

**Engineered Biomolecules with Improved
Functionalities
For Bone Tissue Engineering**

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Dedication

This thesis is dedicated to my parents who supported me each step of the way. Thanks for always being there for me. Without their love and support my PhD would not have been made possible.

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Declaration of Originality

This thesis is a record of the work by me in the Department of Materials at Imperial College London. The work reported herein was composed by and originated entirely from me unless stated otherwise.

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Abstract

Biomolecules such as bone growth factors, extracellular matrix proteins, bioactive factor-derived peptides, and other mineralisation-promoting proteins are gaining increasing attention in bone tissue engineering due to the fact that they can play an important role in cell attachment, growth, differentiation, and migration, and therefore be used to enhance bone formation and develop bone substitutes.

This study is focused on the development of engineered biomolecules with improved functionality, and their combination with supporting matrix materials for bone tissue engineering applications. Two types of biomolecules are studied: The first are short peptides derived from the growth factor bone morphogenetic protein 7 (BMP-7), which induces bone regeneration, and regulates bone formation. The second is an engineered recombinant metalloenzyme *E. coli* alkaline phosphatase (*E. coli* ALP), which hydrolyses organic phosphate and generates the inorganic phosphate required for the mineralisation of hard tissue. Titanium (Ti) implants and polycaprolactone (PCL) scaffolds were used to incorporate these engineered biomolecules to verify the improvement of their functionality and biological efficiency. Results indicate that the BMP-7-derived peptides have a limited function and are not promising candidates for bone tissue engineering applications; while the mutant *E. coli* ALP shows superior activity compare to the wild-type ALP (nearly 25 times) and bovine ALP (more than 9 times), and is shown to be a promising factor to enhance bone formation. The activity of *E. coli* ALP can be affected by many factors including NaCl, lyophilisation, histidine, and imidazole. When the ALP is combined with either a Ti implant or an ALP/PCL scaffold, it demonstrates outstanding mineral-promoting ability, making it a potential candidate for bone tissue engineering applications. In conclusion, this study demonstrates the potential of engineering biomolecules with improved functionalities for bone tissue engineering applications.

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List of Abbreviations

α -MEM	Alpha-modified Eagle's medium
Amp	Ampicillin
ALP	Alkaline phosphatase
APS	Ammonium persulfate
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
β -GP	β -glycerophosphate
Ca	Calcium
CaCl ₂	Calcium chloride
CaP	Calcium phosphate
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DTT	Dithiothreitol
ECM	Extracellular matrix
<i>E. coli</i>	<i>Escherichia coli</i>
EDS	Energy dispersive spectroscopy
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
FGF	Fibroblast growth factor
Fmoc	Fluorenylmethyloxycarbonyl
FPLC	Fast protein liquid chromatography
GCAP	Germ cell alkaline phosphatase
GST	Glutathione S-transferase
HA	Hydroxyapatite
His	Histidine
HOBT/HBTU	1-Hydroxybenzotriazole/O-(Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HPLC	High-performance liquid chromatography
IAP	Intestine alkaline phosphatase
IGF	Insulin-like growth factor
IPTG	Isopropylthio- β -galactoside
Kan	Kanamycin
LB	Luria-Bertani
MALDI	Matrix-assisted laser desorption/ionisation

min	Minute
Mg	Magnesium
NaOH	Sodium hydroxide
NaCl	Sodium chloride
NGF	Nerve growth factor
Ni-NTA	Nickel-nitrilotriacetic acid
OCPC	o-cresolphthalein complexone
OP-1	Osteogenic protein-1
P	Phosphate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEO	Poly(ethylene oxide)
PCL	Poly(ϵ -caprolactone)
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
Pi	Inorganic phosphate
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
PLAP	Placental alkaline phosphatase
PLGA	Poly(lactic-co-glycolic acid)
PLLA	Poly(L-lactic acid)
PPi	Pyrophosphate
PVDF	Polyvinylidene fluoride
pNPP	p-Nitrophenylphosphate
rbs	Ribosomal binding site
RGD	Arginine-glycine-aspartic acid
ROP	Organic phosphate monoesters
rpm	Rounds per minute
SBF	Simulated body fluid
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
Taq	<i>Thermus aquaticus</i>
TBE	Tris/borate/EDTA buffer
TEMED	N,N,N,N'-tetramethylenediamine
TGF	Transforming growth factors
TFA	Trifluoroacetic acid
Ti	Titanium
TMA	Tetramethylammonium
TNAP	Tissue nonspecific alkaline phosphatase
VEGF	Vascular endothelial growth factor
Wt	Wild-type

Amino acid residues

A (Ala)	Alanine	M (Met)	Methionine
C (Cys)	Cysteine	N (Asn)	Asparagine
D (Asp)	Aspartic acid	P (Pro)	Proline
E (Glu)	Glutamic acid	Q (Gln)	Glutamine
F (Phe)	Phenylalanine	R (Arg)	Arginine
G (Gly)	Glycine	S (Ser)	Serine
H (His)	Histidine	T (Thr)	Threonine
I (Ile)	Isoleucine	V (Val)	Valine
K (Lys)	Lysine	W (Trp)	Tryptophan
L (Leu)	leucine	Y (Tyr)	Tyrosine

CHAPTER 1

Introduction

1.1 Scope of the thesis

This thesis is concerned with the development of biomolecules with improved functionalities to stimulate mineralisation and bone formation. Two types of biomolecules are studied. First are short peptides derived from the bone growth factor bone morphogenetic protein 7 (BMP-7) which induces bone regeneration, and regulates bone formation. Second is an engineered recombinant metalloenzyme *E. coli* alkaline phosphatase (*E. coli* ALP) that hydrolyses organic phosphate and generates inorganic phosphate required for mineralisation of hard tissue. Titanium (Ti) implants and polycaprolactone (PCL) scaffolds have been used to incorporate these engineered biomolecules to verify the improvement of the functionality and biological efficiency.

The Introduction chapter provides a general review of bone tissue engineering and covers different approaches of developing advanced tissue through cells, scaffolds and biologically active molecules. Focusing on biomolecules, some widely used biomolecules such as bone growth factors, extracellular matrix proteins, and other mineralisation-promoting proteins are reviewed in Section 1.4. The background knowledge of engineered recombinant proteins and immobilisation methods of biomolecules are presented in Section 1.5 and 1.6.

In Chapter 2, some short BMP-7-derived peptides which are reported to possess similar bone inducing properties to whole BMP-7 are studied. In order to immobilise the peptides onto titanium implants, a His-tag is added into the short BMP-7-derived peptides. After the synthesis and characterisation of the BMP-derived peptides, *in vitro* cell culture studies are performed to determine the effect of peptides on C2C12 and MC3T3-E1 cells.

The following chapters focus on the second type of biomolecule: *E. coli* ALP. Chapter 3 provides a description of producing and purifying the wild-type and mutated recombinant *E. coli* ALP with improved biological activity. After genetic modification, the mutant *E. coli* ALP shows higher biological activity than the normal wild-type *E. coli* ALP. Therefore the mutant *E. coli* ALP is promising for the construction of biomaterials for bone regeneration.

In Chapter 4, modification of the mutant recombinant *E. coli* ALP is performed to achieve a controlled biomolecule immobilisation. In order to maximally expose the active sites of ALP upon immobilisation, recombinant *E. coli* His-ALP is engineered. The production and purification of the engineered recombinant His-ALP are described in Section 4.2.

Chapter 5 describes and discusses two applications of bone tissue engineering with *E. coli* ALP, one being a Ti implant with recombinant ALP coating, and the other being an ALP/PCL scaffold. Preparation methods of both biomaterials are introduced in Section 5.2. Soaking experiments are performed to determine the mineral formation efficiency.

Finally, some concluding remarks and following research strategies of this study are presented in Chapter 6.

1.2 Tissue engineering

Due to the increase of human life expectancy, there is an increasing need for materials to substitute for tissues. Millions of patients suffer tissue loss or failure in the United States every year [1]. Examples of tissues needing replacement include liver, skin [2], bone [3], cartilage [4], lung, heart, kidney, and blood vessels[5]. Traditionally, the treatment for tissue damage is a transplantation that uses autologous or allogeneic materials and surgical reconstruction. However, both materials are limited by the

scope of their use and available resources. For example, although transplants are considered an effective treatment for liver failure [6], many patients die while waiting for a suitable replacement due to the severe shortage of donors [7]. Therefore, it is necessary to develop new approaches for tissue repair so that the need for transplantation is reduced or even eliminated.

Tissue engineering is a thriving field of medical science that addresses tissue loss or failure [8, 9]. As defined by Langer and Vacanti, tissue engineering is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" by manipulating cells *via* their extracellular microenvironments [1]. The aim of tissue engineering is to create substitute tissues or organs that can replace or enhance tissue which has been damaged by disease, injury, or ageing [10]. Although tissue engineering covers a broad range of applications, it can be mainly divided into two applications: 1) therapeutic application, for which a substitute tissue is either grown inside or outside a patient and then transplanted; 2) diagnostic applications, where a substitute tissue is made *in vitro* and used for testing drug metabolism, toxicity, and pathogenicity [11].

1.2.1 General principle

The general principle of tissue engineering is illustrated in Figure 1. The approach combines cells, scaffolds, and bioactive factors. Patient-derived cells, following expansion *in vitro*, are mixed with bioactive factors and then seeded in a scaffold to induce functionality. The engineered scaffold usually needs to undergo a pre-implantation *in vitro* culture before implantation *in vivo* for tissue regeneration.

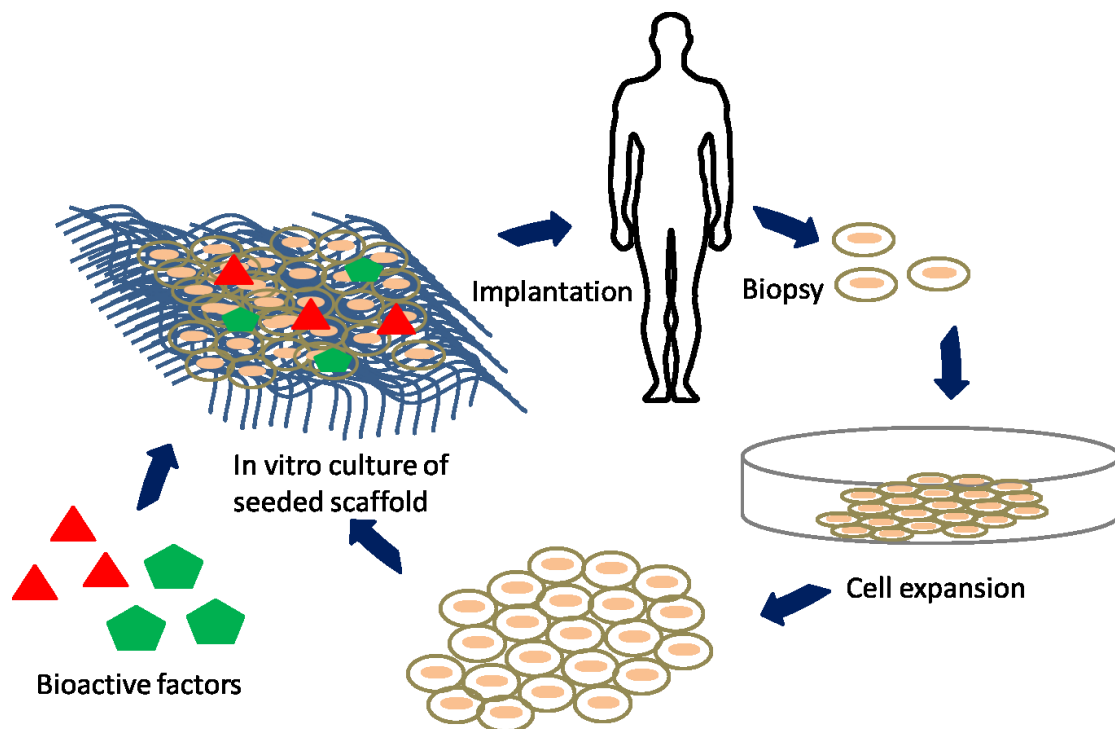


Figure 1. General principle of tissue engineering. Cells are isolated from the patient, expanded in cell culture, and then seeded on a scaffold material with bioactive factors to induce functionality. The scaffold is cultured *in vitro* before implantation in the defect area in the patient [12].

According to the definition, tissue engineering includes the following research areas:

(1) Cells: Living cells play important roles in tissue engineering. Cell studies include determining the ideal cell source, and researching the mechanism of cell biology such as proliferation and differentiation [13, 14]. Cell based strategies to enhance bone regeneration have a rapid development since the understanding of musculoskeletal cell behaviour improves [15].

(2) Scaffolds: Scaffolds are artificial structures made by biocompatible materials to enhance tissue formation. The surface of the scaffolds should permit cell attachment and reorganisation, and should finally form a substitute tissue following proliferation, extracellular matrix synthesis, and migration [16]. Three-dimensional scaffolds with high porosity suitable for cell attachment and migration in bone tissue engineering have increasingly gained attention [17, 18].

(3) Bioactive factors: In addition to choosing cells and fabricating scaffolds, other aspects such as biomechanical properties [19], or bioactive molecules such as growth

factors that show positive effects on tissue formation [20] are critical for developing substitute functional tissues [12].

These research areas are not isolated from each other. Combined approaches of mixing cells, scaffolds, and bioactive factors together are also highly interesting in bone tissue engineering [21]. For example, by optimising the initial cell density of bone marrow-derived cells seeded on three-dimensional scaffolds, an early differentiation and mineralisation was achieved within 3 weeks, and the new bone tissue was formed after 6 weeks [22]. The cell-scaffold system can be further combined with bioactive factors, such as an RGD-containing peptide that promotes osteoblast adhesion and spreading [23] to benefit bone tissue regeneration.

1.2.2 Bone biology

Bone is a living tissue with an outstanding ability to continuously remodel throughout the lifetime of an individual [24, 25]. Mature bone contains two morphologically distinct types of tissue: a hard outer layer called cortical or compact bone, which is strong, dense and tough, and a spongy inner layer called trabecular or cancellous bone, which is lighter, less dense and less elastic than compact bone [26]. Trabecular bone can bear more strain than cortical bone. Bones are responsible for providing structural support for the body. In addition, it supports muscular contraction, protects internal organs, and serves as a mineral store of Ca^{2+} and P^{3-} ions in the body [26]. Bone is composed of inorganic mineral, organic matrix, and water. The absolute amounts of these components may change with certain factors, such as species, age, and sex. The inorganic mineral, which is mainly composed of calcium hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), is responsible for rigidity and hardness of bone as well as being a mineral store [26]. The organic part of the bone (see more information in Section 1.4.2), which is mainly collagen fibres, provides nucleation sites of hydroxyapatite crystals and makes the bone resilient [27].

Cells are also important components of bone and are responsible for bone formation, maintenance and (re)modelling. Typically, there are several types of cells in the bone originating from two lineages: the mesenchymal stem cell lineage and the hematopoietic stem cell lineage. The mesenchymal stem cell lineage produces undifferentiated cells or preosteoblasts, osteoblasts, bone lining cells, and osteocytes. The hematopoietic stem cell lineage provides circulating or marrow monocytes, preosteoclasts, and osteoclasts. These cells are widely used in bone tissue engineering. For example, human mesenchymal stem cells were grown on a highly porous and degradable poly(ϵ -caprolactone) scaffold to develop bone grafts for bone tissue engineering [28]. Figure 2 shows the function and location of cells in the bone matrix [29, 30].

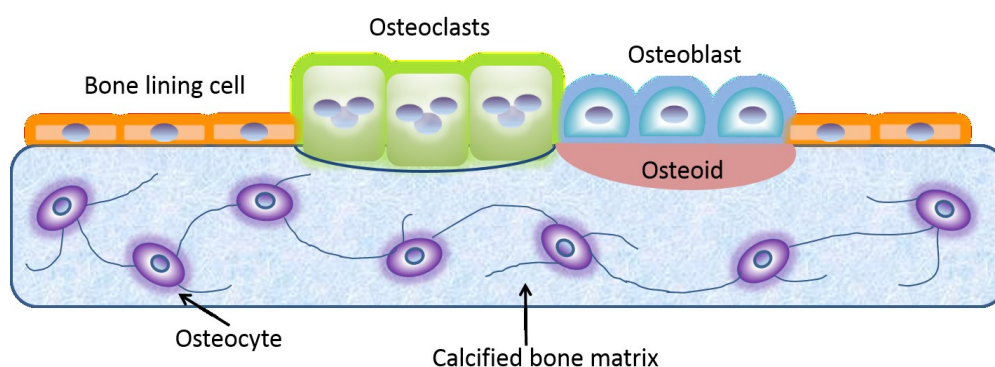


Figure 2. Schematic representation of cells in calcified bone matrix. Osteoblasts are responsible for bone matrix synthesis and mineralisation. When surrounded by bone matrix and incorporated within the newly formed osteoid, osteoblasts eventually transform into osteocytes. Osteoclasts are responsible for bone resorption. Only osteocytes are situated deep in bone matrix, other cells such as osteoblasts, bone-lining cells, and osteoclasts are situated on the bone surface [29].

Cells play important roles in the bone modelling and remodelling process. Bone modelling is an essential process associated with the growth and re-shaping of bones in childhood and adolescence [31]. Meanwhile, bone remodelling is a life-long process consisting of resorption and ossification. Its purpose is to support skeletal integrity, shape, and mass [31]. 25% of trabecular bone volume is renewed every year by osteoclasts and osteoblasts during remodelling [32]. The remodelling process consists

of four phases: first, preosteoclasts are stimulated and differentiated into osteoclasts under the influence of bone growth factors. Then osteoclasts digest old mineral matrix (resorption). When the resorption is finished, osteoblasts start to synthesise new bone matrix (ossification). Finally, osteoblasts transform into bone lining cells situated on the newly formed bone surface.

1.2.3 Approaches toward bone tissue engineering

Compared to other tissues, the requirement of bone substitutes is of particularly high importance. Although bone has a high regenerative ability to heal the most minor fractures by itself, large bone defects still require a template for regeneration, which often involves operations [24, 33]. Bone substitutes, which act as templates, are often necessary in a widely range including trauma, congenital and degenerative diseases, cancer and cosmetics [34, 35]. Approximately 500,000 operations requiring bone substitutes are carried out every year in the United States [34]. Currently available bone substitutes, including autologous grafts (from the patient), allogeneic grafts (from a donor), demineralised bone matrices, as well as a wide range of synthetic biomaterials such as metals, ceramics, polymers, and composites are far from ideal and have many associated problems [36]. For example, autologous grafts have limited sources and may lead to significant donor site morbidity, while synthetic materials do not behave like native bone [37]. Therefore, bone tissue engineering has emerged as the alternative strategy to the current therapies for bone regeneration or substitution.

As described in Section 1.2.2, bone has a complex structure and function. In order to operate, close interactions between bone and cells, matrix, bioactive factors are of vital importance. Therefore engineering bone requires a comprehensive approach with broad expertise [38]. Bone tissue engineering is based on the understanding of principles of bone formation and regeneration, and aims to combine living bone cells with biocompatible scaffolds as well as appropriate bioactive factors to induce new

functional bone tissue. Cells, scaffolds, and bioactive factors, especially biomolecules which induce bone functionality, are the main research areas in bone tissue engineering. Different approaches for developing biological bone substitutes are shown in Figure 3.

(1) Cells: Various types of cells have been considered as promising candidates in bone tissue engineering including autologous cells, allogeneic cells, xenogeneic cells, stem cells, and genetically engineered cells. Autologous cells, such as periosteal cells, cortical cells, marrow cells, and cells derived from the surrounding soft tissues are preferred because of their non-immunologic effect [39]. However, patients are frequently unable to provide enough cells, especially those who have end-stage organ diseases.

Stem cells are considered an alternative cell source because their outstanding ability to undergo osteogenic differentiation. The types of stem cells used in bone tissue engineering include embryonic stem cells, bone marrow-derived mesenchymal stem cells, umbilical cord blood-derived mesenchymal stem cells, adipose tissue-derived stem cells, muscle-derived stem cells, and dental pulp stem cells [40]. For example, embryonic stem cells can differentiate into the osteoblast lineage by supplementing the serum-containing media with ascorbic acid, β -glycerophosphate, and/or dexamethasone/retinoic acid, or by co-culture with fetal murine osteoblasts [41]. Moreover, pre-osteoblasts and osteoblasts, which are important for the development and maintenance of bone, have been widely used in bone tissue engineering. For example, pre-osteoblast MC3T3-E1 cells were seeded on polymer scaffolds to enhance bone regeneration [42].

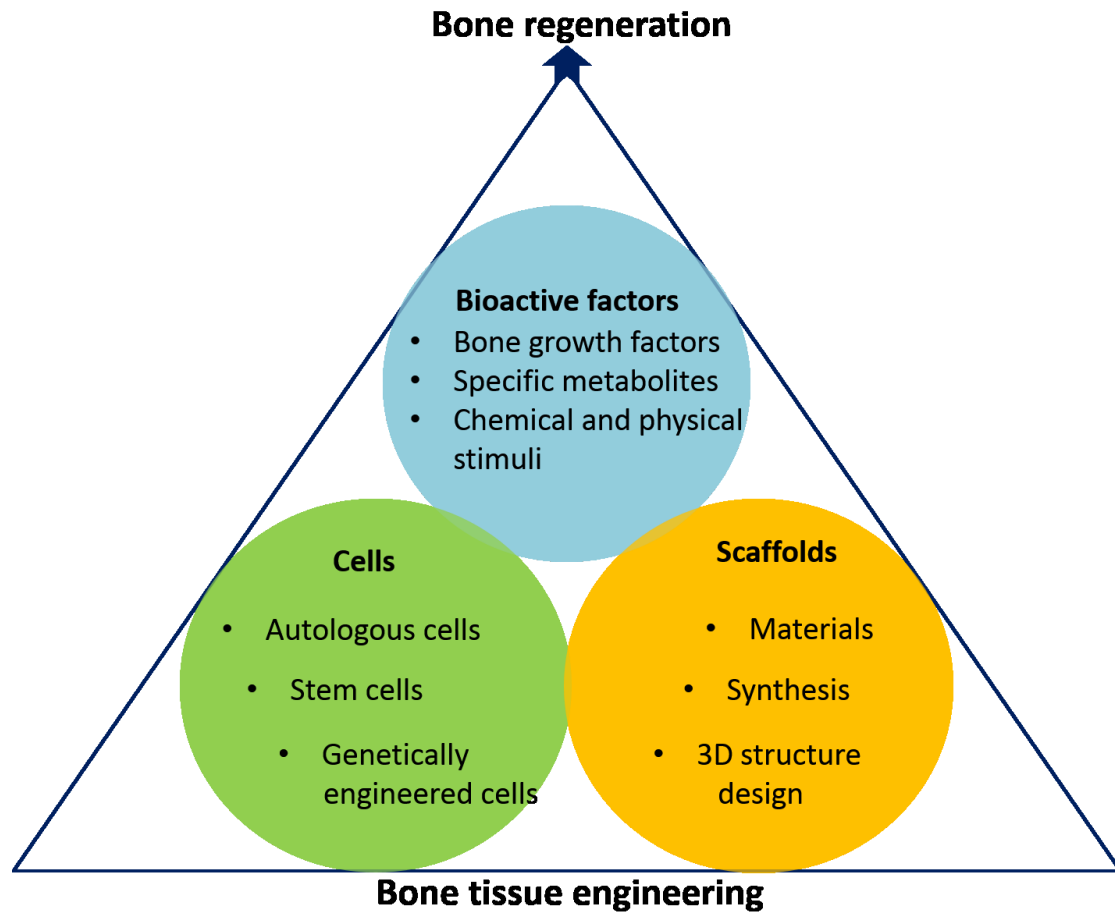


Figure 3. Schematic representation of three main research areas in bone tissue engineering: cells, scaffolds, and bioactive factors. In each area, there are several different approaches that can be used individually or in combination to achieve bone regeneration.

(2) Scaffold: A good scaffold must facilitate cells to attach, proliferate, and develop in a predictable and controlled way. At the same time, the scaffold needs to mimic the mechanical properties of the natural bone tissue, allow the diffusion of nutrients and waste products, and avoid an immune response [43]. Additionally, the scaffold should allow an efficient release and delivery of bioactive factors to induce bone formation [44]. In order to meet these requirements and to develop novel biocompatible and bio-functional scaffolds for bone tissue engineering applications, various natural and synthetic biocompatible materials have been developed and investigated. A brief review of porous material scaffolds used in bone tissue engineering is in Section 1.3.

(3) Bioactive factors: Cells need certain signals to survive and develop in their new environment after being seeded on scaffold. In order to stimulate the correct biological signals and induce the desired functionality, a number of bioactive factors including growth factors, hormones, specific metabolites, and chemical and physical stimuli are required for bone regeneration [45]. In the area of bone tissue engineering, biomolecules such as bone growth factors, extracellular matrix proteins, bioactive factor-derived peptides and other mineralisation-promoting proteins are of high interest. For example, bone morphogenetic proteins have been widely used in bone tissue regeneration because of their ability to promote differentiation and regulate cellular processes [46]. Examples of the biomolecules related to bone tissue engineering is reviewed in Section 1.4.

1.3 Scaffolds for bone tissue engineering

In vivo, the extracellular matrix provides an environment for cells to attach, proliferate, and differentiate. In tissue engineering, scaffolds are required to undertake this responsibility [47]. Three important steps are necessary for fabricating a suitable scaffold for bone tissue engineering applications [25]. The first step is to fully understand and consider the essential properties (listed in Table 1) of the scaffold for bone tissue engineering. Such properties affect not only cell survival, signalling, growth, propagation, and reorganisation, but also gene expression and phenotype [48].

Property	Requirements
Biocompatibility	• Well integrated in the surrounding tissue • No immune response
Porosity	• With large surface area to volume ratios • Fully interconnected geometry • Well balanced with the mechanical needs
Pore size	• Within the 200-900 μm range for bone tissue engineering [49, 50]
Surface properties	• Well controlled chemical and topographical properties such as the surface roughness for easy adhesion of cells and proteins [51]
Osteoinductivity	• Controlled release of bioactive factors • Induction of osteogenic differentiation pathway
Mechanical properties	• Comparable to the living bone
Biodegradability	• Well matched degradation rate with the growth rate of the new bone

Table 1. Essential properties of scaffolds for bone tissue engineering.

The next step is to select the most appropriate materials to produce a scaffold. This is a very important step since the properties of the material will largely determine the properties of the scaffold [52]. Numerous materials have been considered including metals, polymers from natural and synthetic origins, and ceramics. Despite their lack of biodegradation, metal implants, particularly titanium implants, have been widely used in reconstructive surgery, orthopaedic surgery, and oral implantology as scaffolds for load-bearing, bone replacing, and contacting applications [53]. The purpose of using titanium implants is to obtain a life-long secure anchoring of the implants in the surrounding bone tissue [54]. A brief summary of titanium implants and surface modification methods of titanium implants is shown in Section 1.3.1 and 1.3.2,

respectively. In addition to metal, natural polymers such as fibrin, collagen, chitosan, and hyaluronic acid, and synthetic polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA), poly(ϵ -caprolactone)(PCL), and poly(l-lactic acid) (PLLA) are well-studied scaffold candidate materials [55-57]. Table 2 reviews some polymers commonly used in bone tissue engineering [55, 58-60]. Among them, poly(ϵ -caprolactone) (PCL), which has been widely used as a model polymer, is reviewed in Section 1.3.3.

The final step of fabricating a suitable scaffold is to develop or choose an appropriate processing technique. The chosen technique should not only be accurate and consistent, but also have no negative side effects on the materials properties [48]. Some common techniques are summarised in Table 3 including nano-fibrous electrospinning, solvent casting, gas foaming, and freeze drying [36].

Origin	Material	Positive features	Negative features
Natural	Fibrin	• Promotes cell migration and vascularisation • Promotes osteoconduction	• Poor mechanical properties
	Collagen	• Low immune response • Promotes osteoconduction	• Poor mechanical properties
	Chitosan	• Promotes osteoconduction and wound healing	• Not soluble in water and organic solvents
	Hyaluronic acid	• Minimal immunogenicity • Promotes osteoconduction	• Poor mechanical properties
Synthetic	PLA	• Easily metabolised in the body • Good mechanical properties	• Strongly hydrophobic
	PGA	• Good mechanical properties • Quick degradation	• Not soluble in most organic solvents
	PLGA	• Good mechanical properties • Adjustable degradation rate	• Unstable compared to homopolymers
	PCL	• Easily metabolised in the body • Low chemical versatility • Promotes osteoconduction	• Slow degradation, unsuitable for short term applications

Table 2. Natural and synthetic polymers used for bone tissue engineering.

Technique	Advantage	Disadvantage
Nano-fibrous electrospinning	• Simple operation • Adequate control of pore size and porosity • Fibre diameters ranging from several microns down to several hundred nanometers	• Usually sheets or cylinders, not 3D scaffold shapes
Solvent casting	• Simple operation • Adequate control of pore size and porosity	• Limited pore shape and thickness • Limited thickness ranging from 0.5 to 2 mm [61] • Limited inter-pore connectivity
Gas foaming	• Highly porous foams without the use of organic solvents	• Closed-pore structure, with only 10–30% of interconnected pores [62]
Freeze drying	• Highly porous foams (over 90%) • Pore size ranging from 20 to 200µm	• Low mechanical stability • Pore sizes in the range of 100 µm • Closed-pore structure [63]

Table 3. The processing techniques for fabricating a suitable scaffold for bone tissue engineering.

1.3.1 Bone substitute implants

An implant is an artificial medical device made from a biocompatible material that is

designed and fabricated for substitution of a missing or damaged tissue [64]. Common applications include bone implants [65], dental implants [66], and cardiac implants [67].

Titanium (Ti) is one of most widely used bone substitute implant materials, especially in load-bearing applications because of its superior properties, such as high mechanical strength, low weight, outstanding corrosion resistance, and very importantly, its biocompatibility. The biocompatibility of Ti implants is attributed to the 3-10 nm thick stable oxide layer on the implant surface which is formed by reactions with oxygen [68]. The oxide layer not only prevents the formation of fibrous tissue around the implant, but also creates direct contact with osseous tissue [53]. Ti used for medical implants began as early as the 1940s [69]. Since then, the usage of Ti implants has continually increased worldwide, with more than 1,000 tonnes of Ti implanted into patients every year [70]. Ti implants are also widely used in bone tissue engineering. For example, titanium foams, which have a pore distribution architecture, show excellent biomechanical properties and bioactivity to form a bone-like apatite layer [71]. However, although Ti shows good biocompatibility, implant fixation delay may be caused by the non-physiological surface that is directly exposed to a physiological environment of the native surrounding bone tissue without any physiological transition after implantation [72]. This can seriously diminish the quality of life for the patient. To enhance the interface between Ti and the surrounding tissue, and to reduce the time needed for bone implant fixation, surface modification of the Ti implants is believed to be an effective approach. Surface modification improves implant properties by modifying the implant surface characteristics such as composition, structure, and morphology, but leaving the mechanical properties intact [73]. Through surface modification, the tissue compatibility of the surface layer can be improved.

1.3.2 Surface modification of titanium implants

Implant surface topography, surface biochemistry, and mechanical loading are believed to affect new bone formation [53]. For example, the surface roughness of Ti implants affects osteoblast growth and adhesion [74]. Surface modification is now a key consideration in developing functional bone implant materials.

Several reviews summarise a variety of surface modification approaches for Ti or Ti alloys in the biomedical field (Figure 4) [54, 66, 75-78]. The approaches can be divided into two parts. First, surface topography approaches, which are achieved mainly through mechanical methods such as grinding, machining [79], and polishing [80], and chemical methods such as acid etching [81] and alkali etching [82]. Surface topography approaches have been widely used in industry because they are easy to perform. Second, surface biochemistry approaches which use coating techniques to create biologically active implant surfaces to promote bone formation [83, 84]. Research has been focused on biochemical methods to produce bioactive coatings that induce a specific cellular response and promote osseointegration. For example, chitosan was used as a biocompatible bioactive coating on Ti implant to enhance osteoblast attachment and growth [85]. There are mainly two bioactive coating types on titanium implants: inorganic coatings, such as calcium phosphate (CaP) coatings which mimic the mineral phase in nature bone [86], and organic biomolecule coatings which are fabricated from bone-related biomolecules such as growth factors, extracellular matrix proteins, and other mineralisation-promoting proteins. Growth factors such as platelet-derived growth factor and insulin-like growth factor on the Ti implant stimulated bone regeneration [87] and alkaline phosphatase, an important enzyme in bone formation, accelerated mineralisation when coated on Ti implants [72], improved the osteogenic response [88], and enhanced periprosthetic bone formation [89]. Some peptides derived from these biomolecules can also be immobilised onto Ti implant surfaces to enhance bone regeneration. For example, the well-known peptide sequence Arg-Gly-Asp (RGD), which plays an important role in osteoblast adhesion,

was coated on Ti implant to enhance tissue integration [90] and stimulate bone formation [91].

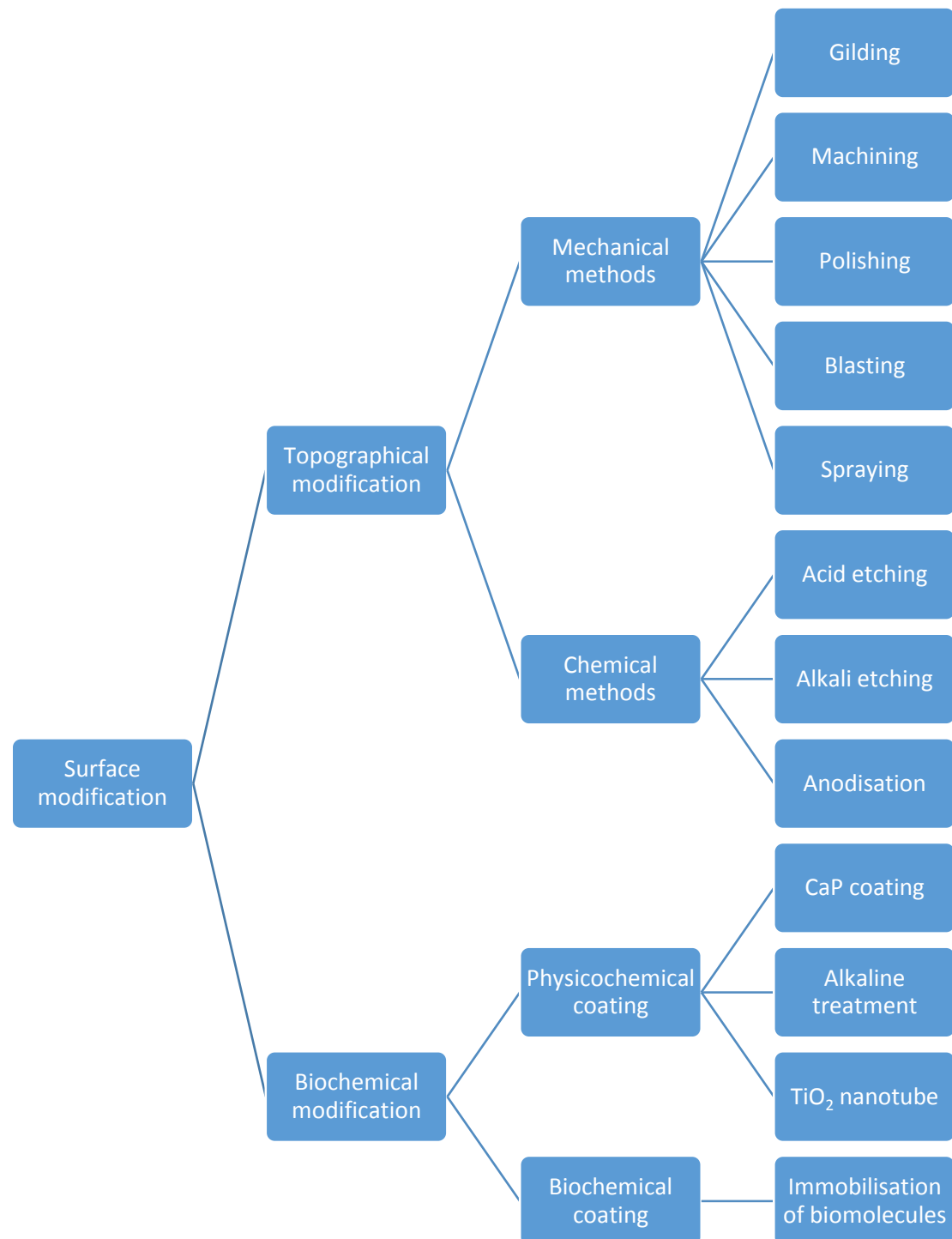


Figure 4. Surface modification techniques of titanium implants used in bone tissue engineering to improve biocompatibility [54, 66, 75-78].

1.3.3 PCL scaffolds for bone tissue engineering

Poly(ϵ -caprolactone) (PCL) is one of the most widely studied polymers and has been successfully used as a scaffold in bone tissue engineering applications [92, 93]. PCL is a fully biodegradable, semi-crystalline polymer with a low glass transition temperature (-60°C), a low melting temperature (59 to 64°C) and a high decomposition temperature (350°C) [94]. PCL degrades at a lower rate than PLA and PLGA due to its high hydrophobicity and crystallinity [95], making PCL suitable for long term, load-bearing applications. Additionally, PCL is cheaper, more stable, and available in larger quantities than other polymers.

In addition to using it alone, PCL can also be combined with various other polymers and biomolecules. Numerous studies have been carried out using PCL biocomposites and copolymers with polymers and proteins [96, 97]. For example, the enzyme alkaline phosphatase was directly encapsulated in PCL microtubes fabricated by co-electrospinning [98]. And an encapsulated lipase in PCL preserved 50% of its original activity after the 10th run in the transesterification reaction in hexane medium [99].

1.4 Biomolecules for bone tissue engineering

In many bone tissue engineering applications, simply culturing cells on scaffolds is insufficient for bone regeneration. This is because cells require appropriate bioactive factors to induce functionality and to thereafter develop tissue. As described in Section 1.2.3, biomolecules such as bone growth factors, cellular matrix proteins, bioactive factor-derived peptides, and other mineralisation-promoting proteins can be used as bioactive factors to enhance bone formation and develop bone substitutes [38]. These biomolecules may play a role in the steps towards bone formation including modulating cell attachment, growth, differentiation, and migration.

Bone formation is an extremely complex process involving a large number of biomolecules that therefore have the potential to be used in bone tissue engineering. Here, the highly studied ones are reviewed, including bone growth factors, in particular bone morphogenetic proteins and derived peptides, cellular bone matrix proteins, and other mineralisation-promoting proteins such as alkaline phosphatase.

1.4.1 Bone growth factors

Growth factors, which play a key role between cells and the microenvironments, have the ability to stimulate proliferation and differentiation, and to regulate various cellular processes including cellular development, matrix production, and tissue regeneration [20]. With regard to bone growth factors, various bone-derived growth factors have been investigated, including insulin-like growth factor I and II (IGF-I, IGF-II), transforming growth factors (TGF-beta 1, TGF-beta 2), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and bone morphogenetic proteins (BMPs). The origin, functions, signalling pathways, and cellular target of these bone growth factors are listed in Table 4. They are mainly isolated from bone matrix extracts and bone cells, or bone organ culture media [100]. All of these bone growth factors play important roles in the local regulation of bone formation, but they may target different cells of the osteogenic lineage, and have a specific role in different stages of regulating cell proliferation and differentiation [101]. For example, IGF-I stimulates bone matrix synthesis in calvariae through its stimulatory effect on DNA synthesis [102]. Basic FGF stimulates calvarial DNA synthesis, which causes an increased number of collagen-synthesizing cells, and eventually enhances bone formation [103].

