Development of an Antimicrobial Hydroxyapatite Cement Using Human Defensins-Like Antimicrobial Peptides

By

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ABSTRACT

Bone cements are used in orthopaedics mainly as fixation devices in hip joints. One of the most important bone cements is Hydroxyapatite (HA) cement. It is widely used due to its advantages that include biocompatibility, bioactivity and low setting temperatures. HA cements can absorb bacteria leading to infection during surgery. In order to solve this problem, antimicrobial hydroxyapatite bone cements need to be developed.

Antimicrobial peptides (APs) are low molecular weight natural compounds. APs with a broad spectrum of antimicrobial activity act as the first line of defence against bacterial invasion in almost all forms of life. Human defensins represent one type of the most important antimicrobial peptides. The main advantage of human defensins is that they can avoid bacterial resistance which is a significant problem when antibiotics are used. However, the sequence of human defensins is very long and therefore very difficult to synthesise high purity antimicrobial peptides. If synthetic defensins with a short peptide sequence can still exhibit antimicrobial properties, synthetic defensins could be used. This work focuses on isolating fragments from the antimicrobial core of human defensins that can have significant antimicrobial properties and incorporating them in HA cements in order to make antimicrobial bone cements.

Four peptide sequences were selected from α- and β- human defensins. One peptide sequence with the best antimicrobial property was selected to incorporate with HA cements. Mass spectrometry and HPLC were used to identify the molar mass and purity of the peptide, respectively. The antimicrobial properties were studied by

measuring the minimum inhibitory concentration (MIC) of the peptides. The pH, antimicrobial properties, peptide release and mechanical properties (compressive strength) of bone cements were measured against different concentrations of the peptide.

All four fragments from the antimicrobial core of human defensins showed antimicrobial activity against *E.coli*, *S.aureus* and *P.aeruginosa*. Peptide AP4 with the shortest sequence (9-amino acid) had the best purity and the lowest MIC. In this research all pH values of HA cements with different concentration of peptides were at 7 indicating that all cement pastes underwent phase transition to HA. With the concentration of peptide increasing, the amount of peptide released was increased. For each concentration, during the first 0.5 hour, the release rate was higher than between 0.5 hour and 3 hours. After 48 hours, the peptide release concentration reached a plateau with no significant change. When the concentration of AP4 in the cement was increased, the compressive strength was decreased. The 24 hours compressive strength of each concentration was lower than 48 hours. The antimicrobial property of HA cements was increased with increasing the concentration of AP4. However, HA cements with highest concentration of 8 wt% of antimicrobial peptide could not completely eliminate bacteria. The antimicrobial property between 24 hours and 48 hours was not very different. An attempt to calculate the surface concentration of the antimicrobial peptide assuming homogeneous distribution of the peptide in the HA cement lead to the conclusion that the concentration of peptide on the surface was lower than the MIC of AP4. It can be concluded that the AP4 was not a very good choice of antimicrobial peptide to be added in bone cements. Other sequences might have been more effective. This study however can be used as a good example and reference to conduct further work in this area.

DEDICATION

谨以此论文献给我敬爱的父母，感谢他们长久以来的信任与支持。特别感谢我的小伙伴程序、石保生、张思齐和郑淑国。在那段科研最黑暗的时期，感谢你们无条件的帮助，鼓励与陪伴，让我走到了现在。爱你们。

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TABLE CAPTIONS

ABBREVIATIONS

A,Ala	Alanine
ABCs	Acrylic bone cements
Ahx	6-Aminocaproic acid
APs	Antibacterial peptides
β-$Ca_3(PO_4)_2$, β-TCP	β-tri-calcium phosphate
C,Cys	Cysteine
$Ca_{10}(PO_4)_6(OH)_2$,HA	Hydroxyapatite
$Ca_{10}HPO_4(PO_4)_5(OH)_2$	Calcium-deficient hydroxyapatite
℃	Degree Celsius
Ca/P	Calcium/ phosphate
$CaCO_3$	Calcium carbonate
$CaHPO_4$	Calcium phosphate dibasic
CFU	Colony forming unit
CPCs	Calcium phosphate cements
CS	Compressive strength
D, Asp	Aspartic acid
DCM	Dichloromethane
DCPD	Dicalcium phosphate dihydrate, brushite
DIPEA	N,N-Diisopropylethylamine
DMF	N,N-Dimethylformamide
E,Glu	Glutamic acid
E.coli	Escherichia coli

Et_2O	Anhydrous diethyl ether
F, Phe	Phenylalanine
FAM	Carboxy-fluorescein
FITC	Fluorescein isothiocyanate
Fmoc	Fluorenylmethyloxycarbonyl, N-terminal protection group
G,Gly	Glycine
g	Gram
H, His	Histidine
h	Hours
HBTU	O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate
HPLC	High pressure liquid chromatography
HNP	Human Neutrophil antimicrobial Peptides
HBD	Human Beta-Defensin
I,Ile	Isoleucine
K,Lys	Lysine
L,Leu	Leucine
LMW	Low molecular weight
M, Met	Methionine
mg	Milligram
MIC	Minimum inhibitory concentration
min(s)	Minute(s)
mL	Millilitre
mm	Millimetre

m/z	Mass to charge
N, Asn	Asparagine
$NaH_2PO_4 \cdot 2H_2O$	Sodium phosphate monobasic dihydrate
nm	Nanometre
P, Pro	Proline
P.aeruginosa	Pseudomonas aeruginosa
PBS	Phosphate buffer saline
P/L	Ratio, powder phase to liquid phase
PMMA	Poly (methyl methacrylate)
PTFE	Polytetrafluoroethylene
Q, Gln	Glutamine
R,Arg	Arginine
rpm	Rounds per minute
S,Ser	Serine
SPPS	Solid peptide phase synthesis
S.aureus	Staphylococcus aureus
T,Thr	Threonine
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane
UV	Ultraviolet and visible light
μL	Microlitre
μm	Micrometre
V, Val	Valine
W, Trp	Tryptophan
wt%	Weight percent

XRD	X-ray powder diffraction
Y, Tyr	Tyrosine

CHAPTER1: INTRODUCTION AND LITERATURE REVIEW

1.1 Bone cements and fillers

Today trauma and increased life expectancy result in an increase of bone loss contributing to a significant raise of the artificial bone market [1]. On the one hand, bone defects have usually irregular shape and the gap between the damaged sites is often very narrow. On the other hand, minimally invasive surgical techniques are now required in orthopaedic surgery in order to avoid infection. Therefore injectable bone cements have attracted a lot of attention. There are two main types of bone cements used today; acrylic bone cements(ABCs) and calcium phosphate cements(CPCs) [2]. Poly (methyl methacrylate) (PMMA) bone cement is one of the most successful ABCs developed by Charnley in the 1960s and has played an important role in bone cements for many years [3]. ABCs are prepared by mixing a liquid phase and a powder phase which form a solid through a polymerization reaction [2]. Although they have been widely used, ABCs have many drawbacks. One of them is the exothermic polymerization temperature which ranges from 40 to 110^{o}C leading to bone necrosis. Also, the exothermic polymerisation reaction makes incorporation of temperature sensitive drugs such as antibiotics very difficult [4]. Another disadvantage is the possibility of un-reacted monomer to be released in the bone before polymerisation starts leading to chemical necrosis of bone. The biggest problem of ABCs is that they can contribute to implant aseptic loosening which may lead to the lack of secondary fixation as well as mechanical failure of bone cements [5]. Thus, there was a need for the development of other types of bone cements. In 1982, LeGeros *et al* introduced the concept of CPCs [6] and later Brown and Chow

obtained the first patent on self-setting CPCs in 1986 [7-9]. Since 1996, the Food and Drug Administration approved CPCs to be used for the repair of human cranial defects [8]. They can be used for bone repair as substitutes or regeneration materials in areas such as the treatment of fracture defects, dental and orthopaedic surgery, craniofacial or maxillofacial reconstruction, surface biomaterials for artificial joints, etc. They can also be used as drug delivery systems [10-14]. Several significant advantages of CPCs have been recognised:

(1)CPCs are injectable. Although this is a common feature with ABCs, it is an important breakthrough in the area of bioceramics. The excellent mould ability, that CPCs exhibit results in a perfect fit of cements around the shape of the bone cavity and implant. This represents an optimum tissue-biomaterial contact leading to decreased possibilities for invasive surgery and can result in bone ingrowth stimulation [15]. In this case, CPCs and ABCs are called bone cements, but the properties, the chemical nature, and applications are very different, where the CPCs show their own superiority. The differences are summarized in Table 1.1 [2].

(2)The most outstanding feature of CPCs is their biocompatibility (ISO 10993) and bioactivity. The biocompatibility and bioactivity can allow the CPCs to perform the requested function in the host and do not induce any unacceptable harm in the beneficiary of the medical therapy. At the same time, the tissue of the implant recipient forms a direct bond with the material [16,17]. In this case, the CPCs also show osteo-conductivity offering a good substrate for osteo-genesis [3, 14,18]. Some authors reported that CPCs such as tricalcium phosphate and rhenanite ($CaNaPO_4$), are osteotransductive. Osteotransductive is a term, used to describe the in vivo behaviour of materials such as the above mentioned, that upon implantation they show fast osteointegration but later they are resorbed and slowly replaced by new

bone without leaving any gap between material and bone and without losing mechanical integrity [19].

(3)CPCs can set at body or room temperature and therefore their use cannot lead to bone necrosis by hyperthermia. For this reason, the setting reaction offers good conditions for many types of drugs or biological molecules to be incorporated. Nowadays, CPCs attract a lot of attention in the area of drug delivery [10].

(4)The CPC's setting reaction through the dissolution and precipitation process which can avoid the drawbacks of a polymerisation reaction as in the case of ABCs the polymerisation of which can lead to the release of toxic polymerisation by-products[15].

Table 1.1: Comparison of acrylic bone cements versus calcium phosphate bone cements [2]

	Calcium phosphate bone cements	**Acrylic bone cements**
Material	Ceramic	Polymer
Liquid phase	Water or aqueous solutions	Methyl methacrylate monomer
Powder component	Calcium phosphate	Three basic ingredients: PMMA (89wt%),Benzoyl peroxide(0.75wt%) & Barium sulphate or zirconium dioxide (10wt%)
Setting reaction mechanism	Dissolution-precipitation reaction	Polymerization
Reaction products	Brushite or Hydroxyapatite	Poly-methyl-methacrylate (PMMA)
Exothermic temperature during setting	No-exothermic	Exothermic 40-110 °C
Bioactivity	Bioactive	Inert

1.2 Chemistry of calcium phosphate cements

CPCs can be produced by the mixture of a powder (that may also contain fillers) with water or an aqueous solution containing binders; A paste can be produced by mixing the water and the powder which sets at body or room temperature. During the setting reaction, one or more calcium phosphate crystals form as nuclei and the precipitate sets around the entanglement of the crystal nuclei [3]. The setting process involves 3 stages [20]:

(1) *Dissolution:* the powder dissolves in the liquid phase until saturation of the liquid with calcium and phosphate ions.

(2) *Nucleation:* the concentration of the reactant ions arrives at a critical peak in the liquid phase and leads to the formation of crystallisation nuclei.

(3) *Crystallisation*: crystals of the new phase grow in this plastic paste and the system begins to harden [20].

The process is shown in Figure 1.1. Depending on the setting reaction conditions, there is a possibility of the formation of two products: brushite (DCPD) and precipitated hydroxyapatite (HA).

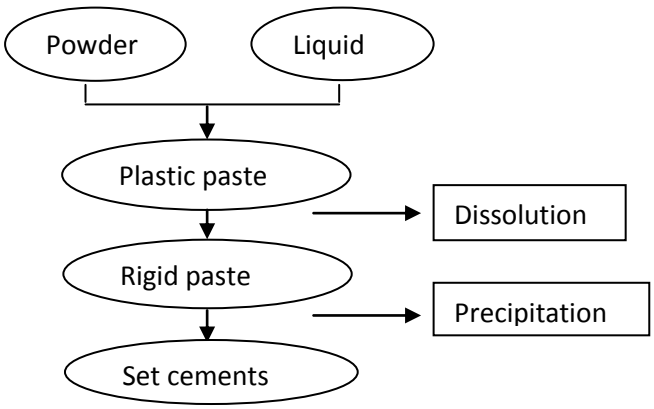


Figure 1.1: The process of CPC's setting reaction

1.2.1 Brushite

Brushite is known as dicalcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$) and is formed via an acid setting reaction. It is more stable when the pH ranges between 2 and 4.2[10]. There are two main ways to form brushite [21,22].

1. $\beta\text{-}Ca_3(PO_4)_2 + Ca(H_2PO_4)_2\ 2H_2O + H_2O \longrightarrow 4CaHPO_4 \cdot 2H_2O$

2. $\beta\text{-}Ca_3(PO_4)_2 + H_3PO_4 + 6H_2O \longrightarrow 3CaHPO_4 \cdot 2H_2O$

The brushite cements are resorbable compared to hydroxyapatite (HA) and their mechanical strength is lower than HA [23]. Furthermore, DCPD also tends to transform into HA after implanted in the body [24].

1.2.2 Hydroxyapatite

The chemical composition of HA [$Ca_{10}(PO_4)_6(OH)_2$] is close to the mineral component of human bones which have the apatitic structure [25]. The apatite formula can be presented as $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$, where x=0-2. The stoichiometric HA has x=0 whereas the calcium-deficient hydroxyapatite has x=1[26]. The big difference in order to distinguish HA from brushite is that apatite is most stable at pH above 4.2 [10]. There are several ways to form HA as follows[27,28]

1. $7Ca_4(PO_4)_2O + 2Ca(H_2PO_4)_2 \cdot H_2O \rightarrow 3Ca_{10}(PO_4)_6(OH)_2 + 3H_2O$

2. $3Ca_4(PO_4)_2O + 3H_2O \rightarrow 3Ca_{10}(PO_4)_6(OH)_2 + 2Ca(OH)_2$

3. $2Ca_4(PO_4)_2O + 2CaHPO_4 \cdot 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 4H_2O$

4. $2\beta\text{-}Ca_3(PO_4)_2 + 2CaHPO_4 + 2CaCO_3 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 2CO_2$

All the above methods could result in HA cement. But considering the experimental conditions, method 4 is the easiest way to synthesise hydroxyapatite in the lab. Although HA cements exhibit all the advantages of CPCs such as excellent mouldability, biocompatibility, bioactivity, osteoconductivity, non-exothermic setting reaction and better mechanical properties compared to brushite, HA cements have one drawback; the porous structure of HA cements can absorb bacteria which can cause potential infections [29].

1.3 Bone infections

A patient can be infected not only due to HA cements that can absorb bacteria, but also due to the surgical site and procedure. Some data show that worldwide, the number of hip replacements can reach approximately 1 million per annum [30]. Based on the large amount of patients with orthopedic implants, the risk of infection is only 0.5-5% for joint infections but due to the large number of patients infections remain a big problem [30, 31]. In order to solve this problem, the use of drugs and antibiotics incorporated in CPCs has been studied [10, 40-47, 59-61].

The most common bacteria that lead to infections during an operation are Gram-positive and Gram-negative bacteria [32-35]. In the report from the Public Health Laboratory Service Communicable Disease Control Centre, 41.2% of the joint infections were caused by Gram-positive bacteria and most of them were caused by Staphylococcus aureus (*S.aureus*); 21.8% were caused by Gram-negative bacteria such as Escherichia coli (*E. coli*) and Pseudomonas aeruginosa (*P.aeruginosa*) [36]. For this reason, *S.aureus*, *P.aeruginosa* and *E.coli* are good candidates in order to test the antibacterial capacity of one material or drug.

1.4CPCs as drug delivery systems

CPCs exhibit two characteristics --low exothermal setting reaction and a self-setting in vivo ability. Both of these two characteristics are advantageous for antibiotics or other temperature sensitive molecules to be incorporated in CPCs [37]. On the other hand, biologically active molecules may change some properties of CPCs. Generally, the physico-chemical properties (e.g. setting time, porosity, pH & mechanical properties) and release kinetics of CPCs have been studied extensively [15].

1.4.1Physico-chemical properties of CPCs incorporating low molecular weight drugs(LMW)

Compared to calcium phosphate ceramics when acting as drug delivery systems, CPCs cannot only adsorb the drug molecules on their surface but also they can incorporate drug molecules throughout the whole volume of the cements by blending for example an antibiotic solid in the powder phase or dissolving them in the liquid phase [29, 38, 39]. When the LMW drug dissolves in the water phase, the drug molecule will be homogeneously distributed within the cement when mixing the liquid phase with the powder phase [10].

Generally, the incorporation of LMW drugs can increase the setting time and porosity of apatite CPCs and if the LMW drug is mixed with the liquid phase, the setting reaction will be longer than if the drug was mixed with the solid phase. Moreover, increasing the concentration of LMW drug could also increase the setting time and the porosity [40,41]. The increase in the setting time may be due to LMW drug chelating Ca^{2+} ions instead of phosphate ions reacting with calcium ions [10,42,43]

The mechanical properties of antibiotic loaded-CPCs have the opposite tendency with setting time. It is believed that addition of antibiotics always decrease the mechanical properties of the cements [44]. Furthermore, an increase in porosity or inhibition of the setting reaction may contribute to the decrease of mechanical properties [38]. During the setting, pH changes may result to partial denaturation of the antibiotic activity. Generally, no significant changes have been observed. However, some authors reported that pH changes during the setting reaction may result in the loss of bactericidal behaviour of antibiotics [45].

1.4.2LMW release kinetics from CPCs

The distribution of the drug in the CPCs determines the kinetics of drug release. When the drugs are incorporated with the powder phase, the paste tends to precipitate a new mineral phase. When the drugs are incorporated with the liquid phase on the other hand, a small amount of drugs may interfere with the crystallisation. The drugs will remain blocked between the entangled crystals in one of the three ways shown in Figure1.2 [10].

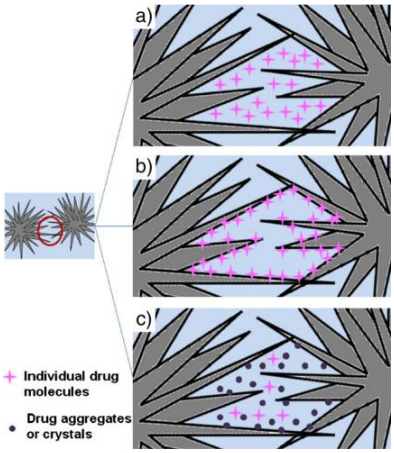


Figure 1.2: Drug in CPCs' matrix; a) together with the liquid phase which is present in the pores between crystal, b) present or bonded with the surface of the new mineral phase, or c) in the form of a solid if the amount added is not all dissolved in the liquid phase [10].

Generally, the LMW drugs do not have any effect on the phase formed during the setting reaction. The setting reaction is governed by diffusion throughout the CPCs' pores. In the first 24 hours, a burst release of the LMW drug from the CPCs occurs [39,46,47] and the LMW drug incorporated in the liquid phase will have higher elution efficiency than the ones incorporated in the solid phase [41].

