (here, 2) $\times 10^4$

Fig A.11. The ruling pattern of the haemocytometer (Improved Neubauer).

Freezing cells:

- Proportions of frozen medium:
 - 60% volume of DMEM/F12 (medium)

- 30% volume of Serum
 - 10% volume of DMSO
- After cells treated with trypsin and centrifuged, pour away the upper medium and resuspend cells in the frozen medium.
- Divide into the freezing vial with capacity of 1.5 ml.
- Refill liquid nitrogen every 3-4 weeks because the nitrogen is easily evaporate.

Appendix C – Protocol for iron colorimetric assay

- Mix the solutions well and keep for overnight:
 - 500 μl of sample.
 - 50 μl of 1 M HNO_3 .
 - 200 μl of 50 mM orthobathophenanthroline (OBP). To prepare the solution, dissolve 29.5 mg of the solid in 1 ml of DI water.
 - 230 μl of 1 M Tris base buffer (pH 8).
 - 20 μl of 1-2 M Dithionite. Any concentration within this range will be successful.
- Measure absorbance: simultaneously measure the absorbance at 538 and 700 nm, the difference between the two is recorded.

To build a calibration curve, iron standard solutions in the concentration range of 1 - 100 ppm have also been prepared. Colorimetric test are done the same as outlined above. The calibration curve for Fe concentration and absorbance value is shown in Fig A.2.

Fig A.12. Calibration curve for Fe concentration and absorbance value.

Appendix D - Protocol for cell fixation for SEM observation

- Rinse the samples with PBS.
- Fix samples:
Fix solution: 2.5% v/v glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3-7.4).
Fix samples for 30 min to 1 h at room temperature or for 2 h in the freezer (4°C).
- Wash samples:
Rinse (immerse) in 0.1 M sodium cacodylate buffer for 5 min.
- Put the samples in 1 w/v% tannic acid for 30 min at 4°C.
To prepare, add 1 g tannic acid in 100 ml sodium cacodylate or PBS.
- Rinse again in 0.1 M sodium cacodylate buffer for 5 min.
- Rinse samples with DI water for 10 min.
- Dehydration:
Dehydrate samples in graded ethanol: 30% v/v ethanol (mixture of ethanol and DI water), 50%, 70%, 85%, 95%, 100%, each for 5-10 min.
- Critical Point Dry Run: Put samples in CPD to dry or
Immerse in 50% v/v HMDS (mixture of HMDS and ethanol), 70%, 100% HMDS for 20 min at RT.
- Remove all the HMDS. Put the samples in the dessicator.

Appendix E - Calibration curve for cell number and MTT absorbance

After osteoblast cells treated with trypsin and resuspended in medium, cells density was counted using haemocytometer as described in Appendix B. Cells were seeded into 24-well plate at a series of cell densities. After 3.5 h, MTT test was carried out, as described in section 3.2.7.5. The following is the calibration curve for cell number and absorbance value of MTT assay.

Fig A.13. Calibration curve for cell number and absorbance value of MTT assay.

Appendix F - Equation for ALP activity calculation

Since alkaline phosphatase (ALP) can catalyze the hydrolysis of colorless substrate, *p*-nitrophenyl phosphate (pNPP) to a yellow product *p*-nitrophenol and phosphate, the ALP activity was measured based on the final quantity of *p*-nitrophenol, using Bear's law:

$$A = \epsilon l C \quad (\text{A.4})$$

where A is the absorbance value at 405 nm, ϵ is the molar absorptivity (extinction coefficient), for *p*-nitrophenol, the value is $18.75 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; l is light path length and C is the analyte (*p*-nitrophenol in this calculation) concentration.

The quantity of *p*-nitrophenol was calculated according to the following equation:

$$\text{quantity} = \frac{A}{\epsilon l} \times V \quad (\text{A.5})$$

where V is the volume of the final solution (pNPP substrate and NaOH solution).

Appendix G - Calibration curve for BCA assay

BCA assay was conducted following the test tube protocol adapted from the protocol of Pierce BCA™ Protein Assay Kit. The following is the calibration curve of protein concentrations with absorbance values at 562 nm.

Fig A.4. Calibration curve for protein concentration and absorbance value at 562 nm.