Bone growth factor	Origin	Main functions	Cellular target
Insulin-like growth factor (IGF)	Bone extracellular matrix, osteoblasts, chondrocytes	• Stimulates type I collagen production • Stimulates cellular proliferation	Tyrosine kinase receptors
Transforming growth factor beta(TGF-beta)	Bone extracellular matrix, platelets	• Stimulates type II collagen production • Stimulates cell replication and the formation of extracellular matrix	Serine/threonine kinase receptors
Platelet-derived growth factor (PDGF)	Osteoblasts, platelets	• Signals inflammatory cells to migrate to fracture site • Promotes angiogenesis • Stimulates cell replication	Tyrosine kinase receptors
Fibroblast growth factor (FGF)	Osteoblasts, chondrocytes, macrophages, mesenchymal cells	• Promotes angiogenesis • Stimulates cell replication	Tyrosine kinase receptors
Bone morphogenetic protein (BMP)	Bone extracellular matrix, osteoprogenitor cells	• Activates mesenchymal cells to transform into osteoblasts • Regulates chondrocytes and bone-marrow stromal cells	Serine/threonine kinase receptor

Table 4. Growth factors used in bone tissue engineering and their main functions [100, 101].

In addition to the five main bone growth factors mentioned in Table 4, there are other regulators of bone listed below. Although they may have other main functions, they are all directly or indirectly related to bone formation. Some of them may have a biphasic effect on bone formation depending on timing, dosage and the duration of administration.

(1) Vascular endothelial growth factor (VEGF): Although VEGF is an essential regulator in the development and modulation of angiogenesis, it is also functional in bone repair. It has been shown that blocking VEGF activity in mice results in an enlarged area of hypertrophic cartilage and impaired trabecular bone formation [104]. VEGF is also believed to regulate growth plate morphogenesis, cartilage remodelling and ossification [105].

(2) Epidermal growth factor (EGF): EGF has biphasic effects on cell growth, proliferation, differentiation, and bone formation. Osteoblast growth and bone formation can be either increased or decreased by controlling the treatment time and dosage of EGF [106-108]. The results indicate that EGF may be functional in the early phase of bone formation process to promote osteoblast growth rather than the late phase involving mineralisation [109].

(3) Nerve growth factor (NGF): NGF is an important regulator for the growth, maintenance, and survival of certain neurons. It is also has the ability to enhance bone regeneration in a fracture model and to stimulate osteoblast differentiation [110].

(4) Erythropoietin (EPO): EPO is an essential factor controlling erythropoiesis. In addition, studies show that EPO induces bone formation either by activating the production of BMPs or by directly activating mesenchymal cells to form osteoblasts *in vitro* [111].

(5) Wnt proteins (Wnts): Wnts are a group of glycoproteins which regulate cell behaviours including cell growth, differentiation, and function. They also regulate the

bone remodelling process [112]. For example, Wnt7b, which is expressed by osteogenic cells, induces osteoblast differentiation *in vitro* and promote bone formation [113].

1.4.1.1 Bone morphogenetic proteins

Among all bone growth factors used to induce bone formation, bone morphogenetic proteins (BMPs) are the most potent inducers of ectopic bone formation described to date. They show clear functions in the regulation of bone formation, which have been examined extensively *in vivo* [114-116]. BMPs are members of the transforming growth factor superfamily, and are inducers of bone regeneration and ectopic bone formation [117], as well as regulators of the key steps of cartilage and bone morphogenesis [118]. BMP was first identified to be responsible for the induction of new bone in the 1960s, when demineralised bone matrix implants in ectopic sites in rats were found to induce bone formation [119]. The role of BMP became clear when the purification and sequence of bovine BMP-3 and cloning of human BMP-2 and -4 were investigated in the late 1980s [120-122].

Similar to other members of the TGF superfamily, BMPs are first synthesised as large precursor proteins containing a hydrophobic leader sequence, followed by the pro-peptide and the mature part of the protein. The precursor protein will cleave to yield carboxyl terminal mature dimers upon dimerisation [121]. The crystal structure reveals that BMPs contain a cysteine knot containing six cysteine amino acid residues in their carboxyl terminal [123]. The cysteine knot is responsible for the formation of dimer through disulphide bonds. In addition, BMPs contain N-linked glycosylation sites [123]. After secretion to the extracellular space, BMPs bind to serine/threonine kinase receptors which contain type I and II subtypes on the cell surface to achieve their signalling function [124]. Type I and II BMP receptors and their downstream molecules Smad-1, 5 and 8 are essential for signal transduction [125].

Approximately 20 BMPs have been identified and characterised to date [126]. The functions of some BMP family members are summarised in Table 5. Based on the amino acid sequence similarities of the mature regions of BMPs, the BMP family members can be broadly classified into several subfamilies [127]. Both BMP-2 and BMP-4 have 80% amino acid sequence homology of molecules; BMP-5, -6, -7 and -8 have 78% amino acid sequence homology; BMP-12, -13 and -14 also have high homology. However, BMP-3 and BMP-11 are significantly different from the any other members and stand as two separate subfamilies [122, 126, 127].

Although various BMPs are naturally existing in the body, researchers have focused on BMP-2 and BMP-7 because of their specialist functions. For example, BMP-2 is one of the most thoroughly investigated BMPs that has been approved for clinical studies to successfully simulate spinal fusion equal to or better than the patient's own bone [128]. This means spinal fusion can be achieved without painful surgery and patients experience fewer side effects and complications. BMP-7, also called osteogenic protein (OP-1), has the ability to stimulate osteoblast differentiation [129], and convert C2C12 cells from myoblasts into osteoblast lineage cells [130]. However, large amounts of BMP-2 are required during the treatment [131-133]. Although BMPs are natural components in the body, high concentrations of BMPs may lead to immunological and carcinogenic effects. In addition, difficulties in the mass production of BMP-2 make it expensive and thus it does not have extensive applications in clinical practice [134, 135]. Furthermore, BMPs have several hundred amino acids, which are too large and complex to chemically functionalise on nanostructured materials (such as particles and fibres) [135, 136].

BMP	Main functions
BMP-2	• Induces osteoblast differentiation • Mediates retinoid
BMP-3	• Inhibits osteogenesis
BMP-4	• Induces osteoblast differentiation • Regulates the formation of teeth, limbs, and bone • Repairs fractures
BMP-5	• Induces chondrogenesis
BMP-6	• Plays a key role in osteoblast differentiation • Induces chondrogenesis • Controls iron homeostasis <i>via</i> regulation of hepcidin
BMP-7 (OP-1)	• Induces osteoblast differentiation • Regulates renal development and repair
BMP-8a	• Induces osteoblast differentiation • Induces chondrogenesis
BMP-8b	• Induces osteoblast differentiation
BMP-9	• Induces hepatogenesis • Plays a role in the nervous system
BMP-10	• Plays a role in the trabeculation of the embryonic heart
BMP-11 (GDF-8)	• Plays a role in mesodermal and neuronal tissues
BMP-12(GDF-7)	• Induces tendon-iliac tissue formation
BMP-13(GDF-6)	• Induces tendon and ligament-like tissue formation
BMP-14(GDF-5)	• Induces chondrogenesis • Enhances tendon healing
BMP-15	• Modifies follicular development

Table 5. BMP family members and their main functions [126, 127].

1.4.1.2 Peptides derived from BMPs

To overcome the problems triggered by the use of full-length BMP proteins, peptides derived from BMPs were designed and used for bone tissue engineering applications as listed in Table 6 [118, 129, 136-140].

Peptide	Source	Main functions
P24 peptide	BMP-2	Enhances the osteoblastic differentiation of bone marrow stem cells
KIPKASSVPTELSAISTLYL	'knuckle epitope' of BMP-2	Increases alkaline phosphatase activity in osteoblasts
KPCCAPTQLNAISVLYFDDS, AISVLYFDDSSNVILKKYRN	BMP-7	Encourages the mineralisation process in osteoblasts
SNVILKKYRN, KPSSAPTQLN	BMP-7	Promotes osteoblast proliferation
KAISVLYFDDS	BMP-7	Increases osteoblast calcium deposition

Table 6. Peptides derived from BMPs used for bone tissue engineering applications.

By using peptide sequences, a specific cellular interaction related to a certain fragment of the entire protein can be targeted without any unwanted responses. In addition, peptide sequences can be easily produced by chemical synthesis, which allows a precise control of the chemical components and avoids the species differences from biological synthesis [141]. Furthermore, small peptide sequences tend to be a more efficient approach especially in surface coating applications because more peptides with active sites can be immobilised on the same surface area than large proteins, therefore exposing more active sites. Even more important, these peptide sequences

possess similar functionalities compared to the proteins. It was reported that specific peptides support bone mineralisation [129] and induce bone formation [142]. For example, the BMP-7-derived peptide KAISVLYFDDS has the ability to increase osteoblast calcium deposition, which is an essential step in bone formation [136]. Therefore, the use of BMP-derived peptides is a promising approach for the construction of biomaterials for bone repair.

1.4.2 Extracellular matrix proteins

Bone matrix is a composite material located between the cells that contains inorganic and organic components. The inorganic component, which is composed of carbonate rich hydroxyapatite (with a carbonate content between 4% and 8%, w/w) [143], provides compressive strength and serves as a mineral store of calcium and phosphate [26]. The organic part of bone matrix contains 90% collagen (primarily type I collagen) and a number of non-collagenous proteins to make up the remaining 10%.

Collagen, which is the most abundant protein in the body, plays critical roles in the structure and function of bone tissue. Cell and molecular evidence indicates that collagen also has direct effect on bone cell function including apoptosis, proliferation, and differentiation [144-146]. For example, type I collagen plays a key role in mediating the signalling cascade for expression of a mature osteoblast phenotype and mineralisation of the extracellular matrix [146]. Non-collagenous proteins, such as osteocalcin, osteonectin, bone sialo-protein, fibronectin, thrombospondin, and certain proteoglycans, are expressed by osteoblasts during osteogenesis. Similar to collagen, many non-collagenous proteins have been shown to have multiple functions on bone cells including regulation of collagen fibril mineralisation, cell division, migration, differentiation and maturation, growth factor activity, and tissue integrity and function [147, 148]. For example, osteocalcin, the most abundant non-collagenous protein in bone (10-20% of total, w/w), is important in bone metabolic regulation and

is thought to play a role in bone mineralisation and calcium ion homeostasis [149]. It has also been used as a clinical marker for bone turnover by measuring its presence in urine. Table 7 gives information about some of the most highly studied bone matrix proteins to date [150].

Bone matrix protein	Main functions
Type I collagen	• Provides tensile strength • Plays a key role in the structure and function of bone tissue • Affects bone cell function
Osteocalcin	• Promote mineralisation and bone formation • Regulates bone metabolism • Binds calcium
Osteonectin	• Induces osteoblast differentiation • Regulates mineralisation
Osteopontin	• Increases angiogenesis • Enhances bone resorption
Bone sialo-protein	• Binds to integrins, may assist cancer cells
Fibronectin	• Regulates osteoblast differentiation
Thrombospondin	• Inhibits bone cell precursors
Proteoglycan	• Responsible for compressive strength • Inhibits mineralisation

Table 7. Bone matrix proteins and their main functions [150].

Because of the importance of bone matrix proteins in the bone formation process, extensive studies have been carried out to functionalise scaffolds with elements of bone matrix proteins in bone tissue engineering. The bone matrix proteins involved in the cell adhesion process including type I collagen, osteopontin, bone sialo-protein, fibronectin, thrombospondin and vitronectin are of particular interest [151, 152]. Cells

need specific adhesion molecules to bind to a surface, the extracellular matrix or another cell. Cell adhesion is essential because it directly influences the following steps of cell behaviour, including growth, differentiation, and migration. One family of well-known cell adhesion molecules are the integrins, which bind to specific amino acid sequences such as arginine-glycine-aspartic acid (RGD) within bone matrix proteins [153]. Thus, bone matrix proteins containing the RGD sequence have been widely considered as bioactive factors for mimicking the nature interface, promoting cell adhesion, and influencing the response of osteoblasts.

Similar to BMPs, it is not only the entire bone matrix proteins that are effective in enhancing cell adhesion, and short peptides derived from them have also been considered as potential bioactive factors. The most widely used peptide sequence in bone tissue engineering is the cell adhesion peptide RGD [154]. Covalent attachment of RGD peptides on different polymers stimulates adhesion and spreading of cells [155]. Combination of RGD peptides with other bioactive factors such as bone growth factors has been considered another promising approach [151, 156].

1.4.3 Other mineralisation-promoting proteins

Bone is formed in two stages: first a protein matrix that can become calcified is synthesised, and then mineral is deposited. The process of depositing apatites and other salts onto extracellular matrix fibres is referred to as bone mineralisation [157]. Although the mechanism of bone mineralisation has not been fully understood, two possibilities for the control of mineralisation are known [30]. First, the process may be controlled by growth factors and other regulators which mediate cell activity of producing the components of mineral. Second, bone mineralisation may also be controlled by the organic component of the bone matrix such as collagen, non-collagenous proteins, alkaline phosphatase, as well as citrates and lipids [30]. Among them, the metalloenzyme alkaline phosphatase (ALP) is the most studied

mineralisation-promoting factor in bone tissue engineering applications [72].

1.4.3.1 Alkaline phosphatases

Alkaline phosphatase (ALP) is a metalloenzyme that can be widely found in most species, from bacteria to humans [158]. They naturally exist as homodimers and each monomeric subunit contains a catalytic site with two Zn^{2+} and one Mg^{2+} . These three metal ions are necessary for enzymatic activity, and also play a role in the conformation of the ALP monomer and indirectly regulate subunit-subunit interactions [159, 160]. A single subunit fails to display any catalytic activity on its own.

There are a total of four kinds of ALPs in mammals, all of which reside on the surface of the plasma membrane by a covalently attached glycosylphosphatidylinositol anchor [161]. Three of them are tissue-specific: placental alkaline phosphatase (PLAP), germ cell alkaline phosphatase (GCAP), and intestine alkaline phosphatase (IAP). The functions are not been clearly understood, but PLAP may play a role in the regulation of cell division and proliferation as well as the mediation of IgG transport through the placenta; GCAP may be related to early embryonic development and germ cell development; IAP is likely to be involved in the intestinal absorption of lipids [161]. The fourth ALP in mammals is tissue nonspecific alkaline phosphatase (TNAP). As its name implies, it can be found in more than one tissue, mainly in bone, liver and kidney.

ALPs were first noted by Robison in 1924, who found that a higher than normal level of ALPs were detected at the sites of mineralisation in bone and calcifying cartilage [162]. The hypothesis derived from the observation was that ALPs have the ability to hydrolyse organic phosphates phosphoric esters and generate inorganic phosphates, thus resulting a local increasing of phosphate ions which were finally precipitated as calcium phosphates at the site of calcification [162]. Over decades, many researchers have gained a deeper understanding of the bone mineralisation process and the

function of ALPs. There are two stages of mineralisation in woven bone and calcifying cartilage: biologic mineralisation and crystal growth [163, 164]. Biologic mineralisation is initiated from specific regions of the outer plasma membrane of osteoblasts, chondrocytes and odontoblasts, which contain high levels of ALPs. ALPs hydrolyse organic phosphatases and provide inorganic phosphate. Then the inorganic phosphate ions are transported into vesicles, which contain calcium binding proteins through Na^+/P_i co-transporters. Calcium binding proteins have the ability to concentrate calcium ions. With the gradual accumulation of both phosphate ions and calcium ions in these particles, the first hydroxyl apatite crystal appears. Once the apatite crystal forms, the vesicle breaks down, which marks the end of the first biologic mineralisation stage. In the next stage of mineralisation, crystal growth continues and spreads throughout the bone matrix with a constant supply of phosphate ions and calcium ions from the body fluid [165].

The process of bone mineralisation indicates that ALPs are essential components that catalyse the hydrolysis of organic phosphate monoesters present in the surrounding body fluids and generate inorganic phosphate ions during the first stage of bone mineralisation. Additionally, ALPs have an equally important function of reducing the level of pyrophosphate, which is an inhibitor of mineralisation that not only poisons the crystals, but also prevents interactions between crystals and the interstitial fluids by covering the crystal surface [166]. Figure 5 shows how ALPs increase the local concentration of inorganic phosphate, a mineralisation promoter, and at the same time, decrease the concentration of extracellular pyrophosphate, an inhibitor of mineral formation [167].

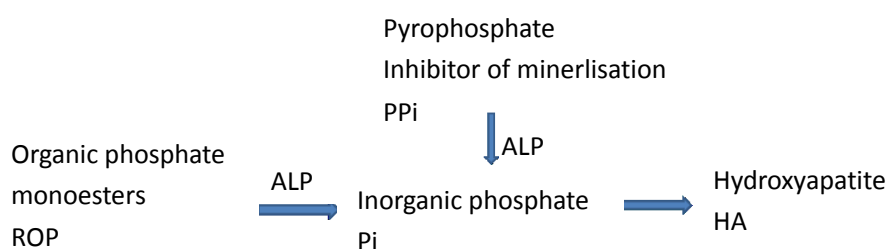


Figure 5. The role of ALPs in the bone mineralisation process. The catalytic action of

TNAP increases the inorganic phosphate (Pi) concentration of hydroxyapatite (HA) by hydrolysis of organic phosphate monoesters (ROP) as well as pyrophosphate (PPi), an inhibitor of mineralisation [167].

ALPs are considered well-conserved enzymes. According to a sequence analysis of mammalian ALPs, PLAP, GCAP and IAP are highly homologous (90-98%) with each other, and TNAP is 50% identical to the others [168]. The same is true for *Escherichia coli* (*E. coli*), and although *E. coli* ALP has a relatively low sequence identity with the mammalian enzymes (25-30%), the residues involved in the active site and the ligands coordinating three metal ions are largely conserved [169]. In addition, the X-ray analysis of the *E. coli* ALP and the mammalian ALPs suggests that all ALPs have a similar fundamental three-dimensional structure [170]. Therefore, based on the sequence and structural homology, *E. coli* and mammalian ALPs are believed to have a similar catalytic mechanism [159]. The general reaction scheme of ALPs is summarised in Figure 6. The whole process occurs in four steps: 1) reaction of the enzyme ALPs and organic substrate to form an enzyme-substrate complex (E•ROP), 2) phosphorylation of the enzyme serine residue to form the covalent enzyme-phosphate complex (E-P), 3) hydrolysis of E-P to form the non-covalent enzyme-phosphate complex (E•P), and 4) dissociation of enzyme-bound phosphate and release of inorganic phosphate [159].

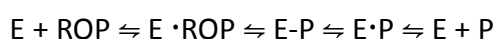


Figure 6. The general scheme for the ALPs-catalysed hydrolysis of phosphate monoesters. E represents the enzyme ALPs, ROP represents an organic phosphate monoester, and P represents the phosphate product. The non-covalent bond is represented by a dot and the covalent chemical bond is represented by a dash. A substrate is first reacted with the enzyme to form an enzyme-substrate complex. Then a temporary covalent enzyme-phosphate complex is formed by attacking a serine residue of the substrate, followed by the hydrolysis of the complex. Finally inorganic phosphate is released.

Although ALPs have commonality in their catalytic mechanism, sequence, and conformation, *E. coli* ALP is smaller, has a lower optimum pH and displays higher heat stability compare to mammalian ALPs [158, 161]. In addition, *E. coli* ALP is located in

the periplasmic space between the cell membrane and cell wall, which makes it more durable and easier to isolate [171, 172].

Similar to other members in the ALP family, *E. coli* ALP (EC 3.1.3.1) is a dimeric metalloenzyme. Each monomer contains 449 amino acid residues and two atoms of zinc and one atom of magnesium at each active site [159]. The enzyme is encoded by the *phoA* gene, and synthesised as a precursor monomer with a signal peptide at the N-terminus. The active enzyme is formed by removing the signal peptide and dimerising two of the resulting mature monomers before transportation to the periplasmic space of *E. coli* [173, 174]. The molecular weight of the dimer has been reported as ranging from 80-100 kDa [173].

Based on the X-ray crystal structure analysis of *E. coli* ALP, the active site can bind the inorganic phosphate by the two zinc atoms and the guanidinium group of arginine 166 [168, 175]. In order to better understand the function of certain conserved amino acid residues around the active site and to investigate the reaction mechanism of *E. coli* ALP, a number of studies have been performed using a site-directed mutagenesis strategy [175-179]. Results suggest the catalytic activity of *E. coli* ALP can either be increased or reduced by different mutations. For example, when arginine 166 is substituted by alanine or serine, the catalytic activity is reduced; but when aspartic acid 101 is replaced by alanine, enzyme activity is increased at alkaline pH which may due to an increasing accessibility of the substrate and a reducing affinity for phosphate [175]. High catalytic activity is always a desired property for ALPs because it means a stronger ability to generate inorganic phosphatase from organic phosphatase. Based on the former research, Xu *et al.* have developed a series of *E. coli* ALP mutations with significantly higher catalytic activity and decreased affinity for the phosphate competitive inhibitor compared to the wild-type (wt) *E. coli* ALP [180]. By applying a directed evolution strategy, mutant *E. coli* ALP 4-186, which contains three amino acid residue replacements (aspartic acid 101 was replaced by serine (D101S), lysine 167 was replaced by arginine (K167R), and serine 374 was replaced by cysteine (S374C))

was engineered with an improved catalytic activity of nearly 35 times than that of wt *E. coli* ALP (Table 8, Figure 7) [180]. These mutated *E. coli* ALPs are promising candidates for bone tissue engineering applications.

<i>E. coli</i> ALP	Amino acid residuals replacement	Specific activity (unit/mg protein) pH 10.05 1M	Activity increased times (compared to wild-type)
Wild-type	None	37.5	1
D101S	D101S	415.8	11.1
2-834	D101S, K167R	539.4	14.4
4-186	D101S, K167R and S374C	1302.1	34.7

Table 8. Biological activity of the wild-type and some mutant *E. coli* ALPs according to Xu *et al.* Among them, mutation 4-186 shows almost 35 times higher activity compare to the wild-type [180].

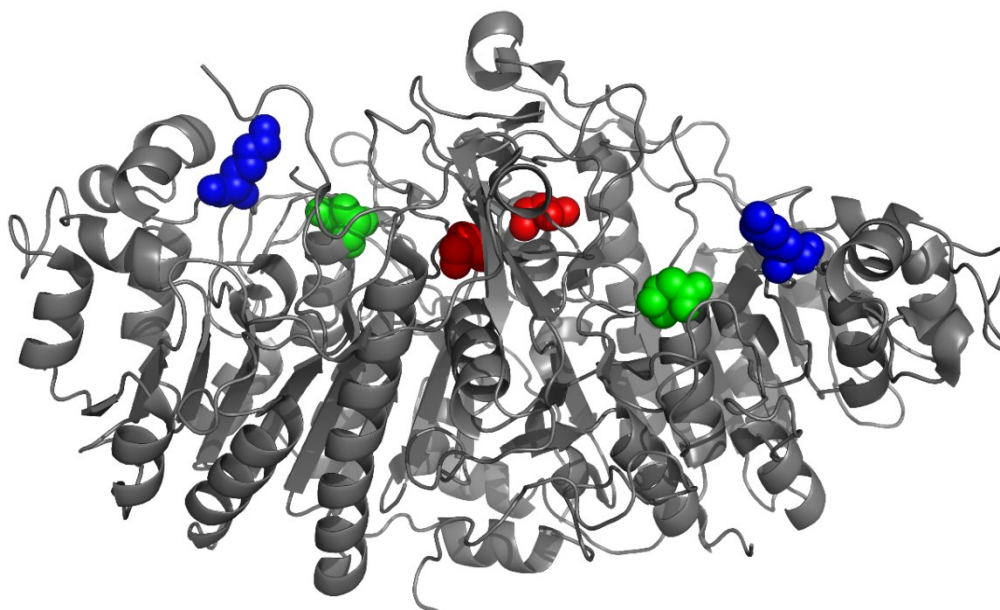


Figure 7. The three-dimensional protein structure of the mutant *E. coli* ALP 4-186 dimer, which contains D101S (green), K167R (blue) and S374C (red) replacements. This figure was drawn using PyMOL (www.pymol.org).

1.4.3.2 Lipids

In addition to the biomolecules mentioned above, lipids are also important components of the bone matrix and may have the potential to be functional in bone tissue engineering. It has been found that a calcium-phospholipid-phosphate complex has the ability to induce crystallisation [181]. Phospholipids are also associated with matrix vesicles in calcifying cartilages [182].

1.5 Engineered recombinant proteins for bone tissue engineering

As introduced in Section 1.4, bioactive molecules such as bone growth factors, cellular matrix proteins, and other mineralisation-promoting proteins play a crucial role in bone formation and regeneration. However, there is the question of how to produce these biomolecules in a large scale to meet the demands for medical treatments and research, while maintaining their native function and activity.

There are two main strategies for obtaining biomolecules for bone tissue engineering [45, 183]. The first is to isolate biomolecules from bone tissue. Since these biomolecules are produced by cells and naturally exist in bone tissue, the straightforward way of using them is to isolate and purify them from bone tissue. It is a classical way of obtaining biomolecules and the functions of these molecules can be preserved. However, many of these biomolecules are naturally expressed at very low concentrations in bone tissue so that the amount of production is limited and the purification process is difficult and time-consuming. The second strategy is recombinant protein synthesis [184, 185]. In order to produce abundant amounts of biomolecules and to overcome the purification problem, recombinant protein synthesis system was developed [183]. A recombinant protein is a manipulated form of a protein that is derived from recombinant DNA [186]. In the following section, the

general principles and production strategies of recombinant proteins are summarised.

1.5.1 General principles

Recombinant proteins are generated in various ways from the expression of recombinant DNA. Recombinant DNA, as the name implies, is a type of DNA that is artificially created by combining one or more strands of DNA into a different set of DNA using cloning methods [187, 188]. The recombinant DNA technique was first developed in the early 1970s after the discovery of restriction enzymes, which have the ability to open a strand of DNA at certain “restricted” points and combine foreign DNA with the carrier DNA at this point [189].

Generally, the cloning and expression recombinant protein can be divided into six steps (Figure 8) [186, 190-192]:

(1) Choose a target gene and a vector. In bone tissue engineering, the target gene usually codes for a bioactive molecule such as a bone growth factor. A vector is a carrier of the target DNA. The most widely used vectors are plasmids. They are small and replicable circular DNA molecules which do not act as a part of the main cellular genome but can carry foreign genes that may entitle host cells with useful properties such as drug resistance, mating ability, and toxin production [173]. Section 1.5.2.2 reviews the most commonly used cloning vectors and expression vectors in genetic engineering to produce recombinant proteins.

(2) Cleave the target gene and the vector with a restriction enzyme. Restriction enzymes recognise and cut the DNA at specific target sequences. After cutting, either sticky ends or blunt ends are produced. Sticky ends, the single-stranded overhang DNA ends at the site of cleavage, are of particular interest because they have identical nucleotide sequences and can pair with other overhangs with the same ends [193]. Therefore, by cutting with the same enzyme, the target DNA and the vector have a

strong possibility of ligation because of the complementary sticky ends.

(3) Construct recombinant DNA. In order to construct a stable recombinant DNA, both hydrogen bonds and covalent bonds need to be sealed. While the hydrogen bonds can be formed between the complementary sticky ends of the target DNA and the vector because of the restriction enzymes, the covalent bonds that hold the sugar-phosphate backbone have to be sealed by an enzyme DNA ligase which catalyses the formation of phosphodiester linkages between nucleotides [194]. This process is called ligation.

(4) Transform into a host cell. A ligation solution containing a population of recombinant DNA is mixed with competent living cells, which are specially treated cells with high acceptability to foreign DNA. This process is called transformation. There are two popular transformation methods: by heat shock and by electroporation [195]. The preparation of competent cells and transformation are described in Section 3.2.7. After transformation, the recombinant DNA molecule will replicate along with the dividing and reproducing of the host cells. Expression systems and host cells are introduced in Section 1.5.2.1.

(5) Screen transformants. The mixture solution of transformed cells is spread out on a Petri dish with selectable medium so that the cells are separated from one another. The vector usually contains a selectable marker such as an antibiotic marker to identify successful recombinant. Only the hosts with the vector will live because they have resistance to the antibiotics. In addition to antibiotics, the substrate of target recombinant protein can be used to select successful recombinant. 5-bromo-4-chloro-3-indolyl phosphate (BCIP) can react with ALPs and generate a blue product. Blue colonies developed on the BCIP-plate indicating the expression of ALPs [175]. Each colony growing on the dish is a cell clone, which is genetically identical and therefore contains the same recombinant vector [196].

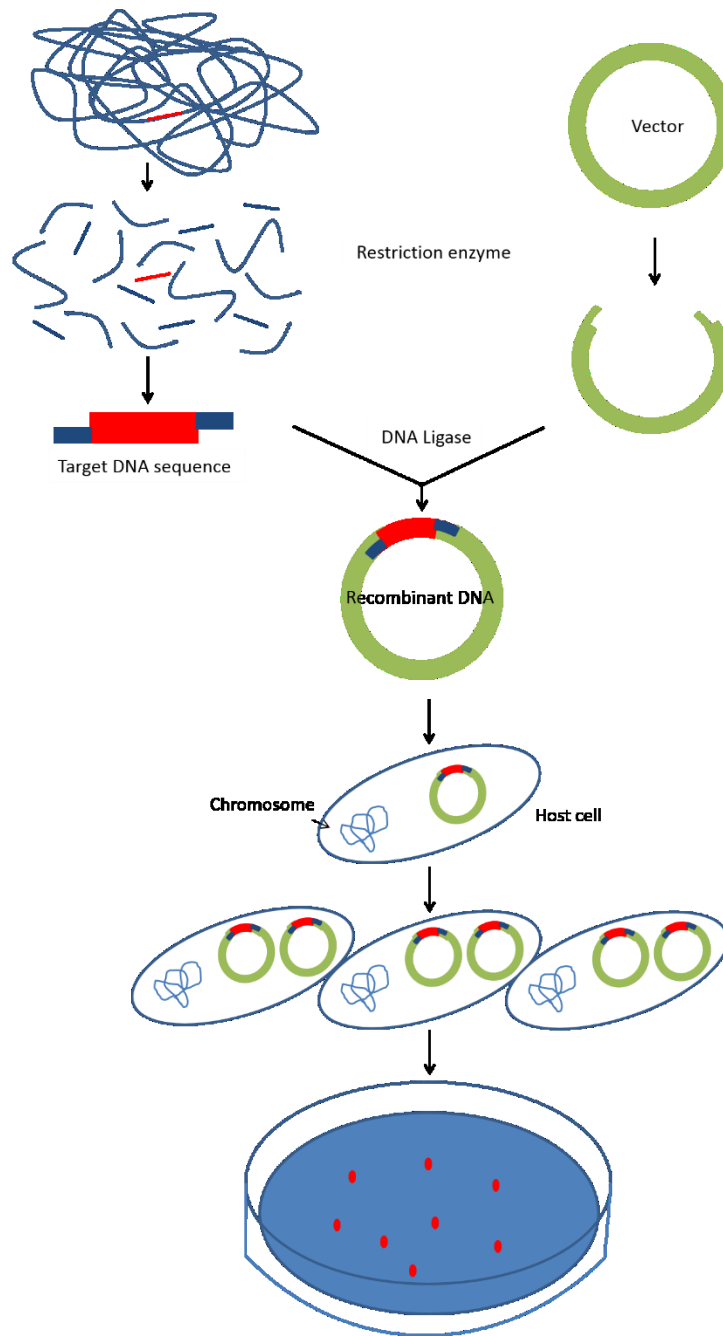


Figure 8. The cloning process to produce recombinant plasmids. The target DNA and the vector are cut with the same restriction enzymes to generate complementary sticky ends. By adding the enzyme DNA ligase, the target DNA and the vector are ligated to form a new recombinant DNA molecule. After transforming into suitable host cells, the recombinant DNA is reproduced along with the host cell DNA.

(6) Recombinant protein production and purification. Recombinant protein production involves inserting the target recombinant DNA into an expression vector following a promoter so that the recombinant protein can be expressed by triggering

the promoter with an inducer. Take one of the most widely used expression vectors pTrc which contains a *tac* (*trp/lac*) promoter for example. If a recombinant DNA is inserted into a pTrc vector and is located after the *tac* promoter, the expression of desired recombinant proteins will be stimulated by the inducer lactose or its analog isopropyl- β -D-thiogalactopyranoside IPTG [197].

1.5.2 Strategies of producing recombinant proteins

The requirement of high quality recombinant proteins used for either laboratory studies or industrial applications has increased dramatically in recent years [198]. According to www.phrma.org, more than 75 types of recombinant proteins have been used as pharmaceuticals, and more than 360 new medicines based on recombinant proteins are being developed [199]. Higher production efficiencies as well as lower costs of the final product are important factors for recombinant protein production [200]. In order to achieve the mass production, different strategies have been applied by using different expression systems and different vectors.

1.5.2.1 Choice of expression system

There are two main systems for the expression of recombinant proteins: prokaryotic (phage, bacteria) expression systems and eukaryotic (mammalian cell, yeast) expression systems [198, 201]. The choice of expression system is very important because it determines the choice of vector and affects the expression and purification method of the recombinant protein. In order to find the most suitable host, the specific requirements and applications for the recombinant protein should be considered, such as cell growth characteristics, expression levels, biological activity of the target protein, and potential toxicity [202, 203]. Table 9 summarises the advantages and disadvantages of some commonly used host systems.

Host	Advantage	Disadvantage
Bacteria <i>E. coli</i>	• Rapid cell growth • Easy induced expression by IPTG • Easy to grow with high yields (up to 50% of total cell protein) • Simple purification	• Easy to form inclusion bodies • No post-translational modification • Biological activity and immunogenicity may differ from natural protein
Bacteria <i>Staphylococcus aureus</i>	• Secretes fusion proteins into the growth media	• Lower expression levels than <i>E. coli</i>
Mammalian cell	• Same biological activity as native proteins • High levels of expression	• Slower cell growth • Manipulated cells can be genetically unstable
Yeast	• Easy to form disulphide bonds and glycosylation • Well established large scale production and downstream processing	• Difficult to control gene expression • Glycosylation not identical to mammalian systems
Baculovirus cells (insect)	• High level of expression	• Product not always fully functional
Fungi	• Secrete large quantities of product into growth media • Cheap	• Low level of expression • No cloning vectors available

Table 9. Advantages and disadvantages of the commonly used host systems for recombinant protein production.

Bacteria expression systems, especially *E. coli* host cells, are believed to be the simplest

and most inexpensive way to produce large amounts of recombinant proteins [186]. That is mainly attributed to the easily achieved high cell density culture of *E. coli* [204]. In addition, because *E. coli* is such a well-characterised system, various approaches for the efficient production have been established. Recent studies have made it possible that recombinant proteins can be secreted into a specific cellular compartment of *E. coli* as shown in Figure 9 [203]. These features will further improve *E. coli* as a host for production of recombinant protein.

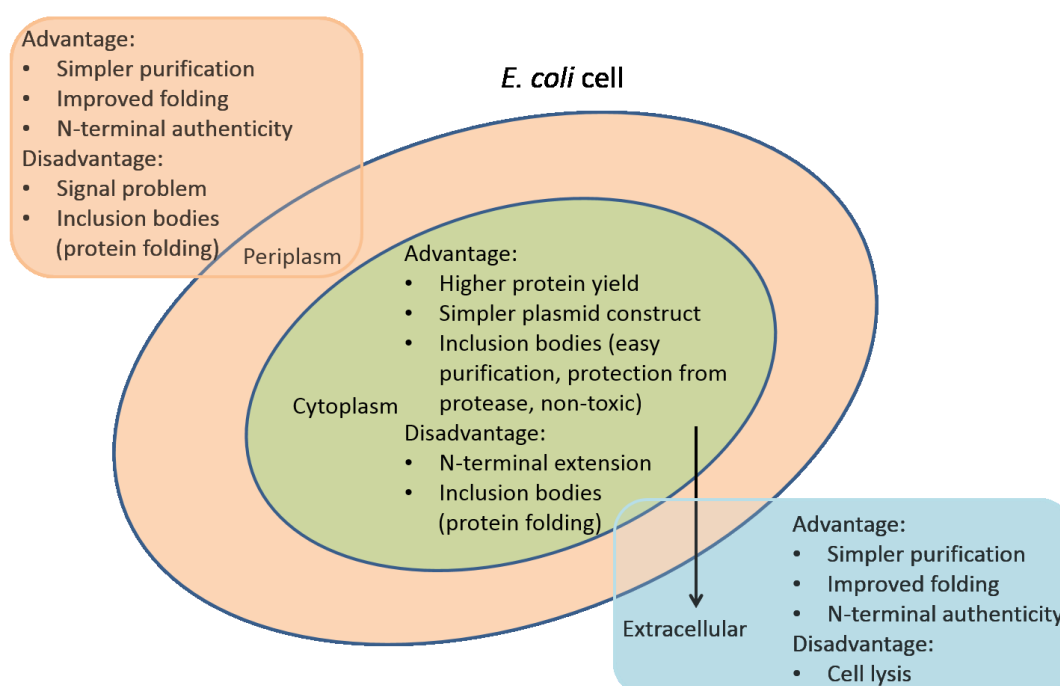


Figure 9. Advantages and disadvantages of the recombinant protein production into each compartment (the cytoplasmic space, the periplasmic space or the extracellular culture medium) of *E. coli* [203].

1.5.2.2 Choice of vectors

In order to transport the target gene into host cells for protein expression, molecular vehicles called vectors are used. Essentially, a vector is a genetic frame which carries the target gene to be cloned, together with a number of necessary parts that will ensure the protein expression in the host [186, 190, 205]. As mentioned above, the choice of vector is determined by the host. Once the host has been selected, matched

vectors are available for consideration. Same as the choice of host system, the final choice of vector should consider the specific requirements of the application and the behaviour of the target protein [206].

Generally, there are two types of vectors: cloning vectors and expression vectors [190, 207]. A cloning vector is designed mainly for cloning purposes. There are many types of cloning vectors, but plasmids are the most commonly used ones. The cloning vectors usually have high copy numbers so that target DNA fragments within the vector will be amplified in the host cells. Once enough copies of the target DNA is achieved, the target DNA fragment may be further subcloned into another vector such as expression vector which is designed for protein expression in cells [207]. To achieve this goal, an expression vector must include a strong promoter, the correct translation initiation sequence such as a ribosomal binding site and start codon, a strong termination codon, and a transcription termination sequence [208]. The pET vector (Figure 10) is one of the most popular expression vectors for recombinant protein expression (invivogen.com) [209].

One important point is that the division of cloning vectors and expression vectors is not absolute. Some cloning vectors have the ability to express recombinant proteins; at the same time, expression vectors can finish cloning as cloning vectors because they contain necessary features for cloning including an origin of replication, a selectable marker, and a suitable site for the insertion of a gene such as the multiple cloning site [205]. Still, it is typical to first clone into a cloning vector and the cloning gene is then transferred to an expression vector [210]. This additional step can ensure the accuracy of the cloned gene.