Nowadays, the most popular biological molecules in the drug delivery systems are antibiotics such as gentamicin, vancomycin, ciprofloxacin or doxycycline [10]. Many researchers have applied these antibiotics in CPC delivery systems and received some excellent antibacterial bone cements. But their bacterial resistance can still be a problem. If the release rate of antibiotics from CPCs is not fast enough to reach the minimum inhibitory concentration (MIC) of the antibiotic this can contribute to bacterial resistance [15,48,49]. To avoid bacterial resistance, antimicrobial peptides have attracted a lot of attention.

1.5 Antibacterial peptides

Antimicrobial peptides (APs) are low molecular weight natural compounds composed of no more than 50 amino acids [50-52]. APs act as the first line of defence against bacterial invasion in almost all forms of life [53]. APs kill bacteria very fast and have a broad spectrum of antibacterial activity. Moreover, they are cytotoxic against fungi, protozoa, cancer cells, and even viruses [51,54-57].

The structure of APs consists of the primary structure, the secondary structure and the tertiary structure. The primary structure is the peptide sequence formed by the reaction of amino acids from the N-terminus (amine) to C-terminus (carboxylic acid)forming a peptide bond (HN-C=O).

The secondary structure is the three-dimensional structure of APs and it is classified into four families: alpha, beta, combined alpha-beta and non-alpha-beta (see Figure 1.3) [93]. The alpha family consists of AMPs with helical structures. The beta family is composed of AMPs with beta-strands. While the alpha-beta family comprises of both helical and beta-strands in the 3D structure, the non-alpha-beta family contains neither helical nor β-strands [93]. The diversity of APs discovered is too great to sort them except categorize them by the second structure [51]. Until February 2015, the Antimicrobial Peptide Database [94-95] (http://aps.unmc.edu/AP/main.php) contained 2497 antimicrobial peptides. Table 1.2 presents the repartition of these 2497 APs among the different classes.

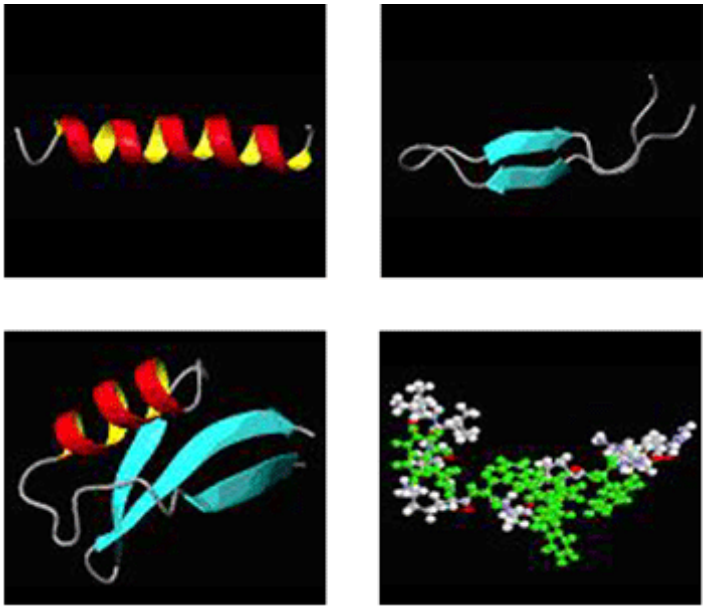


Figure 1.3:3D model structures of the four classes of peptides. (a) alpha peptide (b) beta peptide (c) combined alpha-beta peptide (d) non-alpha-beta peptide [93]

Table 1.2: Repartition of these 2497antimicrobial peptides among the different structural classes obtained from the Antimicrobial Database.

Secondary Structure	APs Number	% of total
α-Helix	349	14
β-Strands	101	4
Combinedα- and β-structure	93	4
Neither α- nor β-structure	470	19
Unknown 3D structure:	1467	59

The tertiary structure is the hydrophilic and hydrophobic properties of amino acids on the peptide, which could induce interaction with membranes. The side-chain can make an amino acid hydrophilic if the side-chain is polar or hydrophobic if the side chain is non-polar. Therefore, as shown in Figure 1.4, if the side amino acid of the chain is hydrophilic, peptides will point out to the polar environment and point in to the non-polar environment. On the other hand, the hydrophobic side chain will point in to the polar environment and point to the non-polar environment. Based on the different reaction mechanism, some researchers suggested that the different hydrophobic or hydrophilic amino acids on the side peptide chain should affect the antimicrobial activity of the peptides [58].

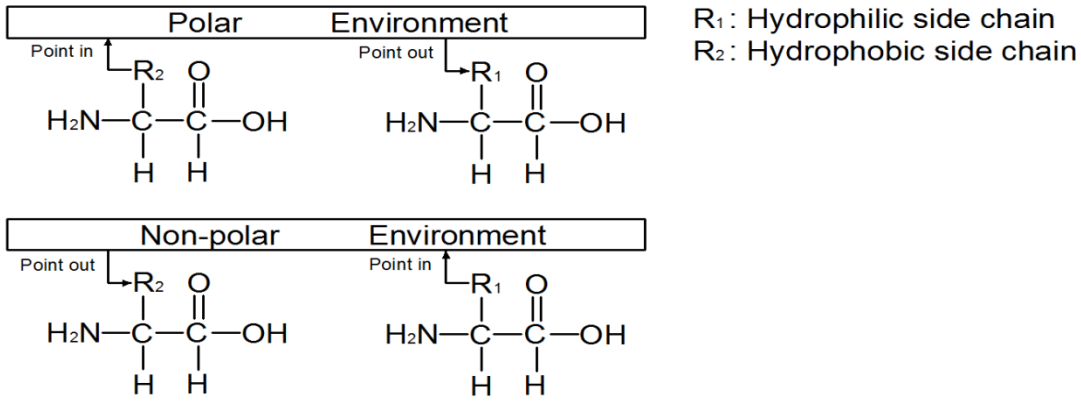


Figure 1.4: The relationship between environment and hydrophilic and hydrophobic amino acid.

Based on the Antimicrobial Peptide Database, there are 2497 antimicrobial peptides that have been discovered, 2061 of which have an antibacterial activity. They were found from different sources like frog, human, sheep, bacteria and etc. [53]. The most APs present a broad activity spectrum and against the *S.aureus, P.aeruginosa* and *E.coli* [51]. Two of them already have been used in bone cements to prevent infection

and their sequence is shown in Table 1.3 [59-61, 71, 81]. Stallmann *et al* [59-61, 71] combined both hLF1-11 and DHVAR-5 with several Calcium Phosphate and PMMA carriers studied in vivo and vitro to prevent the infection [59, 60, 96]. However, their impact on the infection was not ideal due to the poor release of hLF1-11 and DHVAR-5 [59-61, 71, 96]. Human defensins could possibly be an alternative way of tackling bone infection.

Table 1.3: Two antibacterial peptide used in bone cements and their sequence [71]

Peptide	Structure
DHVAR-5	LLLFLLKKRKKRKY
hLF1-11	GRRRRSVQWCA

1.5.1 Defensins

Defensins are small proteins extracted from granulocytes which have 30-40 amino acid residues and a molecular weight of around 3-4KD [62]. The main characteristic of defensins is that they are cysteine-rich and arginine-rich cationic peptides [62,63]. The primarily attractive property of defensins is the broad spectrum of antimicrobial activities, moreover, they widely exist in all vertebrates, invertebrates and plants [62,64,65]. Defensins could be classified as α-defensins and β-defensins which differ in the length of the peptide segments between the cysteines and the pairing of the cysteines that are connected by disulphide bonds [63,65]. The main mechanism of defensins to destroy microorganisms is through permeability of the plasma membrane resulting in leakage of cells content [66].

So far, 10 kinds of different human defensins have been discovered. Six of them are human α-defensins and four of them are human β-defensins [67]. Human α-defensins

are rich in arginine and contain 29-35 amino acids with three disulfide bridges connecting residues Cys^1- Cys^6, Cys^2- Cys^4 and Cys^3- Cys^5. Human β-defensins contain 35 amino acid and the disulfide bridges which are different from α-defensins are Cys^1- Cys^5, Cys^2- Cys^4, and Cys^3- Cys^6. The sequences and disulfide bridges of human defensins are shown in Table 1.4 [68-69, 91]. The letter shown in Table 1.4 is the abbreviation of amino acid, as shown in ABBREVIATIONS part on the page XIII to XVI.

The main advantage of human defensins is that they were found in the human body and they exhibit a mechanism of resistance which is very old in evolutionary terms. In addition, unlike conventional antibiotics such as penicillin, which microbes readily circumvent, the defensins could result in minimal induction of bacterial resistance. [53, 70-71]. It is shown clearly from Table 1.4 that the sequence of some of the defensins is still too long compared to the antimicrobial peptides used in bone cements before (Table 1.3) [59,60,71,72]. Recently, there is a lot of interest in mimicking the structures of natural defensins and synthetic defensins comprised of 7-15 amino acids [73].

Table 1.4: The human defensins sequences [68-69, 91].

Name	Sequence
α-defensins	
HNP1	ACY---CRIPACIAGERRYGTCIYQGRLWAFCC
HNP2	CY---CRIPACIAGERRYGTCIYQGRLWAFCC
HNP3	DCY---CRIPACIAGERRYGTCIYQGRLWAFCC
HNP4	VCS---CRLVFCRRTELRVGNCLIGGVSFTYCC TRVD
HD5	ARATCY---CRTGRCATRESLSGV C EISGRLYRLCC R
HD6	TRAFTCH---CRR--SCYSTEYSYGTCTVMGINHRFCC L
β-defensins	
HBD 1	GNFLTGLGHRSDHYNCVSSGGQCLYSACPIFTKIQGT CYRGKAKCCK
HBD 2	GIGDPVTCLKSGAICHPVF CPRRYKQIGTCGLPGTKCCKKP
HBD 3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK
HBD 4	EFELDRICGYGTARCRKK CRSQEYRIGRCPNTYA CCLRKWDESLLNRTKP

1.6 Aims and objectives

The main aim of this thesis is to isolate short fragments from the antimicrobial core of human defensins that can have significant antimicrobial properties and incorporate them in CPCs in order to make antimicrobial bone cements.

Based on the advantages of human defensins mentioned above, α- and β- human defensins are the target defensins to be isolated. To select the sequence from human defensins, 2 principal elements are taken into account. Firstly, as in cysteine-rich cationic peptides mentioned above, the peptide sequence should have at least one cysteine. Secondly, for the reason shown before that the hydrophobic or hydrophilic amino acid on the side of the chain may bring different antibacterial activity, four kinds of different amino acids on the sequence side will be developed; hydrophobic-

hydrophobic, hydrophilic-hydrophilic, hydrophobic-hydrophilic and hydrophilic-hydrophobic. Thus, four peptide sequences were selected from α- and β-defensins shown in Table 1.5.

In this study, mass spectrometry and HPLC were used to identify the molar mass and purity of peptides. The antibacterial property was studied by measuring the minimum inhibitory concentration (MIC) of peptides against *S.aureus, P.aeruginosa* and *E.coli*. The pH, antibacterial properties, peptide release and mechanical properties (compressive strength) of bone cements were measured against different concentrations of peptides.

Table 1.5:Antibacterial peptide sequences studied in this project

Code	Sequence
AP1	FAM-Ahx-PACIAGERRYG
AP2	FAM-Ahx-CATRESLSGVC
AP3	GTCGLPGTKCC
AP4	FAM-Ahx-CRVRGGRCA

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

All of the materials used in the peptide synthesis were purchased from Sigma-Aldrich and Novabiochem, UK. All N-terminals of the amino acids were protected with Fluorenyl-methyloxy-carbonyl (Fmoc) groups and the side chain was also protected with proper protecting groups. The amino acid and resin beads which were purchased from Sigma-Aldrich were: Fmoc-Arg-OH, Fmoc-Tyr-OH, Fmoc-Ser-OH, Fmoc-Val-OH, Fmoc-Thr-OH, Fmoc-Glu-OH, Fmoc-Ile-OH, Fmoc-Cys-OH as well as the solvents and reagents used for SPPS (Solid Peptide Phase Synthesis) and cleavage cocktail components: anhydrous dichloromethane (DCM,≥99.8%), anhydrous N,N-dimethylformide (DMF, ≥99.8%), N,N-diisopropylethylamine (DIPEA, Biotech. grade,≥99.5%), Carboxyfluorescein (FAM, BioReagent, HPLC≥95%), piperidine (Biotech. grade, HPLC≥99.5%), ninhydrin (Reagent grade), trifluoroacetic acid (TFA, HPLC grade, ≥99%) and triisopropylsilane (TIPS,≥99%). The amino acid and amino acid preloaded Wang resin beads were obtained from Novabiochem were: Fmoc-Ala-OH, Fmoc-Ala-Wang, Fmoc-Gly-OH, Fmoc-Gly-Wang, Fmoc-Cys-Wang, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH and Fmoc-Pro-OH. The purity of all amino acids was above 98%. The anhydrous diethyl ether (Et_2O) of HPLC grade for the precipitation of peptides was purchased from Fisher Scientific. The Phosphate Buffer Saline (PBS) pH=7.4 used for the release studies and dissolving peptides was purchased from Sigma-Aldrich. The vessels with cap and fritted disc named Aldrich® system 45™ which were used in peptide fluorescence labelling and resin cleavage, were purchased also from Sigma-Aldrich.

The materials used for HA bone cements were Calcium phosphate dibasic ($CaHPO_4$), β-tri-calcium phosphate [β-$Ca_3(PO_4)_2$], Calcium carbonate ($CaCO_3$) and Sodium phosphate monobasic dihydrate ($NaH_2PO_4\ 2H_2O$). All of them were purchased from Sigma-Aldrich and with the purity of over 97%. All of the materials were used as received. A Hydrion® Insta-Check® 0-13 pH test paper was used for the cement pH measurements test and was purchased from Sigma-Aldrich.

2.2 Antibacterial peptide synthesis

The peptide synthesis was based on the reaction between the amine group (N-terminal) and the carboxylic acid (C-terminal) from two different amino acids to form an amide bond which is also known as a peptide bond. Based on the fact that each amino acid has both C- and N- terminals, the results of two amino acids reaction cannot be controlled in a particular way. In order to solve this problem, Fluorenyl-methyl-oxycarbonyl (Fmoc) based Solid Peptide Phase Synthesis (SPPS) was used here. [74]

2.2.1 Peptide synthesized by the Fmoc-SPPS method

The principle of SPPS is shown in Figure 2.1. All of the amino acids were N-terminal protected by a base-labile Fmoc group in SPPS which could be removed by the use of piperidine. The C-terminal of the first amino acid as well as all the peptide side chains were protected by an acid-labile protecting group, which could be cleaved by trifluoroacetic acid (TFA) [74]. This method has the advantage that the Fmoc removal mechanism is different from the side chain and the Wang resin beads. [74] Thus, the peptide formed should have the desired amino acid sequence. After loading the first amino acid, the unprotected N- terminal of the first amino acid reacts with the unprotected C-terminal of the second designed amino acid to form a peptide bond.

Following this strategy, the peptide sequence assembled from C-terminal to N-terminal step by step. Before every amino acid was added in, piperidine was added to cleave the Fmoc protection groups from the former amino acid. Furthermore, the amino acid could not couple automatically at room temperature and therefore O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and N,N-Diisopropylethylamine (DIPEA) were used as coupling reagents to form reactive intermediates of carboxylic acid assisting the amino acid reaction at ambient temperature [75].

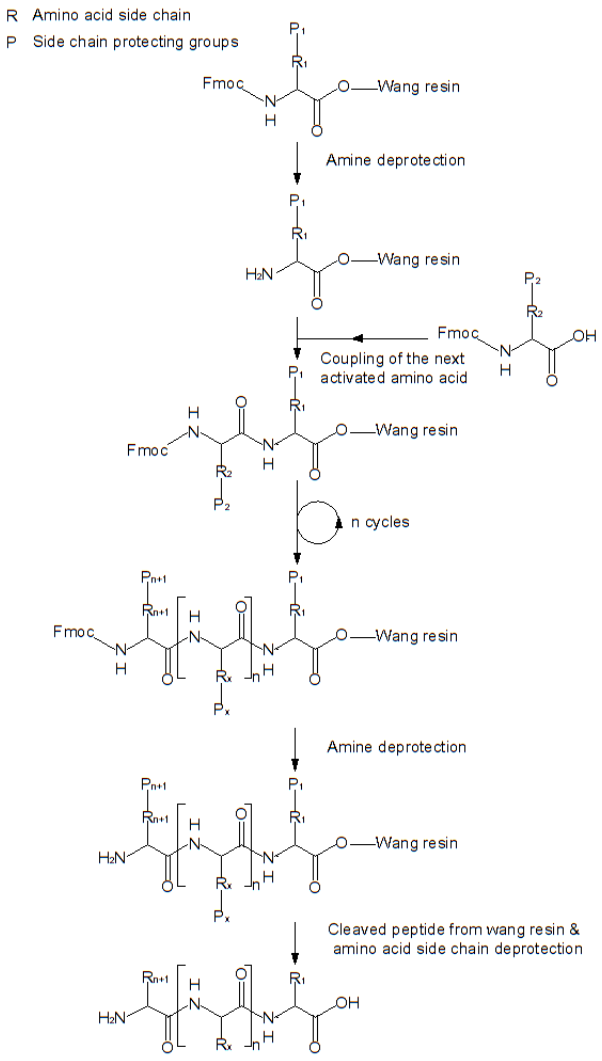


Figure 2.1: Principles of SPPS [74].

In this study, the C-terminal of the first amino acid was protected by Wang resin. All amino acids and the Wang resin beads used were already preloaded with the Fmoc-groups. Peptides were synthesized using a CEM Liberty 1 microwave peptide synthesizer using standard SPPS with Fmoc strategy mentioned above. The synthesizer was kindly offered by Dr Anna Peacock in the School of Chemistry, University of Birmingham. The peptide sequences are shown in Table 1.5. AP1, AP2 and AP4 were fluorescence labelled at the N-terminal of the peptide sequence with carboxyfluorescein (FAM). Considering the bulky FAM may have affected the function of the peptide sequence, the 6-Aminocaproic acid (Ahx) was loaded to decrease the impact.

2.2.2 Fluorescence labelling of the peptide sequence

The final peptide product synthesized by the microwave peptide synthesizer, contained the Wang resin, the Fmoc group of the last amino acid and all the side chain protecting groups. The first step was to remove the Fmoc groups, then coupling the linker (Ahx) and labelling with FAM at the end.