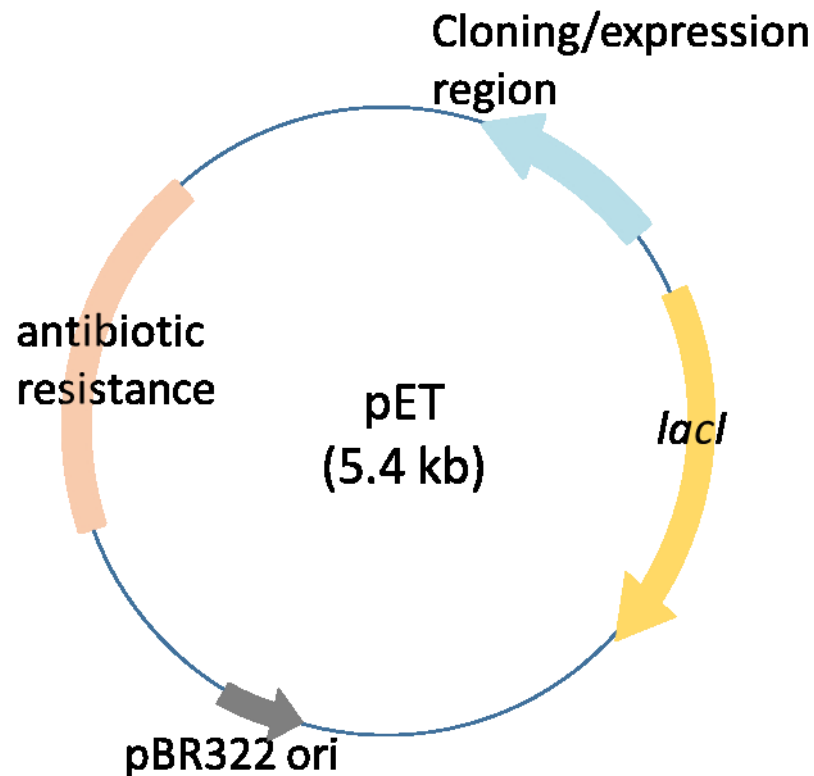


Figure 10. The map of pET expression vector. An antibiotic resistance marker is shown in pink, a *lacI* gene is shown in orange, and a pBR322 origin is shown in grey. The blue arrow shows the cloning/expression region. It contains a T7 promoter, a *lac* operator, a ribosomal binding site (rbs), the multiple cloning sites, and some functional tags such as His-tag.

In expression vectors, the promoter is of vital importance because it controls the expression of the cloned gene. A strong and tightly controlled promoter is always desired. The promoters are normally inducible so that protein expression is only initiated when required by the introduction of an inducer [211]. Figure 11 illustrates the induction principle of T7 promoter in pET expression vector by IPTG [212-215].

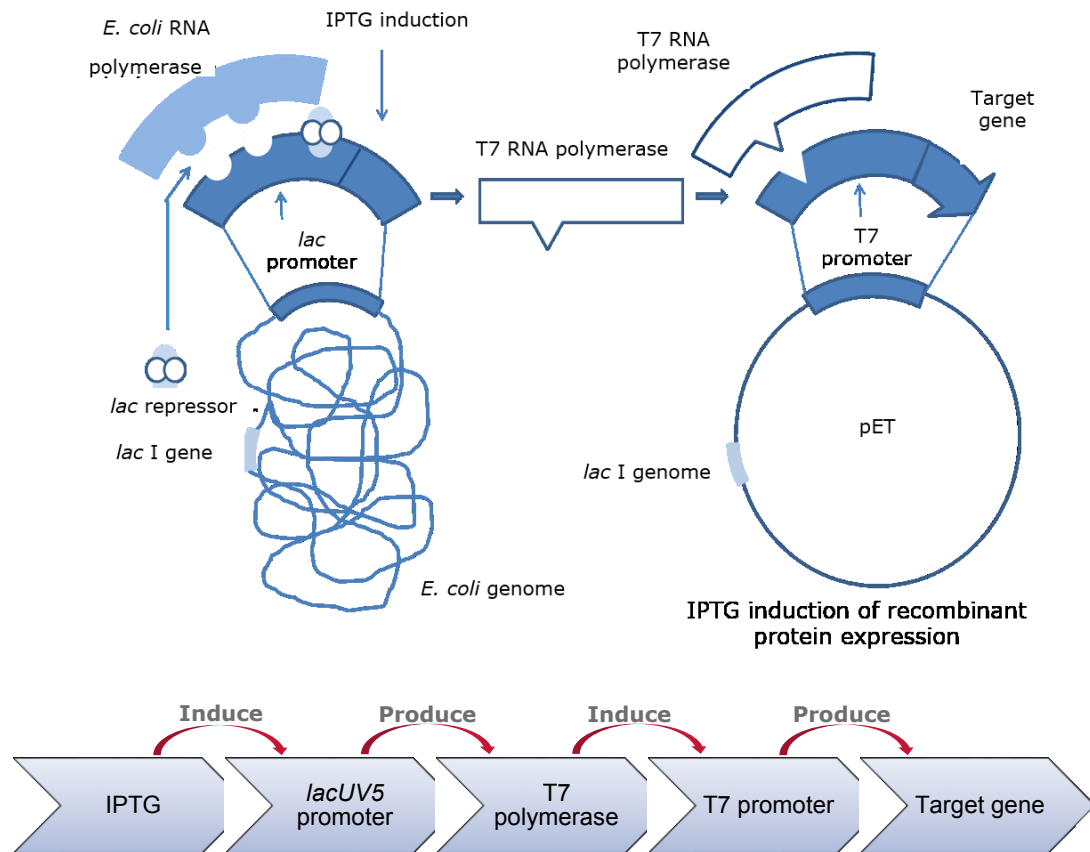


Figure 11. The general principle of IPTG induction of T7 promoter-driven expression of a recombinant protein from *E. coli* BL21(DE3) host cell contains pET plasmid [215]. *lac* repressor prevents expression of T7 RNA polymerase gene by cellular RNA polymerase. By addition of IPTG, *lac* repressor is dissociated from the operator allowing expression of T7 RNA polymerase gene from *lacUV5* promoter. Then the T7 RNA polymerase binds to the T7 promoter and initiates expression of the target protein. Therefore, IPTG induces *lacUV5* promoter to transcribe the T7 RNA polymerase, which in turn transcribes the target DNA in the plasmid.

In addition to the choice of the promoter, one other key factor to be considered when choosing a vector is a fusion tag, which is a small sequence with certain biological functions [216, 217]. It has been widely used because it can greatly simplify subsequent isolation, purification and detection [208, 218]. Table 10 and 11 summarises a list of expression vectors containing fusion tags and reviews the main features of both fusion protein and non-fusion proteins that may influence the final choice of vector [219-222].

Expression vector	Tag
pGEX	Glutathione S-transferase (GST-tag)
pQE	His-tag
pET	His-tag
pEZZ18	IgG binding domain of protein A
pRIT2T	IgG binding domain of protein A

Table 10. Common expression vectors containing fusion tags.

	Advantages	Disadvantages
Fusion protein	• Cell compartments can be targeted • Provides a marker for expression • Simple purification using affinity chromatography • Easy detection • Refolding achievable on a chromatography column • Ideal for secreted proteins as the product is easily isolated from the growth media	• Tag may interfere with protein structure and affect folding and biological activity • Cleavage site is not always 100% specific if the tag needs to be removed
Non-fusion protein	• No cleavage steps necessary	• Difficult to purify and detect • Problems with solubility, reducing potential yield

Table 11. Advantages and disadvantages of fusion protein and non-fusion proteins expressed by expression vectors.

His-tag is one of the most commonly used tags. In the following section, more information about the His-tag and His-tag fusion protein will be reviewed.

1.5.3 His-tag fusion proteins

The DNA sequence of five to nine histidine (His) residues is frequently used in expression vectors for recombinant protein production. The expressed recombinant protein with poly His-tag at its N- or C-terminus is called the His-tag fusion protein [208, 216]. The choice of either N-terminal His-tag or C-terminal is dependent mainly on the characteristics of the protein [223]. An end cannot be chosen if it is folded inside the structure or is important for the function or structure of the protein.

There are several advantages of using a His-tag [219, 224, 225]. First, it can be used in any expression system and has a low metabolic burden to host cells. Although it is more common to use in *E. coli* and other prokaryotic expression systems, it also can be used in a mammalian host. Second, a His-tag can greatly simplify the purification of the fusion protein through an affinity purification procedure because it has a high affinity for metals ions such as nickel, cobalt, and copper [226]. Thus a His-tag fusion protein can be easily and quickly purified by using affinity chromatography at any scale (Figure 12) [227]. Third, the His-tag has a low likelihood of affecting the structure and the function of the fusion protein because it only contains several amino acids. If required, it can be easily cleaved by site-specific proteases. In addition, the His-tag can be easily detected using an immunoassay.

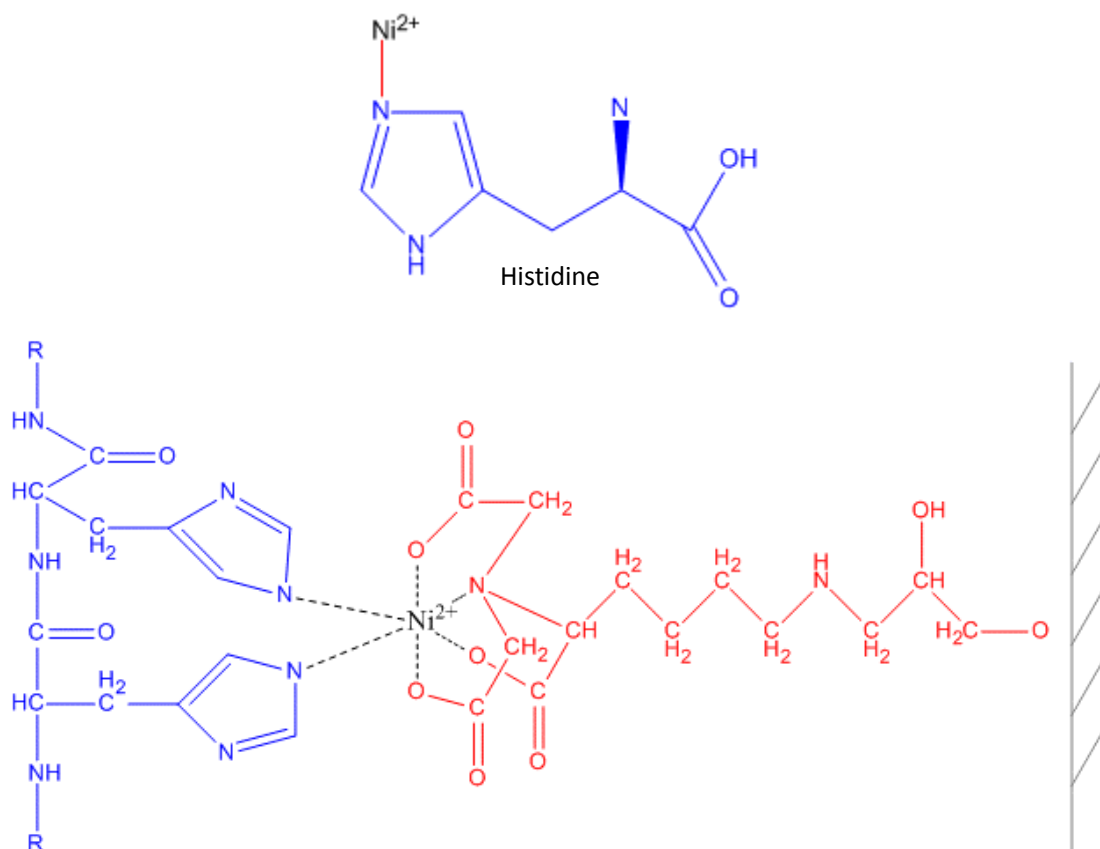


Figure 12. Interaction between a His-tag fusion protein and nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrix. The blue part represents the His-tag and the red colour represents NTA. Ni has six chelating sites, four of them bind to NTA beads, and the other two metal coordination sites are free to interact with the functional groups of His-tag fusion proteins[227].

His-tag has a wide range of applications. First of all, it has been widely used to purify fusion proteins [225]. Take the *E. coli* expression system for example as shown in Figure 13 [228]. After expression of the His-tag fusion protein in *E. coli*, the cell pellets are collected and lysed. By adding the bacterial lysate, which contains various cell proteins as well as the fusion protein to a nickel affinity column, and washing out unbound proteins, the His-tag fusion protein should bind the column and can be eluted by imidazole.

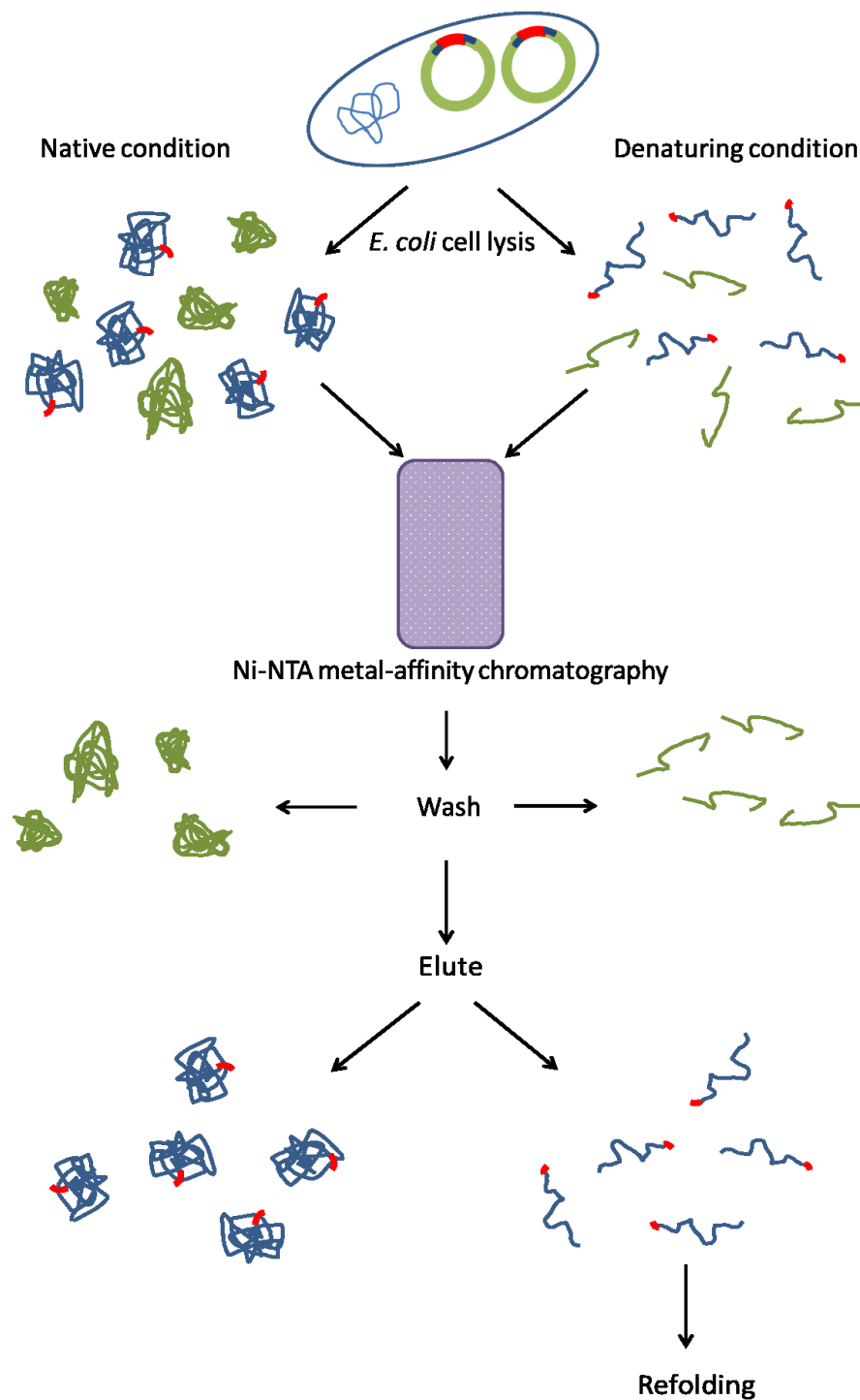


Figure 13. Purification process of the His-tag fusion protein from an *E. coli* expression system under both native and denaturing conditions. The blue colour represents the fusion protein and the red represents His-tag. *E. coli* proteins are shown in green. His-tag fusion proteins are first absorbed on the Ni-NTA metal-affinity column, then eluted by imidazole [228].

This is a one-step purification procedure with high purification efficiency because very few natural proteins contain histidine regions, so the target fusion protein is the only

one that binds to the column in most situations [229]. The His-tag can also be used to detect the fusion protein through anti-His-tag antibodies or by in-gel staining with fluorescent probes bearing metal ions in ELISA, western blotting or other analytical methods [230]. In addition, the His-tag can be used for the immobilisation of proteins on implant surfaces such as nickel-coated implants [231]. More information about immobilisation will be discussed in Section 1.6.

There are two main methods to construct a His-tag fusion protein (Figure 14). The first is through a His-tag vector such as pET24 (Figure 14A) [216]. Proteins expressed through these vectors will automatically contain a His-tag. The other method is by using polymerase chain reaction (PCR) (Figure 14B) [232, 233]. The PCR is a rapid way of amplifying (duplicating) specific DNA sequences (Figure 15) [234, 235]. A brief introduction of PCR is described in Section 4.2.3. If a primer used in PCR contains histidine codons (CAT or CAC) next to the START or STOP codon, the encoding protein will contain a His-tag.

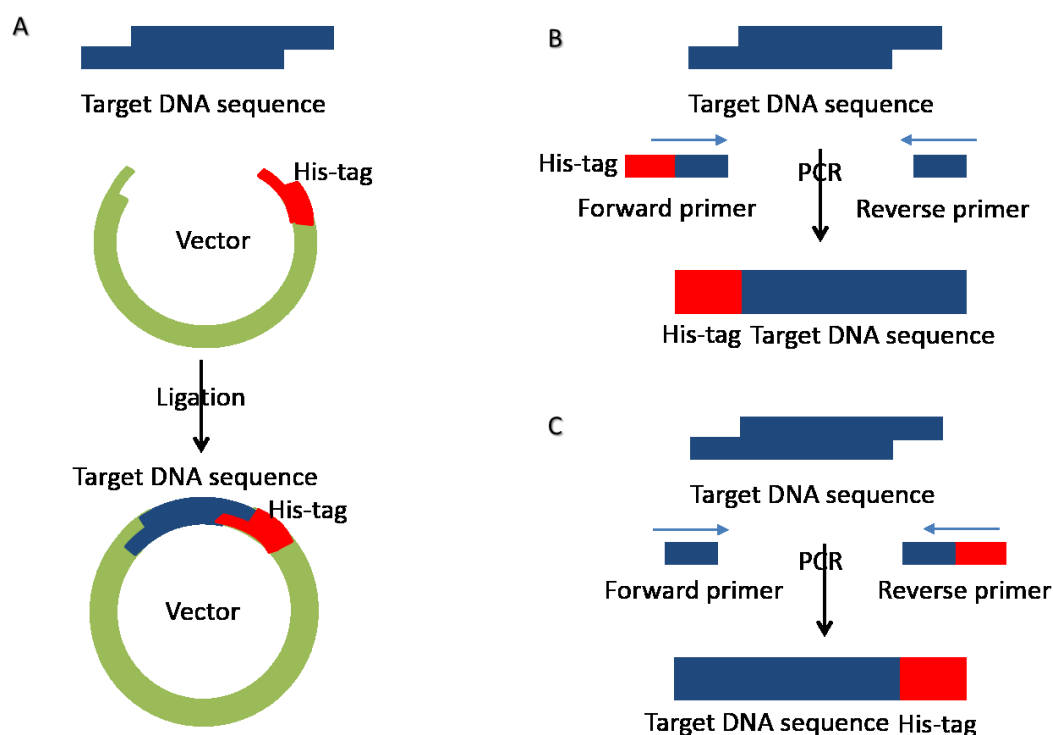


Figure 14. Two methods to construct a His-tag recombinant DNA: by inserting the

target DNA sequence encoding a protein of interest in a His-tag vector (A), by using either forward primer or reverse primer containing the His-tag to obtain either N-terminal or C-terminal His-tag fusion protein after a PCR reaction (B and C).

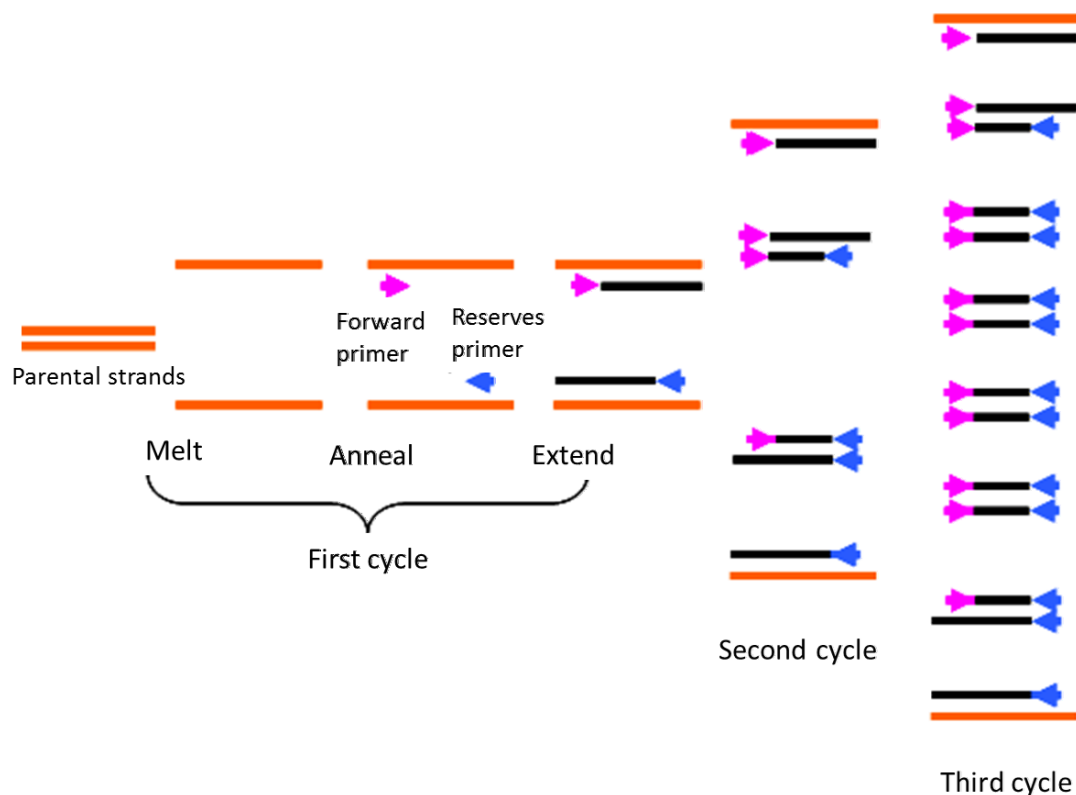


Figure 15. General principle of the PCR cycle. The first three cycles of PCR are shown. In the first cycle, double-strand DNA is denatured at 94-96°C to generate two single chains. Each single strand DNA then binds to the primers at 54-65°C. The temperature is raised again to 72°C to start the synthesis of new complementary strands by DNA polymerase. After the first cycle, two gene copies are generated. When the cycle repeats, more identical gene copies will be amplified. At the end of 32 cycles 1 billion gene copies will be amplified [236].

1.6 Immobilisation of biomolecules on bone substituting materials

As described in Section 1.2.3, bone tissue engineering combines living cells, biocompatible scaffolds, and appropriate bioactive factors especially biomolecules to induce new functional bone tissues. Although free bioactive factors can be used

directly in bone tissue engineering and have been proven as effective, they display some disadvantages compared to the use of immobilised bioactive factors on solid substrates [237]. First, the initial burst of the free bioactive factors may cause uncontrolled bone formation and undesired inflammation [238]. Second, a large amount of bioactive factors are wasted by diffusion. In addition, the activity of free bioactive factors is not as stable as the immobilised ones. The combination of biomolecules and scaffolds not only offers better control of biomolecules and increases their stability and efficiency, but also provides an extracellular environment for cells to modulate bone regeneration [239]. Therefore, extensive research has focused on bioactive scaffolds with immobilised biomolecules. A brief review of immobilisation methods is summarised in the following sections.

1.6.1 General principles

Immobilised biomolecules refer to biomolecules physically attached to a solid support or enclosed in a network to allow exchange but maintain separation from the bulk phase of substrate, inhibitor, and other molecules [240, 241]. They can be used repeatedly and continuously while their biological activity remains. Based on their superior properties, immobilised biomolecules have been widely used not only in bone tissue engineering, but also in the development of biosensors, bioprocessing for the chemical industry and the pharmaceutical industry, and bioremediation [242, 243]. Table 12 lists the advantages and disadvantages of immobilised biomolecules. In order to eliminate or at least reduce the disadvantages of immobilised biomolecules, choosing a suitable immobilisation method is of vital importance.

Advantages	Disadvantages
• Multiple or repetitive use and continuous use • More stability, longer half-lives and less degradation • Better process control and less labour intensive • Reduced reaction time • Less chance of contamination in products and easy separation • High loading rate	• Loss or reduction of biological activity • Diffusional limitation • Additional cost

Table 12. Advantages and disadvantages of immobilised biomolecules in bone tissue engineering.

1.6.2 Strategies of immobilising biomolecules on bone substituting materials

Various immobilisation methods have been developed for different biomolecules and supporting scaffolds. Below the most commonly used methods are listed (Figure 16) [242-245].

(1) Adsorption: Adsorption is the simplest and cheapest immobilisation method. It is a reversible process that depends on the conditions such as pH, ionic strength, temperature, and polarity of the solvent. Based on the binding mode of the biomolecules, adsorption can be divided into physical adsorption and ionic binding. In physical adsorption, biomolecules are bound to the scaffold surface by hydrogen bonds, van der Waals forces, or hydrophobic interactions; while in ionic binding, biomolecules are bound to scaffold surface through salt linkages. Both methods cause little or even no structure change of the biomolecules so that the biological activity of biomolecules is well-preserved. However, due to a weak binding force of adsorption,

the immobilised biomolecules may leak from the support scaffolds. BMP-2 immobilised on a nanofibrous chitosan membrane by physical adsorption was found insufficient after 4 weeks of long term incubation [237]. Although ionic binding force is stronger than physical adsorption, leakage of biomolecules still occurs when the ionic strength is too high or the pH is varied [246].

(2) Covalent binding: This method is based on the formation of covalent bonds between the chemical groups of biomolecules and chemical groups on the surface of support materials. It is one of the most widely used methods because it suits a broad range of pH and ionic strength conditions. ALPs were covalently immobilised on microporous nanofibrous fibrin scaffolds to promote mineralisation and osteoblast differentiation *in vitro* and *in vivo* [247] and cell adhesive peptides were immobilised on Ti implants to mediate surface-cell interactions by covalent chemical attachment [248]. Various reactions between the chemical groups have been developed depending on the functional groups available on the matrix including amino-groups, carboxyls, sulhydryls, hydroxyls, imidazoles, phenolics, thiols, threonines and indoles [249]. However, the chemical groups of biomolecules that are essential for their biological functions must be avoided in the covalent linkage. Covalent binding leads to a non-reversible and strong bond between biomolecules and support materials so that no leakage of biomolecules occurs. On the other hand, because the bond is strong, the support materials have to be changed together with the biomolecules once they lose their biological activity, thus increasing their cost.

(3) Cross linking: The covalent binding method is based on the formation of covalent bonds between biomolecules and supporting materials, while the cross linking method is based on the formation of covalent bonds between biomolecules to develop three-dimensional cross-linked aggregates through multifunctional reagents such as glutaraldehyde, diazonium salt, hexamethylene diisocyanate, and N-N' ethylene bismaleimide [250]. More than one biomolecule can be cross linked together to achieve multiple functions [251]. Although it provides strong binding and prevents

leakage, cross linking may cause significant changes in the biological activity of biomolecules, and severely limit diffusion.

(4) Entrapment: The entrapment method is based on enclosing biomolecules within a matrix network in gels [252], fibres [253], or microcapsules [254]. The most commonly employed entrapment matrixes are polymeric gels and natural gels such as agar, gelatin and alginate. By adjusting the pore size of matrix, biomolecules can be trapped while still allowing the penetration of substrates and products [255, 256]. This method is distinguished from the crosslinking and coupling methods described above in that the biomolecule is not chemically bound to the matrix.

(5) Encapsulation: This method involves encapsulating biomolecules in the membrane rather than in the lattice of a polymer matrix like entrapment [257]. It is an affordable and simple method, and a larger quantity of biomolecules per unit volume can be immobilised than any other methods. Encapsulation immobilisation of enzymes in nanofibres by co-electrospinning has been studied [258, 259], and has shown great potential to be used in bone tissue engineering. By using this method, not only a high enzyme loading (up to 50%, w/w) in the fibres can be easily reached, enzymatic activities of the fibres were well-preserved during and following electrospinning [260]. In addition, co-electrospinning nanofibres with a small substrate molecule alkaline phosphatase (80 kDa) acts as an enzyme release device which is a desirable feature in bone tissue engineering [98].

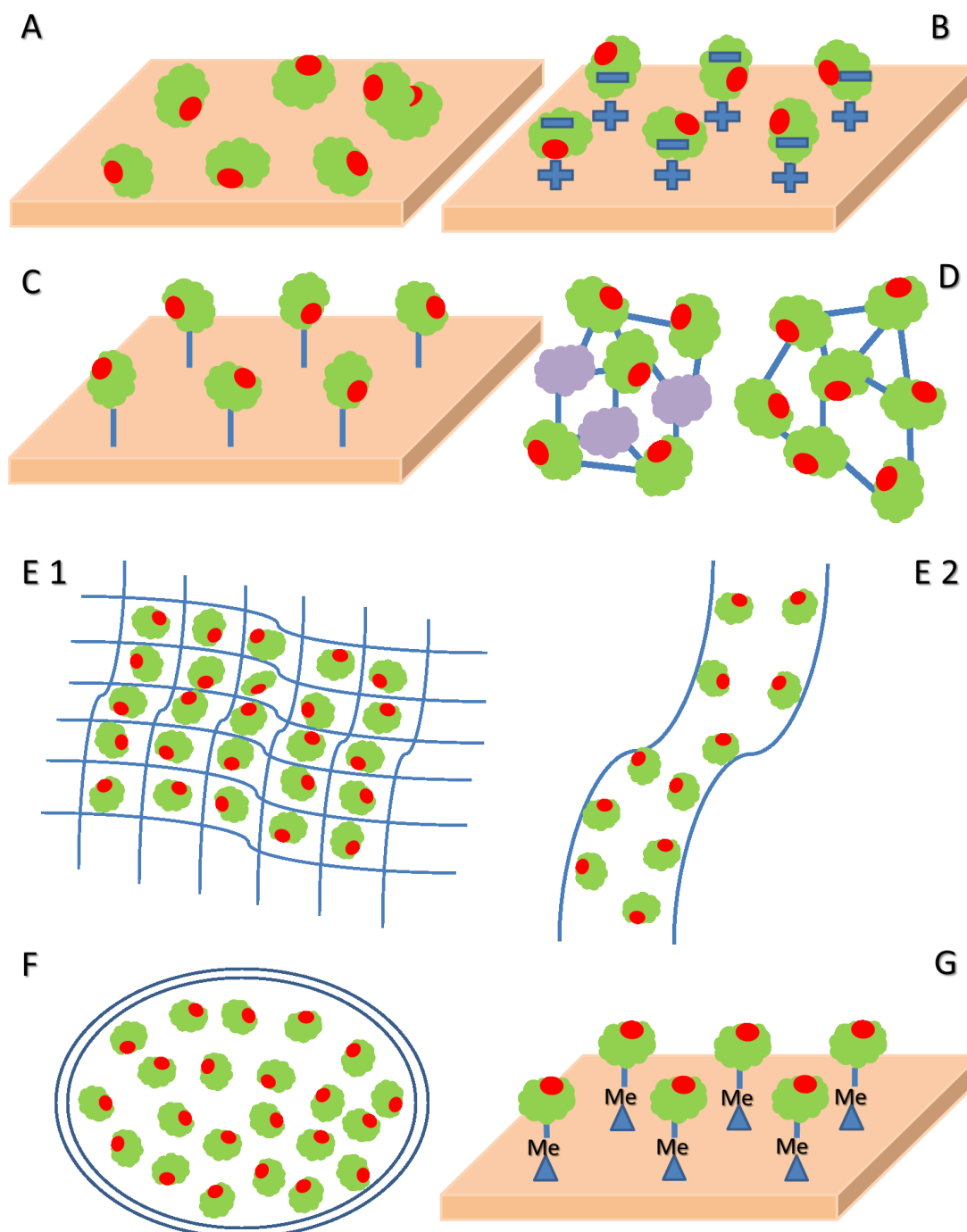


Figure 16. Schematic representation of the main biomolecule immobilisation methods. (A) Physical adsorption. Biomolecules (shown in green) are randomly attached on the supporting surface (shown in pink). Biomolecules may overlap each other, and the active site of biomolecules (shown in red) may hide inside. (B) Ionic binding. Positively charged supporting materials non-covalently interact with negatively charged biomolecules and *vice versa*. (C) Covalent binding. Covalent bonds are formed between the chemical groups of biomolecules and chemical groups of supporting materials. (D) Cross linking. A three-dimensional cross-linked aggregate is generated by the formation of covalent bonds between biomolecules. Either one type of biomolecule or more than one type (other biomolecules show in purple) can be cross-

linked. (E) Entrapment. Biomolecules are trapped within the lattice of a polymer matrix in gel (E1) or in fibres (E2). (F) Encapsulation. A larger quantity of biomolecules is enclosed within membrane. Method E and F have little effect on the conformation and the activity of biomolecules, but may have severely limited diffusion. (G) Chelation. Biomolecules are immobilised by the formation of coordinate bonds with metal ions on the supporting materials. It is an orientation-controlled immobilisation so that the active site of biomolecules may be maximally exposed.

(6) Chelation: Chelation or metal binding is based on the formation of two or more separate coordinate bonds between a polydentate ligand and a single central atom [261]. A well-known example is the Ni-NTA complex and the His-tag interaction shown in Figure 12. Four chelating sites of Ni bind to NTA beads, the other two metal coordination sites can interact with functional groups of His-tag to immobilise fusion proteins on various supporting scaffolds such as titanium implants [231], nanoparticles [262, 263] and gold surfaces [264]. It is a simple and reversible method. The binding is highly specific and strong. High purity fusion proteins can be easily eluted by imidazole. In addition, an orientation-controlled immobilisation can be achieved through this method. Most methods mentioned above lead to a non-specific random immobilisation, which may decrease the biological activity of biomolecules. Chelation, on the other hand, can immobilise biomolecules in a controlled, orderly way so that the active site of biomolecules may be maximally exposed.

The above immobilisation methods can be classified according to different standards.

(1) Physical methods: adsorption, entrapment and encapsulation; chemical methods: covalent binding, cross linking and chelation. (2) Reversible methods: adsorption and chelation; irreversible methods: covalent binding, cross linking, entrapment and encapsulation. (3) Surface immobilisation: adsorption, covalent binding, cross linking and chelation; encapsulation immobilisation: cross linking, entrapment and encapsulation. To make it easy to compare, Table 13 summarised the main features of each immobilisation method. Each has different strengths and weakness. Choosing the immobilisation method is based on the characteristics of the biomolecules and supporting scaffolds, as well as the specific application.

	Advantages	Disadvantages
Physical adsorption	• Little or no conformational change of the active sites • Simple and cheap	• Desorption from changes in temperature, pH, ionic strength • Non-specific
Ionic binding	• Milder conditions than chemical method. • Little change in the conformation and the active site • High activities reserved in most cases	• Leakage from the carrier may occur in substrate solution of high ionic strength or upon variation of pH
Covalent binding	• Stable method • Prevents elution of protein into the production stream • Suits a broad range of pH, ionic strength and other variable conditions • Site-specific binding	• Relatively expensive • Low activity yields due to harsh environments or toxic reagents • Active site may be modified through the chemical reactions used to create covalent bonding.
Cross linking	• Very little desorption, strongly bound • Helps stabilising biomolecules • Relative high loading rate	• May cause significant changes in the active site • Severe diffusion limitation • Affects biological activity

Table 13. Main features of immobilisation methods used in bone tissue engineering.

	Advantages	Disadvantages
Entrapment	• Relative high loading rate • Helps stabilising biomolecules • Little change in the conformation and the active site	• Leakage of low molecular weight biomolecules • Diffusion limitation
Encapsulation	• Cheap and simple • Highest loading rate • Easy tailored system	• Severe diffusion limitation • May cause significant changes in the active site
Chelation	• Site-specific binding • The binding is highly specific and strong • Orientation-controlled immobilisation	• Restrict biomolecules contains selective tags • Fusion tags may affect biological activity

Table 13. (Continued) Main features of immobilisation methods used in bone tissue engineering.

1.7 Hypotheses

The hypotheses tested was that two engineered biomolecules, short peptides derived from BMP-7, and recombinant metalloenzyme *E. coli* alkaline phosphatase, have the ability to enhance mineralisation, induce bone regeneration, and stimulate bone formation. In this respect these biomolecules may have potential applications in the field of bone tissue engineering. In order to test these hypotheses, the following questions have been addressed in the thesis:

- (1) What is the biological activity of these biomolecules?
- (2) What is the effect of these biomolecules on osteoblasts?
- (3) What is the improvement of the functionality and biological efficiency when these biomolecules are incorporated with supporting materials?

CHAPTER 2

BMP-derived peptide for surface modification of bone substituting implants

2.1 Introduction

In this chapter, BMP-7-derived peptides for surface modification applications of bone substituting implants are described. The concept of developing peptide coated implants is to mimic the natural environment of bone formation, and to enhance implant fixation with the surrounding tissue (Figure 17). Since a non-physiological surface of the implant is exposed in a physiological aqueous environment, specific requirements are imposed on the implant coating to mediate these different structural entities [53]. The coating should facilitate the attachment of proteins and cells while maintaining their native conformation and biological activity. Ideally, a bioactive coating also has the ability to induce a specific cellular response, and to promote bone formation. This chapter demonstrates the main concepts and principles of engineered BMP-7-derived peptide coating for bone tissue engineering.

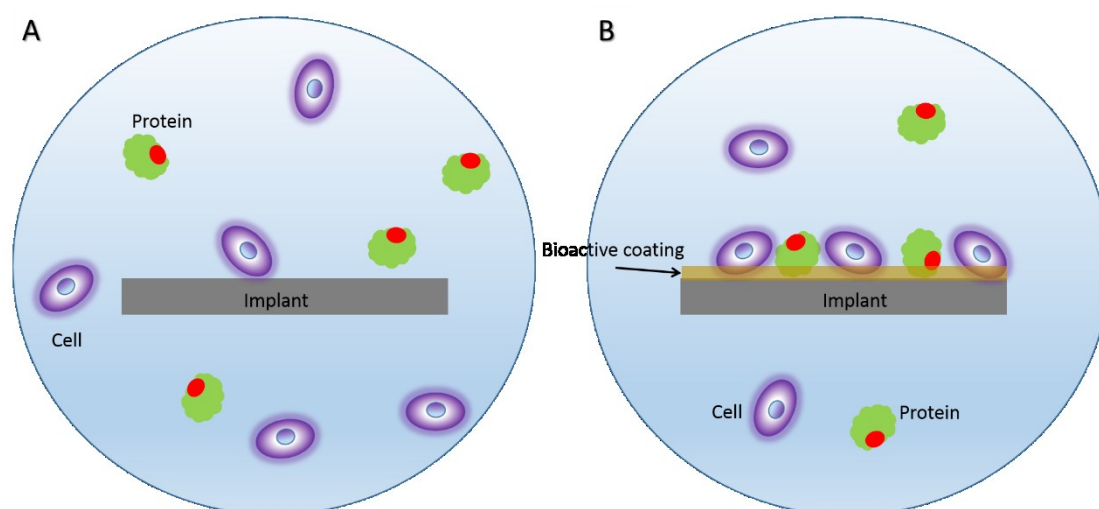


Figure 17. Concept of the development of implants with bioactive coating. A non-physiological surface of an implant (A) lacking interactions with proteins and cells,

which are necessary for bone regeneration. However, a bioactive coating (B) on an implant, which induces a specific cellular response, can reduce the time required for implant fixation and enhance the interaction between implant and the surrounding tissue, thus enhancing bone formation.

2.1.1 Engineered BMP-derived peptides

Among the relevant biomolecules involved in bone tissue engineering, bone morphogenetic proteins (BMPs) are one of the most studied bioactive factors because their outstanding contributions towards bone formation. As mentioned in Section 1.4.1.1, BMPs play an important role in ectopic bone formation and regulate the key steps of bone and cartilage morphogenesis [116]. Exclusive studies have been carried out to immobilise BMPs on the implants to fabricate bioactive coatings [265, 266]. For example, BMP-2 coatings markedly increased the osteoinductive potential of Ti implants [267]. BMP-4 immobilised on Ti-6Al-4V alloy by plasma surface modification strategy induced alkaline phosphatase activity in pluripotent C3H10T1/2 cells [268]. However, the applications of full-length BMPs are limited due to their large structure, potential immunological and carcinogenic risks, low chemical stability, and high cost [134-136]. In addition, the active site of BMPs can be easily hidden during immobilisation due to its complex structure.

One promising solution is to use peptides derived from BMPs for bone tissue engineering applications. As mentioned in Section 1.4.1.2, compared to full-length proteins, peptides offer easier production, more precise control of the chemical components and a higher utilisation efficiency while preserving the entire or a certain part of the protein function [118, 136, 137]. Immobilising a part of BMPs on an implant to produce a peptide coating has been investigated. For example, a synthetic receptor binding motif mimicking BMP-2 immobilised on Ti surfaces enhanced the bone healing rate when compared with untreated Ti surfaces [269]. Biological evaluations showed that the peptide coated implants enhanced cell growth rate, protein expression, and

alkaline phosphatase production in MC3T3-E1, and significantly improved bone growth in the canine mandibles [269].

This chapter is focused on a short peptide KAISVLYFDDS derived from BMP-7 (Figure 18) [129, 136]. Amino acids AISVLYFDDS are derived from the 111-120 amino acid residue sequence of BMP-7, and the K terminal amino acid is designed for an easier functionalisation on nano-materials [270, 271]. There are several reasons to choose this specific peptide. First, this peptide is derived from the Finger 2 domain of BMP-7, which contributes to the ligand-receptor binding interactions [272]. Second, this peptide has been shown to increase osteoblast calcium deposition, which is an essential step in bone formation [129, 136]. In addition, this peptide only has 11 amino acids, which is easy to synthesise and engineer.

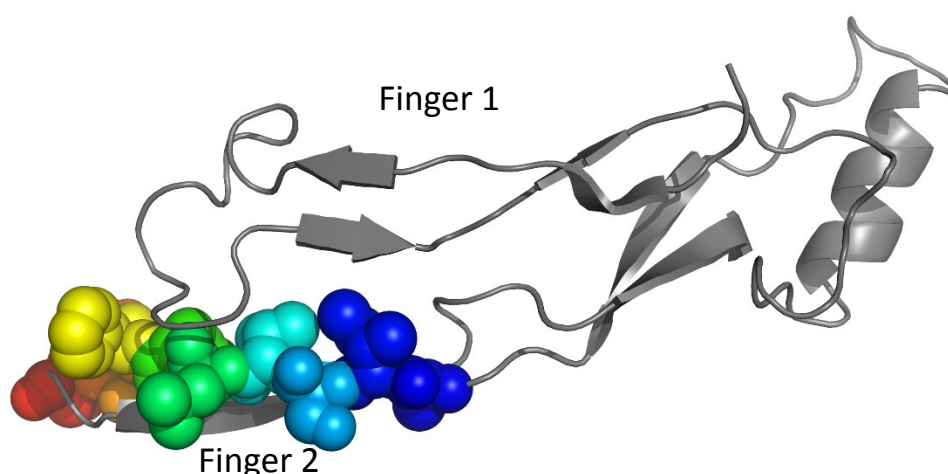


Figure 18. The three-dimensional protein structure of BMP-7. It contains two bioactive areas, Finger 1 and 2 domains, which contribute the ligand-receptor binding interactions. The amino acids shown in colourful dots in Finger 2 domain represent the peptide sequence AISVLYFDDS (111-120 amino acid residue sequence of BMP-7). This figure was drawn using PyMOL (www.pymol.org).

In order to immobilise KAISVLYFDDS on Ti implants in a highly efficient way without affecting its biological activity, two immobilisation methods are chosen: adsorption and chelation (see Section 1.6.2). Chelation immobilisation requires an engineered BMP-7-derived peptide with a sequence of six histidine residues (His-tag) that bind to

nickel chelates. The concept is inspired by the His-tag fusion protein in which a protein containing a His-tag can be easily immobilised on nickel matrix [231] (Figure 12). Similarly, a His-tag peptide can bind to nickel pre-treated Ti implants. By using chelation, a higher loading rate and efficiency is expected compare to adsorption. Figure 19 is a schematic representation of the immobilisation of the BMP-7-derived peptides on Ti implants. Figure 19A shows the peptides KAISVLYFDDS are randomly immobilised on the un-treated Ti implant by physical adsorption. After treatment with a nickel solution, nickel ions on the Ti implant showed a specific affinity for His-tag peptides so that peptides can be orderly immobilised (Figure 19B).

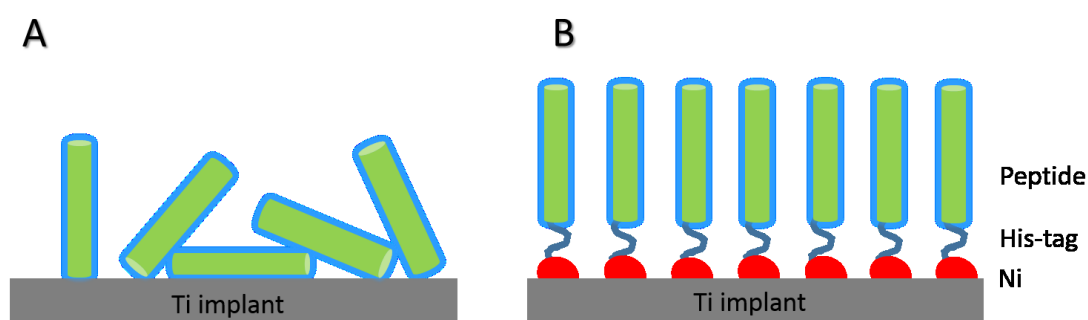


Figure 19. The immobilisation of BMP-7-derived peptides KAISVLYFDDS on Ti implants by (A) adsorption and (B) chelation. The BMP-7-derived peptides are shown in green, His-tags are shown in blue, red dots represent the nickel (Ni) ions on the Ti implant.

2.1.2 C2C12 cells

Previous studies indicates that the C2C12 myoblast cells are useful models to investigate the biological activity of BMPs [273, 274]. BMPs inhibit myotube formation and induce osteoblastic differentiation of C2C12 [130, 275, 276]. In addition, C2C12 cells express an extremely high level of alkaline phosphatase activity when treated with BMPs. In this study, C2C12 cells were used to verify the biological activity of the BMP-7-derived peptides.

2.1.3 Scope of chapter

The objective of this chapter is to re-engineer the short BMP-7-derived peptides for bone tissue engineering with improved functionality. In this chapter, some short BMP-7-derived peptides which are reported to possess similar bone inducing properties to whole BMP-7 are studied. In order to immobilise the peptides onto Ti implants, a His-tag is added into the short BMP-7-derived peptides. After the synthesis and characterisation of BMP-derived peptides, an *in vitro* cell culture study is performed to determine the effect of peptides on C2C12 and MC3T3-E1 cells.

2.1.4 Hypotheses

The hypotheses tested was that BMP-7 derived peptides have the ability to increase osteoblast calcium deposition, and may have potential applications in the field of bone tissue engineering. In order to test these hypotheses, the following questions have been addressed in this Chapter:

- (1) What is the molecular weight and purity of BMP-7 derived peptides after synthesis?
- (2) What is the biological activity of these BMP-7 derived peptides? What is the effect of these biomolecules on C2C12 myoblast cells?
- (3) What is the effect of these peptides on MC3T3-E1 pre-osteoblasts?

2.2 Materials and methods

2.2.1 BMP-derived peptides synthesis and characterisation

2.2.1.1 Materials

DMSO (99.9%), tetramethylammonium hydroxide pentahydrate (TMA) (97%), 3-

mercaptopropionic acid, EDTA, amino acids, lyophilised bovine serum albumin (BSA) (96%), nickel sulphate (99.99%), Bradford reagent were purchased from Sigma-Aldrich (Gillingham, UK). Chloroform (99%), Tris buffer and glycerol were purchased from VWR (Poole, UK).

2.2.1.2 BMP-derived peptides design

Peptide KAISVLYFDDS consists of two parts: the amino acids AISVLYFDDS are derived from the 111-120 amino acid residue sequence of BMP-7, and the K terminal amino acid is added to make the peptide easier to eventually functionalise on nanomaterials [136].

In order to immobilise the peptide on Ti implants by chelation, a His-tag (HHHHHH) that strongly interacts with the nickel-Ti implant surface *via* metal-affinity coordination was added. His-tag was added on both C-terminal (KAISVLYFDDSHHHHHH) and N-terminal (HHHHHHKAISVLYFDDS) to compare the immobilisation effects.

2.2.1.3 BMP-derived peptides synthesis

In peptide synthesis, multiple amino acids are linked *via* peptide bonds, which are covalent bonds that form between amino acids when the carboxyl group (C-terminus) of one amino acid reacts with the amino group (N-terminus) of the other [277]. Chemical peptide synthesis starts from the C-terminal and ends at the N-terminus of the peptide.

There are two types of peptide synthesis methods: liquid-phase peptide synthesis and solid-phase peptide synthesis. The first is the classical approach, and mainly used in large-scale industrial peptide production. The second one, solid-phase peptide synthesis (Figure 20 [278]), is now widely used in laboratory-scale manufacturing. The

core principle of solid-phase peptide synthesis is a repeating deprotection-wash-coupling-wash cycle [279]. First, an N-protected peptide is covalently attached to a small solid bead (resin). Then the peptide is deprotected by removing the Fmoc, an α -amino protecting group, and by revealing a new N-terminal amine to which the next amino acid can be attached. Next, the free N-terminal amine of this peptide is coupled to a single N-protected but C-activated amino acid. The peptide chain is building up by repeating this cycle. Once the synthesis is completed, peptide chain can be cleaved from the resin by trifluoroacetic acid (TFA).

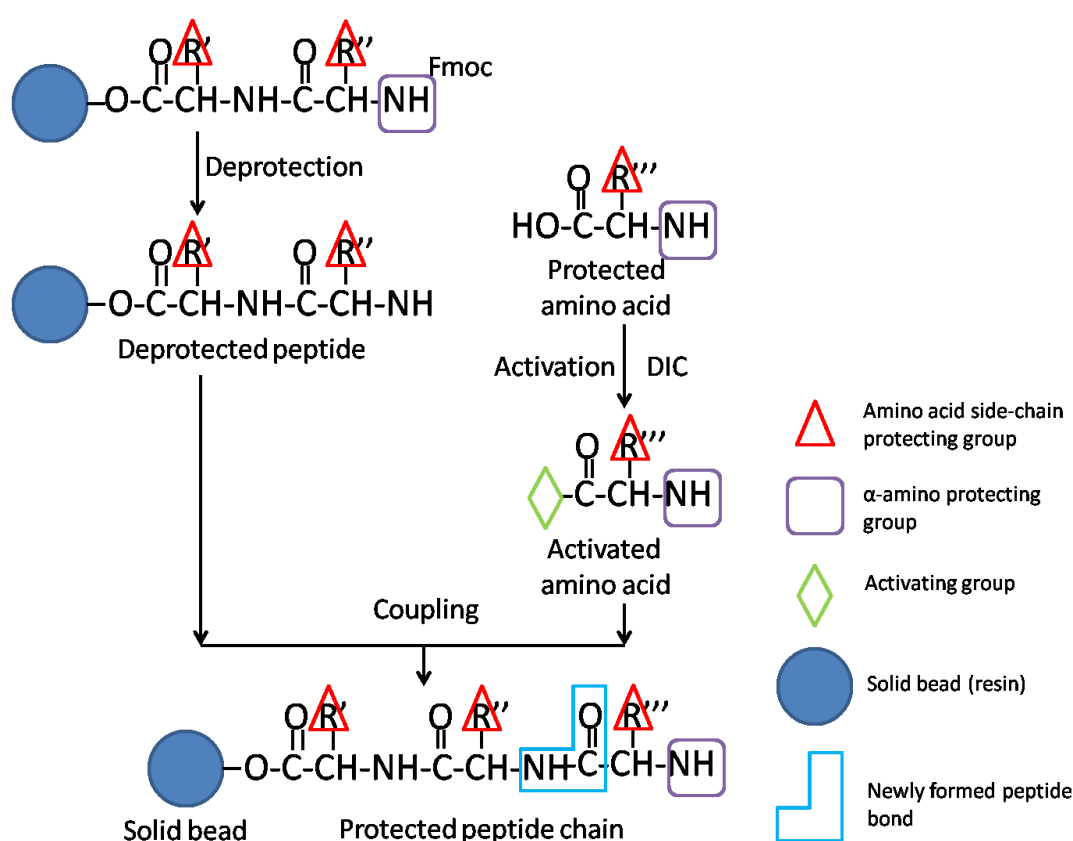


Figure 20. The process of solid-phase peptide synthesis. A resin bead acts as the solid-phase. 1-hydroxybenzotriazole/o-(benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HOBt/HBTU) is used to activate the carboxyl group before coupling the amino acids. Fluorenylmethyloxycarbonyl (Fmoc) functions as an α -amino protecting group[278].

In this chapter, three peptides (KAISVLYFDDS, KAISVLYFDDSHHHHHH, and HHHHHHKAISVLYFDDS) were synthesised by solid phase peptide synthesis method

[279] using Fmoc-protected amino acid monomers and HOBt/HBTU activation on a QUARTET multiple synthesiser (Protein Technologies. Inc., USA). In each coupling step, a 10-fold excess of amino acids was used. Cleavage from the resin and side-group deprotection was performed by using TFA and triisopropylsilane. The peptides were lyophilised and analysed by matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry (MALDI micro MX (Micromass, UK)) from the Chemistry Department, Imperial College London. The lyophilised peptides were stored at -20°C.

2.2.1.4 HPLC

High performance liquid chromatography (or high pressure liquid chromatography, HPLC) is a chromatographic technique that can separate compounds from a mixed solution. It is widely used in biochemistry and analytical chemistry applications to identify, quantify and purify the individual components from the solution [280].

The HPLC instrument consists of a reservoir of mobile phase, a pump that provides higher pressures, an injector, a separation column, and a detector that provides a characteristic retention time for the sample (Figure 21) [281]. Compounds are separated by the column because different components in the mixture have different passing rate.

HPLC (GX-271; Gilson, Sweden), was used to purify the peptides synthesised in this study. A c18 reverse-phase HPLC column was used with a gradient of acetonitrile and water. A flow rate of 9.98 ml/min was used and fractions were collected in Eppendorf tubes.

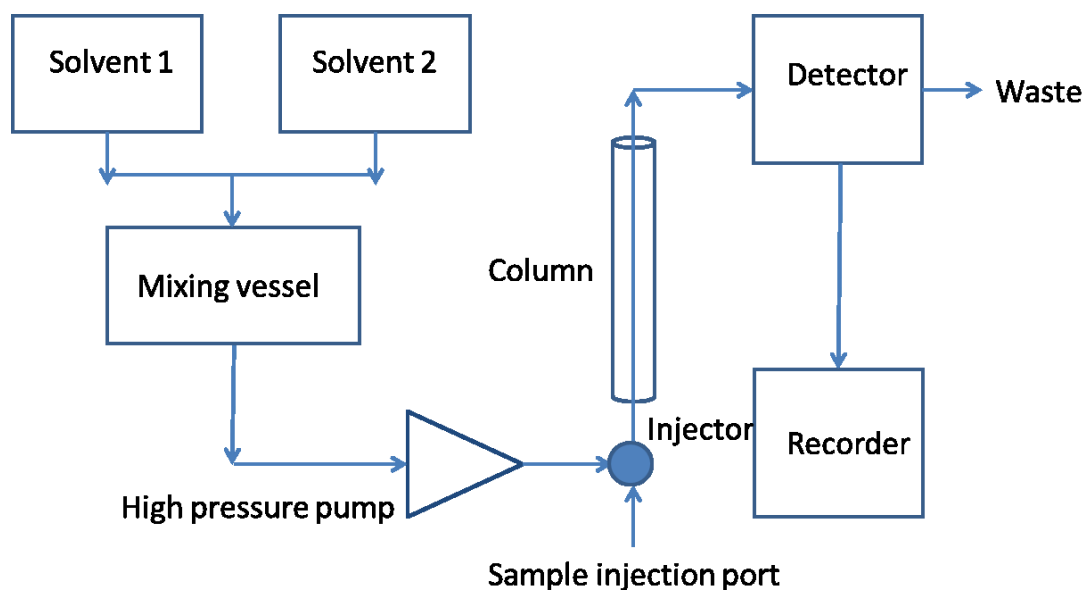


Figure 21. Schematic of an HPLC instrument [281]. (Image adapted from Chemistry Hypermedia Project website).

2.2.1.5 MALDI Mass Spectrometry

Matrix-assisted laser desorption/ionisation (MALDI) is a soft ionisation technique used in mass spectrometry. It is a popular analytical tool for peptides, proteins and other biomolecules which tend to be fragile when ionized by more conventional ionisation methods [282]. MALDI can be used for the identification of biomolecules, and the determination of their molar mass distribution. It is also a very simple and rapid analytical method to determine the molecular weight of biomolecules.

Upon MALDI analysis, the sample is first co-crystallised with a matrix compound, which protects the biomolecules from being destroyed by the direct laser beam and facilitates the later vaporisation and ionisation. Laser radiation results in the vaporisation of the sample-matrix mixture. The energy transfer during a matrix-assisted laser induced desorption provides high ion yields of the intact sample and allows high accuracy and sensitivity measurement of peptide compounds [283].

2.2.2 *In vitro* cell culture study of BMP-derived peptides

2.2.2.1 Materials

Unless stated otherwise, all cell culture reagents and media were purchased from Gibco/Life Technologies, UK. BMP-2 was kindly provided by Professor W. Sebald (Universität Würzburg). Routine cell culture was carried out under aseptic conditions in a class II microbiological safety cabinet (Medical Air Technology LTD, UK). Cells were maintained in a GA 3000 LEEK incubator (LEEK LTD, UK). Cells were routinely viewed on an IX-70 phase contrast inverted microscope (Olympus, UK) and photographed using an Olympus OM-4 mounted automatic camera.

2.2.2.2 C2C12 cell culture

The mouse myoblast cell line, C2C12 was purchased from American Type Culture Collection (ATCC, USA). For each experiment, a vial of frozen cells was thawed and seeded on 75 cm² plastic culture flasks in the growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin), in humidity 5% CO₂ at 37°C until they reached 80% confluence.

The number of cells was quantified using a haemocytometer. The cells were collected by centrifugation at 1600 rpm for 5 min at ambient temperature. The pellet was resuspended in 5 ml growth medium. 20 µl of cell suspension was mixed with 20 µl of 0.4% trypan blue. The mixture was added on a haemocytometer. Cells were counted manually from four different fields and averaged, giving the number of cells/ml.

To examine the effects of BMP-7-derived peptides on C2C12 cells, the cells were seeded at a density of 2.5×10^6 cells/cm² in 24-well plates (4 wells per group). After

confluence had been reached, the growth medium was replaced by treatment media containing different concentrations of peptides and cultured for an additional 3, 6, 9 and 14 days, respectively. The treatment media (with and without the peptides of interest to this study) were changed every 2-3 days. The growth medium containing 300 ng/ml BMP-2 was used as positive control group. The cells were washed twice with phosphate buffered saline (PBS) after removal from the treatment media, and kept in -80°C until their total protein content, total DNA content and ALP activity was analysed. After 3, 14 and 21 days, the cells were fixed in 2.5% (v/v) glutaraldehyde and dehydrated in a graded ethanol series followed by incubation in hexamethyldisilazane. The cells were sputter coated with chromium then visualized by SEM.

2.2.2.3 MC3T3-E1 cell culture

Pre-osteoblasts are widely used to determine the biology activity of BMPs [284, 285]. MC3T3-E1, a mouse pre-osteoblast cell line, was used to determine the biological activity of the BMP-7-derived peptides. MC3T3-E1 cells were seeded on 75 cm² plastic culture flasks in the growth medium (α -minimum essential medium (α -MEM) supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin), in humidity 5% CO₂ at 37°C.

In order to determining the effect of the BMP-derived peptides on MC3T3-E1 cells, the same treatments were performed as above. The growth medium containing 50 ng/ml BMP-2 was used as positive control group. The cells were washed twice with PBS after removal from the treatment medium, and kept in -80°C until their total protein content, total DNA content and ALP activity were analysed. MC3T3-E1 cells were fixed in the same method as above and then visualised by SEM.

2.2.2.4 Scanning electron microscopy

The scanning electron microscope (SEM) images the sample by scanning a focused beam of high-energy electrons to generate signals at the sample surface. The signals derived from electron-sample interactions reveal information of the sample such as the external morphology, the chemical composition, the crystalline structure, and other properties such as electrical conductivity [286]. In this study, SEM is used to observe surface morphologies and cell behaviour after treatment with BMP-7-derived peptides.

2.2.2.5 Energy dispersive spectroscopy

Energy dispersive spectroscopy (EDS) is an analytical technique usually linked to SEM [287]. It uses an emitted X-ray spectrum to identify particular elements (qualitative analysis) and determine their relative proportions (quantitative analysis) in a sample [288]. In this study, EDS was used to analyse the extracellular matrix deposition in cell culture experiments.

2.2.2.6 Biochemical assays

A) Total protein assay

The total protein content of cells was determined by a commercial micro BCA (bicinchoninic acid) protein assay kit (Thermo Scientific Pierce Protein Research Products). The assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by proteins in alkaline conditions with the highly sensitive and selective colorimetric detection of the Cu^{1+} using a unique reagent containing BCA [289]. The purple-coloured reaction product shows a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations from 20 $\mu\text{g/ml}$ to 2000 $\mu\text{g/ml}$ [289]. After three thaw-freeze

cycles, 100 µl of cell lysate was mixed with the assay reagent in a 96-well plate and incubated for 2 hours at 37°C. Light absorbance of these samples was measured at 562 nm on a 96-well plate reader (Labsystems, Life Sciences International Ltd, Hampshire, UK). Experiments were performed independently in triplicate.

B) Total DNA assay

The total DNA content of cells was determined using a commercial Quant-iT™ PicoGreen dsDNA reagent Kit (Invitrogen, UK). Quant-iT™ PicoGreen dsDNA reagent is an ultra-sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) in solution [290]. The amount of dsDNA can be used as a marker for cell proliferation. After three thaw-freeze cycles, 10 µl of cell lysate was mixed with PicoGreen in a 96-well plate. The samples were excited at 480 nm and the fluorescence emission intensity was measured at 520 nm using a 96-well plate reader. Experiments were performed independently in triplicate.

C) ALP activity assay for cells

The ALP activity assay depends on the hydrolysis of p-nitrophenylphosphate (pNPP) by the enzyme ALP and generates p-nitrophenol and inorganic phosphate. p-nitrophenol is converted to a yellow product under alkaline conditions and shows a strong absorbance at 400-420 nm. After specified days of culture, the cells were washed with PBS before stored at -80°C. After three thaw-freeze cycles, 80 µl of cell lysate was mixed with pNPP (Sigma, UK) in a 96-well plate and incubated for 60 min at 37°C, and the reaction was stopped by adding 100 µl 1 M NaOH. Light absorbance of these samples was measured at 405 nm on a 96-well plate reader. A standard curve was constructed using p-nitrophenol (Sigma, UK) as a standard. Experiments were performed independently in triplicate, and the ALP activity of each sample was normalised to protein content and expressed as mean standard deviation.

2.2.2.7 Statistical analysis

Statistical analyses on the data of the *in vitro* experiments of BMP-derived peptides were performed by a one-way variance (ANOVA) with Tukey-Kramer Multiple Comparisons post-test. A $p < 0.05$ was considered as statistically significant. Calculations were performed in Graphpad InStat 3 (Graphpad software Inc., San Diego, USA).

2.3 Results and discussion

2.3.1 BMP-derived peptide synthesis

The BMP-7-derived peptide KAISVLYFDDS, and the other two peptides with His-tag, KAISVLYFDDSHHHHHH and HHHHHHKAISVLYFDDS, were synthesised on a QUARTET multiple peptide synthesiser (Figure 22). The molecular weight and purity of the BMP-7-derived peptides were determined by MALDI (Figure 23) and HPLC, respectively. The theoretical molecular weight of peptide KAISVLYFDDS (11 amino acids) is 1256.60 Da, and those with His-tag (17 amino acids) is 2079.30 Da. The difference between the real molecular weight and the theoretical molecular weight is insignificant. The results indicate that the peptide synthesis technique is accurate and reliable. In addition, all peptides synthesised on the QUARTET multiple peptide synthesiser have a more than 90% purity according to the results of HPLC.

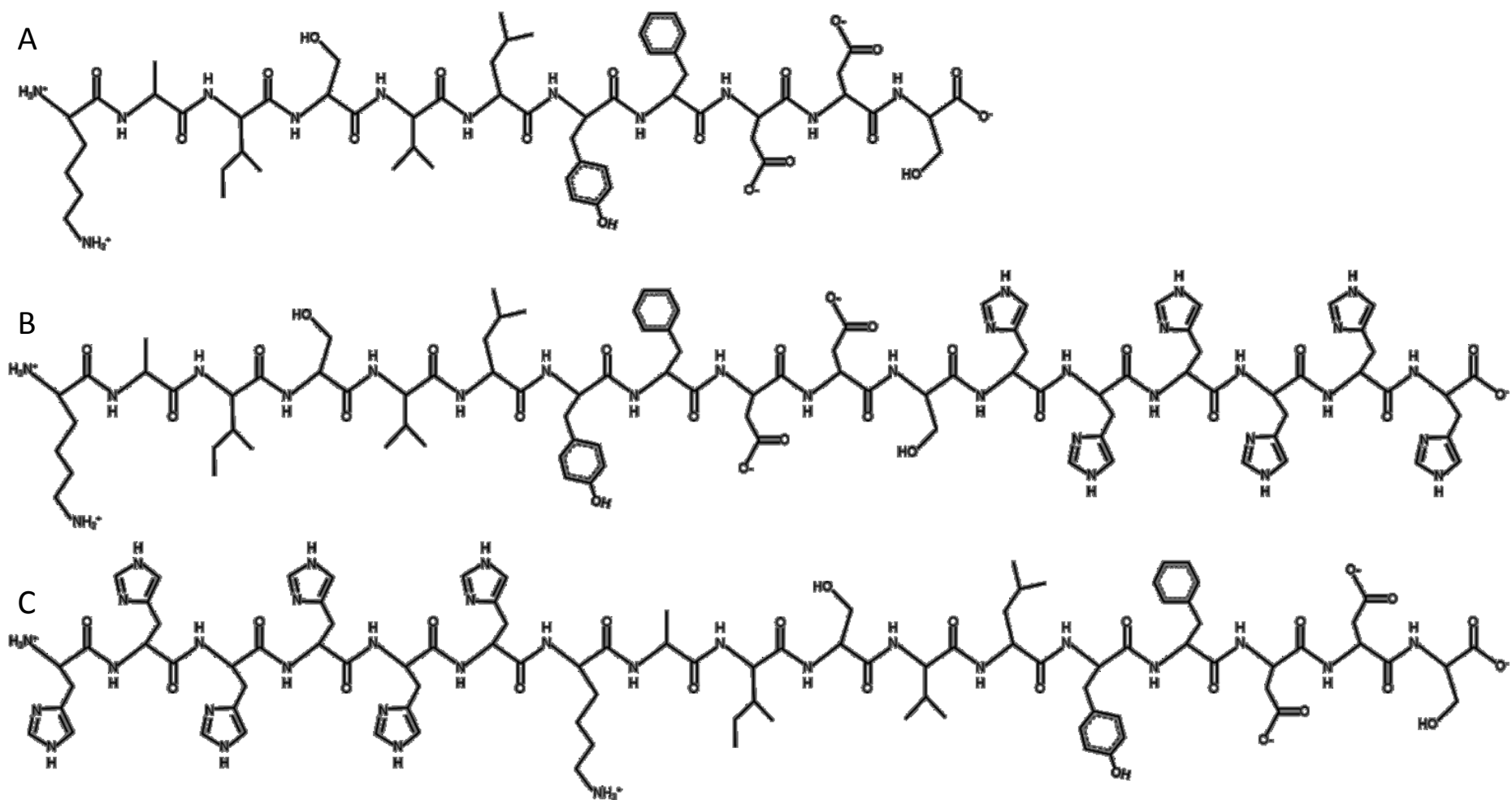
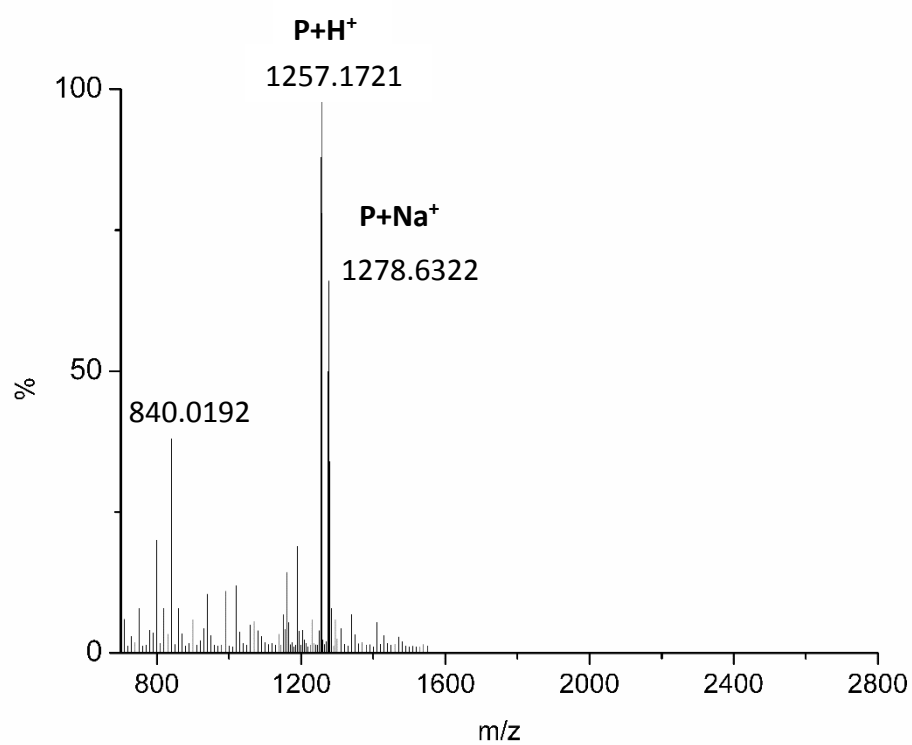
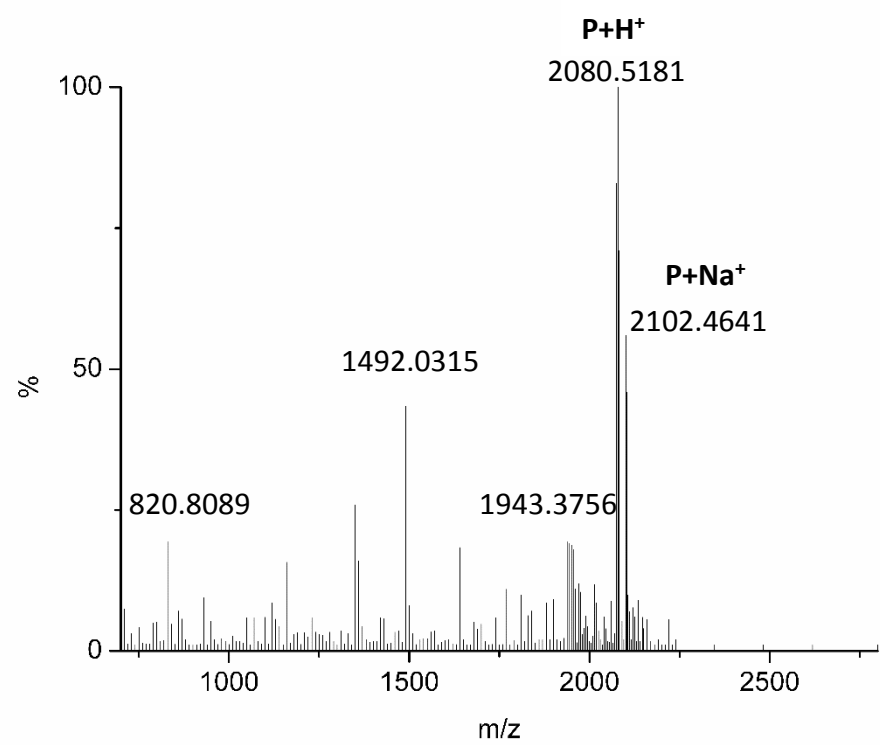


Figure 22. Chemical structure of BMP-7-derived peptides KAISVLYFDDS (A), and KAISVLYFDDSHHHHHH (B) and HHHHHHKAISVLYFDDS (C).

A



B



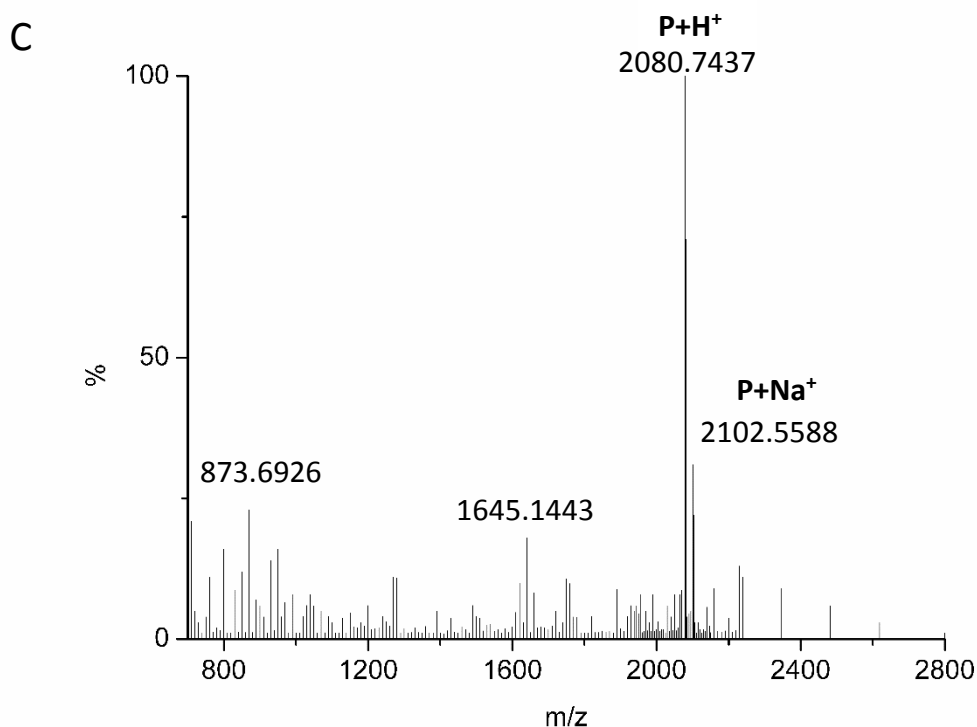


Figure 23. MALDI mass spectral analysis of BMP-7-derived peptides KAISVLYFDDS (A), KAISVLYFDDSHHHHHH (B) and HHHHHHKAISVLYFDDS (C). The P+H⁺ peak corresponds to monoprotonated mass (H⁺) on MALDI mass spectrum indicating a peptide molecular weight. The P+Na⁺ peak corresponds to Na⁺ salt.

2.3.2 Assessment of biological activity of BMP-derived peptides on C2C12 cells

2.3.2.1 Cell proliferation

Three independent runs of C2C12 cell culture experiments were carried out. Figure 24 shows the proliferation of C2C12 cells cultured in treatment media containing different concentrations (0, 0.1, 10, and 1000 µg/ml) of BMP-7-derived peptide KAISVLYFDDS by measuring the total protein content and total DNA content. In Figure 24A, all groups cultured in the presence of the peptide showed a continuous increase in total protein content along with the time for 14 days, although the increase in the group cultured in the presence of 0.1 µg/ml peptide between day 9 and day 14 was insignificant. Only the control group with no addition of peptide showed an early protein content peak

around day 9. On day 14, significantly decreased protein content values were observed for the control group compared to the groups of C2C12 cells treated with 0.1 µg/ml ($p < 0.05$), 10 µg/ml ($p < 0.001$) and 1000 µg/ml ($p < 0.001$) BMP-7-derived peptide. In addition, C2C12 cells treated with higher BMP-7-derived peptide concentrations (10 µg/ml and 1000 µg/ml) showed significantly increased protein content compared to the low peptide concentration group (0.1 µg/ml) ($p < 0.001$). Most importantly, total protein content in 10 µg/ml and 1000 µg/ml peptide groups was significantly increased ($p < 0.001$ and $p < 0.01$, respectively) compared to the peak value in the control group. However, there was no significant difference of protein content between two higher BMP-7-derived peptide concentration groups on day 14.

Total DNA content of C2C12 cells showed similar increasing trends when cells were treated with 10 µg/ml and 1000 µg/ml BMP-7-derived peptide within 14 days. However in addition to the control group, the cells treated with 0.1 µg/ml BMP-7-derived peptide also showed an early DNA content peak around day 9. A significant decrease occurred from day 9 to day 14 in the same group ($p < 0.001$). On day 14, total DNA content values of C2C12 cells were increased along with the concentration of BMP-7-derived peptide presented in the treatment media. There was a significant increase between the groups containing BMP-7-derived peptide ($p < 0.001$). In addition, total DNA content in 10 µg/ml and 1000 µg/ml peptide groups on day 14, as well as the peak value of total DNA content in 0.1 µg/ml peptide groups on day 9 was significantly increased ($p < 0.001$) compared to the peak value in the control group.