After taking out the peptide from the synthesizer, the peptides designated by fluorescence label were moved into a test tube to be weighed and then transferred to a vessel for amine de-protection. Peptides were let to swell in 2mL DMF for 1 hour and then reacted with piperidine in a DMF solution (1:5, v:v) for 30 mins to remove the Fmoc groups. The reaction solution was shaken by an orbital shaker at 200 rpm. After shaking, the solution was vacuum filtered and the remaining solution was discarded. The peptide beads were triple washed by DMF (3mL), then, triple washed by DCM (3mL) and triple washed by DMF (3mL) again. For the labelling of the peptides the following procedure was followed: Fmoc-Ahx-OH (5 eq.) and HBTU (4.9 eq.) were

dissolved in DMF (2mL) in a test tube with a vortex mixer for pre-activation. Two minutes later, DIPEA (10eq.) was added in the same test tube. Then, the mixture was added to the vessel with the peptide for reaction under shaking conditions for 1-2 hours depending on the results of the Ninhydrin test [92]. Ninhydrin test was applied here in order to test whether the peptide coupling was complete. The procedure requires that 2mL of aqueous ninhydrin solution (5g/L) was added into a small amount of resin beads in a test tube. The test tube was left in boiling water for 3mins and then the colour change of the beads was recorded. If there was no colour change or the beads colour changed to brown, the coupling was complete. If the colour of beads turned to blue, the coupling step was incomplete. After the coupling reaction, the beads were vacuum filtered and the remaining solution was discarded. The resin beads were washed 3 times × DMF (3mL), 3 times × DCM (3mL) and 3 times × DMF (3mL).

FAM (2 eq.) and DIEPA(16 eq.) were dissolved in DMF (2mL) solution in a test tube with a vortex mixer for 10 minutes.[74] This solution was added in the vessel to react with the resin beads overnight under shaking conditions at 200 rpm. Considering that FAM is sensitive to light, aluminium foil was used to cover the vessel during the reaction. The reaction was also tested by the ninhydrin test, the procedure of which was described above. After all coupling was completed the resin beads were washed 4 times in DMF (3mL) and 4 times in DCM (3mL). Then vacuum filtration was used to dry the resin beads for 15 minutes.

2.2.3 Wang resin cleavage and side chains de-protection from the peptide

As mentioned above, the Wang resin and the amino acid side chain protecting groups were all acid-labile by TFA. The procedure is described below: Firstly, the dry resin

was weighed and placed in a clean round bottom flask. Then the cleavage cocktail made by mixing trifluoroacetic acid (TFA), triisopropylsilane (TIPS) and deionised water (95:2.5:2.5, v:v:v) was added to the dry resin in the flask (10mL per 100mg of resin) and the mixture was left to be stirred for 2 hours [74]. Aluminium foil was wrapped around the flask to prevent the light access.

2.2.4 Precipitation of peptides

The round bottom flask used to filtrate the peptide solution, was washed by TFA cocktail solution. Then the peptide solution was filtrated and the resin beads were removed by filtration. The solution was then left in vacuum for 2-3 minutes for TFA to evaporate. The solution was added to ice cold diethyl ether (Et_2O) for precipitation and the precipitate was left in a freezer at -20℃ for 2 hours. The peptide solid was then separated from the supernatant by centrifuging at a frequency of 13,500 rpm for 3 minutes in a test tube and then air dried in a freezer for one night -20 ℃. All of the synthesized peptides were stored in a freezer at -20 ℃.

2.3 Peptide characterisation

2.3.1Mass spectrometry

Electrospray mass spectrometry was used to analyse the peptide sequences. The analysis was performed in a Micro mass LCT instrument situated in the mass spectrometry facility of the School of Chemistry.

2.3.2High pressure liquid chromatography

High pressure liquid chromatography (HPLC) is a technique with which the peptide can be identified and purified. With this technique, the solution containing the peptide mixture with gradient of solvents (mobile phase) was pumped through a column

containing solid adsorbent materials (stationary phase). Each component in the peptides can interact with the adsorbent in a different way and therefore the flow rates of each component will be different. In this way, the components can be separated at different flow times out from the column which will also correspond to a different peak in the spectrum.

HPLC using the analytical Dionex Summit equipment was applied to study the purity of the peptides. Preparative HPLC was used for the crude peptide purification. The above equipment is located in the School of Chemistry, University of Birmingham. During this technique, the stationary phase was the silica bonded by octadecyl carbon (C18) chain and the mobile phase consisted of a gradient of solvents, inorganic and organic solvents. In this case, the inorganic solvent consisted of water with 0.05% TFA and the organic solvent consisted of acetonitrile with 0.05% TFA. UV (λ = 210 nm) was used to measure eluted molecules, which matches the peptide absorption region.

For the analytical HPLC, each 10 μL of the aqueous peptide solution was injected into the column and the column was run for 60 minutes. In these 60 minutes, a linear solvent gradient where the 100% was inorganic solvent and 0% organic solvent was used. After that, 40% water and 60% acetonitrile were applied. By increasing the organic solvent, more hydrophobic molecules were eluted from the column. The calculation of purity was based on the integration of peak areas.

The preparative HPLC follows a similar procedure with the analytical HPLC. Different peaks represent different components. In order to purify the peptide, a particular component flows out of the column and is collected. Only fluorescence

labelled peptides were collected. The collected peptides were identified by mass spectrometry.

2.3.3 Minimum inhibitory concentration (MIC)test of peptides

2.3.3.1 Preparation of bacteria

In order to test the antibacterial properties of peptides, bacterial solutions were made. *S.aureus (NCTC 8532)*, *P.aeruginosa (PA01)* and *E.coli (MG165S)* strains were on the agar plate separately. The bacteria were kindly provided by Dr Mark Webber in the School of Immunity and Infection. One bacterial bead from the bacterial agar plate was taken into a sterile plastic bottle containing 5mL of sterilisation broth. One sterile bottle containing 5 mLof sterilisation broth was the control sample. All bottles with the different bacteria and the control sample were placed in an oven at 37 ℃ overnight under shaking conditions. The next day, if the broth solution in the control bottle was clear and the one in the bacteria bottle was cloudy, the bacterial preparation was successful and the bacteria culture was ready to use.

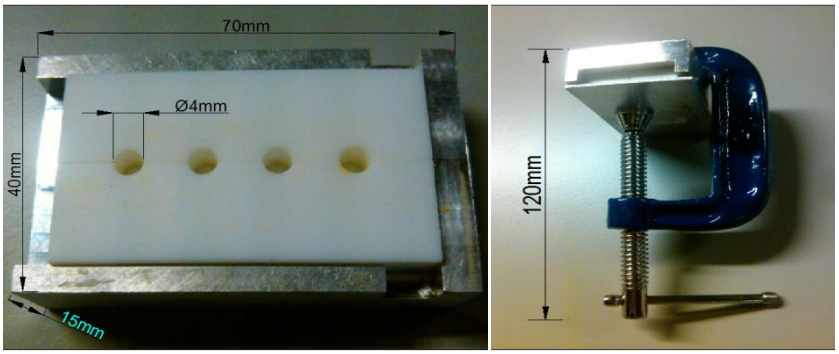
2.3.3.2 MIC test of peptides

Peptides were dissolved in deionised water. A 96 well cell plate was used in order to perform an MIC test. Using a multi-stepper pipette, 50μL of broth were added to the wells from the second column to the end. 50μL of peptide solution was added to the first and second column. Then using a multi-channel pipette 50μLof double diluted peptide solution was added from the second column to the last second one and the remaining 50μL of peptide solution were removed and thrown away. The bacteria (*S.aureus*, *P.aeruginosa* or *E.coli*) broth culture had to be diluted twice to reach a concentration of 10^7 CFU/mL (1:100). By using a 100μL Gilson pipette, 50μLof

diluted organism solution was transferred to the first raw of the well plate ready to examine. Finally, a sterile lid was used to cover the plate that was left in the oven for 18 hours at 37 ℃. After 18 hours, the MIC was the concentration of the last clear well. All peptides were tested three times.

2.4 Hydroxyapatite bone cements preparation

The HA cements used in this study was made by mixing the powder and liquid phase together. Considering the stoichiometric ratio of HA is Ca/P = 1.67, the powder phase consisted of equal moles of dicalcium phosphate anhydrous, calcium carbonate and β-tricalcium phosphate (β-TCP). All these three powders were mixed together and were shaken for 1 hour [28]. The liquid phase was 3 wt% of NaH_2PO_4 $2H_2O$in distilled water. The ratio of the powder to liquid phase was P/L= 0.4 mL/g. The powder was then mixed with the liquid phase in the ratio of 0.4 mL/g for about 1 min and then the mixture was poured into PTFE moulds [Figure2.2 (a)] [28]. The mould was covered by a stainless steel cover and the mould was fixed into place for 48 hours at 37^{o}C with the use of a G-clamp device shown in Figure 2.2 (b).



(a) (b)

Figure2.2: (a) HA cements PTFE mould. (b) The HA cements PTFE mould covered by a stainless steel cover and fixed with a G-clamp device.

2.5 Characterization of HA bone cements

2.5.1 X-ray powder diffraction (XRD)

X-ray Powder Diffraction (XRD) was used in order to identify the phase of HA bone cements. Before testing, HA cements were ground into a powder. A Philips X-Pert XRD with a continuous scan between 2θ = 10° to90° and a step size of 2θ = 0.02 °was used.

2.6 Peptide loading in HA cements.

Different concentrations of APs2, 6 and 8 wt%, were added in the liquid phase of bone cements before mixing. This procedure was performed in a container covered by aluminium foil in order to protect the solution from light exposure. The cements were then prepared following the steps described in section 2.4.

2.7 Characterization of antibacterial HA bone cements

2.7.1 Compressive strength mechanical testing

The compressive strength (CS) of antibacterial HA bone cements was performed on a screw-driven Instron machine (Model 1195, Instron Corporation, High Wycombe, UK) filled with a load cell of30kN and using a crosshead speed of 1 mm/min. As chematic illustration of the force distribution in the CS testing is shown in Figure 2.3[76]. All of the cements were packed in a PTFE mould (4 mm × 6mm). After the cements setting at 37^{o}C as described above, samples were kept in a Phosphate Buffer Saline (PBS) for 1 day or 2 days. During the CS study 15 samples of each concentration was tested. The compressive strength was calculated by the equation below:

$$CS = (4 \times P)/(3.14 \times d^2)$$

where, **P** represents the maximum force applied at fracture and **d** represents the diameter of the sample cylinder.

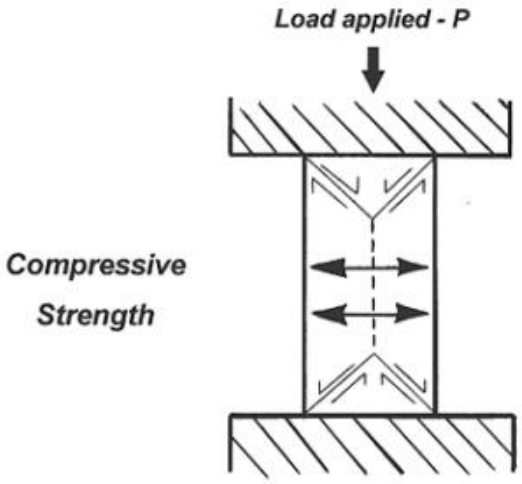


Figure 2.3: Schematic representation of the force distribution in the compressive strength testing [76].

2.7.2 In vitro release experiments

HA cements with 0, 2, 6 and 8wt% of antibacterial peptide were placed (one disc per well) in the 24 well cell culture plates and immersed in 1mL of PBS at 37℃. Each time 50μL of solution was taken out from each cell after 0.5, 1, 1.5, 2, 2.5, 3, 4, 8, 12, 24 and 48h. The solution taken out was placed in a 96 well cell plate and analysed with a fluorescence micro plate reader. The test excitation wavelength was 485 nm and the emission was at 535nm. During the release study three samples of each

concentration were tested. The solutions were protected from the light by aluminium foil.

A calibration curve was made in order to calculate the concentration of the release solution. A 50μL peptide solution with the concentration already known was tested by a fluorescence micro plate reader. The data in the curve was fitted to a straight line. In addition, the linear fitting equation is y=13,923,915.86 x. (R^2=0.98)

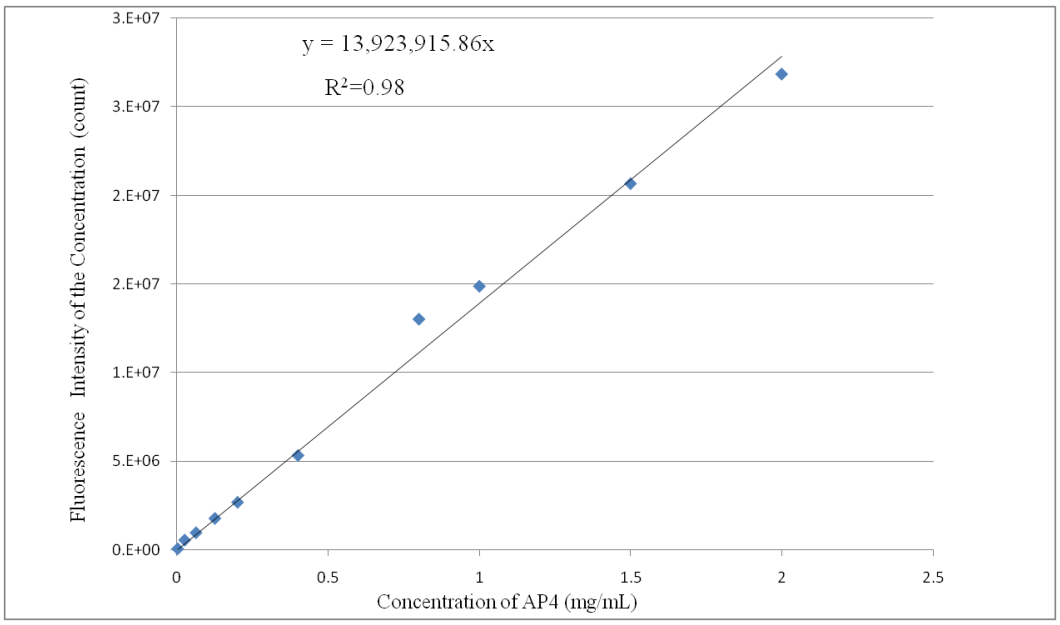


Figure 2.4: Calibration curve of peptide concentration.

2.7.3Antibacterial test of HA cements

The antibacterial properties of cements and surface of cements were both studied. The antibacterial property was measured at two time points, 24 and 48h after setting. The method used to test the antibacterial properties was kill kinetics: viable counting using the Miles & Misra method [77]. The bacteria used for the antibacterial test were prepared as mentioned before in section 2.3.3.1. HA cements with 0, 2, 6 and 8 wt% of AP4 were placed one disc per well in the 24 well cell culture plates and immersed in 1mL diluted bacterial solution. The 0 wt% of antibacterial peptides was studied as a

control sample. A sterile lid was placed on the plate and parafilm was used to cover the surface of the plate. Then the plate was placed in the oven for 24h at 37 ℃.

After 24 hours, the broth solution around the sample was diluted for 12 times and 10μL of the last four dilute solutions were spread on agar plates and these plates were placed into an oven overnight at 37 ℃. The Colony Forming Unit (CFU) on the plate was checked the next day.

In order to test the bacteria concentration on the surface of the cements, the test sample was taken out to be placed into a new 24 well cell plate and the samples were immersed in 1mL of PBS. Using 1mL pipette, the bacteria were washed out from the surface of the cements into the PBS solution. Then the PBS solution in the well was diluted in the same way as the broth solution described above. Ten micro litres of the last four dilute solutions were spread on agar plates and these plates were placed into an oven overnight at 37 ℃. The CFU on the plate was checked the next day.

2.7.4 pH studies of HA bone Cements

The pH was measured for all cements just after mixing the solid and liquid phase and before the cement pastes were placed into the mould. The pH was measured by a pH test paper and compared with the colour chart supplied by the pH paper manufacturer.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Characterisation of solid peptides

3.1.1 Solid phase peptides synthesis

The peptides were synthesised by the method of Solid Phase Peptide Synthesis (SPPS). The sequence of peptides and the molar mass are presented in Table 3.1 below. It is worth mentioning, that only AP1, AP2 and AP4 were FITC labelled whereas AP3 was not labelled. The reason for this was the antimicrobial activity which was believed to be influenced by the FITC labelling. In order to ensure that the synthesised peptides had the desired amino acid sequence, mass spectrometry was used. In addition, High Pressure Liquid Chromatography (HPLC) was applied in order to determine the purity of the synthesized peptides and finally purify the peptides.

Table 3.1: Peptides sequence and theoretical molar mass of peptides

Code	Peptide sequence	Theoretical molar mass (g/mol)
AP1	(FAM)-Ahx-PACIAGERRYG	1663.85
AP2	(FAM)-Ahx-CATRESLSGVC	1596.78
AP3	GTCGLPGTKCC	1039.27
AP4	(FAM)-Ahx-CRVRGGRCA	1448.6

The mass spectrum of AP1 is presented in Figure 3.1. The Y-axis represents the relative intensity and the X-axis represents the detected ion molecular weight divided by the charge of the ion (m/z). The ionization of the mass spectrum equipment causes the molecules to acquire charges by protonating with one or multiple hydrogens (H, $2H^+$,...nH^+). Other ions such as sodium adduct or sulphate adduct are also possible.

The ion product with different amount of positive charge was detected. The peak position represented the detected ion molecular weight divided by the charge of the ion. Each peak represents one product. It is also observed that, the peaks of the same product but with different isotopic atoms in the molecule result in doublets or triplets and the difference between them will be in the range of one neutron. Due to this, the charge carried by the ion molecule will be known from the difference between the numbers of peaks in the spectrum. For example, if the ion molecule carries 2 charges, one neutron divided by the charge 2 will be 0.5, and if the ion molecule carries 3 charges, one neutron divided by the charge will be 0.33333 and the mass spectrum can approximate this value to 0.3. Therefore, from the difference of isotopic atom peaks of one product such as 0.3, 0.4 or 0.5, the charge carried by each molecule will be known. Based on the above, the molar mass was calculated following the equation below:

$$\textbf{Molar weight} = A \times C - C$$

where A= the X-axis value of the peak and C= the charge carried by the molecule.

The calculated molar mass of AP1 is shown in Table 3.2. From Table 3.2, it can be concluded, that the produced peptide product was a mixture of sequences with molecular weights of 1029, 1387 and 1745g/mol. The theoretical molar mass of AP1 was calculated to be 1664 g/mol (Table 3.1). Unfortunately, none of the measured molecular weights matched the theoretical value leading to the conclusion that the synthesised sequence was not the desired peptide sequence or that the synthesised peptide was not pure.