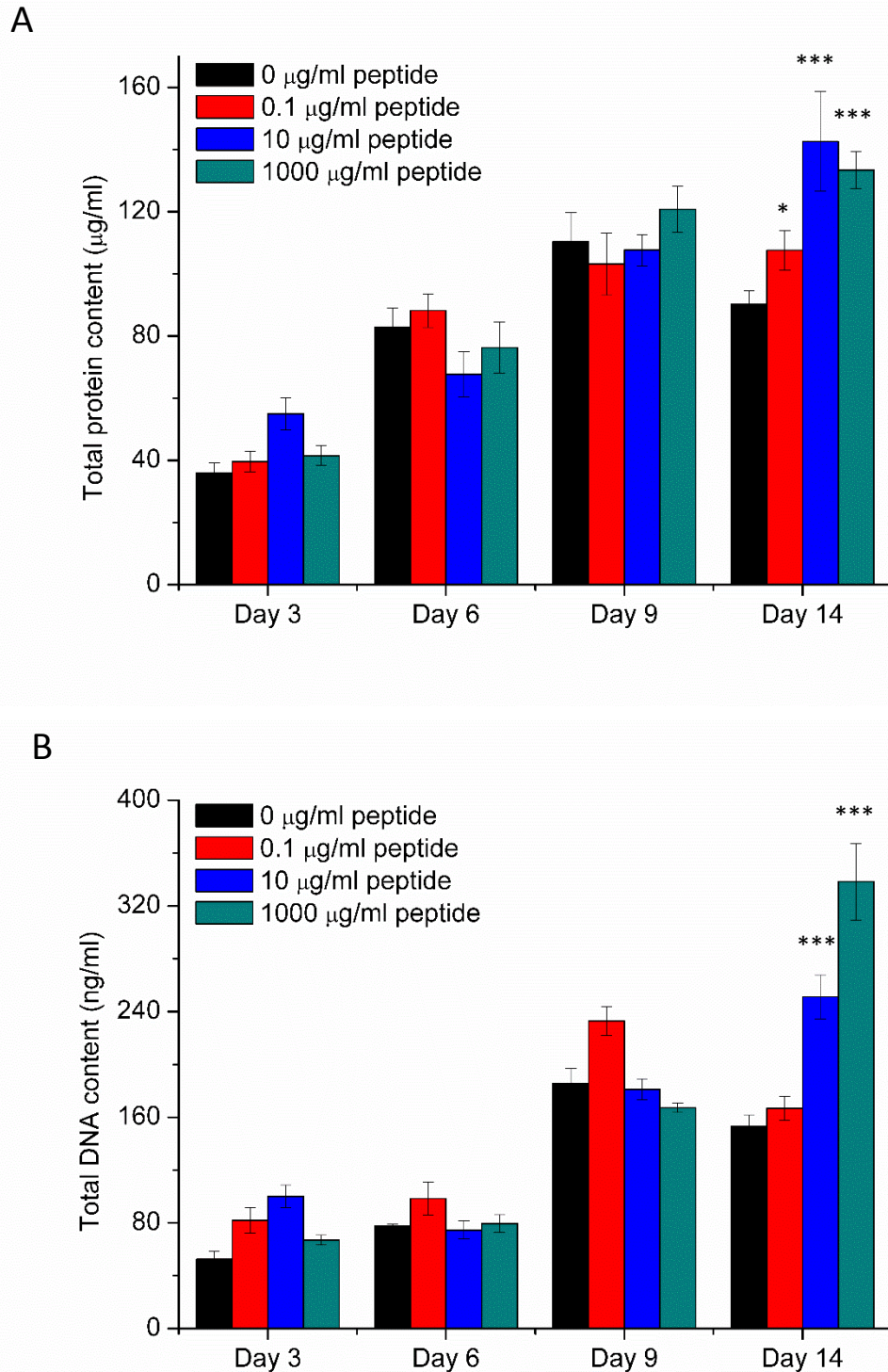


Figure 24. Effect of BMP-7-derived peptide KAISVLYFDDS on the proliferation of C2C12 cells by measuring the (A) total protein content and (B) total DNA content. 0 µg/ml peptide is the negative control group which contains no BMP-7-derived peptide in the treatment medium. 0.1 µg/ml peptide, 10 µg/ml peptide and 1000 µg/ml peptide represents the concentration of BMP-7-derived peptide in the treatment medium was 0.1 µg/ml, 10 µg/ml and 1000 µg/ml, respectively. * $p < 0.05$ and *** $p < 0.001$

indicating significant differences between the respective group with the control group on day 14.

The cell culture experiments revealed a normal cell growth in all experimental groups. It demonstrated that the BMP-7-derived peptide KAISVLYFDDS did not affect C2C12 cell growth. Both total protein content and total DNA content results indicated that the BMP-7-derived peptide in treatment media supported the proliferation of C2C12 cells.

In particular, higher concentration of peptide (10 µg/ml and 1000 µg/ml) in the media may increase the total protein content and total DNA content more than the low concentration groups. At the same time, a delayed proliferation was observed for the higher concentration of peptide groups (from day 9 to day 14). In order to improve the proliferation of C2C12 cells while minimising the side effects on cellular behaviours by the BMP-7-derived peptide, a concentration of 10 µg/ml of BMP-7-derived peptide was chosen for the next studies.

The same cell culture experiments were performed with the other two BMP-7-derived peptides contain His-tag, KAISVLYFDDSHHHHHH and HHHHHHKAISVLYFDDS, and statistically similar results to the peptide KAISVLYFDDS were obtained.

2.3.2.2 Cell morphology

The cell morphology of C2C12 cells cultured in treatment media containing different BMP-7-derived peptides (KAISVLYFDDS, KAISVLYFDDSHHHHHH and HHHHHHKAISVLYFDDS) was evaluated by light phase contrast inverted microscopy at day 3, day 6, day 9 and day 14 (Figure 25). On day 3, no significant differences were observed, with cells attaching and proliferating normally. On day 6, however, the phenotype of C2C12 cells in the presence of BMP-7-derived peptides as well as the negative control group displayed obvious changes. Some spindly fibre-like thin

myotubes formed by cells fusing with each other were beginning to emerge in these groups. Only the cells treated with BMP-2 remained unfused, with mononuclear polygonal cell morphology. Striking morphological changes of cells treated with the peptides of interest as well as the negative control group occurred over the course of day 6 to day 9. On day 9, all cells in the four groups eventually fused into mature multinucleated myotubes.

The cells treated with BMP-2, however, revealed osteoblast-like morphology as expected. On day 14, more osteoblast-like morphology was observed in the BMP-2 treated group while numerous myotubes were formed in the other groups. Some cell clusters were also found in these groups due to their high confluency. No significant differences of cell morphology among the three BMP-7-derived peptide groups was found. In addition, cells cultured in the presence of BMP-7-derived peptides exhibited similar morphology features as the negative control group.

The purpose of the cell morphology study was to determine whether or not the BMP-7-derived peptides have the same ability to influence the differentiation of C2C12 myoblasts into osteoblasts as BMPs. The results of cell morphology proved that 300 ng/ml BMP-2 in the growth medium has the ability to inhibit myotube formation and induce osteoblast trans-differentiation. However, 10 µg/ml of BMP-7-derived peptides failed to convert the differentiation, even though these peptides were derived from the active site of BMP-7 and shown the ability to increase osteoblast calcium deposition. The phenotype of cells in the presence of BMP-7-derived peptides was similar as the negative control, which had no additives in the culture medium. More studies were carried out to further investigate the effect of BMP-7-derived peptides on C2C12 differentiation.

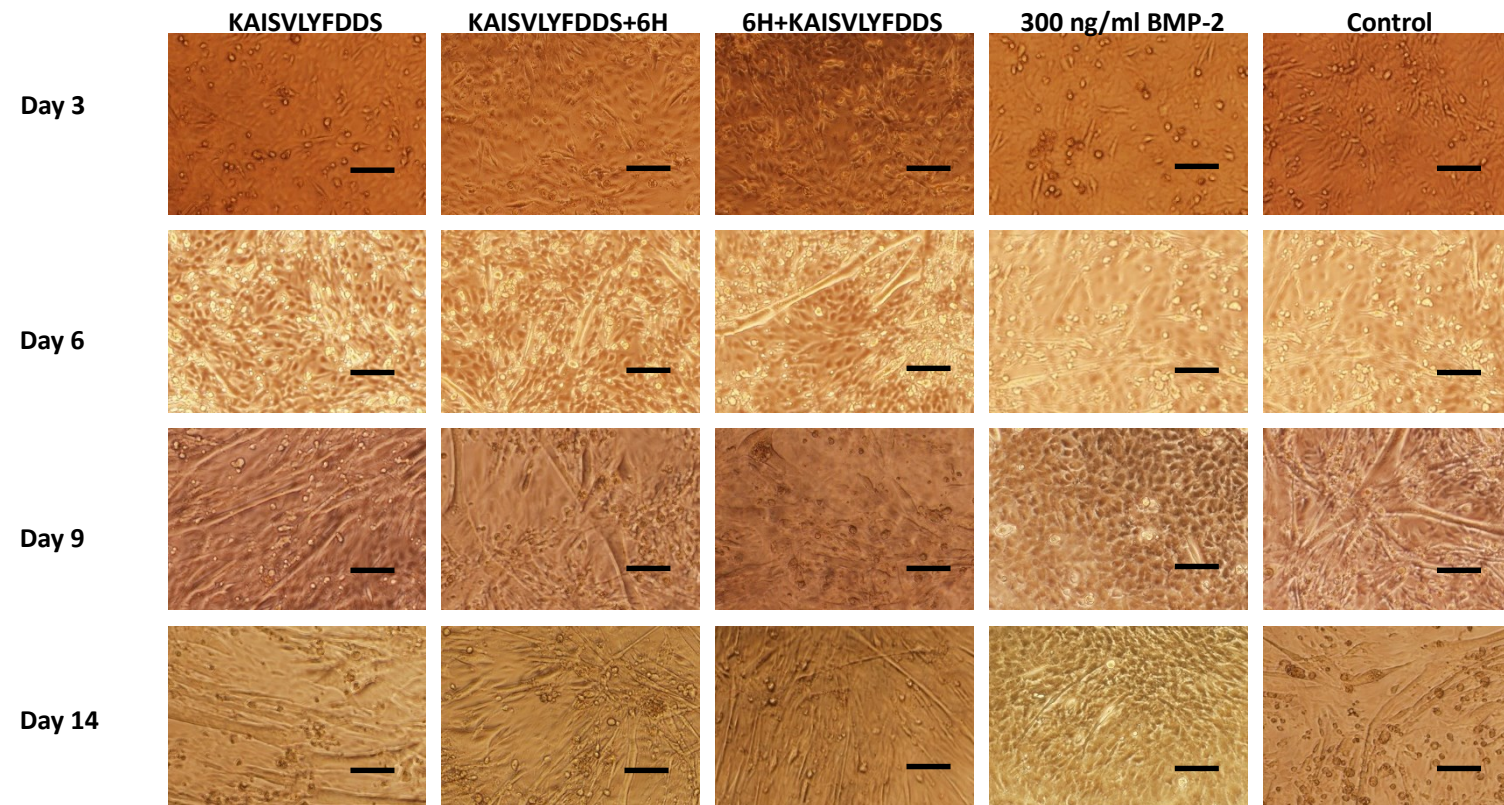


Figure 25. Gross changes in cell morphology of C2C12 cells cultured in treatment media with 10 $\mu\text{g/ml}$ BMP-7-derived peptides KAISVLYFDDS (first column), KAISVLYFDDSHHHHHH (second column) and HHHHHHKAISVLYFDDS (third column) after 14 days in culture. Cells treated with 300 ng/ml BMP-2 was the positive control (fourth column). The control (negative) group represents cells were cultured in growth medium with no additives (fifth column). Each row represents a time point from day 3 to day 14. Scale bars represent 200 μm .

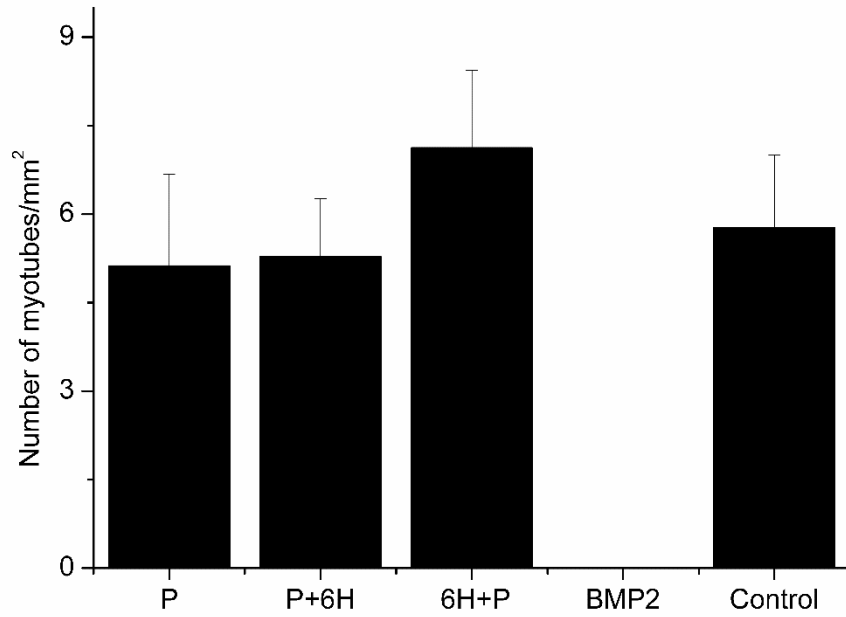
2.3.2.3 Cell differentiation

Figure 26A shows the number of myotubes generated after 6 days of culture in treatment media containing BMP-7-derived peptides. C2C12 cells cultured for 6 days in the presence of 10 µg/ml BMP-7-derived peptides differentiated into myoblasts and formed myotubes similar to the control group. The number of myotubes formed from the three peptides and the control were statistically insignificant. However, BMP-2 in the culture media changed the differentiation pathway of C2C12s so that no myotubes were found in the BMP-2 groups.

The results of the ALP activity measurements are showed in Figure 26B. ALP indicates the early differentiation of C2C12 cells. Cells treated with 300 ng/ml BMP-2 expressed significantly higher ALP activity compared with other groups at each time point ($p < 0.001$). Cell treated with 10 µg/ml BMP-7-derived peptides as well as the control group expressed consistently low ALP activity from day 3 to day 9. On day 14, a slight increase of ALP activity was observed in the BMP-7-derived peptide groups. Among them, peptide HHHHHHKAISVLYFDDS showed the highest value. In addition, all of the values exceeded the control group, but the difference between the groups was statistically insignificant.

These results support the conclusion drawn from the cell morphology results, which is that 10 µg/ml of BMP-7-derived peptides fails to convert the differentiation pathway of C2C12 myoblasts to osteoblasts. Unlike BMP-2, BMP-7-derived peptides generated no burst of ALP activity when applied to C2C12 cells.

A



B

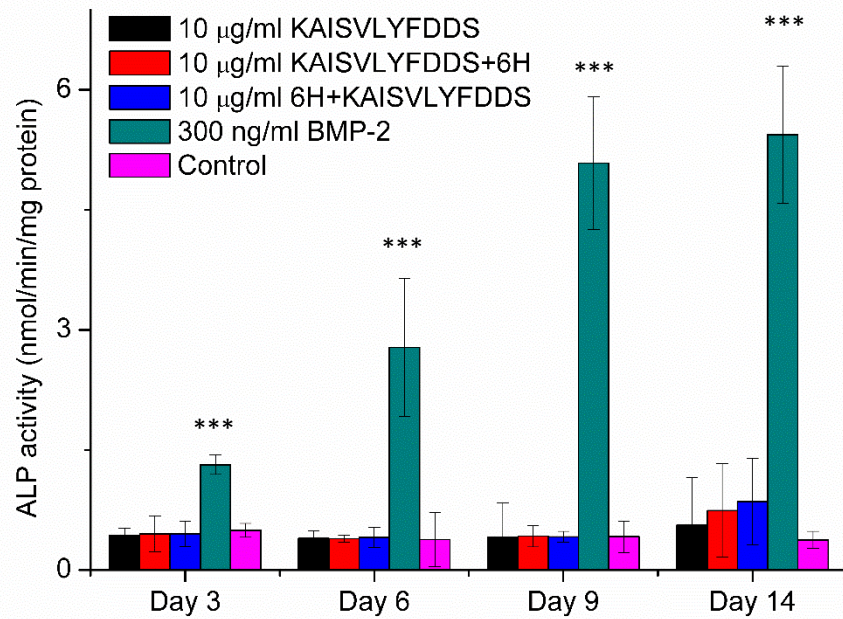


Figure 26. The effect of BMP-7-derived peptides on myotube formation and ALP activity in C2C12 cells. Control group has no additives in the culture media. (A) The number of myotubes were counted after culturing in the presence of 10 µg/ml P (represents BMP-7-derived peptide KAISVLYFDDS), P+6H (represents peptide KAISVLYFDDSHHHHHH) and 6H+P (represent HHHHHHKAISVLYFDDS), and 300 ng/ml BMP-2 as positive control for 6 days. (B) The ALP activity of C2C12 cell lysate treated

with 10 µg/ml BMP-7-derived peptides and 300 ng/ml BMP-2 for 14 days was measured using pNPP as a substrate. *** $p < 0.001$ indicates statistically significant differences between the BMP-2 group with the other groups on the respective day.

The purpose of assessing the biological activities of BMP-derived peptides on C2C12 cells was to compare the biological activity of peptides with that of BMP proteins. Although BMP-derived peptides were shown to have similar activity as the full-length proteins, no literature has reported the effect of BMP-7-derived peptides on C2C12 cells. This study indicates BMP-7-derived peptides support the proliferation of C2C12 cells, but 10 µg/ml of BMP-7-derived peptides failed to convert the differentiation pathway of C2C12 myoblasts to osteoblasts, in contrast to the activity of BMP-2. The possible reason is the peptide KAISVLYFDDS is not derived from the responsible active site of BMPs. Although the mechanism of regulation of C2C12 differentiation by BMPs is not fully understood, it may be related to the DNA binding and/or transcriptional activities of MyoD, which is an important regulator of myogenic differentiation in C2C12 cells [275]. Peptide KAISVLYFDDS, however, is derived from the Finger 2 domain of BMP-7 that is responsible for ligand-receptor binding interaction.

2.3.3 Determining the effect of the BMP-derived peptides on MC3T3-E1 cells

2.3.3.1 Cell proliferation

Figure 27 shows the proliferation of MC3T3-E1 cells cultured in treatment media containing 100 µg/ml of different BMP-7-derived peptides (KAISVLYFDDS, KAISVLYFDDSHHHHHH, and HHHHHHKAISVLYFDDS) by measuring the total protein content and total DNA content within 21 days. In Figure 27A, all groups cultured in the presence of peptides showed a continuous increase in total protein content between days 1 and 14 after cell seeding. Around day 14, a maximum was reached for those groups, after which the total protein content decreased until day 21. The control group containing no peptide showed an early protein content peak around day 10. Before day 14, there was no significant difference in total protein between the control group

and the groups treated with BMP-7-derived peptides. On day 14 and day 21, significantly decreased protein content was observed for the control group compared to the peptide treated groups ($p < 0.001$). On day 14, cells treated with 100 $\mu\text{g/ml}$ KAISVLYFDDS showed the highest protein content value among all conditions, though there was no statistically significant difference between the three BMP-7-derived peptides at day 14 and day 21. It is worth noting that large experimental variability was measured in the late time points of culture, which may have been due to the high degree of cell confluence.

Total DNA content of MC3T3-E1 cells (Figure 27B) showed similar patterns for three BMP-7-derived peptides treated groups within 21 days. The total DNA content values were generally increased in the first 14 days and reached their peak values, then rapidly decreased on day 21. The control groups, however, showed an early DNA content peak around day 7, and maintained the peak value for 7 days, after which the value decreased until day 21. Different from the total protein content, only on day 14 a significant decrease of total DNA content values was observed for the control group compared to the peptide treated groups ($p < 0.001$). Although no significant difference of DNA content between the three BMP-7-derived peptides was found, cells cultured in the presence of KAISVLYFDDSHHHHHH and HHHHHHKAISVLYFDDS showed more similarity compared to the other BMP-7-derived peptide without a His-tag.

The results demonstrated that the BMP-7-derived peptides KAISVLYFDDS, KAISVLYFDDSHHHHHH, and HHHHHHKAISVLYFDDS do not affect MC3T3-E1 cell growth. Both total protein content and total DNA content results indicated BMP-7-derived peptide in treatment media supported the proliferation of MC3T3-E1 cells.

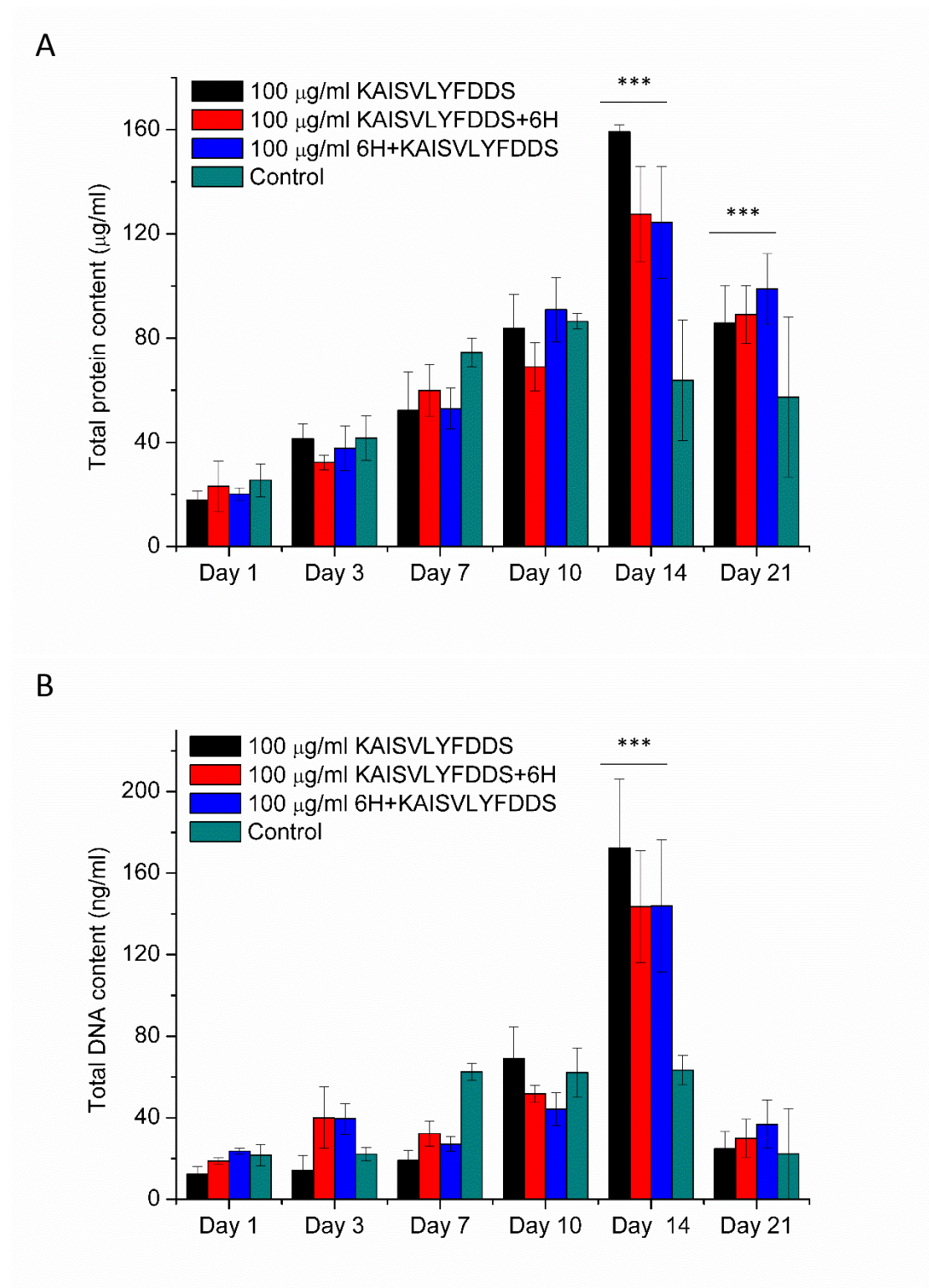


Figure 27. Effect of 100 µg/ml BMP-7-derived peptide KAISVLYFDDS, KAISVLYFDDSHHHHHH (KAISVLYFDDS+6H) and HHHHHHKAISVLYFDDS (6H +KAISVLYFDDS) on the proliferation of MC3T3-E1 cells by measuring the (A) total protein content and (B) total DNA content. *** $p < 0.001$ indicates statistically significant differences between the experimental group and the control group on the respective day.

The cells cultured in the presence of KAISVLYFDDS showed the peak values of total protein content and total DNA content on day 14, although no significant difference between three BMP-7-derived peptides was found due to the large experimental variability. The cells cultured in the presence of two His-tag peptides (KAISVLYFDDSHHHHHH and HHHHHHKAISVLYFDDS) showed similar responses compared to the other BMP-7-derived peptide. The results indicated that a His-tag in the peptide in treatment media do not lead to a significant difference in cell proliferation. In addition, the N- or C-terminal His-tag had a minimal effect on MC3T3-E1 cell proliferation.

2.3.3.2 Cell morphology

Cell morphology of MC3T3-E1 cells cultured in treatment media containing 100 µg/ml BMP-7-derived peptide KAISVLYFDDS, KAISVLYFDDSHHHHHH and HHHHHHKAISVLYFDDS was evaluated by light phase contrast inverted microscopy (Figure 28) and SEM (Figure 29) after 14 days. According to Figure 28, no differences were observed in cell morphology between all the experimental groups on day 14 including the positive control which cells were treated with 50 ng/ml BMP-2, and the negative control group which cells were cultured in growth medium with no additives. Figure 29 shows cells interacted with each other and were surrounded by fibrous extracellular matrix in all groups after 14 days. EDS analysis revealed no mineral deposit was formed from cells cultured in treatment media containing 100 µg/ml BMP-7-derived peptides, nor cells cultured in growth medium with no additives. Only the positive control, which cells were treated with BMP-2, showed a signal of mineral deposit (Ca/P ratio between 1.6 and 1.8) (Figure 30).

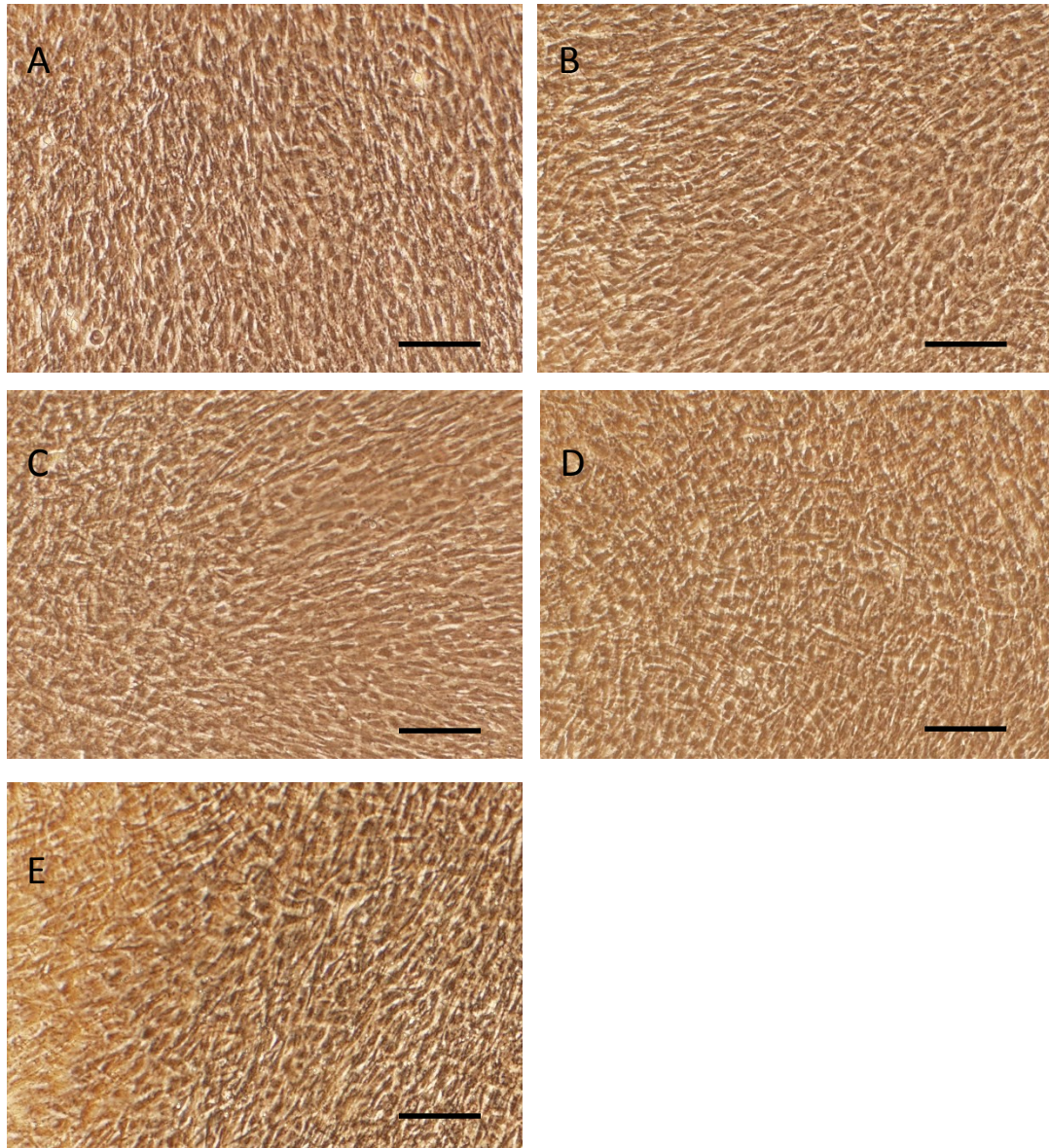


Figure 28. Light phase contrast inverted micrograph of MC3T3-E1 cells cultured in treatment media with 100 µg/ml BMP-7-derived peptides KAISVLYFDDS (A), KAISVLYFDDSHHHHHH (B) and HHHHHHKAISVLYFDDS (C) after 14 days in culture. Cells treated with 50 ng/ml BMP-2 was the positive control (D). The control (negative) group represents cells were cultured in growth medium with no additives (E). Scale bars represent 10 µm.

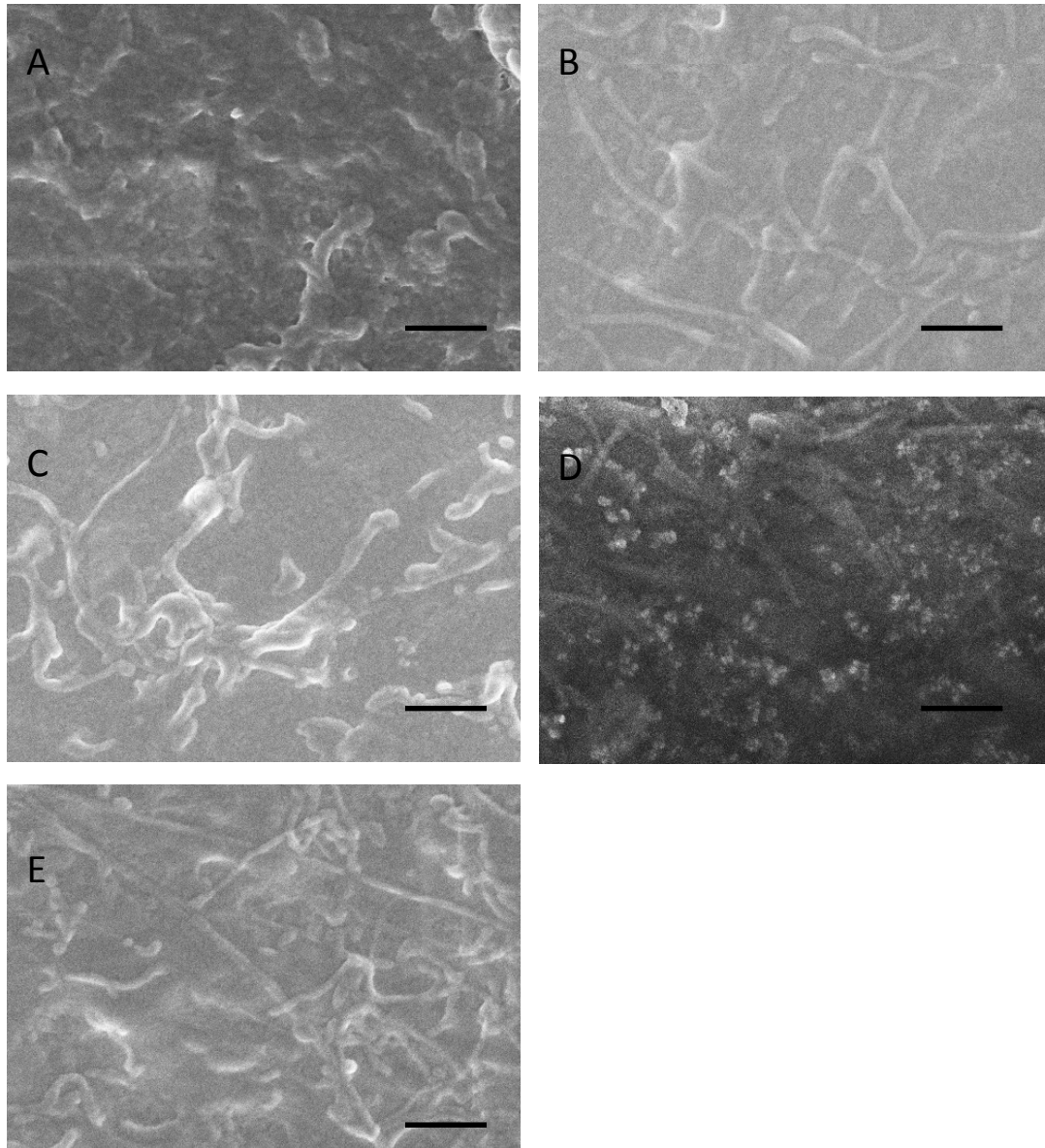
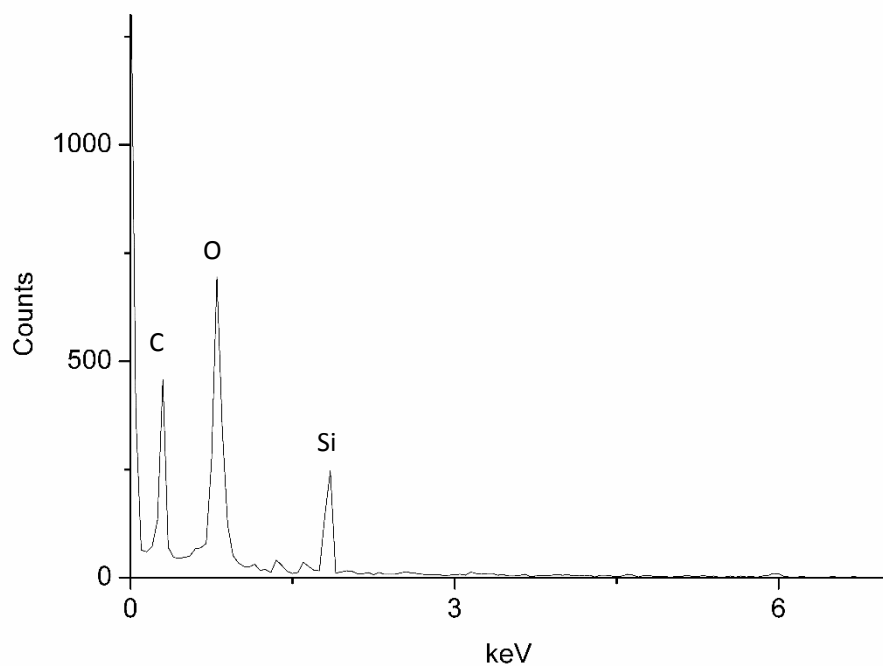


Figure 29. Scanning electron micrographs of MC3T3-E1 cells cultured in treatment media with 100 µg/ml BMP-7-derived peptides KAISVLYFDDS (A), KAISVLYFDDSHHHHHH (B) and HHHHHHKAISVLYFDDS (C) after 14 days in culture. Cells treated with 50 ng/ml BMP-2 was the positive control (D). The control (negative) group represents cells were cultured in growth medium with no additives (E). Scale bars represent 1 µm.

A



B

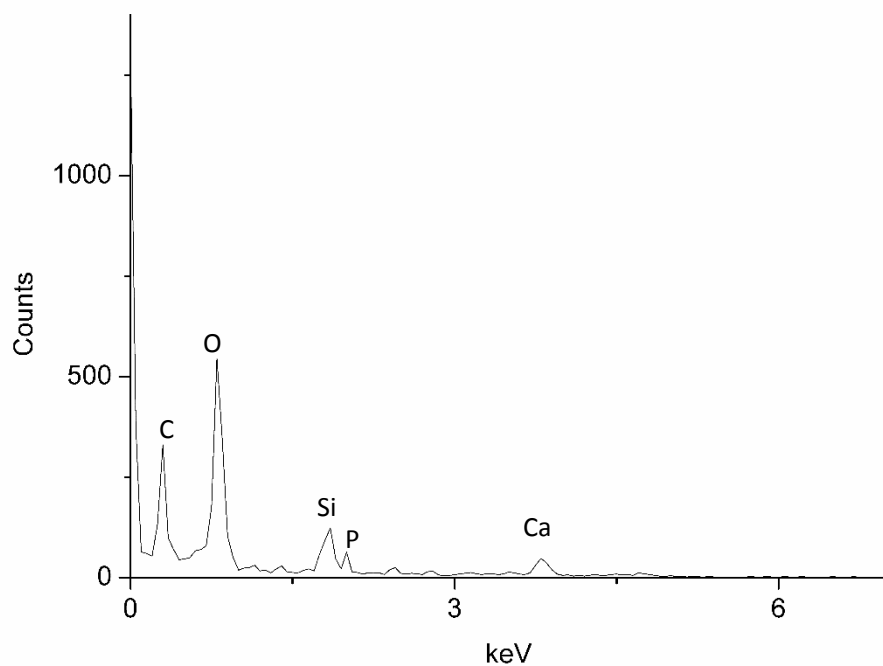


Figure 30. EDS analysis of mineral depositions from cells treated with 100 $\mu\text{g/ml}$ BMP-7-derived peptide KAISVLYFDDS (A), and cells treated with 50 ng/ml BMP-2 (B) after 14 days in culture.

Unlike C2C12 cells, MC3T3-E1 pre-osteoblasts can only differentiate into osteoblasts. Therefore whether MC3T3-E1 cells were treated with BMP-7-derived peptides or not

had no effect on the cell differentiation pathway. The results of cell morphology study indicated that 100 µg/ml BMP-7-derived peptides did not affect the morphology of MC3T3-E1 cells. The EDS results indicate 100 µg/ml BMP-7-derived peptides did not possess the same ability as BMP-2 to induce mineral deposit.

2.3.3.3 Cell differentiation

Figure 31 shows the differentiation of MC3T3-E1 cells cultured in treatment media containing 100 µg/ml of different BMP-7-derived peptides (KAISVLYFDDS, KAISVLYFDDSHHHHHH, and HHHHHHKAISVLYFDDS) within 21 days by measuring the ALP activity, which is considered an early marker of osteoblast differentiation. Cells treated with 50 ng/ml BMP-2 expressed significantly higher ALP activity compared with all other groups on day 7 and 10 ($p < 0.001$). On day 14, although BMP-2 groups still shown significantly higher values ($p < 0.001$ for peptide KAISVLYFDDS and HHHHHHKAISVLYFDDS groups, $p < 0.001$ for peptide KAISVLYFDDSHHHHHH and control group), the ALP activity of cells in the presence of BMP-7-derived peptides gradually increased. In particular, peptide KAISVLYFDDS and HHHHHHKAISVLYFDDS groups showed a rapid increase in the ALP activity. On day 21, the ALP activity of the BMP-2 groups only showed a significant increase compared to the control group ($p < 0.001$). The differences between other groups, however, were not significant due to large experimental variance. All of the values of BMP-7-derived peptide groups exceeded the control group after day 14. Among the three BMP-7-derived peptides, HHHHHHKAISVLYFDDS resulted in the highest ALP activity on day 14 and 21. However, there was no statistically significant difference between these peptides.

The cell differentiation experiment results showed that 100 µg/ml BMP-7-derived peptides in the culture media of MC3T3-E1 cells induced significantly lower amounts of ALP activity compared to BMP-2 within 14 days. On day 21, although the difference was not significant, a lower ALP activity in the presence of BMP-7-derived peptides was observed than BMP-2 groups. Therefore 100 µg/ml BMP-7-derived peptides did not possess the same ability as BMP-2 to induce osteoblast differentiation.

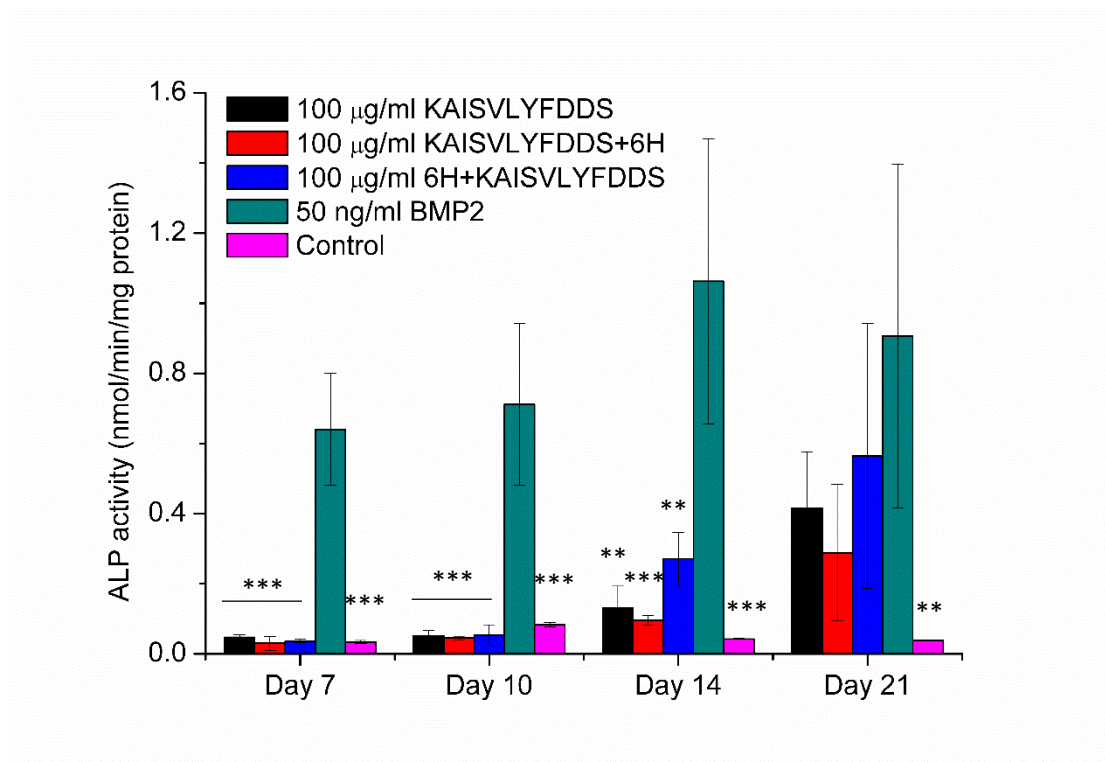


Figure 31. The ALP activity of MC3T3-E1 cell lysate treated with 100 µg/ml BMP-7-derived peptides and 50 ng/ml BMP-2 for 21 days was measured using pNPP as a substrate. ** $p < 0.01$ and *** $p < 0.001$ indicate statistically significant differences between the indicated group and BMP-2 group on the respective day.

According to Chen and Webster, 100 µg/ml peptide KAISVLYFDDS in culture media increased alkaline phosphatase activity in human fetal osteoblasts after 14 days compared to negative controls and BMP-7 [136]. Although different cells (human fetal osteoblast progenitor cell line vs. mouse pre-osteoblast cell line) and positive control (BMP-7 vs. BMP-2) were used here, comparable results were expected in this study. However, negative results were obtained from the ALP activity assay and peptide KAISVLYFDDS induced significantly less alkaline phosphatase activity in MC3T3-E1 after 14 days compared to BMP-2. One possible explanation is due to the different cell line. Although MC3T3-E1 is a popular pre-osteoblast cell line and has been widely used to determine the biological activity of BMPs [284, 285], it may fail to respond to the short peptides used in this study. Another possible reason is the concentration of peptides (100 µg/ml) was too low to induce cell differentiation in MC3T3-E1. Either of these reason indicates that BMP-7-derived peptides in this study have a restrictive application and do not warrant further study. While the ALP activity is believed to be

an early marker of osteoblast differentiation, the deposition of calcium is a late differentiation marker. Chen and Webster also reported peptide KAISVLYFDDS increased calcium deposited by osteoblasts after 7, 14, and 21 days compared to controls and BMP-7 [136]. However, no calcium assay to detect mineral deposition was performed in this study based on the negative EDS and ALP activity results.

2.4 Conclusion

In this study, BMP-7-derived peptide KAISVLYFDDS, and two His-tag peptides KAISVLYFDDSHHHHHH and HHHHHHKAISVLYFDDS were successfully synthesised and characterised. Their biological activities were determined by the mouse myoblast cell line C2C12. The results indicate BMP-7-derived peptides support the proliferation of C2C12 cells, but fail to convert the differentiation pathway of C2C12 from myoblast to osteoblast and to induce high ALP activity when compared to BMP-2. A further cell culture study to determine the effect of the BMP-derived peptides on a mouse pre-osteoblast cell line MC3T3-E1 was carried out. The results indicate BMP-7-derived peptides support the proliferation of MC3T3-E1 cells, but do not process the same ability as BMP-2 to induce osteoblast differentiation. In conclusion, these BMP-7-derived peptides show limited function and are not promising candidates for bone tissue engineering applications.

CHAPTER 3

Production and characterisation of engineered recombinant ALP with improved biological activity

3.1 Introduction

In this chapter, the production and purification of the wild-type and mutated recombinant *E. coli* ALP with improved biological activity are described. The motivation of this study was to develop a practical approach for the expression and purification of recombinant *E. coli* ALP for a future application in bone tissue engineering. As reviewed in Section 1.4.3.1, mammalian ALPs, especially TNAP, are essential components of the bone formation process that generate the inorganic phosphate ions needed for mineral deposition, and hydrolyse pyrophosphate, a mineralisation inhibitor [139, 143]. *E. coli* ALP, despite different features compared to mammalian ALPs such as protein size, optimal pH, and heat stability [135, 138], shares a similar catalytic mechanism of hydrolysing phosphate monoesters with mammalian ALPs [136]. In addition, *E. coli* ALP is durable and relatively easy to isolate [148, 149], which makes it a promising candidate for bone tissue engineering applications. This study demonstrates the production of mutated *E. coli* ALP with improved biological activity in the *E. coli* recombinant protein expression system.

3.1.1 Engineering recombinant alkaline phosphatase

After demonstrating the positive function of *E. coli* ALP in the bone formation process, numerous studies have focused on investigating its reaction mechanism [150-156, 159, 291]. Results suggest the catalytic activity of *E. coli* ALP can be improved by substituting

certain amino acid residues and developing mutations through site-directed mutagenesis [152, 155-157, 292]. High catalytic activity of *E. coli* ALP is desirable because it increases the dephosphorylation efficiency and may cover the activity loss caused by immobilisation [157]. In this research, a mutant *E. coli* ALP 4-186 was studied [157], which shows an improved catalytic activity of nearly 35 times higher than the wide-type. This mutation, which contains three amino acid residue replacements (D101S, K167R, S374C) (Table 8, Figure 7), was developed by Xu *et al.* through a directed evolution strategy, and expressed by an *E. coli* strain SM547 containing an ampicillin resistant plasmid pEK48/4-186 [180]. The SM547 strain has deleted the *phoA* gene from the chromosome, and contains a mutation in the *phoR* regulatory gene [175]. Therefore, a *phoA*-containing plasmid, such as pEK48, in this strain will overexpress ALP from the *phoA* gene on the plasmid even in a rich phosphate medium.

The plasmid pEK48 was originally developed by Chaidaroglou *et al.* and contains a complete wild-type *phoA* gene encoding the wide-type *E. coli* enzyme ALP [175]. The construction process of pEK48 has been divided into three steps, and is described below.

(1) Construction of pHI-1 (Figure 32) [293]. A plaque-forming specialised transducing phage containing the *phoA* gene $\lambda p(phoA-proC)$ [294] was first digested with restriction enzyme *Pst*I to produce a 6.4 kb fragment carrying the entire *phoA* gene. The fragment was then inserted into a cloning vector pBR322, which has a unique *Pst*I site, to form a new plasmid named pHI-1 (10.7 kb). Vector pBR322 is a classical widely used *E. coli* vector for DNA cloning. It provides the new plasmid pHI-1 ampicillin and tetracycline resistance markers, and some restriction cleavage sites. When the pHI-1 was digested with restriction enzymes, for example, with *Hind*III, four fragments were produced: a 2.35 kb fragment, a 0.05 kb fragment, and two 4.15 kb fragments. One of the 4.15 kb fragments contains the *phoA* gene.

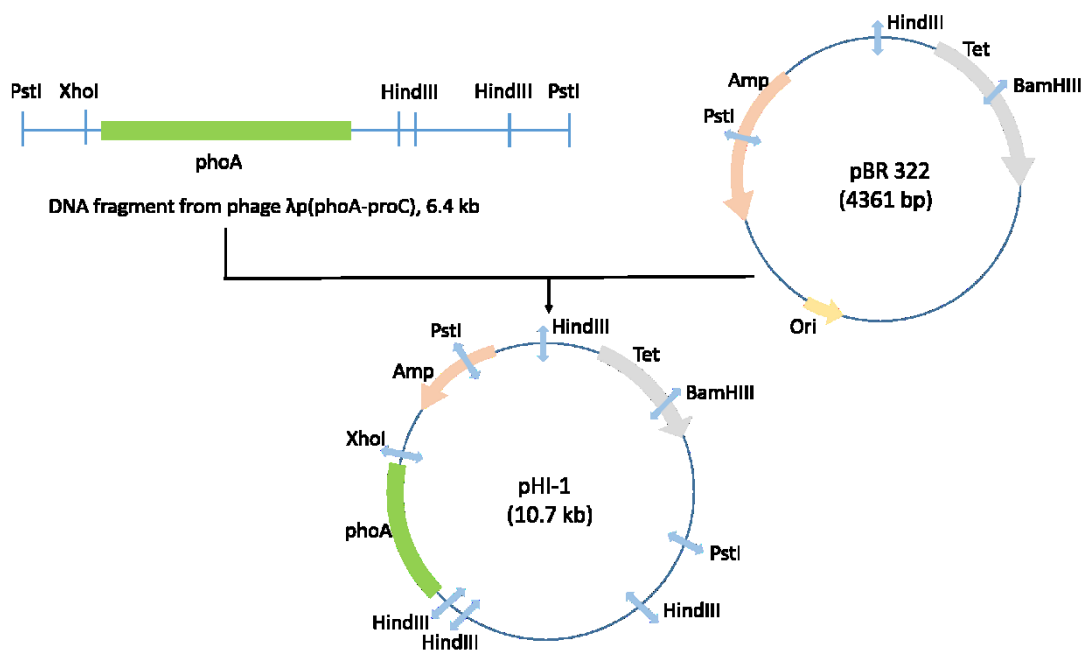


Figure 32. Construction process of plasmid pHI-1. After digestion by *Pst*I, a 6.4 kb DNA fragment that contains the entire *phoA* gene (shown in green) was isolated from phage λ p(*phoA-proC*) DNA. When the phage DNA fragment was mixed with *Pst*I treated pBR322, a new recombinant plasmid pHI-1 was ligated. An ampicillin resistance marker is shown in pink, and a tetracycline resistance marker is shown in grey. Blue arrows represent restriction cleavage sites.

(2) Construction of pEK29 (Figure 33) [175]. When the plasmid pHI-1 was double digested with *Hind*III and *Xho*I, five fragments were produced. Among them, a 2.5 kb fragment that contains the entire *phoA* gene was isolated and combined with a plasmid pGC1 pretreated with *Hind*III and *Xho*I to form a new plasmid named pEK29 (5.2 kb). pGC1 is a GC-clamp vector that is composed of 1900 bp from the pBR322 plasmid including an ampicillin resistance marker and a *colE1* origin, 671 bp from the bacteriophage M13 origin of replication, a GC-clamp, and a list of restriction cleavage sites [295]. The GC-clamp is a GC-rich DNA sequence usually next to target gene designed for an easy detection of single-base mutations in the melting domain. The bacteriophage M13 origin of replication, which is preserved in plasmid pEK29, allows the production and isolation of single-stranded DNA in a large quantity.

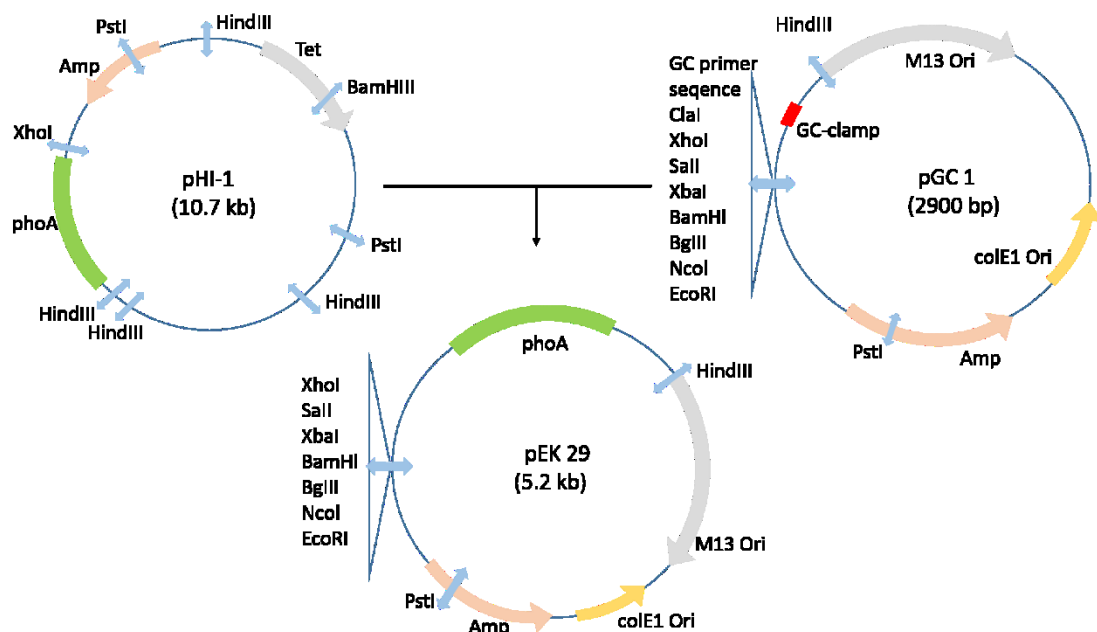
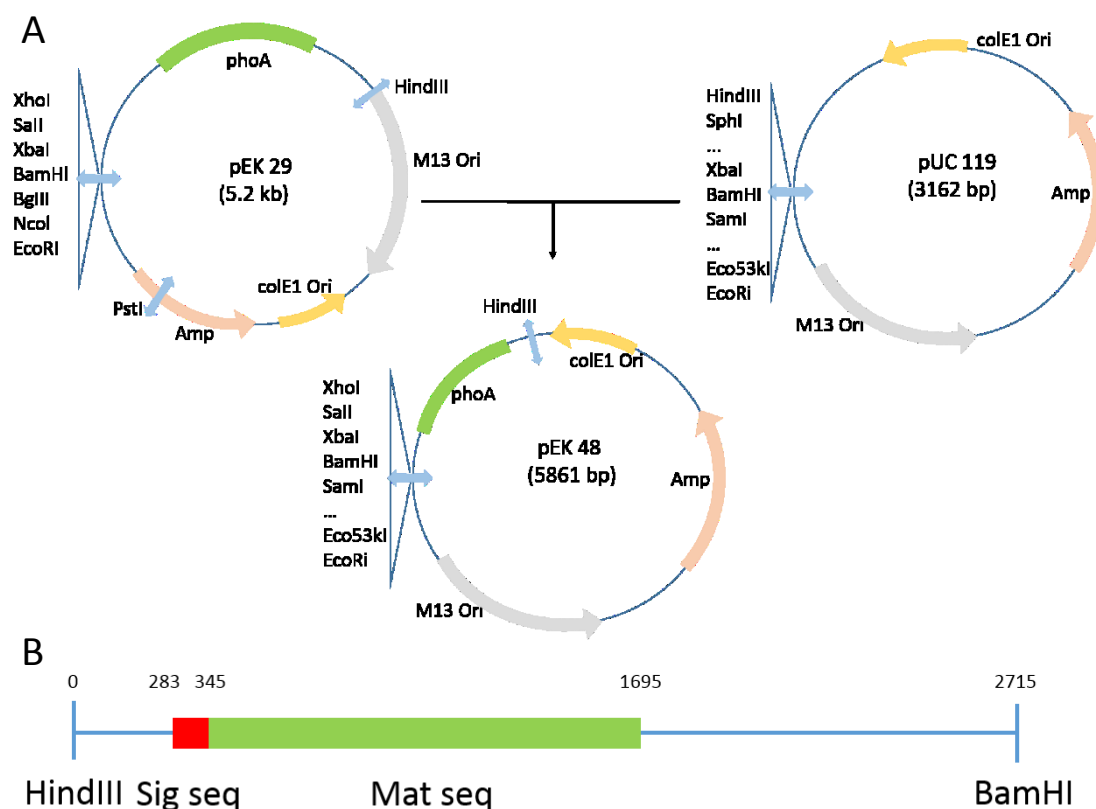


Figure 33. Construction process of plasmid pEK29. After double digestion by *Hind*III and *Xho*I, a 2.5 kb DNA fragment that contains the entire *phoA* gene (shown in green) was isolated from the plasmid pHI-1. The DNA fragment was mixed with the same restriction enzymes as pGC1, and a new recombinant plasmid pEK29 was ligated. An ampicillin resistance marker is shown in pink, a *colE1* origin is shown in orange, a bacteriophage M13 origin is shown in grey, and a GC-clamp is shown in red. Blue arrows represent restriction cleavage sites.

(3) Construction of pEK48 (Figure 34) [175]. The *Hind*III-*Bam*HI fragment of pEK29 which contains the entire *phoA* gene was recloned into a pUC119, which had been double digested with the same two enzymes to form a new plasmid pEK48 (5861 bp). pUC119 is a popular cloning vector. It provides the new plasmid pEK48 an ampicillin resistance marker, a *colE1* origin, a M13 origin of replication, and a multiple cloning sites. The *phoA* gene inserted in one of the multiple cloning site can be expressed under control of the *lac* promoter.



E. coli alkaline phosphatase (*phoA*) gene, complete cds.

Figure 34. Construction process of plasmid pEK48 and the complete coding sequence (cds.) of the *E. coli* alkaline phosphatase gene. (A) Plasmid pEK48 and pUC119 DNA were double digested with *Hind*III and *Bam*HI, mixed, and ligated to form a new recombinant plasmid pEK48 containing the entire *E. coli* ALP *phoA* gene (2715 bp) between the *Hind*III and *Bam*HI restriction sites shown in the green. An ampicillin resistance marker is shown in pink, a *colE1* origin is shown in orange, and a bacteriophage M13 origin is shown in grey. Blue arrows represent restriction cleavage sites. (B) The *phoA* gene contains a signal sequence (nucleotide residues 283-345, 62 bp, shown in red), a mature sequence (nucleotide residues 346-1695, 1.35 kb, shown in green) and linking sequences.

In this study, plasmid pEK48/4-186, which contains a mutant *phoA* gene encoding the 4-186 mutant ALP, as well as the wild-type ALP plasmid pEK48/*phoA*, were transformed into different host stains, such as DH5 α and BL21 to produce and purify wild-type and mutant recombinant *E. coli* ALP with improved biological activity.

3.1.2 Scope of the chapter

This chapter focuses on the production and purification of wild-type and mutant

recombinant *E. coli* ALP with improved biological activity. Described herein are the steps taken to design and produce recombinant mutant *E. coli* ALP showing higher biological activity than the wild-type *E. coli* ALP. Some effects including sodium chloride (NaCl) and lyophilisation on recombinant ALPs are investigated. Different host cells such as BL21 and DH5 α were considered to improve the production of *E. coli* ALP.

3.1.3 Hypotheses

The hypotheses tested was that recombinant mutant *E. coli* ALP have outstanding biological activity compared to wild-type ALP, and may be used in bone tissue engineering. In order to test these hypotheses, the following aims have been addressed in this Chapter:

- (1) Verify the recombinant mutant *E. coli* ALP gene, in particular the mutations.
- (2) Confirm the expression of recombinant mutant and wild-type *E. coli* ALP.
- (3) Purify the recombinant mutant and wild-type *E. coli* ALP from bacterial culture media.
- (4) Determine the specific activity of recombinant mutant and wild-type *E. coli* ALP.
- (5) Investigate the effect of sodium chloride (NaCl) and lyophilisation on recombinant ALPs.
- (6) Investigate the strategies to improve the expression of recombinant mutant and wild-type *E. coli* ALP.

3.2 Materials and methods

3.2.1 Materials

Unless stated otherwise, all chemicals, restriction enzymes, and bacterial cell culture reagents were purchased from Sigma-Aldrich (Gillingham, UK). QIAprep plasmid purification kit was purchased from Qiagen. SYBR Safe DNA Gel Stain was purchased from Invitrogen. GeneRuler 1kb DNA Ladder was purchased from Fermentas. Page

Ruler Prestained Protein Ladder was purchased from Thermo Scientific. The NativeMark Unstained Protein Standard was purchased from Invitrogen. DC Protein Assay kit was purchased from Bio-Rad. Anti-alkaline phosphatase antibody (ab354) was purchased from Abcam, UK. ECL anti-rabbit IgG horseradish peroxidase-linked whole antibody (from donkey) was purchased from GC Healthcare.

Bacterial strains SM547 ($\Delta(phoA-proC)$, *phoR*, *tsx::Tn5*, Δlac , *gal K*, *leu*, *Str^r*), DH5 α (*F⁻*, $\phi 80dlacZ\Delta M15$, $\Delta(lacZYA-argF)$, U169, *deoR*, *recA1*, *endA1*, *hsdR17*(*rk⁻*, *mk⁺*), *phoA*, *supE44*, λ^- , *thi⁻¹*, *gyrA96*, *relA1*), and BL21 (*F⁻*, *dcm*, *ompT*, *hsdS*(*r_B⁻* *m_B⁻*), *gal* [*malB⁺*]_{K-12}(λ^S) were used as the host for gene expression. All strains and plasmids pEK48/*phoA* and pEK48/4-186 were kindly provided by Professor A. E. G. Cass (Imperial College London).

3.2.2 *E. coli* bacterial culture

A vial of frozen bacterial cells was thawed and plated on Luria-Bertani (LB) medium plates (see Appendices) containing 100 $\mu\text{g/ml}$ ampicillin and 40 $\mu\text{g/ml}$ 5-bromo-4-chloro-3-indolyl phosphate (BICP), which can react with ALPs and generate a blue product. Blue colonies developed on the plate indicating the expression of ALP. Single blue colonies on the plates carrying the wild-type or mutant *phoA* gene were picked and suspended in 4 ml of LB media containing 100 $\mu\text{g/ml}$ ampicillin. The cells were cultured in an incubator at 37°C, 200 rpm overnight. 1 ml of overnight culture medium was aseptically transferred into 100 ml of LB broth to incubate at 37°C with continuous shaking until the stationary phase of the culture was reached.

The cells were harvested by centrifugation at 6500 rpm for 15 min. The cell pellet was washed twice with cold 0.01 M Tris-0.03 M NaCl washing buffer and the periplasmic protein *E. coli* ALP was released by a modified osmotic shock method according to Brockman and Heppel [296]. Briefly, the cell pellet was suspended in 2 ml 0.03 M Tris suspension buffer containing 20% (w/v) sucrose solution and 1mM EDTA. After 10 min incubation at ambient temperature, the mixture was centrifuged at 10000 rpm for 20

minutes at 0°C. Next, the supernatant was removed and the cells were resuspended in 10 ml ice cold water for 15 min allowing the *E. coli* ALP to be released into the supernatant. The supernatant was collected by centrifugation at 10000 rpm for 10 min. The precipitation of the proteins from the cold water was done by gradually adding ammonium sulphate to the supernatant to a final concentration of 608 mg/ml (85% saturation). The pellet was collected by centrifugation at 10000 rpm for 20 min at 0°C, and resuspended in 2 ml TMZP buffer [297]. Dialysis was carried out to remove the ammonium sulphate in the suspension. The dialysed solution containing *E. coli* ALP was filtered through a 0.45 µm filter before purification on a GE Healthcare ÄKTA explorer using Q-Sepharose Fast Flow anion exchange chromatography column (Pharmacia). Finally, proteins bound to the column were eluted with a linear gradient of 0-0.2 M NaCl in TMZP buffer. Pure *E. coli* ALP fractions were collected by detecting the absorbance at 280 nm, identified by electrophoresis, and characterised by biochemical assays. The proteins were lyophilised and stored at -80°C.

3.2.3 Plasmid DNA Purification

Plasmid DNA in bacterial cells was extracted using a QIAprep Spin Miniprep Kit [298] following the recommended protocol. The procedure is based on the lysis of bacterial cells followed by adsorption of DNA on a unique silica membrane in the presence of high salt [298]. Bacterial cells from a single colony were cultured in 4 ml LB medium in an incubator at 37°C, 200 rpm for 12 hours. The cell pellet was collected by centrifugation at 6500 rpm for 10 min at 4°C and resuspended in the resuspension buffer by vortexing. Then the lysis buffer was added to the cells and gently mixed to achieve a homogeneously blue suspension. Next, the neutralisation buffer was added into the mixture and immediately vortexed for 1 min until the suspension became colourless, which indicates the SDS in the lysis buffer has been completely precipitated. The samples were centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was transferred to a QIAprep spin column, and centrifuged at 12000 rpm for 1 min at 4°C allowing the binding of plasmid DNA to the silica membrane of the column. The column was washed twice with washing buffers to improve the purity of the plasmid

DNA. Finally, the plasmid DNA was eluted in to a clean Eppendorf in 25-50 µl of water depending on the quantity. The concentration of the plasmid DNA was determined by a NanoDrop 2000c (Thermo Scientific, USA). Plasmid DNA isolated from bacterial cells can be kept at -20°C up to six months.

3.2.4 Gel electrophoresis

Gel electrophoresis techniques are widely used to separate and purify charged molecules such as nucleic acids and proteins with different size, charge, and conformation [299]. The separation is based on the different molecular motilities under an electric field. DNA separation is usually performed in an agarose gel, while proteins are more often separated by polyacrylamide gel electrophoresis (PAGE) with or without sodium dodecyl sulphate (SDS).

A) Agarose gel electrophoresis

Agarose is a natural polymer extracted from seaweed. It can be dissolved in hot water, but when cooled down, the polymers crosslink with each other and form networks of pores. The pore size is dependent on the percentage of agarose. The DNA in the gel is forced to move from the negative electrode to the positive electrode during electrophoresis. Under the same strength of the electrical field, and the percentage of agarose gel, the only factor that can affect the moving speed of DNA is its size. Smaller DNA molecules move faster in the gel than larger DNA molecules because they more easily go through the pores. Although DNA is not directly visible in the gel, the position of DNA molecules can be visualised by the use of a dye under UV radiation.

In this study, 1% (w/v) agarose gel was made with a TBE running buffer containing SYBR Safe DNA gel stain. Samples were mixed with 6X sample loading buffer before loading on the gel. The agarose gel electrophoresis was performed in a gel box (Bio-Rad, USA) at 100-150 V for 1.5 to 2 hours. The gels were visualised and imaged using a BioSpectrum 500 fluorescence reader from UVP (CA, USA).

B) Polyacrylamide gel electrophoresis

A crosslinked polymer network can be formed by mixing the acrylamide with bisacrylamide as well as a polymerising agent, ammonium persulphate (APS), and a catalyst, TEMED (N,N,N,N'-tetramethylethylenediamine). The polymerisation process is inhibited by oxygen. The pore size in the polymer network can be controlled by the concentration of acrylamide and bisacrylamide. The original PAGE was developed by Ornstein and Davis in a discontinuous gel system [300]. The samples were first loaded on a short stacking gel which has a larger pore size to concentrate the samples into a tight band before entering the following running gel [301]. The separation of proteins in native PAGE is based on the size and charge of proteins. In order to simplify the separation process and increase the number of applications, a modified electrophoresis method SDS-PAGE containing a reducing agent, sodium dodecyl sulphate (SDS), was used. SDS covers the protein with a uniformly negative charge and denatures the proteins allowing the separation of proteins based on the size of proteins only. Once the PAGE is finished, protein bands can be visualised directly in the gel by staining, or by transferring to a membrane for analysis by Western blotting.

In this study, proteins produced by bacterial cells were analysed using SDS-PAGE on an 8% (w/v) polyacrylamide gel. Gel buffers used in electrophoresis are described in the Appendices. Samples were denatured by heating at 95°C for 10 min in the presence of a sample buffer containing a reducing agent dithiothreitol (DTT) before loading on the gel. The polyacrylamide gel electrophoresis was performed in a gel box (Bio-Rad, USA) at 100 V for 1 to 2 hours. The gels were stained by a ProteoSilver™ silver stain kit (Sigma-Aldrich, UK), and visualised and imaged on a BioSpectrum 500 fluorescence reader from UVP (CA, USA).

3.2.5 Western blotting

Western blotting, also named protein blotting or protein immunoblot, is a widely used technique to detect and analyse specific proteins. The method was first described by Towbin *et al* in 1979 [302]. It contains four steps [303, 304]. First, gel electrophoresis.

Proteins are separated and immobilised by gel electrophoresis as described in Section 3.2.4. Second, transfer. The gel-resolved proteins are electrophoretically transferred to a membrane, usually made of nitrocellulose or polyvinylidene fluoride (PVDF), so that the proteins on the membrane keep the same pattern of separation as they had on the gel. Third, antibody probing. The membrane is first blocked by incubating with a generic protein, and then probed by adding a primary antibody. The primary antibody is specific but non-labelled. In order to detect and visualise the target proteins, a labelled secondary antibody is used. Finally, detection. The location of the antibody is revealed by using chemiluminescence, chemifluorescence, fluorescence, chromogenic or radioisotopic detections [305].

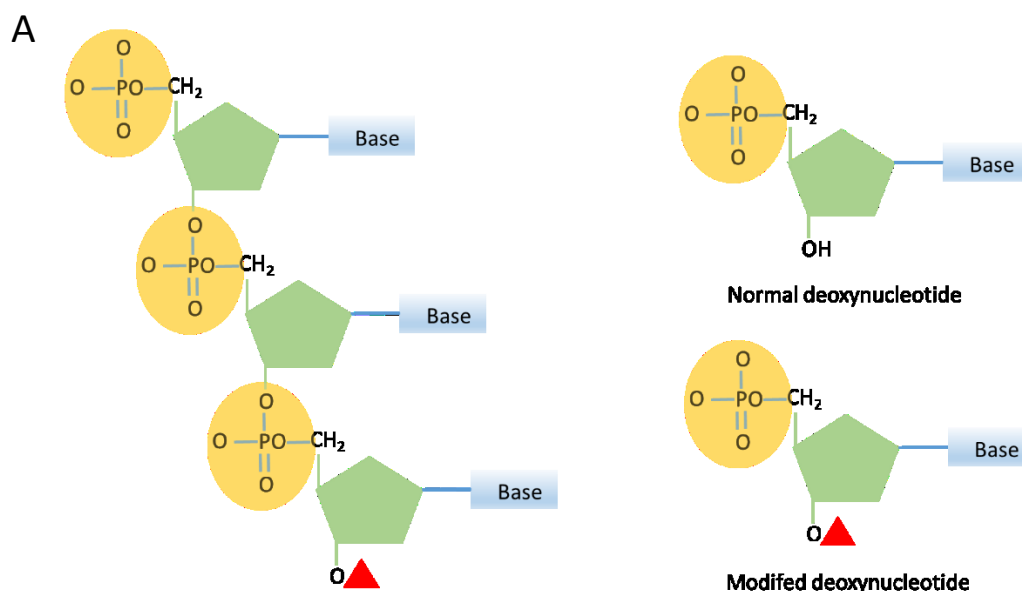
In this study, proteins produced by bacterial cells were first separated using SDS-PAGE gel electrophoresis on an 8% (w/v) polyacrylamide gel. The method is described in Section 3.2.4. Once the electrophoresis is finished, the gel is transferred to PVDF membranes at 100 V for another 1 hour. The membranes with protein bands are first incubated in 5% milky blocking solution with shaking at ambient temperature for 1 hour, and then incubated in primary antibody solution (1/1000) with shaking at 4°C overnight. Next, the membranes are soaked in secondary antibody solution (1/20000), and incubated with shaking at ambient temperature for 1 hour. Finally, the membranes are washed, visualised and imaged on a BioSpectrum 500 fluorescence reader from UVP (CA, USA).

3.2.6 DNA sequencing

DNA sequencing is an essential molecular biology technique to determine the precise order of nucleotides in a DNA molecule. There are two different methods to sequence DNA, one is a chain termination method developed by Sanger and Coulson; the other is a chemical degradation method developed by Maxam and Gilbert. The chemical degradation method is not frequently used because it depends on the extensive use of toxic chemicals to cleave nucleotides. The chain termination method, also called Sanger sequencing method, is an easy and simple way for DNA sequencing. It depends

on the enzymatic synthesis of DNA chains in the presence of chain-terminating inhibitors [306].

DNA sequencing reactions in the chain termination method are similar to the PCR reactions for replicating DNA described in Section 1.5.3, Figure 15. The sample DNA is mixed with polymerase, free deoxynucleotides, and primers. The new DNA chains are generated according to the template sample DNA. However, differing from the PCR, some modified deoxynucleotides (Figure 35A), which lack hydroxyl group at their 3' position to bond to the next deoxynucleotide, are used in addition to normal deoxynucleotides [307]. Once a modified deoxynucleotide is added on the chain, the chain is terminated. After enough time of reactions, all of the copies are terminated by a modified deoxynucleotide, and the copies are stopped at each possible deoxynucleotide to produce a series of DNA chains (Figure 35B). The separation of the products is carried out by gel electrophoresis to determine the length of each strand. The modified deoxynucleotides are either radioactively labelled or fluorescent labelled so the DNA sequence can be determined.



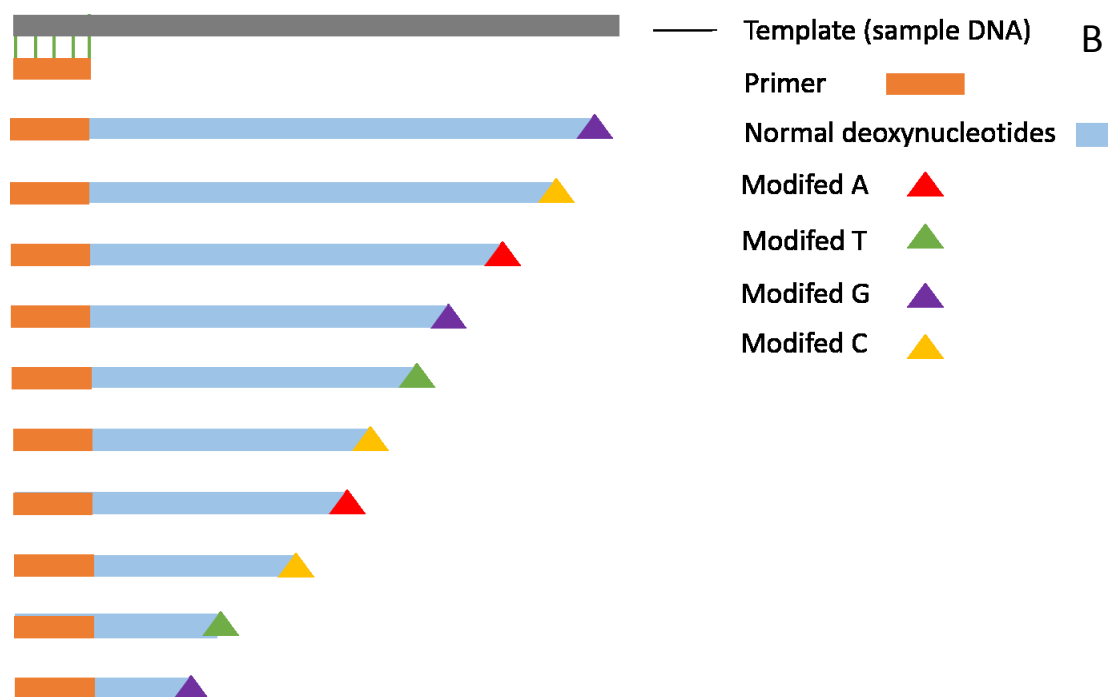


Figure 35. General principle of DNA sequencing by the chain termination method [307]. (A), Modified deoxynucleotide lacks the hydroxyl group at their 3' position so it cannot bind to the other deoxynucleotide to extend the DNA chain. (B) All of the copies produced by the chain termination method are terminated by a modified deoxynucleotide. These modified deoxynucleotides are labelled so the DNA sequence can be determined by a colour match.

In this study, all DNA sequencing were performed by GATC Biotech Ltd. using the chain termination method.

3.2.7 Preparation of competent cells and transformation

Competent cells have the ability to accept foreign DNA or plasmids. One treatment to prepare competent cells is by electroporation, which provides high transformation efficiency [308]. However, special equipment is required in this process. Another method is a chemical method that relies on the treatment of calcium chloride (CaCl_2) to change the permeability of the cell membranes. Although the transformation efficiency of the competent cells produced by the chemical method is relatively low, it is easy to perform so has been widely used in laboratory scale.

Before the CaCl_2 treatment, bacterial cells from a single colony were cultured in 4 ml

LB medium in an incubator at 37°C, 200 rpm overnight. The overnight culture medium was diluted 100 fold into a fresh LB medium (see Appendices) and cultured at 37°C, 200 rpm until the absorbance at 600 nm reached 0.5. The culture was centrifuged at 6500 rpm for 10 min at 4°C, and the cell pellet was collected and incubated on ice for 10 min. The cells were resuspended in 40 ml treatment buffer 1 which contains CaCl₂ at 4°C with gentle mixing for 1 hour. The cell pellet was collected and resuspended again with 20 ml treatment buffer 2 followed by incubation on ice for 15 min. The competent ability of these cells can be maintained for six months at -80°C.

An aliquot of competent cells was gently mixed with the plasmid DNA, and incubated on ice for 25 min. Then the mixture was heat shocked by incubating at 42°C for 50 seconds followed by incubation on ice for 2 min. The bacterial cells were cultured in 1 ml SOC medium in an incubator at 37°C, 200 rpm for 1 hour. The cell pellet was collected and plated on LB plates containing selection antibiotic to screen for the successfully transfected cells.

3.2.8 ÄKTA explorer purification system

ÄKTA explorer purification system is a fully automated liquid chromatography system using a fast protein liquid chromatography (FPLC) technique designed for protein purification [309]. Compare to HPLC, the FPLC system has a lower pressure but a faster flow rate.