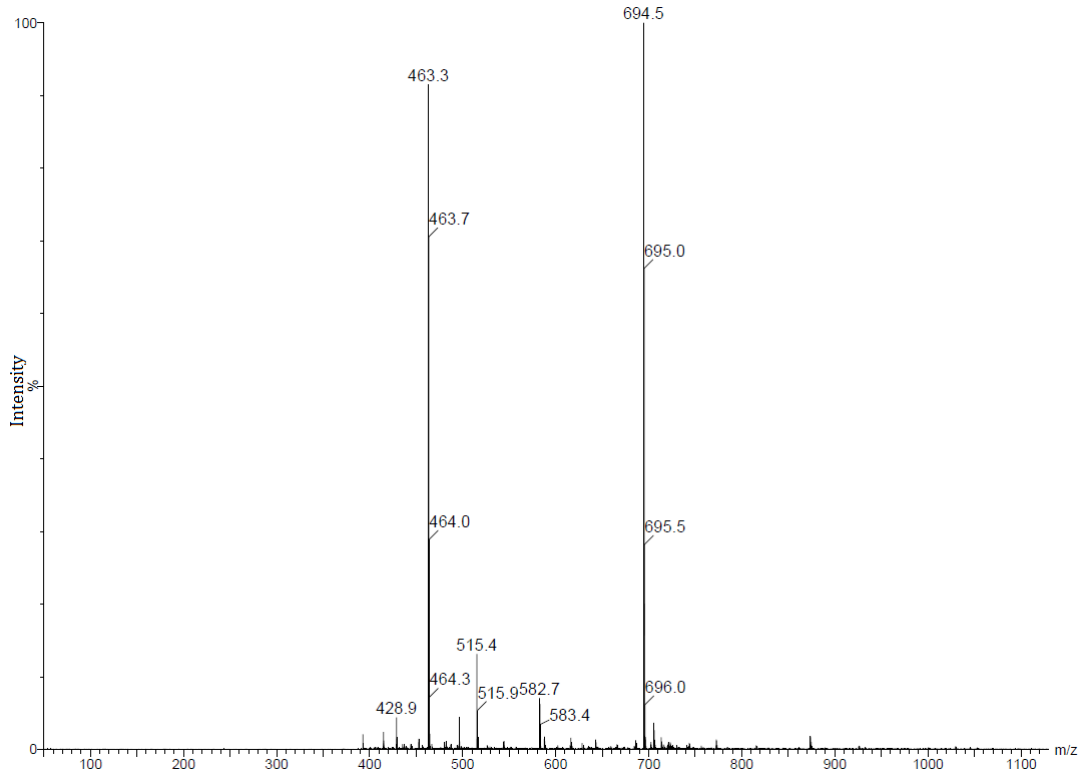


Figure 3.1: Mass spectrum of AP1

Table 3.2: Molar mass of AP1 calculated from the mass spectrum

	Mass/charge	Calculated molar mass (g/mol)
$[M+2H]^{2+}$	515.4	1028.8
	694.5	1387
$[M+3H]^{3+}$	463.3	1386.9
	582.7	1745.1

Figure 3.2 shows the HPLC of AP1. Each peak corresponds to one kind of component and the relative peak area (Table 3.3) presents the percentage of the component in the product. Due to the presence of impurities, there are a few smaller peaks in the HPLC spectrum associated with impurities. Therefore, the highest peak was assumed to be associated with the main peptide sequence and the area under the highest peak was assumed to correspond to the purity of the peptide product. Considering the mass spectrum of AP1 (Figure 3.1 and Table 3.2), AP1 did not have high purity and required purification by HPLC.

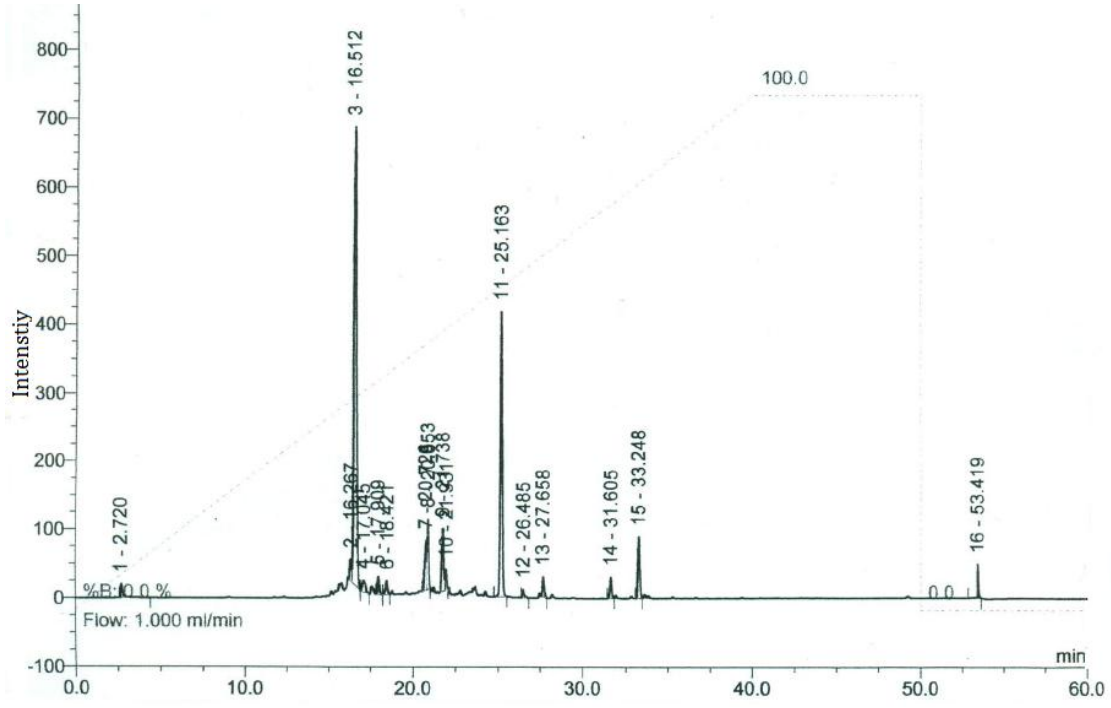


Figure 3.2:HPLC spectrum of AP1

Table 3.3: Calculated relative area under each HPLC peaks from the AP1 spectrum

NO.	Time (min)	Relative Peak Area (%)
1	2.72	1.16
2	16.27	1.52
3	16.51	48.66
4	17.05	1.42
5	17.91	1.33
6	18.42	1.14
7	20.73	3.58
8	20.85	4.62
9	21.74	4.74
10	21.93	1.18
11	25.16	21.78
12	26.49	0.60
13	27.66	1.30
14	31.61	1.46
15	33.25	4.45
Total		100.00

The mass spectrometry measurements of AP2 are shown in Figure 3.3 and the calculated molar weight is shown in Table 3.4. From Figure 3.3 and Table 3.4, it is known that the produced peptide product was a mixture of sequences with molecular

weights of 1073, 1120, 1176, 1219, 1431 and 1534.2 g/mol. The theoretical molar mass of AP2 was calculated to be 1596.78 g/mol (Table 3.1). The calculated value did not match the theoretical molar mass, however, the value of 1534.2 g/mol was the closest to the theoretical value.

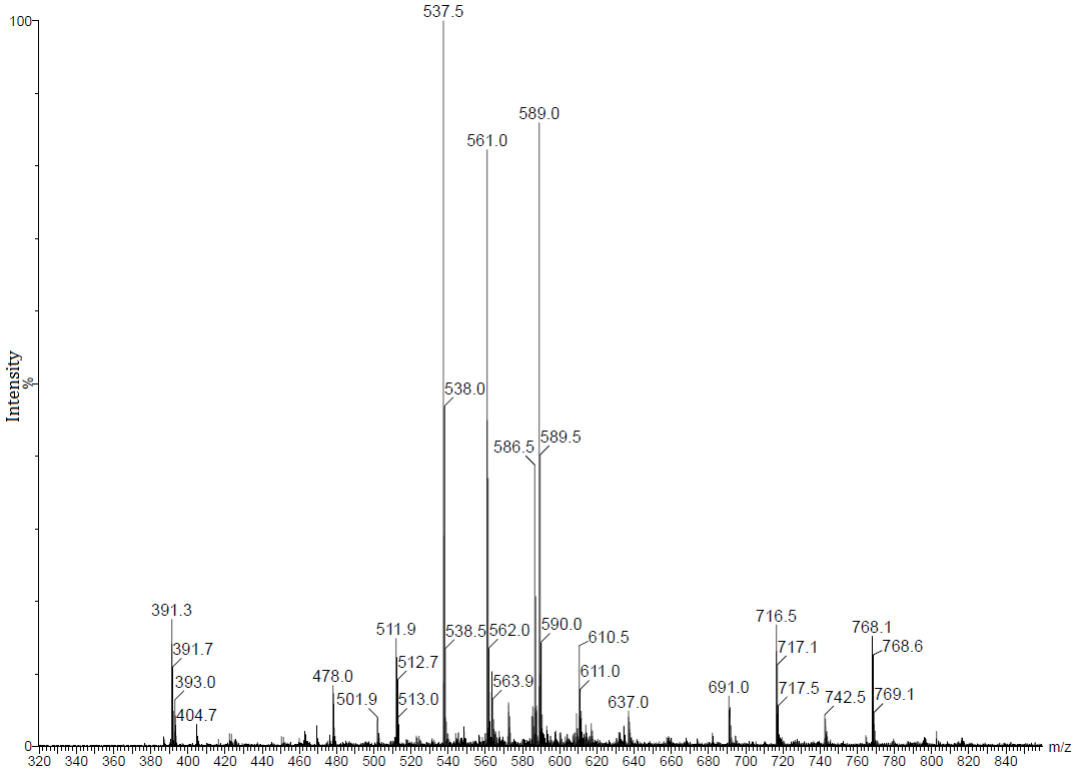


Figure 3.3: Mass spectrum of AP2

Table 3.4: Molar mass of AP2 calculated from the mass spectrum

	Mass/charge	Calculated molar mass (g/mol)
$[M+2H]^{2+}$	537.5	1073
	561.0	1120
	589.0	1176
	610.5	1219
	768.1	1534.2
	716.5	1431
$[M+3H]^{3+}$	391.3	1170.9

The HPLC results of AP2 are presented in Figure 3.4 and the relative percentage of each component is shown in Table 3.5. It was observed, that the AP2 had 16 different components in the product with the largest amount to correspond to 52.05%. Therefore AP2 did not have good purity and it had to be purified.

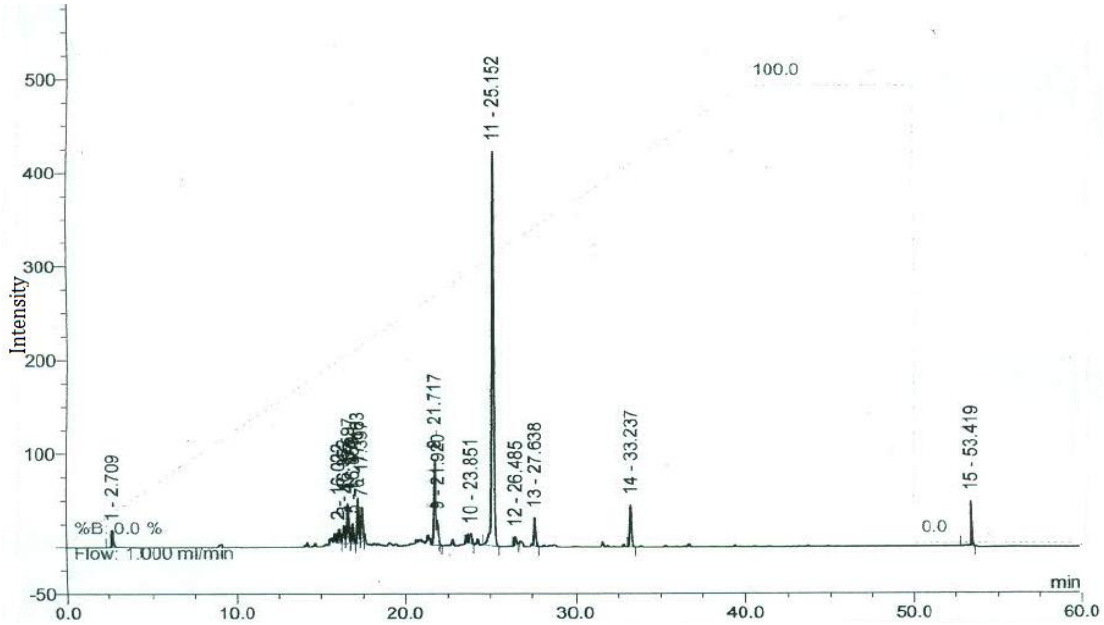


Figure 3.4:HPLC spectrum of AP2.

Table 3.5: Calculated relative area under each HPLC peak from the AP2 spectrum

NO.	Time (min)	Relative Area (%)
1	2.71	2.45
2	16.02	1.47
3	16.35	1.98
4	16.60	4.91
5	16.85	2.01
6	17.16	4.65
7	17.40	3.87
8	21.72	12.43
9	21.92	1.30
10	23.5	1.36
11	25.15	52.05
12	26.49	0.98
13	27.64	3.12
14	33.24	5.06
15	53.42	2.36
16	53.42	1.06
Total		100.00

Figure 3.5 shows the mass spectrum of AP3 and Table 3.6 presents the observed molar mass of AP3 product. From Figure 3.5 and Table 3.6, it can be concluded that the observed molar weight were 1039.7 and 1095.8 g/mol. The observed molar weight of 1039.7 g/mol was very close to the theoretical molar mass of 1039.27 g/mol shown in Table 3.1.

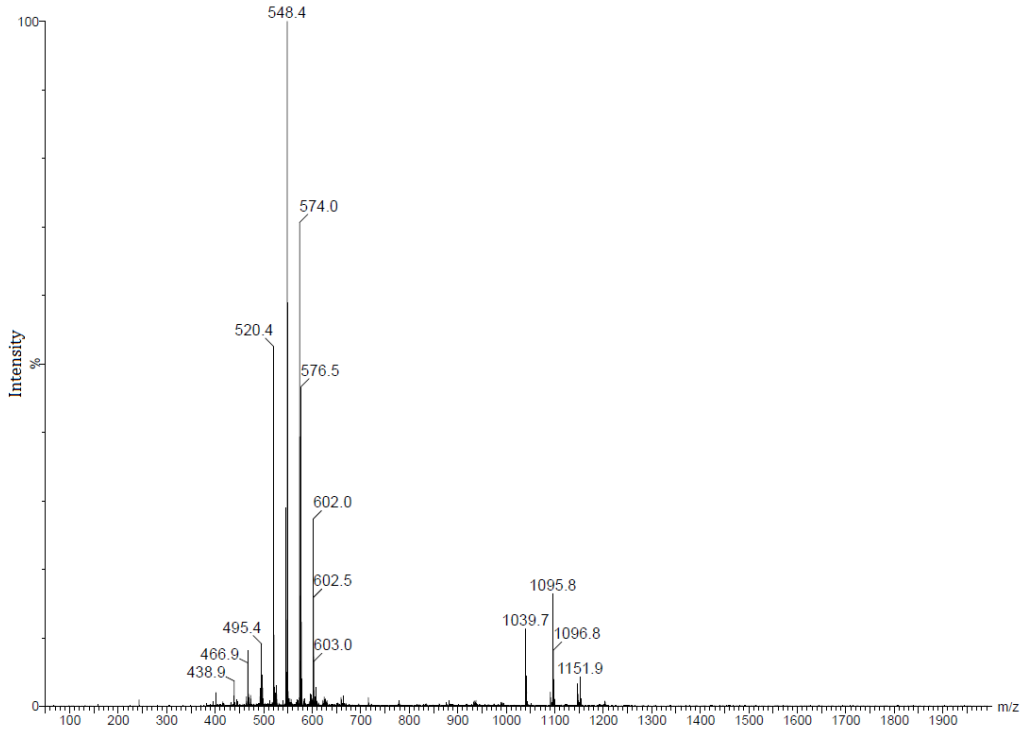


Figure 3.5: Mass spectrum of AP3

Table 3.6: Molar mass of AP3 calculated from the mass spectrum

	Mass/charge	Calculated molar mass (g/mol)
$[M+H]^+$	1039.7	1039.7
	1096.8	1095.8
$[M+2H]^{2+}$	520.4	1039.8
	548.4	1095.8
	574.0	1152
	602.5	1203

The HPLC spectrum of AP3 is shown in Figure 3.6 and the relative percentage of each component is shown in Table 3.7. The AP3 product exhibited 11 different kinds of components. However, from the mass spectrum, it can be noticed that AP3 contained a lot of impurities. Considering that AP3 was not FITC labelled, it could not be used in bone cement as there would not be possible study release rates. For this reason, AP3 peptide was not purified.

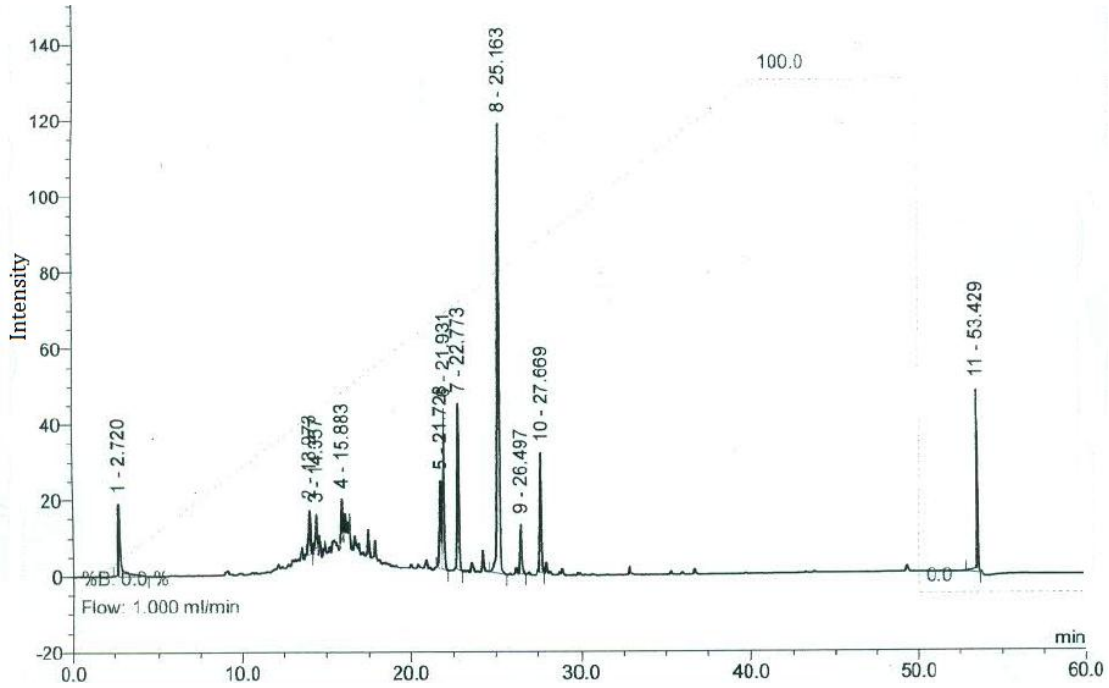


Figure 3.6:HPLC spectrum of AP3

Table 3.7: Calculated relative area under each HPLC peak from the AP3 spectrum

NO.	Time (min)	Relative Area (%)
1	2.72	6.74
2	13.97	3.19
3	14.36	2.42
4	15.88	2.63
5	21.73	7.06
6	21.93	11.86
7	22.77	11.50
8	25.16	37.03
9	26.50	3.45
10	27.67	8.14
11	53.43	6.00
Total		100.00

The mass spectrum of AP4 is shown in Figure 3.7 and the observed molar mass is shown in Table 3.8. From Figure 3.7 and Table 3.8 it can be noticed, that the observed molar mass is 1448.2, 1188 and 1807.2 g/mol. The molar mass at 1448.2 g/mol is the same as the theoretical mass which is 1448 g/mol (Table 3.1).

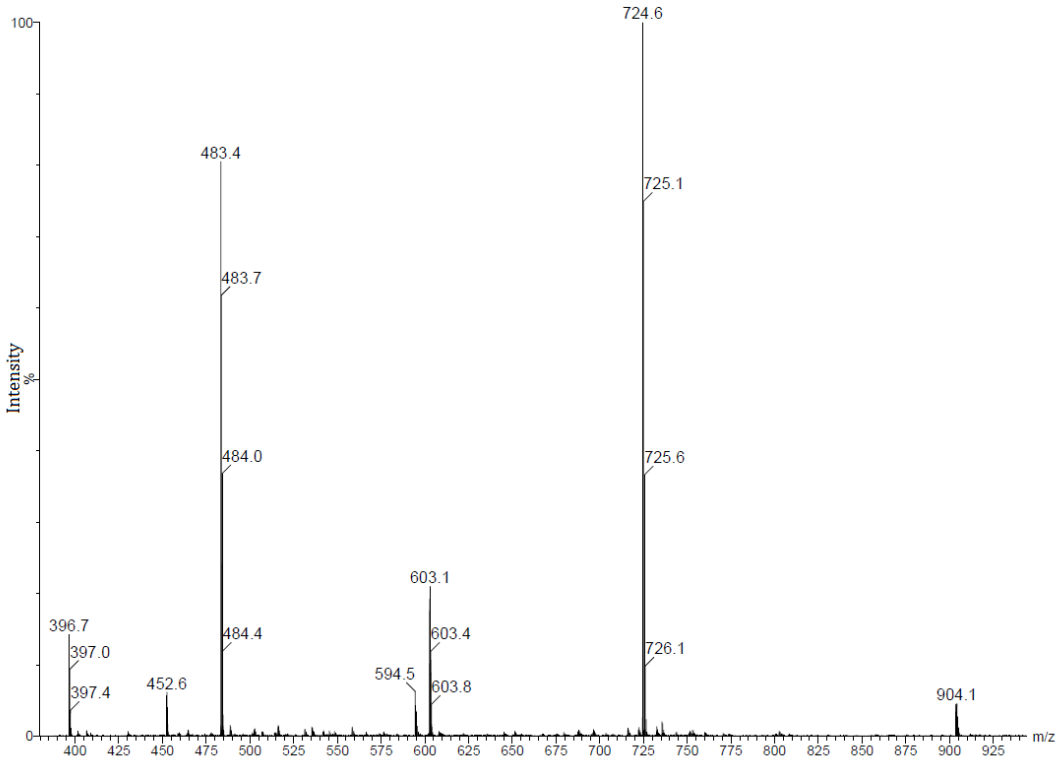


Figure 3.7: Mass spectrum of AP4.

Table 3.8: Molar mass of AP4 calculated from the mass spectrum

	Mass/charge	Calculated molar mass (g/mol)
$[M+2H]^{2+}$	725.1	1448.2
$[M+3H]^{3+}$	397.0	1188
	483.7	1448.1
	603.4	1807.2

The HPLC spectrum of AP4 is shown in Figure 3.8 and the relative component percentage is shown in Table 3.9. From Figure 3.8 and Table 3.9 it can be observed, that there are a lot of impurities in the product. Taking into consideration on the other hand the good match between the calculated molar mass and the theoretical molar mass as well as the very good MIC test result shown in section 3.1.2, purification of the peptide was needed.

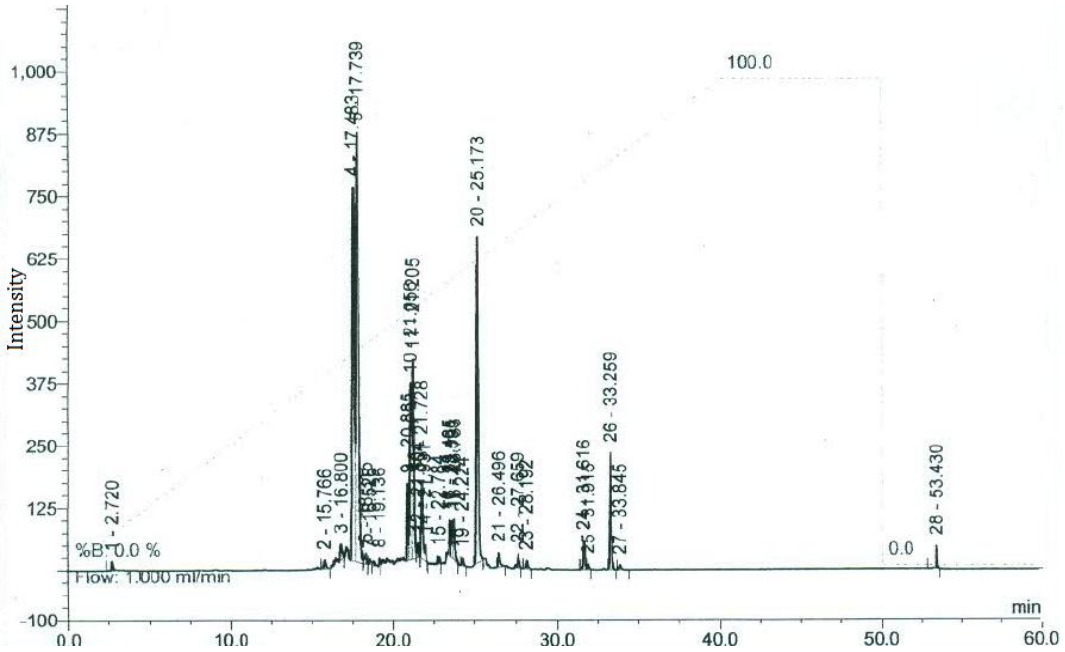


Figure 3.8:HPLC spectrum of AP4

Table 3.9: Calculated relative area under each HPLC peak from the AP4 spectrum

NO.	Time (min)	Relative Area (%)
1	2.72	0.44
2	15.77	0.34
3	16.80	0.87
4	17.48	19.66
5	17.74	29.53
6	18.31	0.52
7	18.53	0.18
8	19.14	0.20
9	20.89	2.15
10	21.06	6.72
11	21.21	8.73
12	21.50	0.55
13	21.73	3.51
14	21.93	0.30
15	22.78	0.26
16	23.43	1.02
17	23.59	1.16
18	23.73	1.49
19	24.22	0.27
20	25.17	13.93
21	26.50	0.62
22	27.66	0.49
23	28.19	0.31
24	31.62	1.15
25	31.91	0.18
26	33.26	4.78
27	33.85	0.21
28	53.43	0.43
Total		100.00

The purification of AP1 was successful. Only one component with fluorescence labelling was observed. Then the product was characterised by mass spectrometry and HPLC. The mass spectrum of AP1 after purification is shown in Figure 3.9 and the calculated molar mass is presented in Table 3.10. The HPLC spectrum is shown in Figure 3.10 and the relative percentage of components is presented in Table 3.11. From Figure 3.10 it could be easily observed that only one main peak was present which reflects to the high purity of the peptide. It is worth mentioning, that the HPLC purification was performed only for 40 mins. The peaks above 40 mins corresponded

to the solution used to wash the HPLC column. For this reason, the percentage of the two peaks NO.8 and NO.9, which have the relative peak area of 0.41 and 63.4, were deleted when the purity of AP1 was calculated. The calculated purity of AP1 after purification was 84.83%. However, Table 3.10 shows that the observed molar mass is 1387.5 g/mol which does not match the theoretical molar mass of 1663.85 g/mol (Table 3.1) and therefore the sequence of AP1 did not correspond to the initial desired sequence.

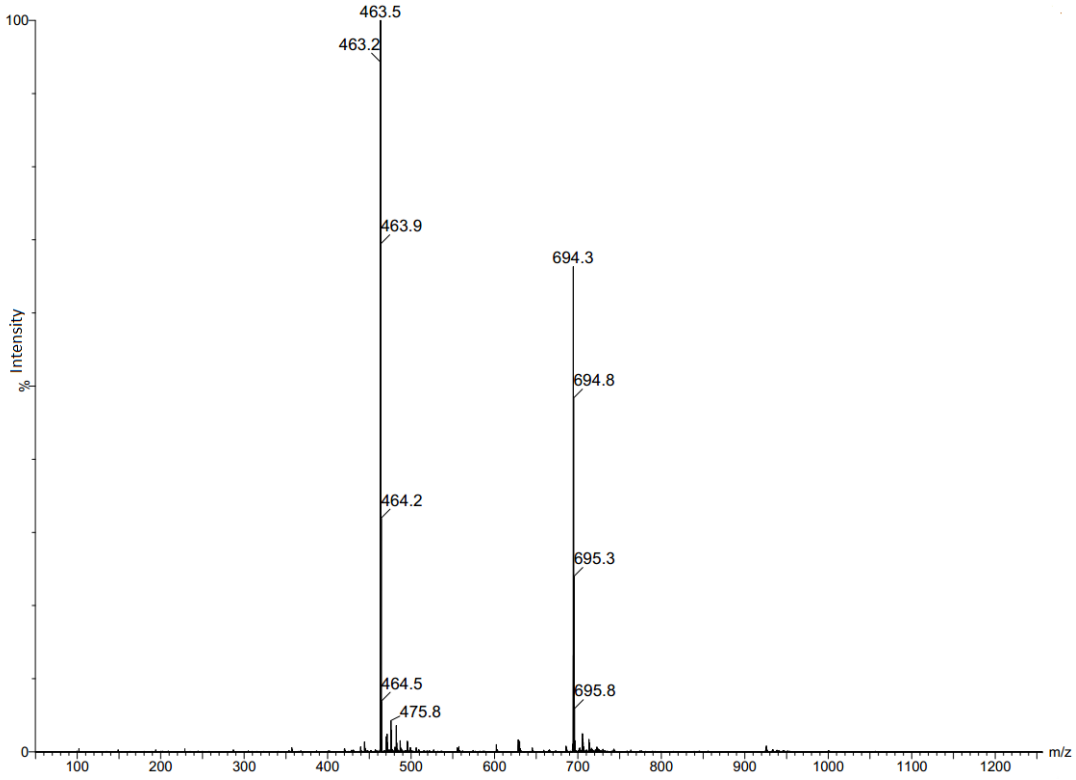


Figure 3.9: Mass spectrum of AP1 after purification

Table 3.10: Molar mass of AP1 calculated from the mass spectrum after purification

	Mass/charge	Calculated molar mass (g/mol)
$[M+2H]^{2+}$	694.8	1387.6
$[M+3H]^{3+}$	463.5	1387.5

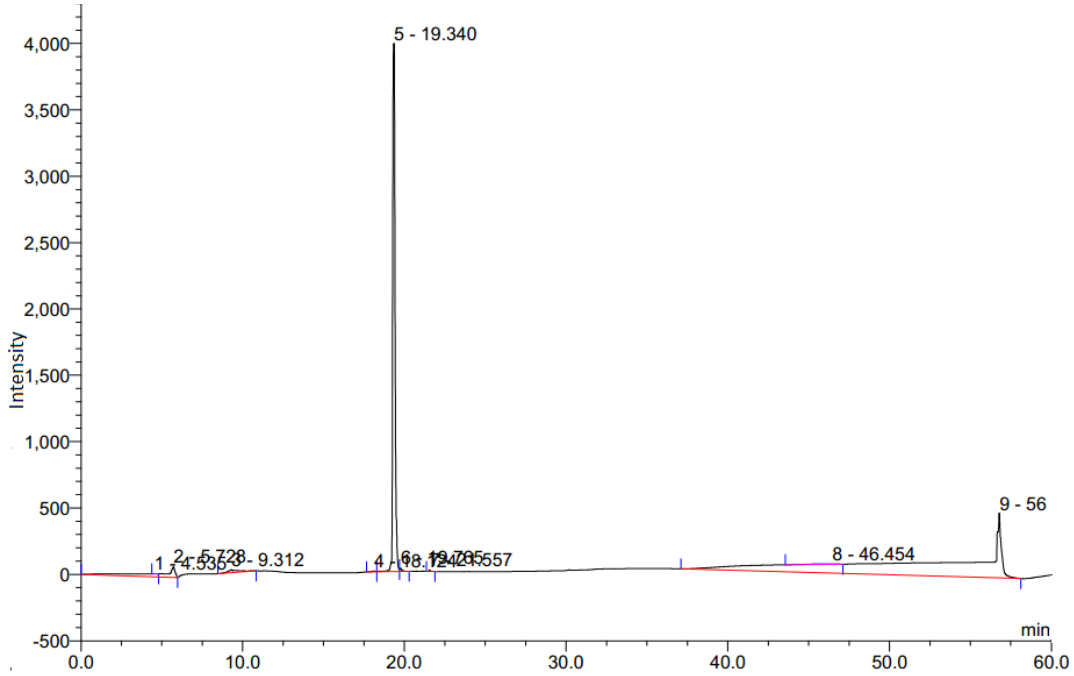


Figure 3.10: HPLC spectrum of AP1 after purification

Table 3.11: Calculated relative area under each HPLC peak from the AP1 spectrum after purification

NO.	Time (min)	Relative Area (%)
1	4.54	0.01
2	5.73	4.40
3	9.31	0.75
4	18.12	0.06
5	19.34	30.74
6	19.77	0.17
7	21.56	0.05
8	46.45	0.41
9	56.79	63.40
Total		100.00

The purification of AP2 peptide was not successful. Fifteen different components were observed by fluorescence labelling in the synthesized peptide. This means that there was only a tiny amount of the expected peptide sequence and the amount left after purification was not enough to conduct any research. The purification of AP4 was successful. Only one product was observed. The mass spectrum of the purified AP4 is shown in Figure 3.11. The calculated molar mass is presented in Table 3.12.

From Table 3.12, it can be noticed that the observed molar mass of the product was 1447.8 g/mol and it matched the theoretical molar mass of 1448.6 g/mol (Table 3.1). Figure 3.12 shows the HPLC spectrum of AP4 after purification and the relative percentage of the components are shown in Table 3.13. The same HPLC method was followed for AP4 as for AP1. Thus, the components present after 40mins corresponded to the peaks from the solution used to wash the HPLC column. The purity of AP4 was 96.55%. The purity of AP4 was sufficiently high to conclude that AP4 exhibits the desired amino acid sequence as given in Table 1.5.

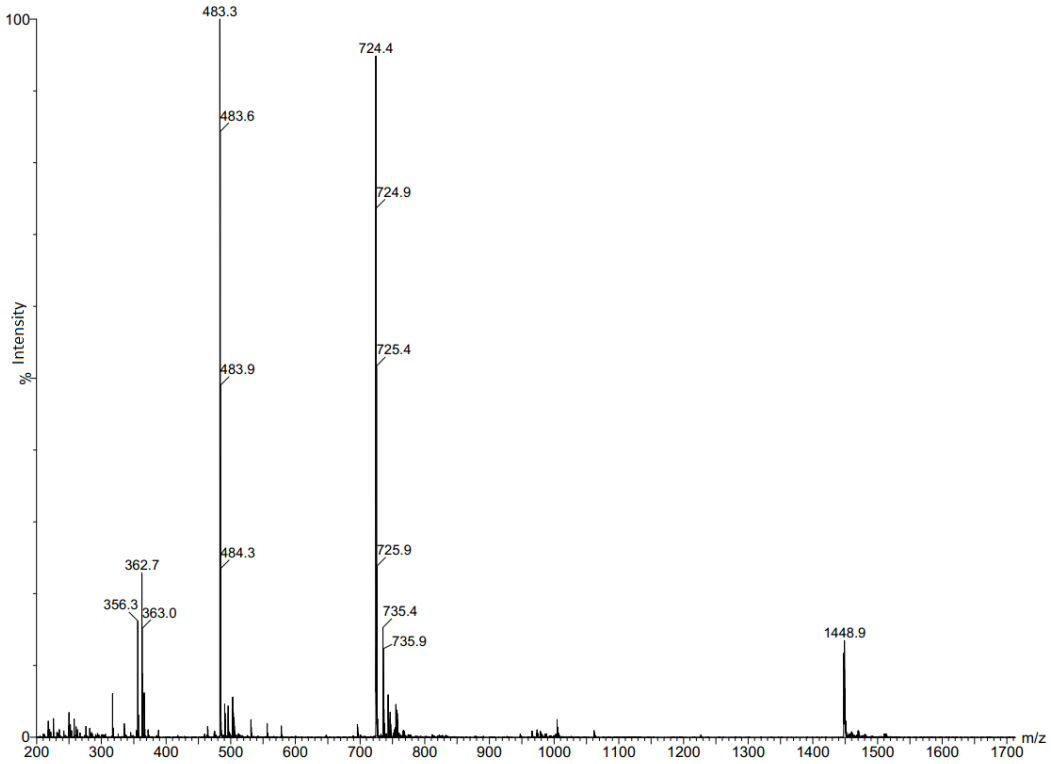


Figure 3.11: Mass spectrum of AP4 after purification

Table 3.12: Molar mass of AP4 calculated from the mass spectra after purification

	Mass/charge	Calculated molar mass (g/mol)
$[M+H]^{+}$	1448.8	1447.8
$[M+2H]^{2+}$	724.9	1447.8
$[M+3H]^{3+}$	483.6	1447.8

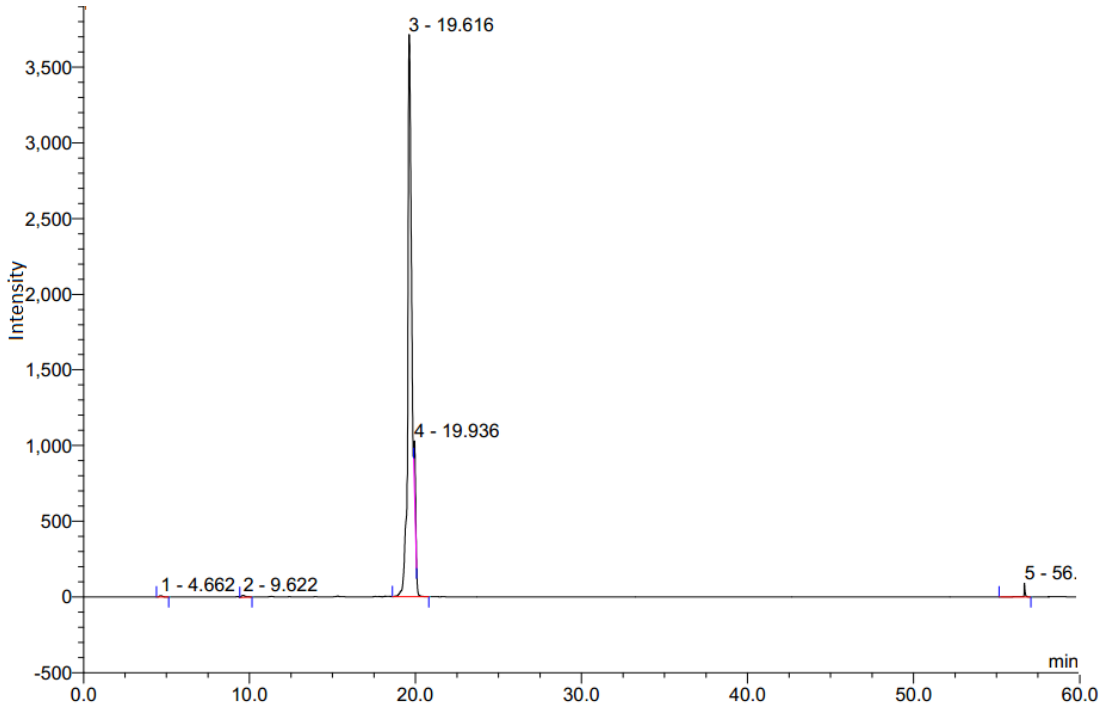


Figure 3.12:HPLC spectrum of AP4 after purification

Table 3.13: Calculated relative area under each HPLC peak from the AP4 spectrum after purification

NO.	Time (min)	Relative Area (%)
1	4.66	0.14
2	9.62	0.30
3	19.62	96.00
4	19.94	2.99
5	56.67	0.57
Total		100.00

The SPPS method used the Fmoc protection group and the Wang resin gave high purity of the sequence. The purity of the peptide decreased as the length of amino acid sequence increased due to the theoretical purity of the product being the exponent of the purity of each amino acid. Because all purchased amino acids had a purity of 98%, the theoretical purity for a 9-amino acid peptide (e.g., AP4) is 0.98^{9} (= 83.37%), for an 11-amino acid peptide (e.g., AP1-AP3) is 0.98^{11}(=80.07%) and for a 17-amino acid peptide is 0.98^{17}(=70.93%). Thus, the length of peptides is a very important factor that can affect the purity of peptides as shown in Figure 3.13 [78]. The shorter the peptide

length, the higher the degree of purity will be. Therefore, the purity of AP4 is expected to be higher than AP1, 2 and 3 as it is a shorter amino acid sequence.