The ÄKTA explorer purification system includes a pump, a UV detector, a pH and conductivity detector, an injector, a mixer, a fraction collector, set of valves, and a monitor. In this study, a Q-Sepharose Fast Flow anion exchange chromatography column was used on ÄKTA explorer to purify wild-type and mutant *E. coli* ALP. The column and system were equilibrated with 5 volumes of TMZP buffer. The proteins were eluted by a linear gradient method with 20 volumes of 0-0.2 M NaCl in TMZP buffer. A flow rate of 1 ml/min was used and fractions were collected in Eppendorf tubes.

3.2.9 Dialysis

Dialysis is a simple separation technique to remove unwanted small molecules from larger molecules in solution by diffusion through a semi-permeable membrane. Figure 36 illustrates the principle of dialysis. Dialysis has been widely used in medicine as an artificial replacement for lost kidney function to remove waste and excess water from the blood. It can also be used in protein purification process to adjust protein from one buffer to another, to change the metal and salt ion concentrations, and to remove unwanted small molecules in protein solution [310].

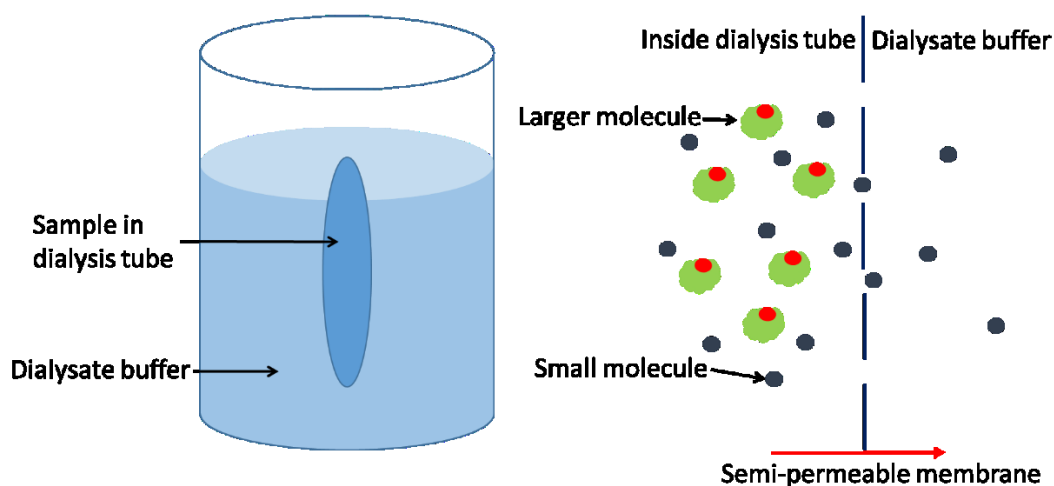


Figure 36. The general principle of dialysis [311]. By using a semi-permeable membrane, which allows small molecules pass through freely while larger molecules cannot, the concentration of those small molecules within the dialysis tube can be effectively reduced. Changing the dialysate buffer regularly allows more small molecules to diffuse into the dialysate buffer.

In this study, dialysis through porous cellulose tubing against TMZP buffer (see Appendix) was used after the salt precipitation to remove the ammonium sulfate salts from the protein. Dialysis was performed at 4°C temperature for 12 h on a stirring plate. Three buffer changes of 10 volumes were performed every 4 h.

3.2.10 Biochemical assays

A) DC Protein assay

The concentration of *E. coli* ALP produced by bacterial cells was determined by a commercial DC Protein Assay Kit (BIO-RAD). The assay is based on the well-known Lowry protein assay where proteins react with an alkaline copper tartrate solution and Folin reagent to produce a blue colour with a strong absorbance between 650 and 750 nm [312]. The DC protein assay has two procedures, one is for standard samples having a protein concentration between 0.1 to 2 mg/ml, and the other is a microassay procedure that can measure protein concentrations from 5 to 250 µg/ml by using only 20 µl of samples for a microplate assay. 20 µl protein samples after purification were mixed with the assay reagent in a 96-well plate and incubated for 15 min at ambient temperature. The light absorbance of these samples was measured at 750 nm on a 96-well plate reader (Labsystems, Life Sciences International Ltd, Hampshire, UK). Experiments were performed independently in triplicate.

B) ALP specific activity measurement of recombinant *E. coli* ALP

Similar to the ALP activity assay for cells, ALP specific activity measurement of *E. coli* ALP depends on the hydrolysis of pNPP by the *E. coli* ALP and generates inorganic phosphate and p-Nitrophenol, which is converted to a yellow product under alkaline conditions and shows a strong absorbance at 400-420 nm. 10 µl of the protein samples after purification was mixed with pNPP (Sigma, UK) in a 96-well plate and incubated at 37°C until a yellow colour was detected by eye, the reaction was then stopped by adding 100 µl 1 M NaOH. Light absorbance of these samples was measured at 405 nm on a 96-well plate reader. A standard curve was constructed using p-Nitrophenol (Sigma, UK) as a standard. Experiments were performed independently in triplicate, and the ALP specific activity of each sample was normalised to protein content and expressed as mean plus/minus standard deviation.

3.3 Results and discussion

3.3.1 Confirmation of recombinant *E. coli* ALP gene

The bacterial strain SM547 containing plasmids pEK48/phoA and pEK48/4-186, which encoding the wild-type and 4-186 mutant *E. coli* ALP, respectively, were cultured in LB media. Both plasmids DNAs were isolated for agarose gel electrophoresis and DNA sequencing to confirm the accuracy of the cloning. Figure 37 shows the agarose gel electrophoresis results of plasmids pEK48/phoA and pEK48/4-186. The difference between the plasmids is 3 amino acid substitutions, therefore they have the same theoretical size of 5861 bp. A clear single band of 5.8 kb was shown when the plasmid pEK48/phoA was digested with the restriction enzyme *Bam*HI (Lane 5); two sharp bands (above 3.1 kb, below 2.7 kb, which contain the *E. coli* ALP gene) were shown when the plasmids were double digested with *Bam*HI and *Hind*III (Lane 2 and 4); several bands were shown when the undigested plasmids were loaded on the gel (Lane 3 and 6) due to their supercoiled structure. The results indicate the real sizes of the plasmids are consistent with the description, and demonstrate that the digestions of the plasmids by restrictive enzymes were correct and efficient.

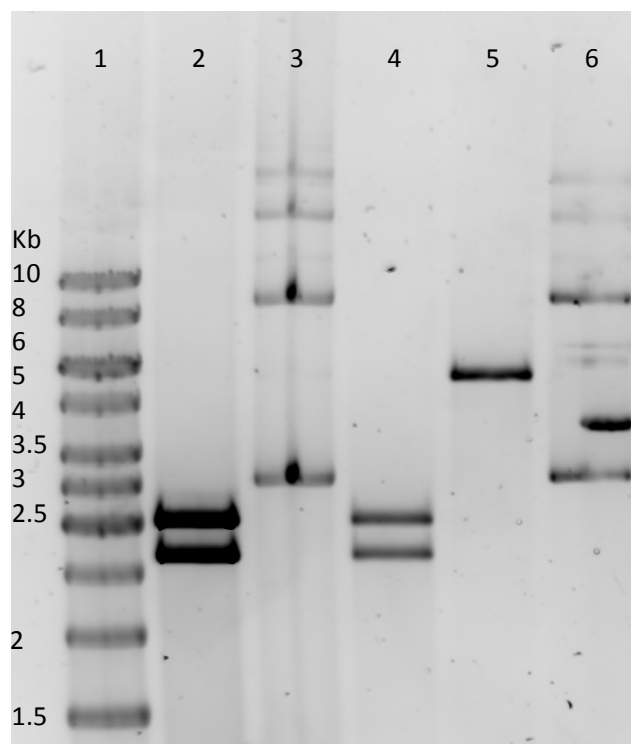


Figure 37. Agarose (1% w/v) gel electrophoresis of restriction enzymes digested plasmids pEK48/4-186 and pEK48/phoA. Lane 1 is a 1 kb DNA ladder; lane 2, the plasmid pEK48/4-186 double digested with *Bam*HI and *Hind*III; lane 3, undigested plasmid pEK48/4-186; lane 4, the plasmid pEK48/phoA double digested with *Bam*HI and *Hind*III; lane 5, the plasmid pEK48/phoA single digested with *Bam*HI; lane 6, undigested plasmid pEK48/phoA.

In order to further confirm the accuracy of the cloning, especially the plasmid pEK48/4-186 with the 3 amino acid mutations, several rounds of DNA sequencing were performed by GATC Biotech Ltd. (Figure 38). After comparing the DNA sequencing results of the 4-186 gene from the plasmid pEK48/4-186 with the current entries in GenBank/NCBI nucleotide data bases, a single data base entry of *E. coli* alkaline phosphatase (*phoA*) gene, complete coding sequence (GenBank: M13345.1), was identified with significant similarity. The aligned sequences show 99% identity over the region of the genes. Six DNA substitutions of the 4-186 were shown exactly as described by Xu et al [180]. One DNA sequencing reaction has an accurate reading limitation of 700 to 1000 bp. Totally, four DNA sequencing reactions were carried out for a complete reading of the entire *E. coli* ALP *phoA* gene of 2715 bp.

```

>|cl|41423 Length=1700 Score = 2036 bits (1102), Expect = 0.0 Identities =
1118/1128 (99%), Gaps = 2/1128 (0%) Strand=Plus/Plus

4-186 1      CAGCGGTTGATTGATCAGGTAGAGGGGGCGCTGTACGAGGTAAAGCCCGATGCCAGCATT 60
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
phoA  57      CAGCGGTTGATTGATCAGGTAGAGGGGGCGCTGTACGAGGTAAAGCCCGATGCCAGCATT 116

...

4-186 541    ATACACTCACTATGCGCTGAATaaaaaaaCCGGCAAACCGGACTACGTCACC-AGTTCGG 599
      |||||||||||||||||||||||||||||||||||||||||||||||||||||
phoA  597    ATACACTCACTATGCGCTGAATAAAAAAACCGGCAAACCGGACTACGTCACC-CACT-CGG 655
                                     649-651

...

4-186 780    TGACCTCGCGCAAGATGCTACGGTCCGAGCGCGACCAGTGAAAAATGTCCGGGTAACGCTC 839
      |||||||||||
phoA  836    TGACCTCGCGCAAGATGCTACGGTCCGAGCGCGACCAGTGAAAAATGTCCGGGTAACGCTC 895
      848

...

4-186 960    AAACGCTGCGTGAACAGGCACAGGCGCGTGGTTATCAGTTGGTGAGCGATGCTGCCTCAC 101
      |||||||||||||||||||||||||||||||||||||||||||||||||||||
phoA  1016   AAACGCTGCGTGAACAGGCACAGGCGCGTGGTTATCAGTTGGTGAGCGATGCTGCCTCAC 1075

...

4-186 1080   ATATGCCAGTGNNCTGGCTAGGACCGAAAGCAACGTACCATGGCAATA 1127
      |||||||||||
phoA  1136   ATATGCCAGTGCGCTGGCTAGGACCGAAAGCAACGTACCATGGCAATA 1183
      1170

```

Figure 38. DNA sequence comparison of the wild-type *E. coli* ALP *phoA* (complete coding sequence, GenBank: M13345.1, lower line) and the mutation 4-186 gene from pEK48/4-184 plasmid (above line). Identical residue matches are indicated by connecting solid lines. DNA mutations are shown in red in blue boxes. The percent similarity value is based on the number of identical and two-point DNA comparisons. N appears at the end of the sequencing representing unidentified nucleotides. Some middle parts of the gene were omitted due to the space limitation. This sequence alignment in one reaction only shows five out of six DNA substitutions (g649a, a650g, c651t, a848g, and g1170t) described by Xu. This comparison was prepared by the BLAST sequence analysis program.

3.3.2 Verifying the recombinant *E. coli* ALP expression

After confirming the DNA sequence of wild-type and mutant alkaline phosphatase gene in the plasmids pEK48/*phoA* and pEK48/4-186, respectively, the proteins were produced from *E. coli* SM547. Figure 39 shows the LB Amp BCIP selective plate with blue colonies of SM547 pEK48/4-186 after culturing for 24 hours in an incubator at 37°C. White colonies were found on the plate after 8 hours of culture, after which they gradually turned to blue. According to Chaidaroglou, bacterial cells after

transformation were first screened by using a selective LB plate containing not only ampicillin antibiotics, but also BCIP, which is a substrate for ALPs [175]. BCIP reacts with *E. coli* ALP expressed from the bacterial cells and forms a blue product, thus turning the colony blue colour. Therefore the colonies grown on the LB Amp BCLP selective plate are cells containing the pEK48 plasmids, while blue colonies developed on the plate indicate the expression of active *E. coli* ALP. The difference between the plates cultured with SM547 pEK48/phoA and SM547 pEK48/4-186 was insignificant.

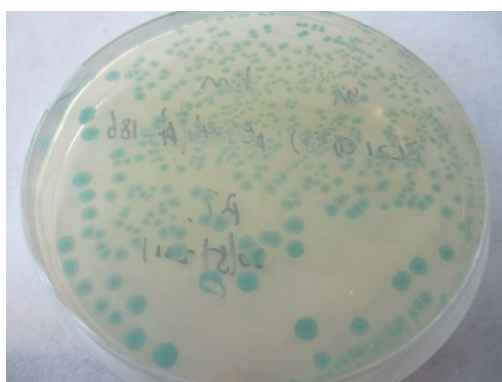


Figure 39. Image of LB Amp BCIP selective plate grown with SM547 pEK48/4-186 after 24 hours incubation at 37°C. All colonies grown on the selective plate contain the pEK48/4-186 plasmids. The blue colour indicates that they are expressing the active form of ALP.

Cell lysates of SM547 pEK48/phoA and SM547 pEK48/4-186 after overnight culture were analysed by SDS-PAGE and western blotting (Figure 40). Several bands were shown on the SDS gel, most of which were under 35 kDa. The *E. coli* ALP showed a weak band at 53 kDa. The result indicates that *E. coli* ALP is not the main protein produced in SM547. Small molecular weight proteins (molecule weight under 35 kDa) were extensively produced by the bacterial cells. However, when the SDS gel transferred to a nitrocellulose membrane and reacted with anti-alkaline phosphatase antibody, one main band of *E. coli* ALP was evident. The western blotting result confirmed the expression of *E. coli* ALP from SM547. Although the same *E. coli* protein expression system was used to produce wild-type and mutant *E. coli* ALP, mutant ALP shown a darker and thicker band than wild-type ALP, which indicated a higher production rate of mutant ALP from SM547 pEK48/4-186. No related data of comparison of protein yield between SM547 pEK48/phoA and SM547 pEK48/4-186 was provided from previous studies.

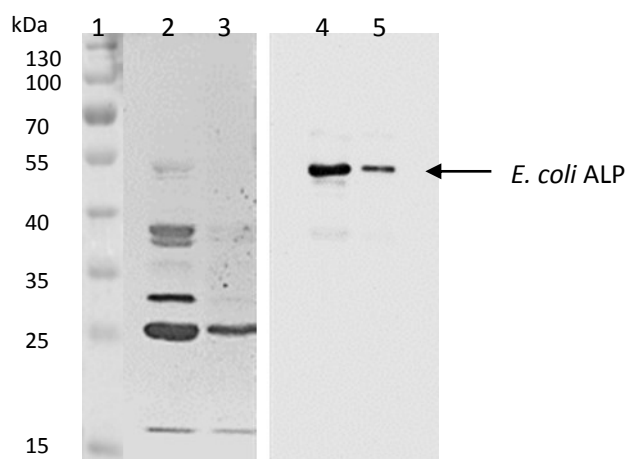


Figure 40. SDS-PAGE (8% w/v) gel electrophoresis (lane 2 and 3) and western blotting (lane 4 and 5) of cell lysates of SM547 pEK48/4-186 and SM547 pEK48/phoA after overnight cell culture at 37°C. Samples were electrophoresed in an SDS gel, then transferred to a nitrocellulose membrane and reacted with anti-alkaline phosphatase antibody. Lane 1, ladder; lane 2, cell lysates of SM547 pEK48/4-186 on gel; lane 3, cell lysates of SM547 pEK48/phoA on gel; lane 4, western blotting analysis of cell lysates of SM547 pEK48/4-186; lane 5, western blotting analysis of cell lysates of SM547 pEK48/phoA. SDS-PAGE gel was stained with Coomassie blue.

3.3.3 ÄKTA explorer purification of *E. coli* ALP

After cells were grown for an appropriate time, they were collected by centrifugation. The periplasmic proteins were released by osmotic shock according to the method described in Section 3.2.2, and the *E. coli* ALP was purified by a Q-Sepharose Fast Flow anion exchange chromatography column on ÄKTA explorer purification system. Figure 41 shows one example of ÄKTA explorer purification analysis of *E. coli* ALP expressed from SM547 pEK48/4-186.

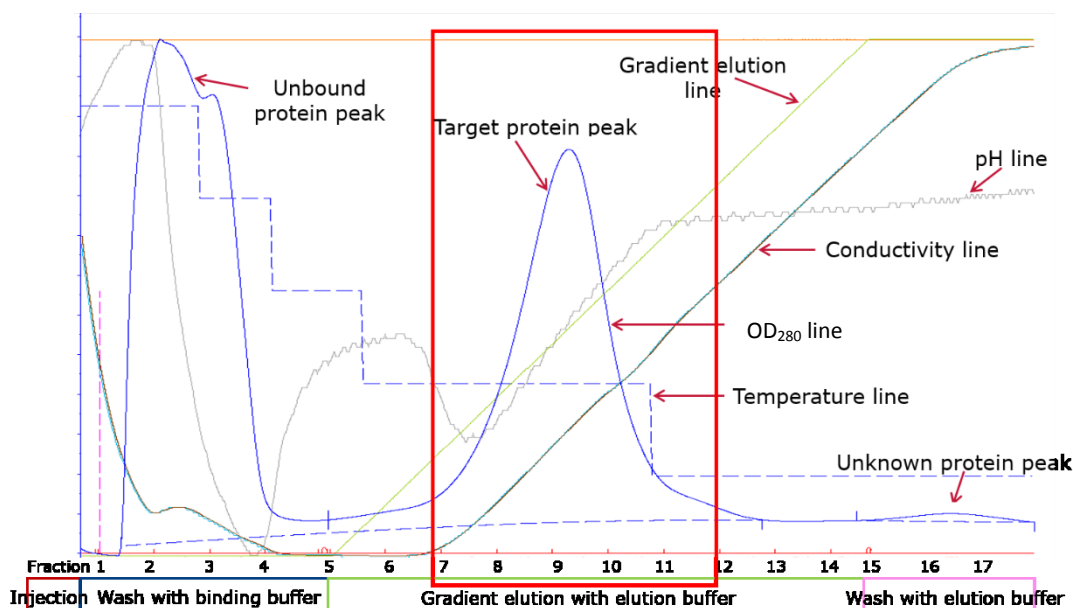


Figure 41. ÄKTA explorer purification analysis of *E. coli* ALP expressed from SM547 pEK48/4-186. The process contains four steps: sample injection, column washing with binding buffer, gradient elution with elution buffer, and column washing with elution buffer. Three main peaks were shown on the OD₂₈₀ line (solid blue line), the unbound protein peak, the target protein peak (shown in the red box), and an unknown protein peak. 17 fractions were collected from the purification.

Temperature, pH, conductivity, and OD₂₈₀ were on-line monitored during the purification. Among them, OD₂₈₀ indicated the protein concentration, so was of the most interest. The ÄKTA explorer purification process consisted of four steps. First, the protein mixture of *E. coli* proteins was injected into the system. Second, protein mixture passed through the column with the binding buffer. *E. coli* ALP was attached on the positively charged solid support so remained inside the anion exchange chromatography column. When the unbounded protein peak went down, which indicated the positive charged proteins were washed away with the binding buffer, the linear gradient started with elution buffer. The target *E. coli* ALP protein was eluted from the column in this step. OD₂₈₀ result indicated that the main protein peak shown in the red box started to increase when the 0.02 M NaCl elution buffer was used in the system, and finished when 0.1 M NaCl elution buffer was used. This was in accordance with references [175, 313]. Other unknown protein peaks were revealed in the last step of column washing when 0.2 M NaCl elution buffer was used.

Figure 42 shows the SDS-PAGE electrophoresis result of purified mutant and wild-type

E. coli ALP after the ÄKTA explorer purification system. Only one band of *E. coli* ALP at 53 kDa was observed on the gel for each sample. The result indicates the purification was successful. The western blotting result confirmed the expression of *E. coli* ALP from SM547.

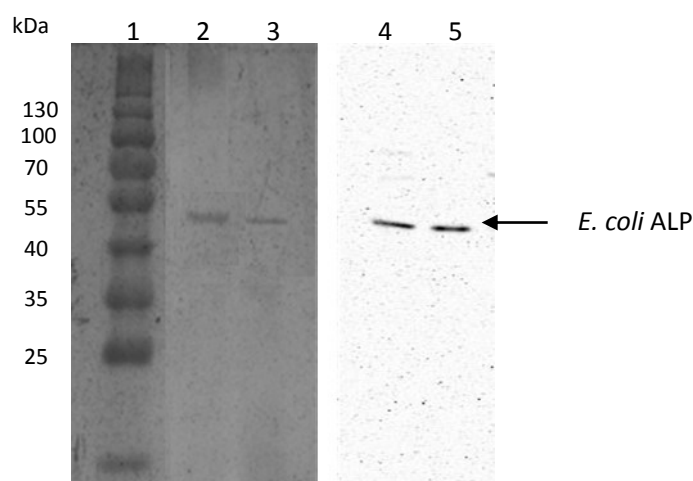


Figure 42. SDS-PAGE (8% w/v) gel electrophoresis analysis (lane 2 and 3) and western blotting (lane 4 and 5) of purified *E. coli* ALP after ÄKTA explorer purification system. Samples were electrophoresed in an SDS gel, then transferred to a nitrocellulose membrane and reacted with anti-alkaline phosphatase antibody. Lane 1, ladder; lane 2, *E. coli* ALP expressed from SM547 pEK48/4-186; lane 3, *E. coli* ALP expressed from SM547 pEK48/phoA; lane 4, western blotting analysis of *E. coli* ALP expressed from SM547 pEK48/4-186; lane 5, western blotting analysis of *E. coli* ALP expressed from SM547 pEK48/phoA. SDS-PAGE gel was stained with Coomassie blue.

3.3.4 Determining ALP specific activity of *E. coli* ALP

In order to determine the activity of *E. coli* ALP, which was normalised to protein content, the protein concentration in the elution buffer was measured. Several protein measurements were considered to determine the ALP concentration including the Bradford protein assay, the BCA protein assay, the Dc protein assay and spectrophotometry on the Nanodrop. The Dc protein assay was chosen based on the reliable results, the wide measurement range (from 5 µg/ml up to 1.5 mg/ml), as well as the small amount of sample required (20 µl of sample for the microplate assay). Other than a normal protein standard of bovine serum albumin, bovine intestinal ALP (lyophilised powder) from Sigma-Aldrich was used as a standard in this study. Bovine

intestinal ALP shows a 26% sequence homology of the active site compare to *E. coli* ALP [314], and is widely studied with implants or scaffolds to develop bioactive materials for bone tissue engineering applications [53]. In order to obtain a comparable result, bovine ALP was used as a positive control in this study to compare with the biological activity of *E. coli* ALP.

Figure 43A is a linear gradient line of *E. coli* ALP from the ÄKTA explorer purification analysis according to Figure 38. 17 fractions were collected and the target *E. coli* ALP should have been contained in the elution fractions from 5 to 14. The ALP specific activities of the *E. coli* ALP were measured by the method described in Section 3.2.10 (Figure 43B).

Initially, the ALP activity assay described in Section 2.2.2.5 was used to determine the *E. coli* ALP produced from bacterial cells. However, most of the fractions shown stronger absorbance signals than the standard after 60 min incubation at 37 °C. In order to suit the demands for the high ALP activity protein, a lower sample amount and shorter incubation time were modified in the new method described in Section 3.2.9. According to Figure 43B, the highest values are from the elution fractions 9 to 12. The results were in accordance with expectations, and indicated that pure *E. coli* ALP with high ALP specific activity was successfully separated. However, all 17 fractions shown ALP specific activities, which indicated the *E. coli* ALP existed in all fractions. This result demonstrates that the efficiency of this purification system was not very high due to a non-specific binding of *E. coli* ALP to the anion exchange column.

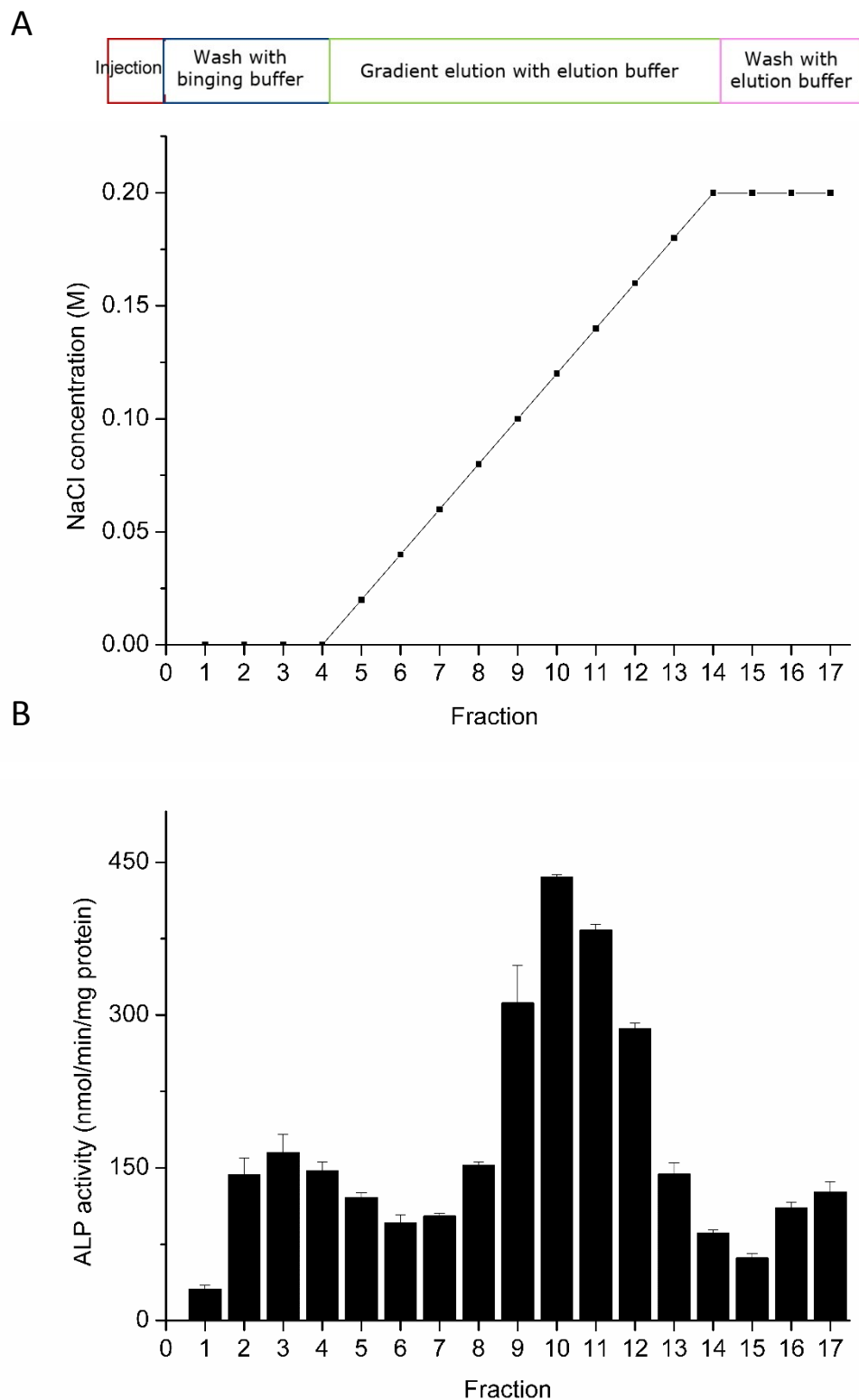


Figure 43. Quantification of ALP activity of 17 fractions after ÄKTA explorer purification analysis of *E. coli* ALP expressed from SM547 pEK48/4-186. (A) The linear gradient line of *E. coli* ALP from the ÄKTA explorer purification system; (B) ALP specific activity of 17 fractions. ALP specific activity was normalised to total protein amount determined by the Dc protein assay.

Figure 44 shows the comparison of ALP activity between the most active fractions of

mutant and wild-type *E. coli* ALP from SM547 pEK48/4-186 and SM547 pEK48/phoA, respectively. Bovine intestinal ALP was considered as a control. The mutant ALP showed a significantly higher activity compared to the wild-type ALP (24.60 ± 3.2675 times) and bovine ALP (9.24 ± 2.3581 times) ($p < 0.001$). The wild-type ALP showed a significantly lower activity than bovine ALP (more than twice) ($p < 0.01$). The results were in line with the reference although lower increasing times were obtained. According to Xu, the mutant ALP showed a nearly 35 times higher activity than the wild-type in pH 10.05, 1 M Tris-HCl buffer. When the activity was measured in pH 10.05, 0.5 M Tris-HCl buffer, the mutant ALP only showed activity 31 times higher than the wild-type [180]. Because the ALP activity is affected by many factors, it is possible that a 25 fold difference was measured in pH 7.4, 0.01 M Tris-HCl buffer.

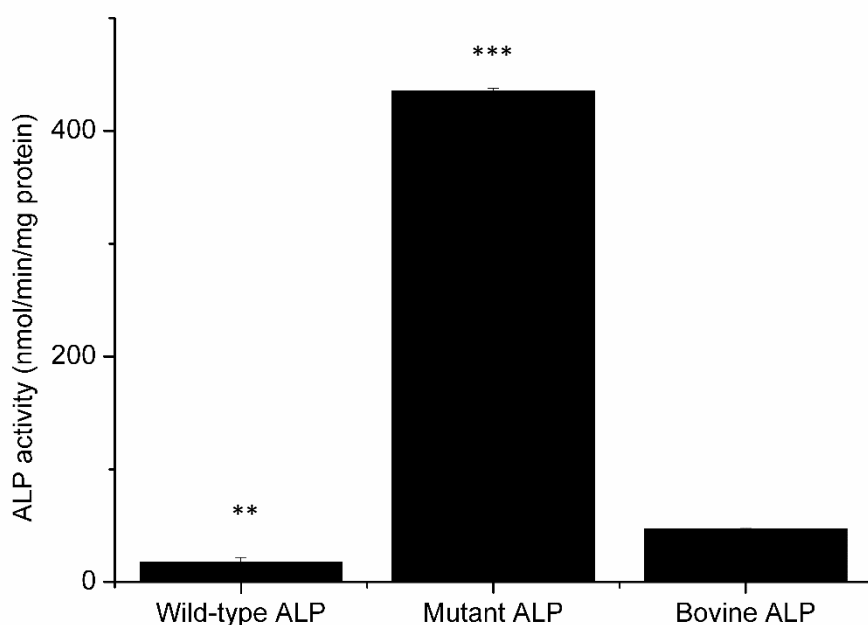


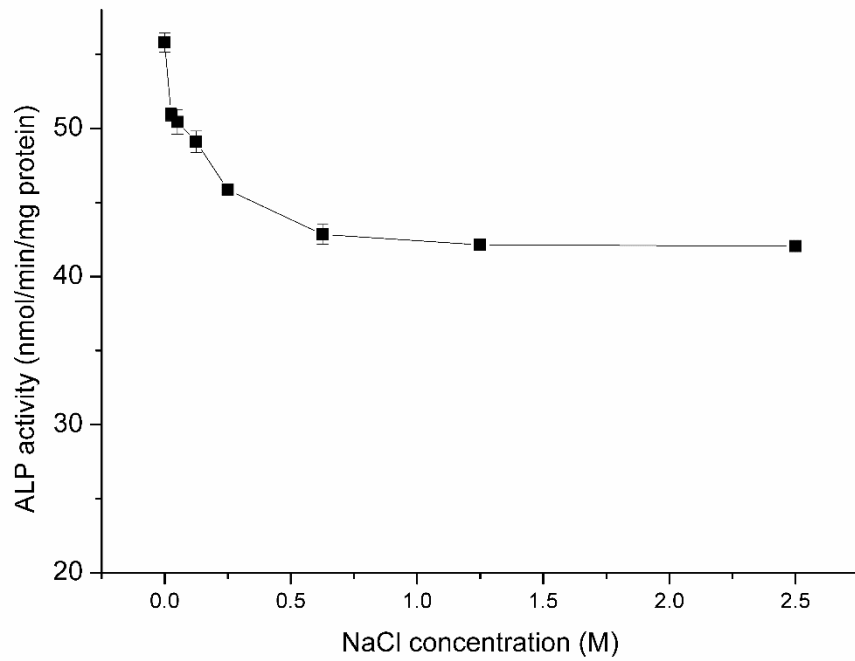
Figure 44. ALP specific activity of purified mutant and wild-type ALP from SM547 pEK48/4-186 and SM547 pEK48/phoA, respectively. Bovine ALP was used as a control. ALP specific activity was normalised by protein amount determined by Dc protein assay. ** $p < 0.01$ indicates statistically significant differences between the wild-type ALP with the bovine ALP. *** $p < 0.001$ indicates statistically significant differences between the mutant ALP with the other two groups.

3.3.5 Effect of NaCl on ALP specific activity

ALP specific activity can be affected by many factors, such as pH, temperature, salt and lyophilisation. The effect of the two most relevant factors in this study, salt concentration and lyophilisation, on ALP specific activity were determined in this Section and Section 3.3.6. Because NaCl was used in the elution buffer to exchange *E. coli* ALP from the column, the influence of NaCl concentration on ALP activity was important to explore. Bovine ALP was used as a model ALP in this measurement. Figure 45A shows the bovine ALP activity in different concentrations of NaCl solution. The activity dropped sharply when the NaCl concentration increased from 0 to 0.025 M. A decreasing rate of ALP activity was observed in higher concentration of NaCl. An elution buffer containing 0.2 M NaCl was used in the ÄKTA explorer purification system, so that the eluted *E. coli* ALP was exposed to a maximum of 0.2 M NaCl. The NaCl concentration may further increase due to lyophilisation.

Figure 45B compares the ALP activity of the freeze-dried mutant and wild-type ALP before and after dialysis. The activities of mutant ALPs were significantly increased after dialysis ($p < 0.001$). A small increase of ALP specific activity was also observed in the wild-type ALP although the increase was not significant. The result indicates that dialysis effectively recovers the ALP specific activity of *E. coli* ALP produced and purified from bacterial cells, especially for the mutant *E. coli* ALP. In order to avoid the reduction of activity, a dialysis was necessary to remove the NaCl in the solution.

A



B

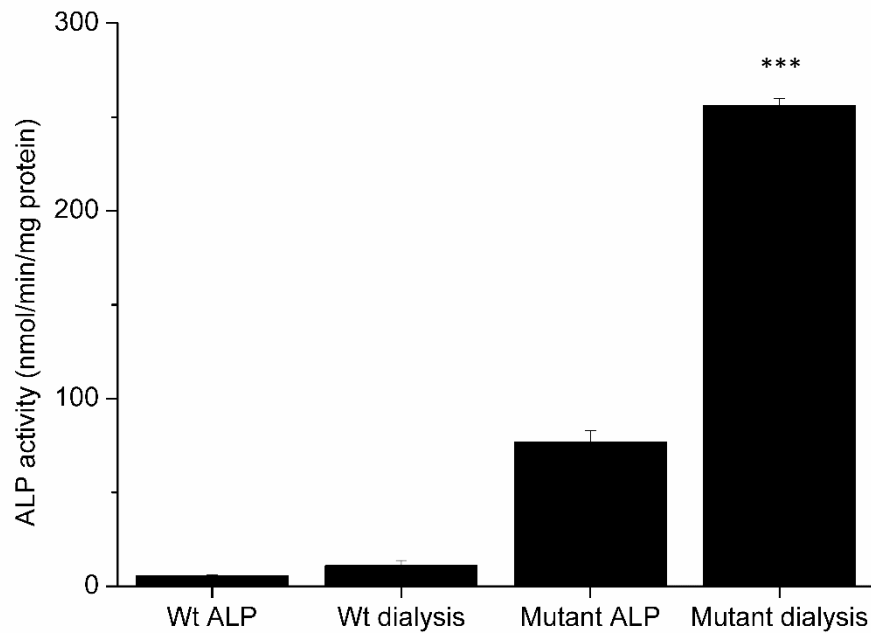


Figure 45. Effect of NaCl on ALP specific activity. (A) 0.125 mg/ml bovine ALP in 0 M, 0.025M, 0.05 M, 0.125 M, 0.25 M, 0.625 M, 1.25 M, and 2.5 M NaCl solution were prepared and the ALP specific activity was measured. (B) Freeze-dried mutant and wild-type ALP from SM547 pEK48/4-186 and SM547 pEK48/phoA before and after dialysis were compared. *** $p < 0.001$ indicates statistically significant differences between the mutant ALP after dialysis with the other groups.

3.3.6 Effect of lyophilisation on ALP specific activity

Figure 46 compares the ALP specific activity of mutant and wild-type ALP before and after lyophilisation. Lyophilisation was used to concentrate the *E. coli* ALP from the elution buffer to increase the protein stability, and to maintain the protein for the long term [315]. However, protein activity may be decreased or lost during lyophilisation. Protein stabilisers such as sucrose, NaCl, and proline can be necessary when freeze-drying the sample. NaCl, which stabilises proteins through the salting-in effect [316], was in the elution buffer and was chosen as the protein stabiliser. The wild-type ALP showed a drop in activity after lyophilisation. The mutant ALP, however, even in the presence of NaCl, had significantly decreased activity after lyophilisation ($p < 0.001$). The possible reason was the acute freezing and drying stresses of lyophilisation damaging the protein [317]. When the protein is rehydrated, denaturation and aggregation of mutations may affect their high activity [318]. In order to avoid the activity reduction, the protein was divided into aliquots and kept at -80°C .

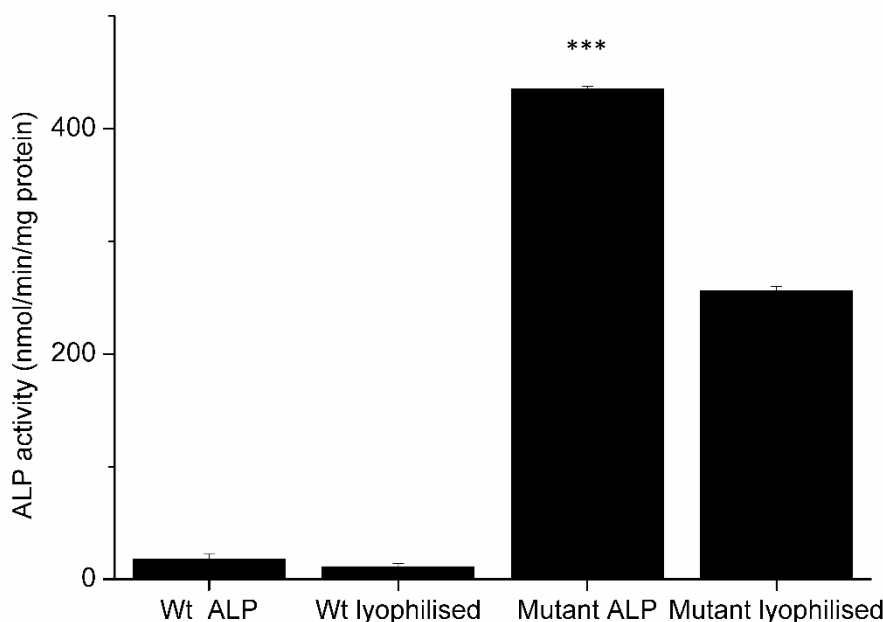


Figure 46. Effect of lyophilisation on desalted mutant and wild-type ALP from SM547 pEK48/4-186 and SM547 pEK48/phoA before and after freeze-drying was compared. ALP specific activity was normalised by protein amount determined by Dc protein assay. *** $p < 0.001$ indicates statistically significant differences between the mutant ALP and

the other groups.