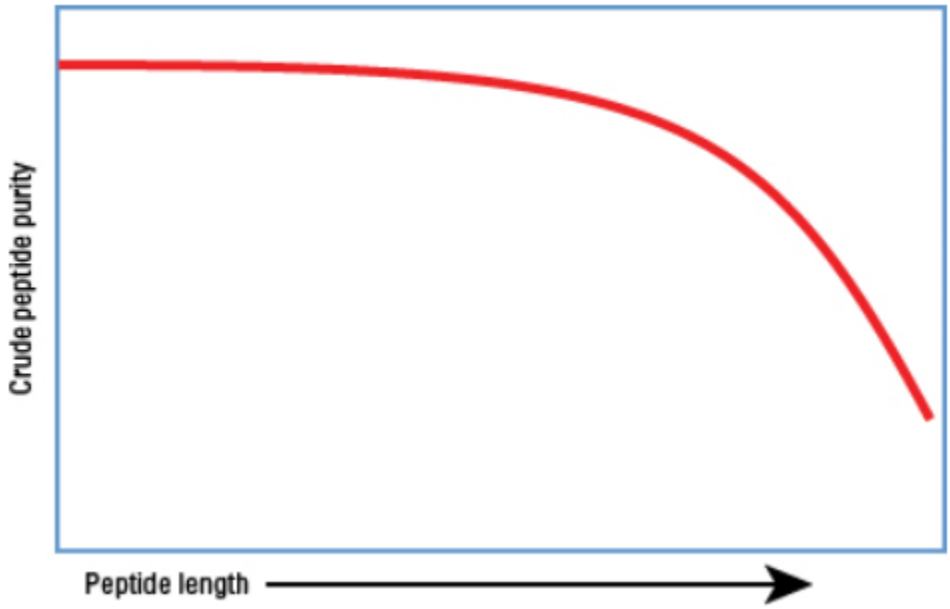


Figure 3.13: Relationship between the length and purity of a peptide[78]

3.1.2 The minimum inhibitory concentration (MIC) of synthesized peptide

MIC was used to identify the antibacterial property of the peptides and the MIC results can help to select the peptide with the best antibacterial property to add into HA cements. As it was mentioned in section 1.3, *E.coli*, *S.aureus* and *P.aeruginosa* are some of the most common bacteria to test the antibacterial ability of the peptides. The MIC test was applied in all non-purified peptides. The MIC results of non-purified peptides are shown in Tables 3.14-3.16. Peptide AP4 showed the lowest MIC with a concentration of 5 mg/mL, 2.5 mg/mL and 5 mg/mL for *E.coli*, *S.aureus* and *P.aeruginosa*, respectively. The MIC values of peptide AP4 were lower than the MIC of AP1, AP2 and AP3. It can be concluded, that AP4 had the best antibacterial property. Furthermore, considering the purification results of synthesized peptides,

AP4 was the only one that was purified satisfactorily. Thus AP4 was the best peptide to add into the HA bone cements.

After purification, the MIC of AP4 was tested against *E.coli, S.aureus* and *P.aeruginosa*, and the results are presented in Table 3.17. From Table 3.17, it could be seen that the MIC of AP4 against *E.coli, S.aureus* and *P.aeruginosa* is 1 mg/mL, 1mg/mL and 2mg/mL, respectively. These values were lower than the MIC before purification showing clearly that the antibacterial property of AP4 was improved after purification.

Table 3.14:MIC values of peptides against *E.coli* before purification

Code	MIC (mg/mL)
AP1	10
AP2	10
AP3	10
AP4	5

Table 3.15:MIC values of peptides against *S.aureus* before purification

Code	MIC (mg/mL)
AP1	10
AP 2	10
AP 3	5
AP 4	2.5

Table 3.16:MIC values of peptides against *P.aeruginosa* before purification

Code	MIC (mg/mL)
AP1	10
AP 2	10
AP 3	5
AP 4	5

Table 3.17:MIC values of AP4 against *E.coli, S.aureus* and *P.aeruginosa* after purification

Bacteria	MIC (mg/mL)
E. coli	1
S. aureus	1
P. aeruginosa	2

3.2 Characterisation of hydroxyapatite bone cements

3.2.1 X-ray powder diffraction (XRD) studies of hydroxyapatite bone cements

X-ray powder diffraction was used to characterise the HA cements. Figure 3.14 shows the X-ray powder diffraction pattern of HA cements produced during this study. It was observed that the cement was crystallized mainly to the phase of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and calcium-deficient hydroxyapatite [$CaHPO_4(PO_4)_5OH$]. A minor phase was calcium carbonate ($CaCO_3$) which could have been remained unreacted during the HA cement formation. However, as shown in Figure 3.14, the main phases in the cement were hydroxyapatite and calcium-deficient hydroxyapatite.

Comparing the XRD pattern of HA cement (Figure 3.14) with the XRD of HA cements reported by Charriere *et al* (Figure 3.15) [79], both of the HA cements have peaks around 2θ=32°and 2θ=26°, which were identified to be associated with HA diffraction pattern. In addition, comparing the XRD pattern of HA cement (Figure 3.14) with the XRD of hydroxyapatite reported by Arsad and Lee [Figure 3.16 (a)][80]. The diffractogram of the HA cement was similar to the hydroxyapatite phase reported in the literature. Both had the strongest peaks around 2θ=32° and the second strong peaks around 2θ=26° and 2θ=50°. In addition, the HA cement sample obtained in this study also had a similar pattern as the HA standard data in the literature [Figure 3.16 (b)] [80].

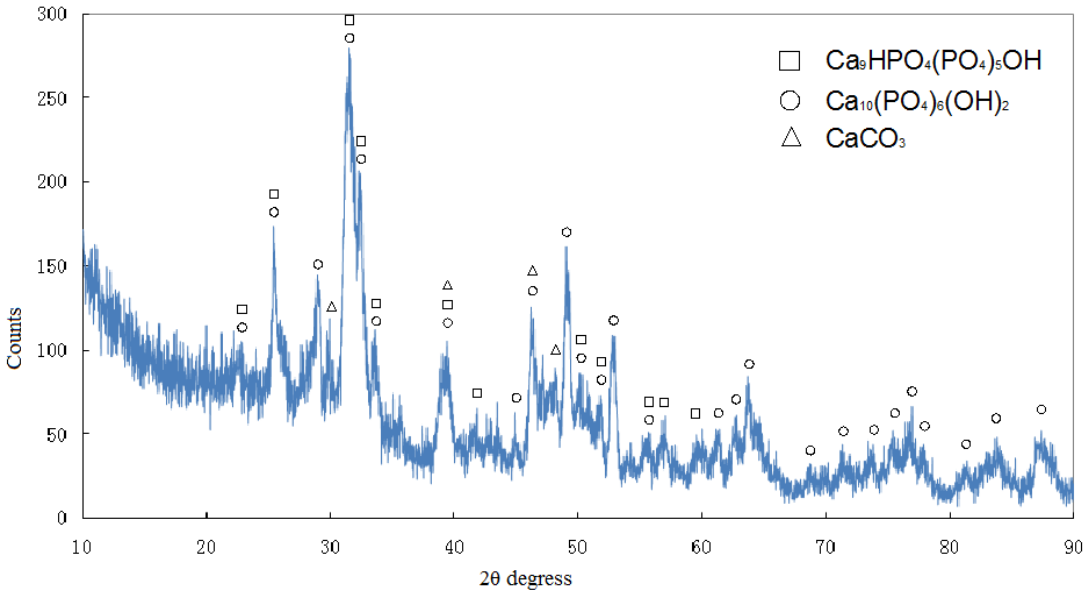


Figure 3.14: X-ray powder diffraction pattern of HA cement sample

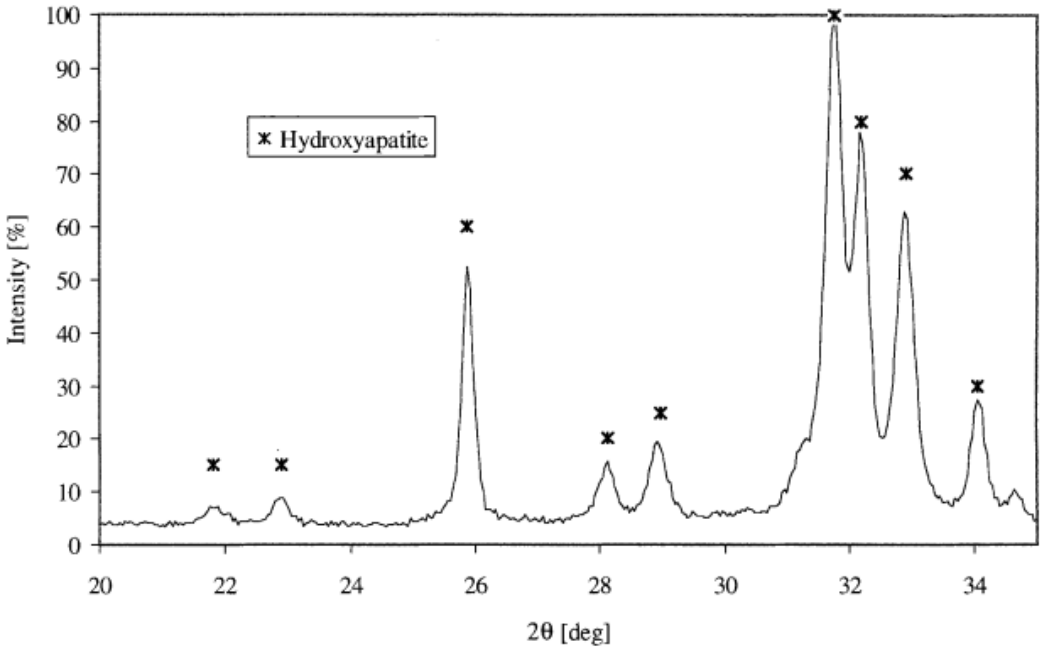


Figure 3.15: X-ray diffraction pattern of HA cements as reported by literature [79]

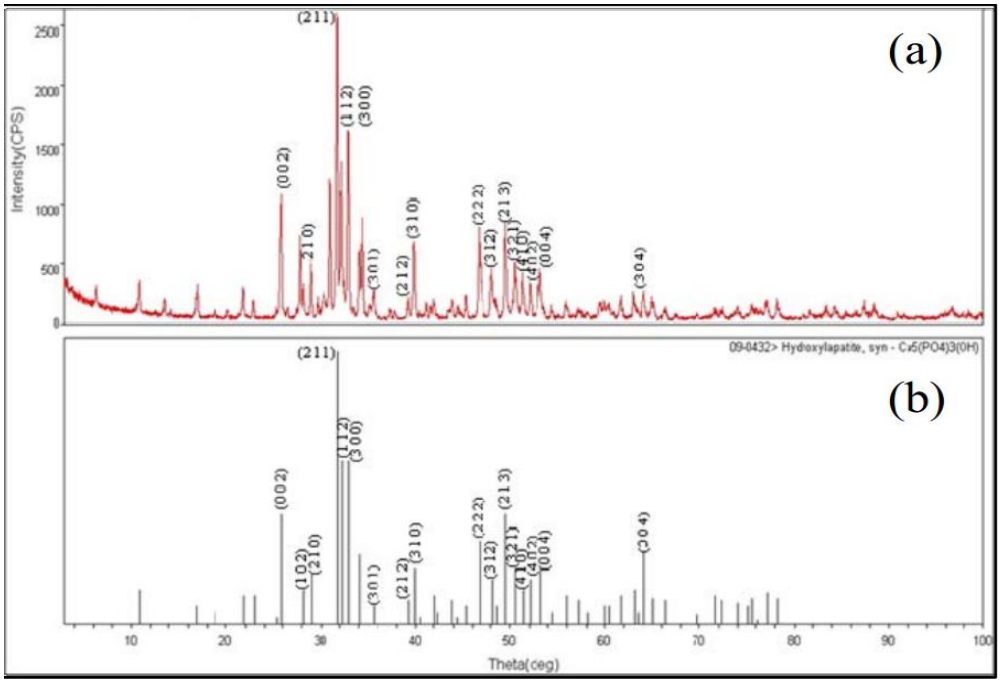


Figure 3.16: X-ray powder diffraction pattern of (a) HA sample in literature and (b) library standard of hydroxyapatite as reported in the literature [80]

3.2.2pH studies of hydroxyapatite bone cements

The pH during hydroxyapatite formation was measured. As mentioned in section 1.2.2, HA phase is crystallised at pH>4.2 [10]. The pH during HA cement formation was 7, which is in the range of HA phase formation. Therefore the phase that is expected to be formed during cement formation is hydroxyapatite.

3.3 Characterization of antibacterial HA bone cements

Based on the purification results and the MIC test results of antibacterial peptides, AP4 was selected to add into the HA cements with the concentration of 0, 2, 6 and 8wt% and the samples were named AB0, AB2, AB6 and AB8, respectively. The AB0 was the control sample in all experiments. All the antibacterial HA cements were prepared by the method mentioned in section 2.6.

3.3.1 pH studies of antibacterial HA cements with peptide

The pH was measured for all cements just after mixing of the solid and liquid phase and before the cement pastes were placed into the mould. The pH was monitored so that to monitor the pH change and whether the pH remained > 4.2 in order for HA to crystallise (precipitate). If the pH was < 4.2, then brushite would have been precipitated instead of HA. In this research all pH values of HA cements with different concentration of AP4 were at pH=7. As mentioned in section 1.2.2, the HA phase was formed in pH above 4.2 and all the pH value of HA cements in the experiment were above 4.2. Even the AP4 had an effect during the HA cements setting, but it could not change the main phase of HA. It was concluded that all the cement pastes underwent phase transition to HA.

3.3.2In vitro release study of antibacterial HA bone cements

The release study was used to analyse the release property of AP4 released from HA cements into solution. All the experiments were performed according to the method described in section 2.7.2. Figure 3.17 shows the change in the fluorescence intensity of the solution (PBS) with time of exposure of cements in PBS. The intensity can be correlated directly to the concentration of released peptides into PBS as described in section 2.7.2. It can be noticed, that the control sample AB0 showed some fluorescence background. AB2, AB6 and AB8 showed an initial burst-release at first 0.5 hours and a gradual release from 0.5 hours to 3 hours reaching a plateau with no significant change up to 48 hours. It is worth noticing, that as the concentration of AP4 increased, the fluorescence intensity increased.

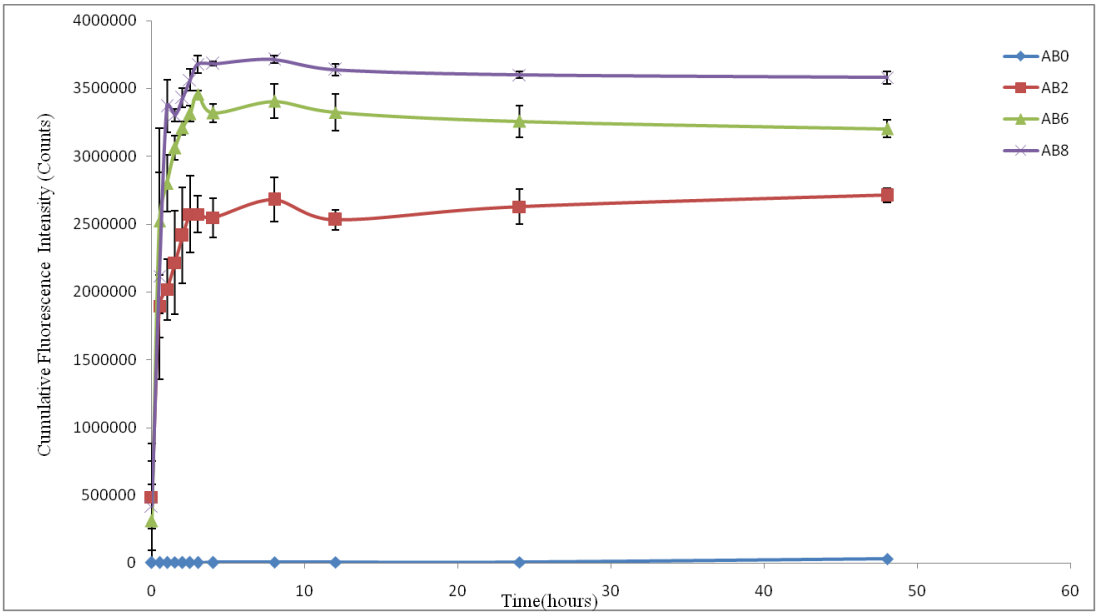


Figure 3.17: Release profile of AB0, AB2, AB6 and AB8. Error bars represent the standard deviation (n= 3)

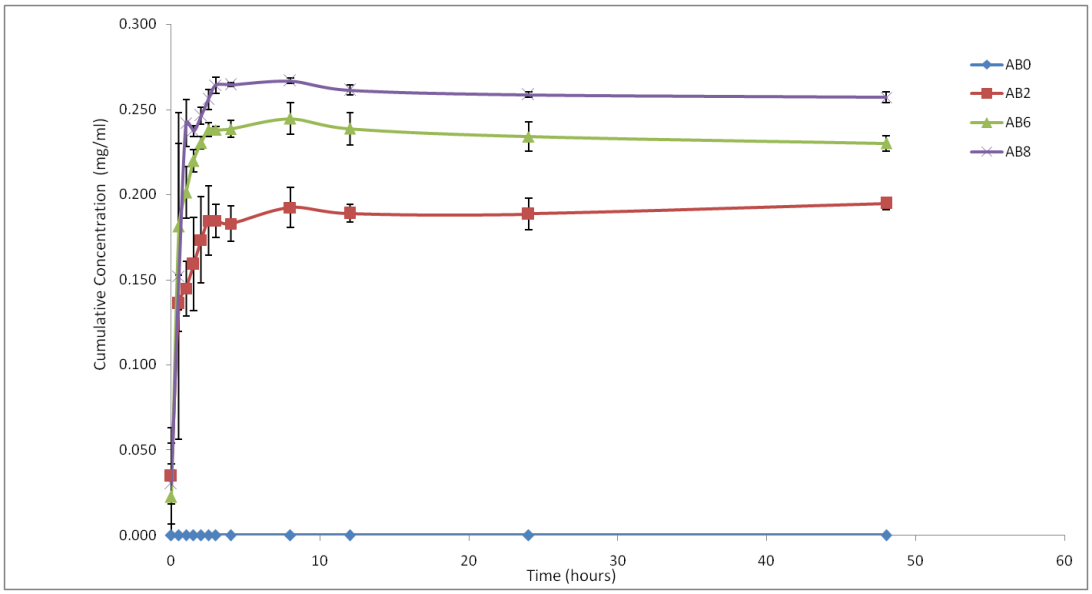


Figure 3.18: Concentration of release solution of AB0, AB2, AB6 and AB8. Error bars represent the standard deviation (n=3)

Figure 3.18 shows the change of concentration of AP4 in PBS with time during the release studies. It can be noticed, that as the release time increased, the peptide

concentration in PBS increased. Similarly with increasing the concentration of AP4 in the HA cements, the concentration of peptides in PBS increased. It is also worth noticing that, the first 0.5 hour the release speed was higher than between 0.5 hour and 3 hours. After 48 hours, the peptide release concentration reached a plateau with no significant change. The peptide concentration at 48 hours of AB2 released in PBS was 0.195 mg/mL, whereas the concentration of AB6 was 0.23 mg/mL and the concentration of AB8 was 0.257 mg/mL. From the peptide concentration and volume of PBS, the mass of the released AP4 can be calculated. The mass of AP4 released from AB2 was 0.178 mg whereas the mass of AP4 released from AB6 was 0.218 mg and the mass of AP4 released from AB8 was 0.240 mg.

AB2 cement contained 0.83 mg of AP4 whereas AB6 contained 2.5 mg of AP4 and AB8 contained 3.33 mg of AP4. Comparing these with the amount of AP4 released from the cements, it can be noticed that the mass of AP4 released out was very small. Similar results were reported by Stallmann *et al.* [59,81]. The antibacterial peptide hLF1-11(GRRRRSVQWCA) was loaded in 6 different types of commercial hydroxyapatite cements in order to study the release property of hLF1-11. A release of small amount of peptides was observed for all loaded cements. One of the main reasons for this behaviour is that parts of hLF1-11 were strongly bound to the hardening cement leading to sequestration and unavailability for release [81]. It is worth noticing that some of the amino acids in AP4 (CRVRGGRCA) and hLF1-11 are similar. For example, both of them have cysteines, a valine, analanine, glycines and arginines, with a positive charge. Based on the above, it could be suggested that the release behaviour of AP4 from HA could be affected by the binding between AP4 and the hardening cements. It is also worth noticing that, during the first 0.5 hour a higher release rate was observed compared to the 0.5 to 3 hours release period. Kuhn

[82] reported an initial burst-release at first 0.5 hours caused by the peptide on the surface of the cements and a gradual release from 0.5 hours to 3 hours caused by the release of peptides within the cements.

3.3.3Mechanical properties of antibacterial HA bone cements

The compressive strength (CS) was measured in order to study the mechanical properties of HA cements containing antibacterial peptides. Compressive strength tests were conducted for all four concentrations of antibacterial HA cements after being immersed in PBS for 24 and 48 hours at 37℃. The reported data were the average values of 15 samples. Figure 3.19 shows the wet compressive strength of the cements with different concentration of antimicrobial peptides soaked in PBS for 24 and 48 hours.

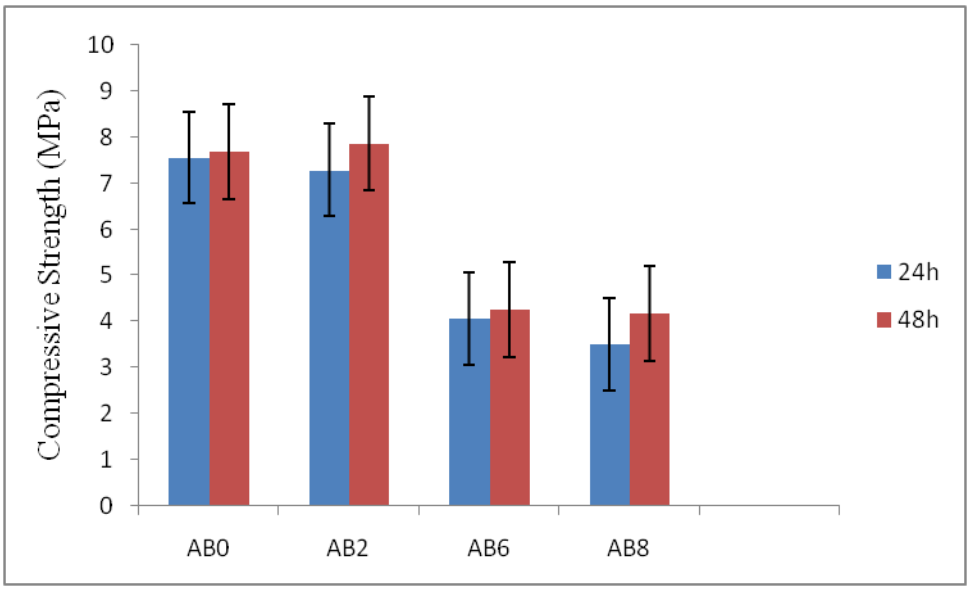


Figure 3.19: Compressive strength of AB0, AB2, AB6 and AB8 for 24 and 48h. Error bars represent the standard deviation (n=15)

As shown in Figure 3.19, the CS of AB6 and AB8 were lower than the CS of AB0 and AB2. The CS of AB2 was some lower but nearly the same with AB0, and the CS

of AB8 was little lower than AB6. It is clear, that as the concentration of the antibacterial peptide increased, the CS of the cement was decreased. But in the range of 2 wt% difference, the CS decrease was not significant.

The setting reaction of cements follows three phases: powder phase dissolution; precipitation and crystallisation. Ratier*et al* [44], reported that the compressive strength of HA cements loaded with tetracycline hydrochloride was decreased above 1% w/w loading. One of the main reasons for this behaviour is that the presence of chloride ions was strongly influencing the setting reaction and the formation of hydroxyapatite. The treatment of tetracycline hydrochloride however with calcium sulphate allowed the loading of the antibiotic up to 7%w/w without decreasing the mechanical properties of the cement (HA formation was not interrupted). In this work, AP4 has three arginines in the sequence, which carry a positive charge. When the powder phase dissolved in the liquid phase, the positive ions might affect the crystallisation of HA. How this is happening is not clear as there is no evidence from the experimental results. However, it is possible that the peptide bound on the hardened cement or the crystallisation of HA was delayed. Most papers in the literature reported compressive strength values of hydroxyapatite cements after 2 days of setting considering the low compressive strength values that normally occur for all HA cements before this time. It is clear, that the compressive strength increased with setting time [28,40,41]. Kisanuki *et al* [41] reported that a cement with high content of dideoxy-kanamycin B exhibited low compressive strength and long setting time. Furthermore, the compressive strength of cements was lowest at the first 3 days and highest at the 7th day of setting. Rabiee *et al*[40], reported a similar observation. Firstly, a long setting time and low compressive strength of HA cements was observed at high loadings of tetracycline hydrochloride. Secondly, the highest

strength was observed after soaking HA cements in SBF for 7 days and the lowest strength was observed for 1 day. One of the reasons for this behaviour was the crystallization of HA as well as the crystal growth were slower at the beginning and faster at a later time. The low compressive strength of HA cements with high peptide concentration could contribute to the long setting time and uncompleted crystallization of cements. From Figure 3.19, it could be noticed that the compressive strength of HA cements at 48 hours was higher than of 24 hours. Considering the results reported by Rabiee *et al* [40] and Kisanuki *et al* [41], higher compressive strength was expected when the antibacterial cements were incubated for a longer time. On the other hand, the porosity could be another reason for the decrease of the compressive strength of HA cements. Drug release could produce more pores in the cements leading to a lower compressive strength of HA. Rabiee *et al*[40], suggested that the drug release increased the porosity of HA cements leading to the decrease in the compressive strength. Barralet *et al*[83] also observed that with the porosity of HA cements increasing, the compressive strength was decreased significantly. In this research, considering the release results, the high concentration of AP4 lead to great amount of peptide released possibly increasing the porosity of the HA cements. However, further work is needed to show the effect of antimicrobial release on the porosity of the cements and consequently the decrease of compressive strength.

3.3.4 Antibacterial assays of antibacterial HA cements

Antibacterial assays were performed in order to study the antibacterial properties of cements as described in Materials and Methods in section 2.7.3. The antibacterial behaviour of the surface of cements immersed in PBS for 24 and 48 h was studied.

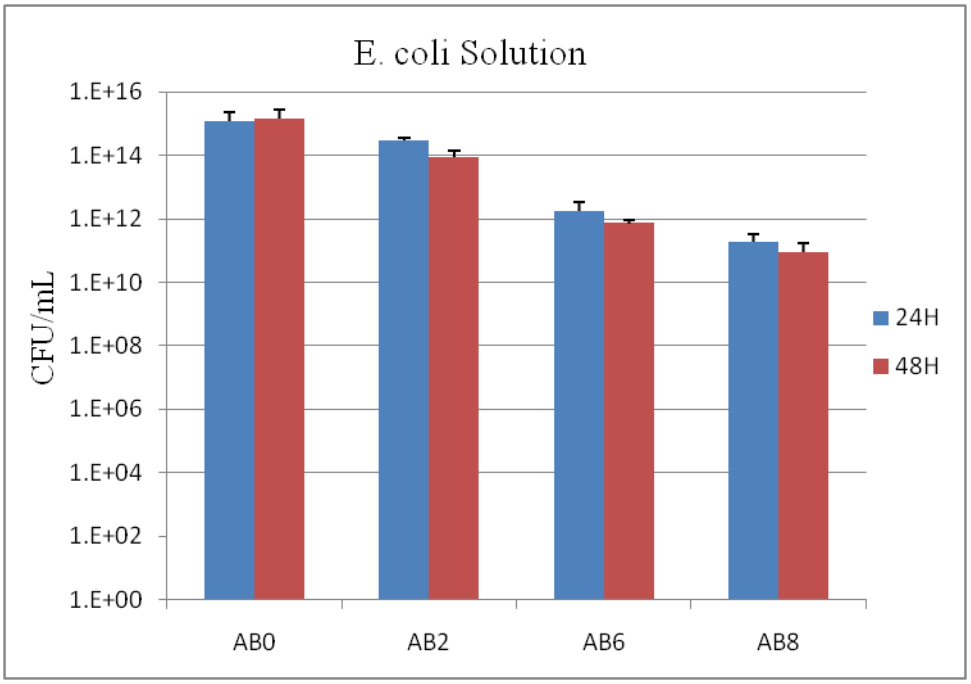


Figure 3.20: *E.coli* CFU numbers in solution of HA cements with different concentration of AP4 (AB0, AB2, AB6 and AB8) for 24 and 48h. Error bars represent the standard deviation (n= 3)

The antibacterial test results of all HA cements after 24 and 48 hours in solution are shown in Figures 3.20 for *E.coli*. It is worth noticing that AB8 had the strongest properties against *E.coli* and AB2 had little ability to eliminate bacteria. It can be concluded that the HA cements with the highest concentration of AP4 can cause stronger effect on *E.coli.* However, none of the surfaces were free of bacteria after 48h. In addition, it should be noticed that the CFU numbers of all test samples have no significant difference between 24 and 48 hours for *E.coli*.

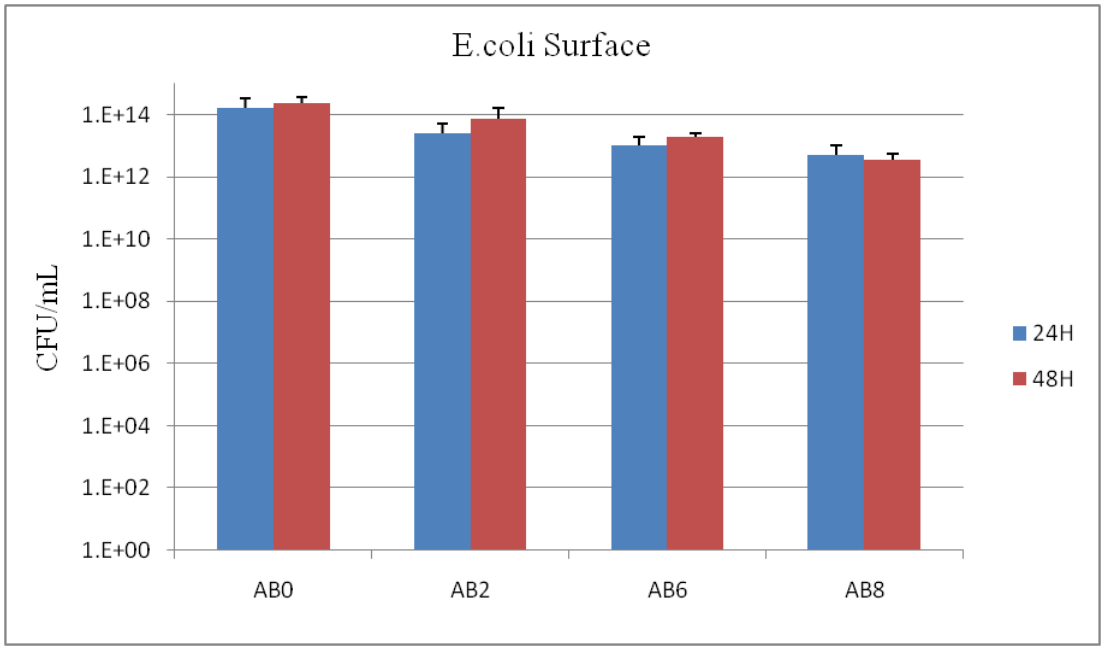


Figure 3.21: *E.coli* CFU numbers on the surface of HA cements with different concentration of AP4 (AB0, AB2, AB6 and AB8) for 24 and 48h. Error bars represent the standard deviation (n= 3)

The antibacterial test results of HA cements after 24 hours and 48 hours on surface for *E.coli* are shown in Figures 3.21. It was observed, that the AB8 had the strongest properties against *E.coli* on the surface and AB2 had nearly no ability to eliminate the bacteria. HA cements with higher concentration of AP4 had stronger effect on *E.coli* on the cements surface. It also can be observed, that the ability of eliminating bacteria was not significant between 24and 48 hours.

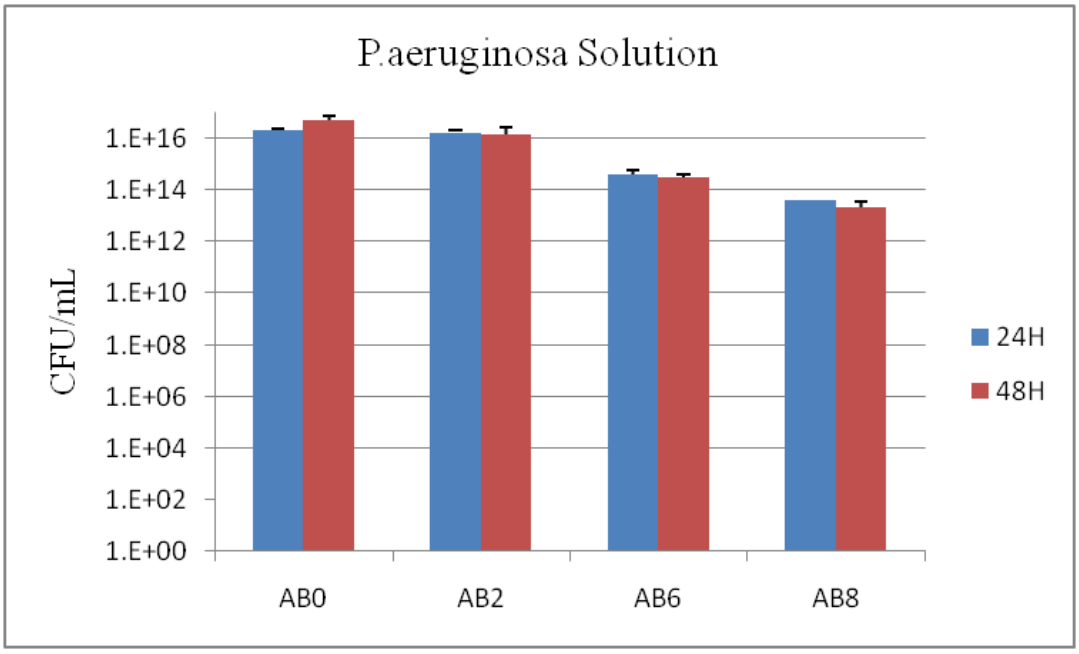


Figure 3.22:*P.aeruginosa* CFU numbers in solution of HA cements with different concentration of AP4 (AB0, AB2, AB6 and AB8) for 24 and 48h. Error bars represent the standard deviation (n= 3)

The antibacterial test results of HA cements after 24 and 48 hours in solution for *P.aeruginosa* are shown in Figures 3.22. It can be observed, that the AB8 had the lowest CFU numbers and AB0 had the highest CFU numbers. It is clear, that HA cements with higher concentration of AP4 can cause stronger effect on *P.aeruginosa*. However, the effect was not very strong. It also can be observed, that the ability of eliminating bacteria was nearly the same for 24 and 48 hours.

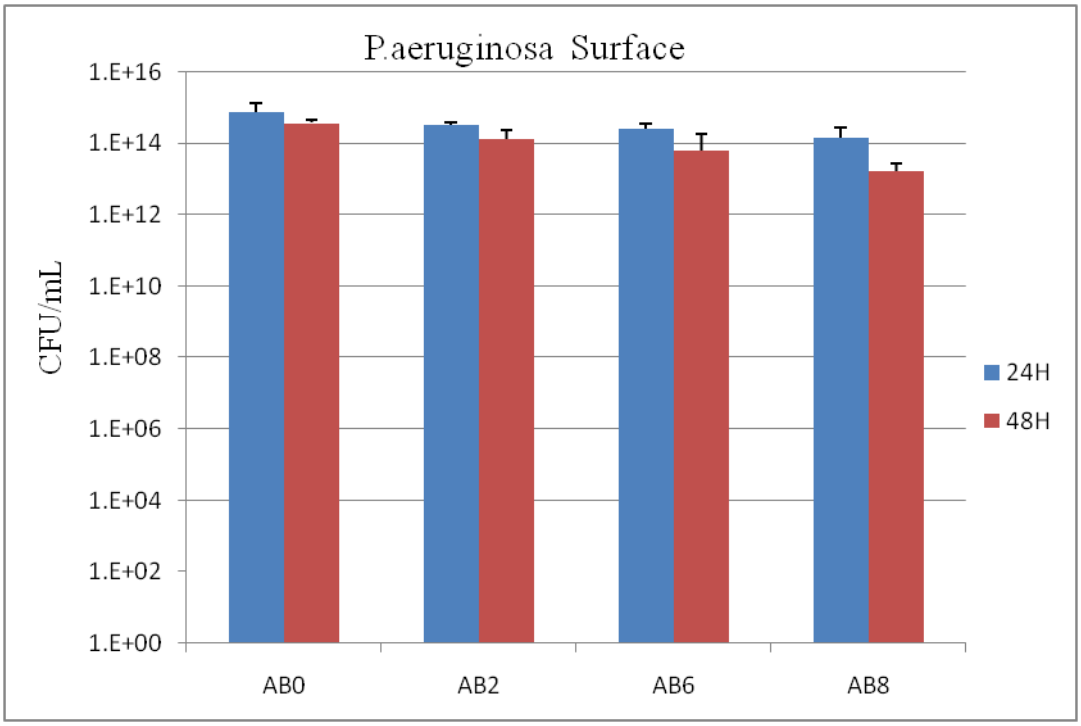


Figure 3.23:*P.aeruginosa* CFU numbers on the surface of HA cements with different concentration of AP4 (AB0, AB2, AB6 and AB8) for 24 and 48h. Error bars represent the standard deviation (n= 3)

The antibacterial test results of HA cements after 24 and 48 hours on surface for *P.aeruginosa* are shown in Figures 3.23. It can be observed, that the AB8 had stronger ability against *P.aeruginosa* than AB2, however, the antibacterial behaviour of them were small. This leads to the conclusion that by increasing the concentration of AP4in HA cements, the ability to eliminate bacteria from the surface was increased. However, the effect was not very strong. And the ability of eliminating bacteria is slightly decreased but nearly the same when the time increases from 24 to 48 hours.

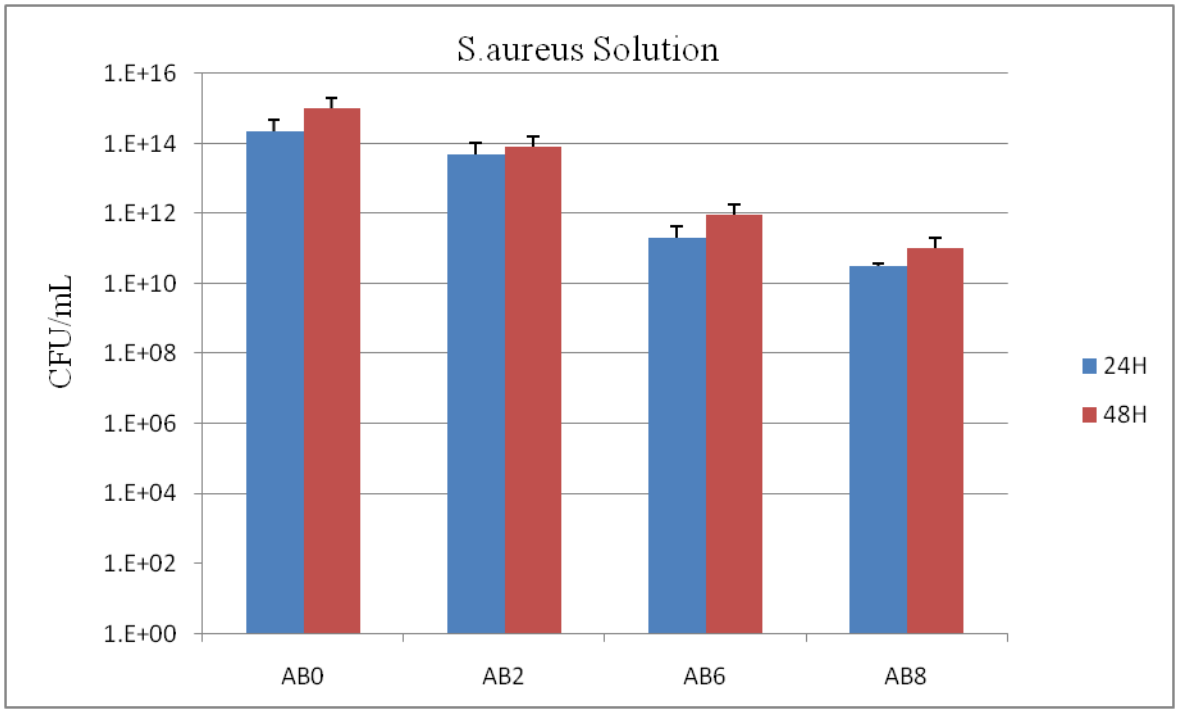
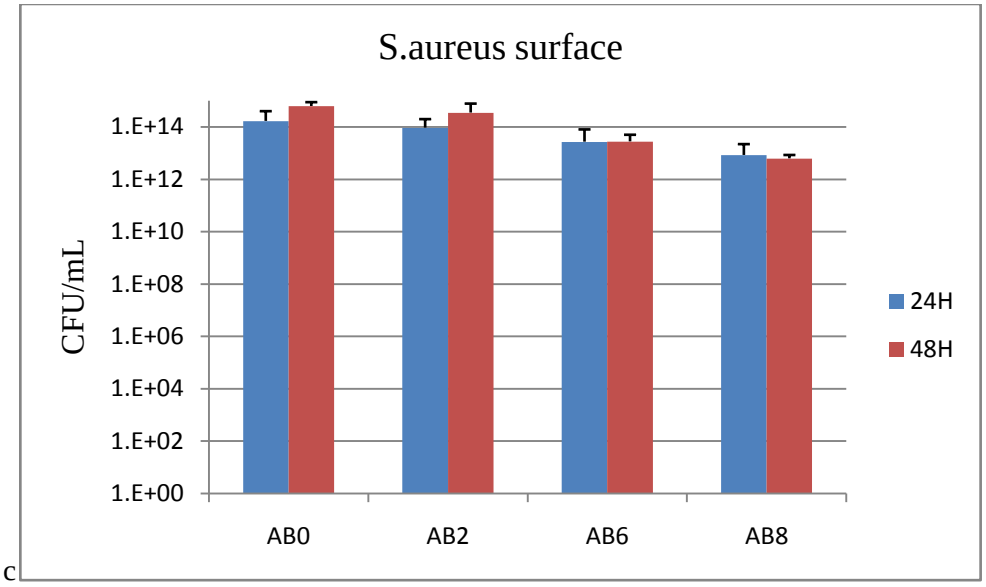


Figure 3.24:*S.aureus* CFU numbers in solution of HA cements with different concentration of AP4 (AB0, AB2, AB6 and AB8) for 24h and 48h. Error bars represent the standard deviation (n= 3)

The antibacterial test results of HA cements after 24 and 48 hours in solution for *S.aureus* are shown in Figures 3.24. It was observed, that AB8 had the lowest CFU numbers and AB0 had the highest. It could be concluded, that the HA cements with higher concentration of AP4 can cause stronger effect on *S.aureus*. It also should be noticed, that the ability of eliminating bacterial had not significantly changed when the time increased from 24 to 48 hours.



c

Figure 3.25:*S.aureus* CFU numbers on the surface of HA cements with different concentration of AP4 (AB0, AB2, AB6 and AB8) for 24h and 48h. Error bars represent the standard deviation (n= 3)

The 24 and 48 hours on surface antibacterial test results of HA cements for *S.aureus* are shown in Figures 3.35 and 3.36. It can be noticed, that AB8 had the strongest effect on *S.aureus* and AB2 had the weakest. It can be concluded, that as the concentration of AP4 in HA cements increased, the ability against *S.aureus* on the cements surface increased. And the ability of eliminating bacterial is almost the same between the time of 24 and 48 hours.

For the antibacterial property in solution, all the results shown above have a similar tendency. When the concentration of AP4 in HA cements increased, the antibacterial ability increased. However, even for AB8, the sample with the highest concentration in antimicrobial peptides was not possible to eliminate all the bacteria. One possible reason could be that the concentration of AP4 released with time from the cements can never reach the MIC of AP4 as calculated in section 3.1.2. The AB2 contained 0.83 mg AP4 in the cements, also the AB6 contained 2.5 mg AP4 and AB8 contained

3.33 mg AP4 in the sample. When the antibacterial test was conducted, all of the samples were immersed in 1 mL bacterial broth solution. Except for the AB2 sample, both AB6 and AB8 cements contained enough peptide to reach the MIC of AP4. However, considering the release results after 48 hours, the peptide concentration at 48 hours of AB2 cement sample in solution was 0.195 mg/mL, whereas the concentration of AB6 was 0.23 mg/mL and the concentration of AB8 was 0.257mg/mL (Figure 3.18). In addition, the MIC of AP4 against *E.coli*, *S.aureus* and *P.aeruginosa* was 1 mg/mL, 1mg/mL and 2mg/mL, respectively. Therefore, even when all HA cements contained the antibacterial peptide AP4 and showed a small effect against the bacteria, the effect was not strong enough to eliminate completely the bacteria from the surface of the samples.

With the concentration of AP4 increasing, the antibacterial property was increased, as a consequence of the increased release of the antibacterial peptide. In the first 0.5 hour, the release was faster compared to the release up to 3 hours. No significant changes were observed from 3 hours to 48 hours (Figure 3.17). This is the reason why the antibacterial ability in the solution was only slightly different between 24 and 48 hours.

Based on the amount of AP4 added in the cements, assuming homogeneous dispersion, the volume of the cylindrical sample and assuming that the surface thickness is ca 1μm, the surface peptide concentration of the sample was calculated. The surface concentration of AB2 was 5.523×10^{-3} mg/cm^2 whereas the surface concentration of AB6 was 0.017 mg/cm^2 and of AB8 was 0.022 mg/cm^2. Comparing the peptide surface concentration of AP4 with the MIC of AP4, it can be noticed that

the peptide surface concentration was much lower than the MIC. Perhaps, this is the main reason why the surface antibacterial property of the cements was so poor.

To our knowledge, there are no other studies exploring the antimicrobial properties of peptides in bone cements except of Stallmann *et al* who reported a study of the release behaviour of hLF 1-11 in HA cements as well as the antibacterial property against *S.aureus* in vivo [59,81]. Both studies concluded that it was difficult for the peptide to be released out of the cement. In this research, the low release rates resulted in an inferior antibacterial behaviour. However, the hLF1-11 could prevent infection better than AP4 due to its better MIC value (6.25mg/L) compared to that of AP4 (1 mg/mL)[59]. On the other hand, the hLF1-11 behaviour against both *E.coli* and *P.aeruginosa* were not reported and therefore it was not possible to compare the antibacterial behaviour of AP4 against these bacteria. In addition, Nibbering *et al*[84] and Hof *et al* [85] suggested, that the immunomodulation can affect the antibacterial peptide behaviour in vivo, which could lead to a lower concentration of peptide capable of eliminating microorganisms and bacteria compared to in vitro studies. It can be expected that, a different concentration of peptides should be considered (lower than the MIC value obtained in the in vitro experiments) for any in vivo studies.

To prevent infection during surgery, different types of antibiotics can be added in calcium phosphate bone cements, e.g. tetracycline hydrochloride, gentamicin, kanamycin etc.[40, 41, 86]. Some of these antibiotics perform well when added in bone cements for two reasons: first, they have shown good release behaviour and second only a small concentration is required to eliminate bacteria. Most of the antibiotics could release in the range of 25 to 85% [40,47,87-89]. Rabiee *et al* [40]

reported, that 25% tetracycline hydrochloride could be released from HA cements. Ethell *et al* [47] reported, that 40% gentamic in could be released and 30-40% amikac in could be released from HA cements. Stallmann *et al*[87] suggested that the release of gentamic in from different commercial cements could vary from 36 to 85%. Takano *et al* [88] also reported, that gentamic in could be released as much as 70% from HA cements. The large amount of antibiotics that can be released contribute to increase the porosity of the cements [10]. The amount of antibacterial peptide released was not possible to reach the amount of antibiotics release maybe because an interaction between the peptide and the cement is possible and a large amount of the peptides are in fact bound to the hardening cement as mentioned in section 3.3.2. Similar observations were made in the case of hLF1-11 added in the HA cements as reported by Stallmann *et al*[81]. If the peptides are not bound perhaps the antibacterial behaviour of cements can be enhanced. Another reason for the low performance of the antibacterial cements in this project may be caused by the high MIC of AP4. In comparison with the MIC values of common antibiotics (in the order of μg/mL) the MIC value for AP4 is much higher in the order of mg/mL. If the antimicrobial peptides used in the cements could reach the MIC value of hLF1-11(6.25mg/L), a better antibacterial property of the cements would be expected. Nevertheless, the use of peptides if compared with the use of antibiotics is always more beneficial as the use of antibiotics often results in bacterial resistance something that has not been observed or discussed in the case of antimicrobial peptides [90].

Another point worth mentioning is that the antibacterial property of the cements was generally poor. The 8 wt% of peptide was already the highest possible amount of peptide that could be added in the cements so that the cements could be mixed effectively. In this work, the peptide was added in the liquid phase with the hope that

the cement could be mixed better to receive homogeneous distribution of the antimicrobial peptide in the cements. For every 1g of powder, 0.4 ml of the liquid phase was needed. The highest possible amount of peptide was 8 wt% of 1g powder mixed with 0.4 ml of the liquid phase. However, 80 mg of the peptide cannot totally be dissolved in 0.4 mL of the solution. When 2 wt% of AP4 was dissolved in the liquid phase of cements, all the peptide dissolved in the liquid solution very well and it was a transparent solution. But when the 8 wt% peptide dissolved in the liquid phase, the solution was not clear which meant that not all of the peptide was dissolved in the liquid phase resulting in a turbid liquid. This was a problem that was difficult to overcome unless the peptide is added in the powder phase. It is possible that a better method to mix the cements could have had an impact on the antibacterial properties of the resulting HA cements.

CHAPTER 4: CONCLUSIONS

The main aim of this project was to isolate fragments from the antimicrobial core of human defensins that can have significant antimicrobial properties and incorporate them in CPCs in order to make antimicrobial bone cements. Mass spectrometry and HPLC were used to identify the molar mass and purity of peptide. The antibacterial property was tested by measuring the minimum inhibitory concentration (MIC) of peptides. The pH, antibacterial properties, peptide release and mechanical properties (compressive strength) of bone cements were measured against different concentrations of peptides.

The following conclusions can be drawn from the above work:

1. All four fragments from the antimicrobial core of human defensins have antibacterial activity against all *E.coli, S.aureus* and *P.aeruginosa*. The length of the peptide could affect the purity and the purity could impact on the antibacterial property of the peptides. Peptide AP4 with the shortest sequence (9-amino acid) had the best purity and the lowest MIC. AP4 was selected to be added into HA cements.

2. The pH was monitored so that to monitor the pH change and whether the pH remained >4.2 in order for HA to crystallise (precipitate). In this research all pH values of HA cements with different concentration of AP4 were at pH=7. Even the AP4 had an effect during the setting of the HA cements, but it could not change the main phase of HA. It was concluded that all the cement pastes underwent phase transition to HA.

c3. The release property was influenced by the concentration of AP4 in the cements. With the concentration of AP4 increasing, the amount of peptide released was increased. For each concentration, during the first 0.5 hour the release rate was higher than between 0.5 hour and 3 hours. After 48 hours, the peptide release concentration reached a plateau with no significant change. In addition, the amount of released peptide was small. Compared to the results from the literature, the main reason for that could be the strong link of the peptide with the hardening HA cement. The mechanism of binding was not clear. Further research is required.

4.The compressive strength was measured to study the effect of the peptide presence on the mechanical properties of HA cements. Compressive strength was affected by the immersion time in PBS and the concentration of AP4. When the concentration of AP4 in the cements was increased, the compressive strength was decreased. The 24 hours compressive strength of each concentration was lower than 48 hours. The decrease of compressive strength with the peptide concentration was probably due to the positive ions on the peptide that could delay the crystallisation of HA. An increase in porosity with the time is also possible due to peptide release. This would affect significantly the compressive strength of the cement. There is no experimental evidence for the real reason for the decrease of compressive strength with peptide concentration and therefore further study is required to answer this question. The increased compressive strength at 48h may be caused by a further crystal growth in the cement.

5. The antibacterial properties of HA cements were affected by the concentration of AP4. The antibacterial properties of HA cements were increased with the concentration of AP4. However, HA cements with highest concentration 8wt% could

not eliminate completely the presence of bacteria. Comparing the results with the literature, it can be concluded that the inferior performance of the peptides used in this study was attributed to the small amount of peptide released with time (lower to the MIC value of the peptide) and the values of MIC that was not low enough compared to other peptides in the literature. The antibacterial property between 24 hours and 48 hours was not very different. The release of peptide was faster at the first 0.5 hour (an initial burst-release was observed) and a gradual release from 0.5 hours to 3 hours and then reaching a plateau with no significant change up to 48 hours. Thus, there is no significant difference in antibacterial properties between 24 and 48 hours. The surface concentration of peptide was calculated and found that the concentration of peptide on the surface was lower than the MIC of AP4. This may be the cause of the poor antimicrobial performance of the cements.

CHAPTER 5: FUTURE WORK

Further investigation should be conducted in order to develop a complete understanding of the mechanism of the effect of peptides on the properties of HA cements:

1. In order to understand the peptide effect on the properties of HA cements, compressive strength change for example should be studied for longer setting times e.g., 15 days. In addition, the porosity of cements should also be studied at the same time length as increase in porosity (perhaps due to peptide release) can affect the mechanical properties of cements. A systematic study of the concentration of peptides in the cements and its effect on the setting of cements should be also conducted.

2. FITC labelled peptides could be used for the antimicrobial cements in order to observe the distribution of peptides in the cements by Fluorescence microscopy.

3. Considering that the antibacterial property of bone cements is not good enough, a wider concentration of the peptide in bone cements can be studied. For the reason that the 8 wt% peptide is already hard to dissolve in liquid phase, the higher concentration of peptide can be added into the powder phase instead of in liquid phase.

4. In this research, the release intensity tested was the cumulative intensity of the release solution. It can be changed to test the fresh release liquid in every time point, separately. This means that the sample will change to a fresh liquid after each time point test and the data will be exactly the released intensity between the last time point and next. This way more accuracy in the release data is expected.

5. Considering that the fluoresce in label may affect the antimicrobial behaviour of peptides, the MIC of peptides with and without fluoresce in label should be both tested.

6. The reason why the antimicrobial properties of the cements were not very good is not clear. For this reason, further study with appropriate experiments is required in order to establish whether the peptides interact chemically with the HA cement and whether this interaction changes the antimicrobial properties of peptides.

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