3.3.7 Strategies to improve the expression of *E. coli* ALP

In order to improve the *E. coli* ALP expression, two strategies were carried out: IPTG induction; and by using different host cells. The first approach is based on the structure of plasmid pEK48 that contains an IPTG inducible *lac* promoter derived from plasmid pUC119. Figure 47 shows the SDS-PAGE analysis of cell lysates after 3 hours IPTG induction. SM547 expressed multiple proteins, and most of them were under 35 kDa. Insignificant differences were found between the lanes (lane 2 and 3; lane 4 and 5) with or without IPTG induction for both wild-type and mutant *E. coli* ALP. This result was in line with the fact that no induction of recombinant protein was described in previous studies of plasmid pEK48. This may be due to the loose-controlling expression system and the weak promoter in the plasmid SM547. Although pUC119 contains essential function of protein expression, it was designed as a cloning vector for producing a high copy number of plasmids. Therefore the protein expression system in pEK48 that derived from pUC119 is not the best choice for the expression of protein at a high level.

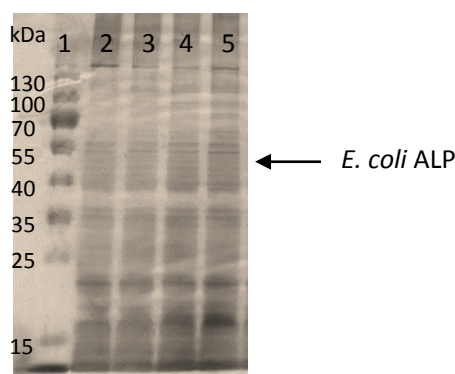


Figure 47. SDS-PAGE (8% w/v) gel electrophoresis analysis of cell lysates of SM547 pEK48/4-186 and SM547 pEK48/phoA after 3 hours IPTG induction. 1 mM IPTG was added when the absorbance at 600 nm of the culture medium reached 0.8. Lane 1, ladder; lane 2 and 3, cell lysates of SM547 pEK48/phoA without and with IPTG induction, respectively; lane 4 and 5, cell lysates of SM547 pEK48/4-186 without and with IPTG induction, respectively. The gel was stained by silver stain kit.

The second approach to improve the *E. coli* ALP expression was carried out by

transforming the plasmid pEK48/phoA and pEK48/4-186 into different host cells. There are different expression systems available to produce recombinant proteins as described in Section 1.5.2.1. However, the *E. coli* expression system is the best choice in this study because of the target *E. coli* ALP protein. In addition to SM547, *E. coli* DH5 α and BL21 were chosen because they are the most commonly used strains in genetic engineering. Table 14 lists the main features between these strains. DH5 α is known for the high production ability of plasmid DNA due to the deletion of *recA* and *endA* gene [319]. It also has the ability to express recombinant protein from the plasmid [320]. In addition, there is a loss of function mutant of *phoA* gene in DH5 α so that the strain lost its ability to express ALP from its chromosome. This feature ensures that all *E. coli* ALP expressed from DH5 α is the recombinant protein encoded by the plasmids. *E. coli* BL21 is a classic host strain for expression of recombinant proteins [321]. It lacks the protease which cleaves proteins so that high level of protein production can be achieved. Although a normal *phoA* gene encoding ALP protein is contained in the strain, recombinant *E. coli* ALP encoded from the plasmids should be in the majority.

Strain	<i>E. coli</i> genotype of <i>phoA</i> gene	Main application
SM547	Deletion	For recombinant protein production
DH5 α	A loss of function mutation	For plasmid DNA production
BL21	Normal	For recombinant protein production

Table 14. Main features of *E. coli* stains used in genetic engineering to produce recombinant proteins.

After transforming the plasmids pEK48/phoA and pEK48/4-186 into competent cells DH5 α and BL21, the successful recombinants were screened on the LB Amp BCIP selective plate. Blue colonies were found on plates with DH5 α and BL21 containing plasmid pEK48/phoA and pEK48/4-186 (Figure 48). However, blue colonies appeared after 48 hours of culture on the selective plate grown with DH5 α strains, while the BL21 strains needed less than 24 hours. Single colonies on these plate were picked and cultured to isolate the plasmids. Agarose gel electrophoresis indicated the correct plasmids were successfully transformed into the host cells.



Figure 48. Image of LB Amp BCIP selective plates grown with BL21 pEK48/4-186 after 24 hours incubation at 37°C. All colonies grown on the selective plate indicating they contain the correct pEK48/4-186 plasmids. The blue colonies indicate they are expressing active *E. coli* ALP.

The biomass concentration of bacterial culture was determined by measuring the cell wet weight. Cell pellets from the SM547, DH5 α and BL21 hosts containing pEK48/4-186 were collected from 500 ml LB media and weighted after 12 hours incubation at 37°C. Among the three host cells, BL21 shown the highest biomass concentration (5.7778 g/l of cell wet weight), followed by SM547 (2.77710 g/l of cell wet weight). DH5 α had only 0.6070 g/l of cell wet weight. The results indicate that two recombinant protein production strains are superior for achieving high density fermentation than DH5 α .

A further study was performed to compare the protein content of purified *E. coli* ALP produced from SM547 and BL21. Figure 49A shows BL21 produced a higher protein content than SM547 in the peak fractions after the ÄKTA explorer purification system. The difference of wild-type ALP between SM547 and BL21 strains was significant ($p < 0.005$). For the mutant protein, although a higher concentration was found in the BL21 group, the difference was insignificant. The ALP specific activity from these four fractions was determined (Figure 49B). Wild-type ALP produced from BL21 strains showed a significantly higher ALP specific activity than the SM547 strain ($p < 0.05$), the same trend as the protein content. However, mutant ALP expressed from SM547 showed a significantly higher ALP specific activity than the BL21 strain ($p < 0.001$). The results indicate that although a higher protein amount was produced from BL21, the protein was not as active as from SM547. It may be that the mixture of wild-type ALP produced by the BL21 strain combined with the mutant recombinant protein reduced the ALP activity.

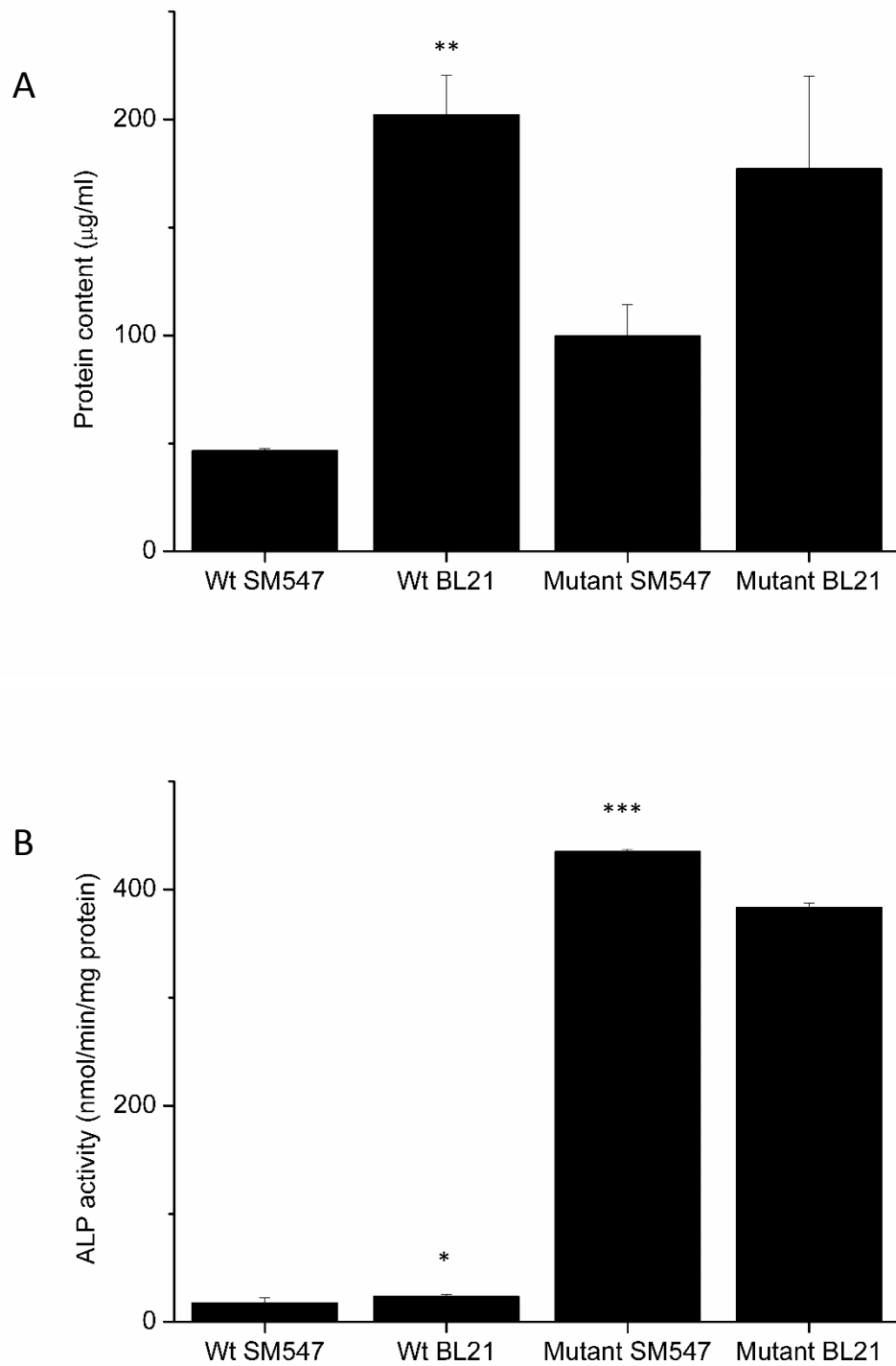


Figure 49. Quantification of total protein amount (A) and ALP specific activity (B) of purified mutant and wild-type ALP from SM547 and BL21. * $p < 0.05$, ** $p < 0.005$ indicate a statistically significant difference of the wild-type ALP between SM547 and BL21 strains. *** $p < 0.001$ indicate a significant difference of the mutant ALP between SM547 and BL21 strains.

3.4 Conclusion

The wild-type and mutated recombinant *E. coli* ALP with improved biological activity were successfully produced by bacterial *E. coli* cells, and purified using the ÄKTA explorer purification system. The mutant ALP produced in this study showed nearly 25 times higher activity compared to the wild-type ALP, and 9 times higher than bovine ALP. The improved biological activity makes the mutant *E. coli* ALP a promising candidate for bone tissue engineering applications. The activity of *E. coli* ALP could be affected by NaCl in the elution buffer and lyophilisation treatment. In order to avoid the activity reduction, a dialysis was necessary after the ÄKTA explorer purification analysis. To improve the protein yield, the plasmids pEK48 were transformed into different host cells. The results indicated that BL21 strain produced the highest protein amount and highest ALP activity of wild-type *E. coli* ALP. For mutant ALP, BL21 produced a higher protein amount, but SM547 expressed the highest ALP activity.

CHAPTER 4

Engineering of recombinant His-ALP with improved functionality for controlled biomolecule immobilisation

4.1 Introduction

Mutated *E. coli* ALP with improved biological activity was produced and purified from the *E. coli* recombinant protein expression system in Chapter 3. Although this mutant ALP showed outstanding high activity compared to the wild-type ALP and bovine ALP, some features warranted further improvement. First, despite the attempts to increase the expression of *E. coli* ALP by IPTG induction or by different bacterial host strains, the protein yield was still low compared to literature reports [322, 323]. A powerful recombinant protein expression system with a strong promoter was therefore desired. Second, the purification step is the bottleneck in the production system. A Q-Sepharose Fast Flow anion exchange chromatography column was used in the ÄKTA explorer purification system to purify *E. coli* ALP. The procedure was time consuming and laborious. In addition, due to the non-specific ionic binding of the anion exchange chromatography column, all negatively charged protein including *E. coli* ALP can be purified from the column. This may reduce the purity of *E. coli* ALP. Therefore, a simple and specific purification method is needed. Third, the recombinant *E. coli* ALP may have limited activity once combined with a scaffold for bone tissue engineering applications. The active sites of *E. coli* ALP may be hidden during random immobilisation, which may negate the outstanding property of high activity. In order to overcome these drawbacks, this chapter explored a new approach of engineering His-tag recombinant *E. coli* ALP with multi-functionality for bone tissue engineering applications.

4.1.1 Engineering His-tag recombinant alkaline phosphatase

As described in Section 1.5.3, His-tag fusion proteins are widely used in recombinant proteins for the superior properties. First, they exhibit an outstanding protein purification ability [225]. By using a Ni-NTA metal-affinity column, which has a strong and specific binding to the His-tag recombinant protein, *E. coli* ALP can be purified directly from bacterial lysates and eluted by imidazole from the column under mild conditions (Figure 13). In addition, the fusion protein can be detected by anti-His-tag antibodies in ELISA, western blotting or other analytical methods [230]. Most importantly, the (His)₆ tail allows an orientation controlled and orderly immobilisation to expose the active site of fusion proteins [231, 233]. For example, the same bioactive factor is immobilised on Ti implants to make active coatings by adsorption (Figure 50A) and by His-tag chelation (Figure 50B), respectively. Due to the random immobilisation upon adsorption, the active sites of the protein may be covered by each other. In addition, only a few proteins can be immobilised because of the complex conformation. However, if a His-tag is added on the protein, the orientation of the His-tag fusion protein is fixed once immobilised on the implant. The active site of the protein can be adjusted to achieve the maximal exposure. Additionally, due to the (His)₆ tail, which acts like a linker between the implant and the proteins, more proteins can be loaded on the surface of implants to reach a higher loading rate and immobilisation efficiency. The activity of site-directed immobilised His-tag ALP was about 1.3-fold higher than random immobilisation [233].

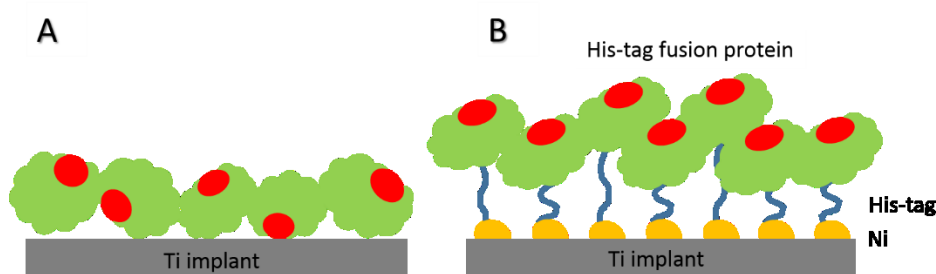


Figure 50. The immobilisation of active factors on Ti implants by (A) adsorption and (B) chelation. The active factors are shown in green, the active sites are shown in red, and His-tag is shown in blue, and orange dots represent the nickel (Ni) ions on the Ti implant.

In order to improve the recombinant protein yield produced from bacterial cells, a new expression system was employed in this study: the pET expression system. The pET expression system is the most powerful system developed so far for the cloning and massive production of recombinant protein from *E. coli* bacterial cells [324]. It is based on a T7 promoter-driven RNA polymerase expression system developed by Studier in the 1980s [325, 326]. As described in Figures 10 and 11, the target gene that is inserted into the cloning/expression region of a pET vector can be highly expressed in the presence of T7 RNA polymerase [212, 324].

There are more than 40 pET vectors available with different features. They vary in promoters, leader signal sequences, fusion tags, and antibiotic resistance markers, but all of them are derived from pBR322 plasmid [327]. Two promoters are provided in the pET system, a T7 promoter, and a T7 *lac* promoter. The latter contains an additional *lac* operator sequence, which can block transcription, and a *lacI* gene, which codes for the *lac* repressor protein, compared to the former. They provide a more tightly controlled transcription to suppress the basal expression in host strains [327]. In this study, a pET vector containing a T7 *lac* promoter is preferred. Based on whether they contain a leader signal sequence or not, the pET vectors can be divided into two parts: the transcription vectors, which lack the translation signal sequence such as pET21, pET23, and pET24; and the translation vectors, which contain the leader translation signal sequence. In this study, since the target *phoA* gene contains its own bacterial translation signals, a transcription vector is needed. Regards to fusion tags, a His-tag is desired because of its purification and immobilisation advantages. A pET24 vector was chosen based on these requirements. The pET24 vector contains a T7 *lac* promoter, a C-terminal His-tag, an N-terminal T7-tag, a kanamycin resistance cassette, a pBR322 origin, and a *colE1* origin (Figure 51) [327]. The T7-tag (11 amino acids) is a functional fusion tag for protein detection and purification.

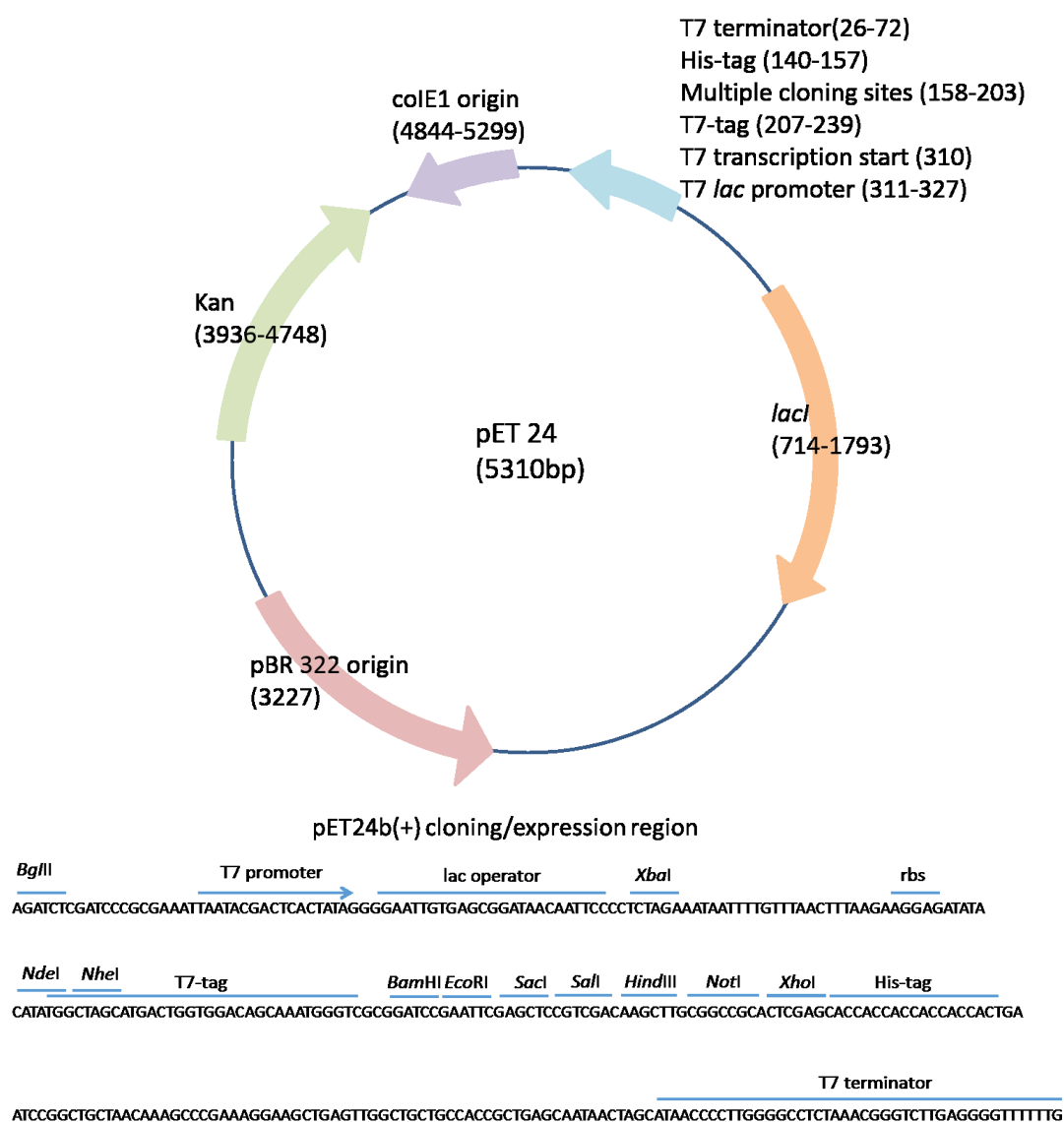


Figure 51. Expression vector pET24 map and its cloning/expression region [327]. The blue arrow shows the cloning/expression region. It contains a T7 promoter, a lac operator, a ribosomal binding site (rbs), the multiple cloning sites, a T7-tag and a His-tag.

The T7 promoter-driven RNA polymerase expression system requires the presence of T7 RNA polymerase in the cell to start transcription. Therefore pET vectors need to be transformed into a λ DE3 lysogen host strain, such as BL21(DE3), in which the host chromosome contains a T7 RNA polymerase gene. The expression of the T7 RNA polymerase gene is controlled by the bacterial *lacUV5* promoter. This system allows a low level of transcription in the non-induced condition [327]. In this study, a general purpose expression host *E. coli* strain BL21(DE3) was used for recombinant protein expression.

Table 15 summarises the major challenges met in the production and purification of recombinant *E. coli* ALP from pEK48 plasmids in bacterial cells and their possible reasons. In order to solve these problems, the mutant and wild-type *phoA* genes were recloned into a pET24 expression vector, which contains a His-tag, to produce the engineered recombinant His-tag *E. coli* ALP in the pET expression system.

Problem	Possible reason	Solution
Low protein yield	Weak expression system	pET expression system
Difficult to purify	Unspecific binding	His-tag chelation binding
Limitations in immobilisation	Random immobilisation	Orientation controlled immobilisation by His-tag

Table 15. Main problems, possible reasons, and solutions in the production and purification of recombinant *E. coli* ALP in bacterial cells.

4.1.2 Scope of the chapter

This chapter demonstrates the development of a multi-functional recombinant His-tag ALP for bone tissue engineering. In addition to the high biological activity possessed by the mutant *E. coli* ALP, the His-tag ALP has the ability to control immobilisation on supporting materials such as a Ti implant. Therefore biomaterials incorporating the His-tag ALP benefit from enhanced biological activity. In this chapter, methods of recombinant His-tag ALP production and purification are described.

4.1.3 Hypotheses

The hypotheses tested was that recombinant *E. coli* His-tag ALP may improve the protein expression, simplify the purification, and has the ability to control immobilisation on supporting materials. In order to test these hypotheses, the following aims have been addressed in this Chapter:

- (1) Construct the recombinant pET24/phoA and pET24/4-186 plasmids.
- (2) Confirm the expression of recombinant His-tag ALP.
- (3) Purify the recombinant His-tag ALP from bacterial culture media.
- (4) Optimise the expression of His-tag ALP.
- (5) Determine the biological activity of His-tag ALP.

4.2 Materials and methods

4.2.1 Materials

Unless stated otherwise, all chemicals and bacterial cell culture reagents were purchased from Sigma-Aldrich (Gillingham, UK). The reagents mentioned in Section 3.2.1 were omitted. Restriction enzymes were purchased from Invitrogen and New England BioLabs. PCR SuperMix High Fidelity was purchased from Invitrogen. Wizard PCR kit was purchased from Promega. Rapid DNA Ligation Kit was purchased from Fermentas. T4 DNA ligase was from New England Biolabs. RapidTransit Transformation kit was from Sigma-Aldrich. Anti-His antibodies were from QIAGEN. ECL Anti-Rabbit IgG Horseradish Peroxidase-linked whole antibody (from donkey) was from GC Healthcare.

Bacterial BL21(DE3) (F^- , *dcm*, *ompT*, *hsdS*($r_B^- m_B^-$), *gal*, λ (DE3)) competent cells were from Agilent Technologies, CA, USA. All other strains and plasmids pEK48/phoA and pEK48/4-186 were kindly provided by Professor A. E. G. Cass.

4.2.2 Design of recombinant pET24/phoA and pET24/4-186

In order to construct the recombinant pET24/phoA and pET24/4-186 plasmids, the target *phoA* and *4-186* genes were inserted into the pET24 vector. Briefly, two restriction cleavage enzymes, *NdeI* and *HindIII*, were chosen to cut the vector and inserted the target gene according to the restriction map of pET24. Because *NdeI* and

HindIII only have one cleavage site in the vector and no cleavage site in the target gene, they ensure a unique combination of the vector and the entire target gene. In addition, the *NdeI* and *HindIII* digestion of pET24 vector can delete the T7-tag which is not needed in this study. Then the target gene was amplified by PCR with the primers containing either of the *NdeI* and *HindIII* sequence so that the target genes produced from PCR contain the *NdeI* and *HindIII* on each end. Finally, the vector and target gene after double digestion with *NdeI* and *HindIII* can be ligated into a new recombinant plasmid with the use of DNA ligase.

In this study, four different target genes were determined for both wild-type and mutant *E. coli* ALP gene (Table 16). The four target genes were different in whether or not they contained a signal sequence, and whether they had a C-terminal or N-terminal His-tag. The pET24 vector provides a C-terminal His-tag, while the N-terminal His-tag was achieved through PCR.

Number	Target gene
1	<i>NdeI</i> + signal sequence + mature sequence of ALP gene + <i>HindIII</i>
2	<i>NdeI</i> + mature sequence of ALP gene + <i>HindIII</i>
3	<i>NdeI</i> + His-tag + signal sequence + mature sequence of ALP gene + STOP codon + <i>HindIII</i>
4	<i>NdeI</i> + His-tag + mature sequence of ALP gene + STOP codon + <i>HindIII</i>

Table 16. Different types of target genes designed in this study.

4.2.3 DNA amplification by PCR

As described in Figure 15, PCR is a rapid way to *in vitro* amplify (duplicate) specific DNA sequences. It is an important technology for research and diagnostic analysis of DNA and RNA [328]. The cycling process contains three steps: (1) denaturation of double-stranded DNA into single strands, (2) annealing of primers for new strand synthesis, and (3) extension of primers to form new strands [234, 235]. Several components are essential for the PCR including a defined DNA segment as a template; two single-

stranded sequence of nucleotide primers, which are complementary to DNA template; excess deoxynucleoside triphosphates (dNTPs); DNA polymerase, and a mixture of buffers. The method was invented for diagnosis of sickle cell anemia in whole genomic DNA by Kary Mullis in 1985, who was awarded the Nobel Prize in 1993 [329]. At that time, the procedure was completed manually, and fresh DNA polymerase was added after each cycle [330]. Later an automatic DNA Thermal Cycler was invented after the discovery of the thermostable DNA polymerase, *Thermus aquaticus* (*Taq*) [331, 332].

In this study, a PCR SuperMix High Fidelity kit was used to amplify target DNA. This reagent contains Mg^{2+} , dNTPs, and a mixture of recombinant *Taq* DNA polymerase and DNA polymerase from *Pyrococcus* species GB-D, and offers a 6-fold improvement in fidelity over *Taq* DNA Polymerase [333]. 45 μ l PCR SuperMix High Fidelity was incubated on ice with 1 μ l 10 μ M forward primer, 1 μ l 10 μ M reverse primer, and 0.5 μ l (approximately 12 ng) template DNA. The reaction was heated at 94°C for 30 seconds, followed by 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 2 min. The reaction products was stored at 4°C until the next step. A touch-down PCR program (Figure 52) was used to overcome amplification of non-specific products, and to increase the reaction's specificity, sensitivity, and yield [334]. The ideal annealing temperature, which is an important parameter in PCR, was determine using a gradient decreasing temperature method during the cycling process for the annealing step [335].

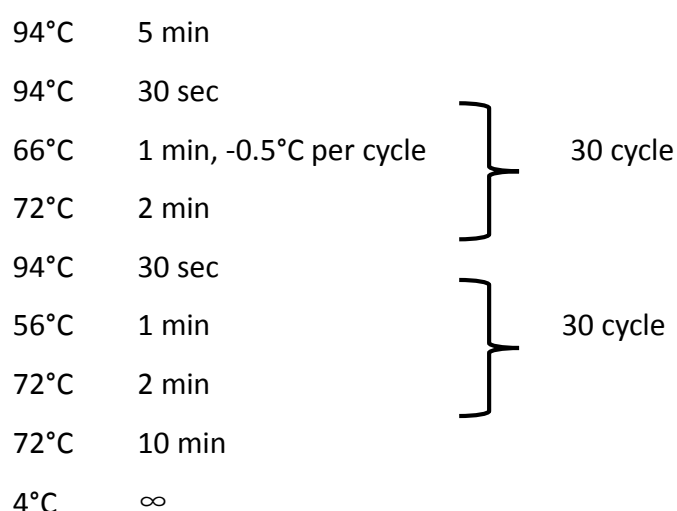


Figure 52. The touch-down PCR programme.

4.2.4 Restriction enzyme digestion

The second step of the production process of recombinant proteins is to cleave the target gene and the vector (Section 1.5.1). This step need to be accomplished with the help of restriction enzymes. Restriction enzymes function by cutting double stranded DNA at specific target sequences to produce either sticky ends or blunt ends [193]. The choice of restriction enzymes were based on the specific features of the vector and the target gene. First, it was necessary to identify the restriction enzymes available on the vector by checking the restriction map. Those restriction enzymes that only have a single restriction site on the vector were chosen. Secondly, the gene sequences of these single-site restriction enzymes were verified, to ensure they are outside the target gene. Finally, two different restriction enzymes were chosen for both ends so that they do not self-ligated [336]. A sticky-end restriction enzyme is preferred due to the higher ligation rate. Ideally, the two enzymes function well in the same digestion conditions to permit a double digestion.

Most of the restriction enzyme digestions in this study were carried out in a buffer containing 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, and 1 mM DTT with a 10 fold excess of enzyme and incubation for 2 hours at 37°C.

4.2.5 Low melt agarose gel electrophoresis and DNA purification

Low melt agarose gel electrophoresis was used to purify the DNA fragments after double digestion. The principle and procedure of low melt agarose gel electrophoresis is exactly the same as agarose gel electrophoresis described in Section 3.2.4. The main difference is this agarose has a low melting (65.5°C) and gelling temperatures (25°C) compared with standard agarose (80°C and 32°C, respectively) [337]. The low melting temperature is below the melting point of most nucleic acids so that DNA fragments can be recovered from the gel without denaturing.

In this study, 1% (w/v) low melt agarose gel was made with a TBE running buffer (see Appendices) containing SYBR Safe DNA gel stain. Samples were mixed with 6X sample loading buffer before loading on the gel. The agarose gel electrophoresis was performed in a gel box (Bio-Rad, USA) on ice at 100 V for 1.5 hours. The gels were visualized using a BioSpectrum 500 fluorescence reader from UVP (CA, USA). The target bands were cut and kept in buffer at 4°C.

A Wizard SV Gel and PCR Clean-Up System was used to purify DNA fragments from the low melt agarose bands. The procedure is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts [338]. The target bands containing DNA fragments of interest were first vortexed and incubated with a membrane binding buffer at 65°C until the gel was completely dissolved. Then the gel mixture was transferred into a column containing the silica membrane, and incubated at ambient temperature for 1 min. Next, the column was centrifuged at 12000 rpm for 1 min at 4°C to bind the target DNA fragment to the silica membrane of the column. The column was washed twice with washing buffers to improve the purity of the plasmid DNA. Finally, the DNA fragment was eluted in a clean Eppendorf in 25-50 µl nuclease-free water, depending on the concentration. The concentration of DNA fragment was assessed on a NanoDrop 2000c (Thermo Scientific, USA). The DNA fragment purified from low melt agarose gel can be directly used for ligation.

4.2.6 Ligation and transformation

As described in Section 1.5.1, the target DNA fragment need to be inserted into the desired vector to construct a new recombinant DNA plasmid [339]. Both hydrogen bonds and covalent bonds of the target DNA and the vector need to be sealed to form a stable plasmid. The former can be achieved by the complementary sticky ends produced by the restriction enzymes. The latter need to be sealed by an enzyme DNA ligase during ligation.

In this study, two ligation reagents were used: a rapid DNA ligation kit from Fermentas,

and the T4 DNA ligase from New England Biolabs. By using the rapid DNA ligation kit, DNA ligation can be accomplished in only 5 minutes at ambient temperature [340]. The vector and target DNA fragment after double digestion were completely mixed (insert/vector molar ratio is 3:1) with T4 DNA ligase and buffer, and incubated at ambient temperature for 5 min. Then the ligation mixture was ready for transformation. By using the second T4 DNA ligase, the vector and target DNA fragment after double digestion were still completely mixed with T4 DNA ligase and buffer, however, a 1:1 insert/vector molar ratio was required, and the incubation was carried out at 16°C overnight.

Once the ligation was accomplished, the next step in cloning was to transform the newly formed plasmid into host cells for replication. The same transformation method was used as described in Section 3.2.7. pET24 recombinant plasmids containing wild-type and mutant alkaline phosphatase genes were transformed into DH5 α and BL21(DE3) competent cells.

4.2.7 Verifying the recombinant plasmids

The transformed cells were streaked on a selectable plate to screen transformants. In this study, the LB Kan BCIP IPTG selective plate was used. Similar to the process of screening *E. coli* pEK48 recombinants, blue colonies developed on the selective plate to indicate the expression of the His-tag ALP from pET24 recombinants. Single blue colonies on the plates were picked and suspended in 4 ml of LB media containing 30 μ g/ml kanamycin. The cells were cultured in an incubator at 37°C, 200 rpm overnight. The plasmid DNA in BL21(DE3) cells was extracted using a QIAprep Spin Miniprep Kit described in Section 3.2.3. In order to verify the size of pET24 recombinant plasmids, the purified plasmids were analysed by restriction digestion and agarose gel electrophoresis.

4.2.8 Expression of the His-tag *E. coli* ALP in BL21(DE3)

A vial of frozen bacterial cells was thawed and plated on LB media plates (see Appendices) containing 30 µg/ml kanamycin, 40 µg/ml BICP, and 0.5 mM IPTG. Single blue colonies on the plates carrying the wild-type or mutant His-tag alkaline phosphatase genes were picked and suspended in 4 ml of LB media containing 30 µg/ml kanamycin. The cells were cultured in an incubator at 37°C, 200 rpm overnight. 1 ml of overnight culture medium was sterilely transferred into 100 ml of LB broth containing 30 µg/ml kanamycin to incubate at 37°C with continuous shaking until the absorbance at 600 nm of the culture medium reached 0.5-1.0. The inducer IPTG was added to a final concentration of 0.2 mM to 1 mM, and incubated either at 30°C or 37°C for another 0.5 hour or up to overnight.

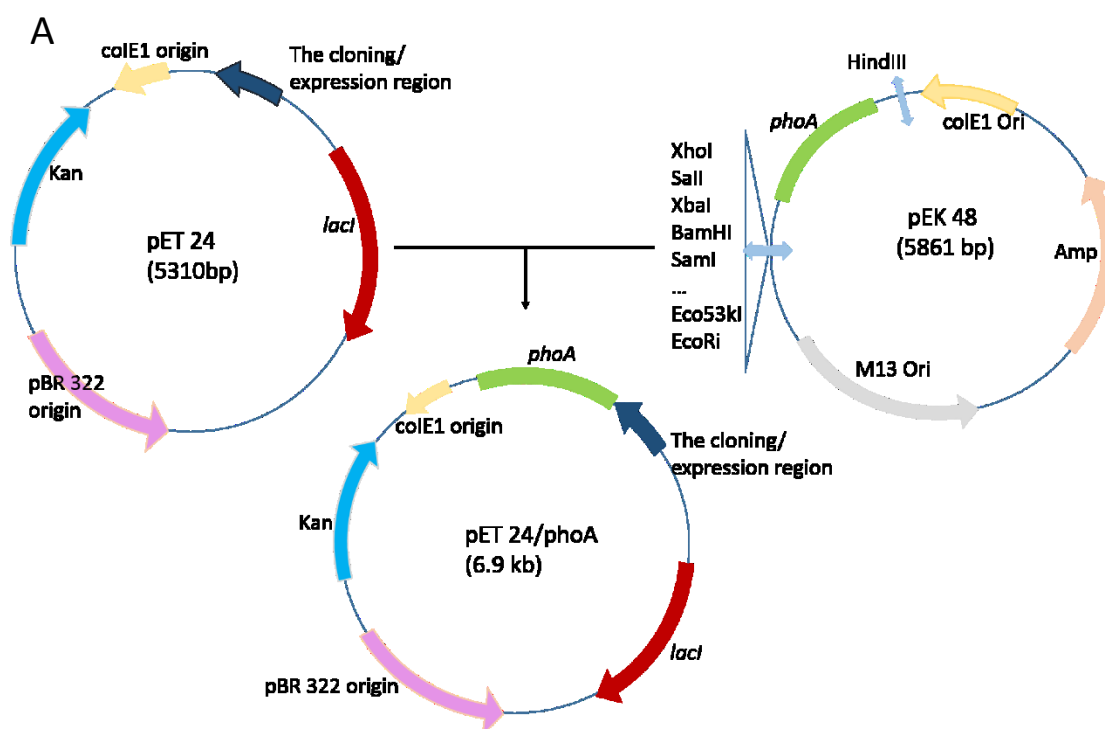
4.2.9 Purification of the His-tag *E. coli* ALP in BL21(DE3)

The cells were harvested by centrifugation at 6500 rpm for 15 min. The cell pellet was washed twice with cold 0.01 M Tris-0.03 M NaCl washing buffer, resuspended in 0.033 M Tris suspension buffer, and disrupted by sonication. Sonication was performed three times for 30 seconds with 1 min intervals for cooling on ice. The sonicated extract was either incubated at 65°C for 30 min and then centrifuged, or directly centrifuged at 12000 rpm for 20 min at 4°C. Similar to *E. coli* ALP, His-tag *E. coli* ALP was purified on the ÄKTA explorer purification system with a HiTrap Ni Sepharose High Performance chromatography column. The supernatant containing His-tag *E. coli* ALP was filtered through a 0.45 µm filter before purification on a GC Healthcare ÄKTA explorer using HiTrap Ni Sepharose High Performance column (GC Healthcare). Proteins bound on the column were eluted with a linear gradient of 0-500 mM imidazole in TMZP buffer. Pure His-tag *E. coli* ALP fractions were collected by detecting the absorbance at 280 nm, identified by electrophoresis, and characterised by biochemical assays as described in Chapter 3. A flow rate of 1 ml/min was used and fractions were collected in Eppendorf tubes.

4.3 Results and discussion

4.3.1 Construction of pET24/phoA and pET24/4-186

The *E. coli* mutant and wild-type ALP were poorly expressed in the SM547 pEK48 expression system. In order to achieve a high-level protein expression, the pET expression system was employed, which is one of the most powerful recombinant protein expression systems in *E. coli*. Figure 53 shows the construction process of pET24/phoA plasmid, which is an example for all the other plasmids containing different target genes. The 1.68 kb DNA fragment containing the entire *phoA* gene with the signal sequence was amplified by PCR from pEK48/phoA by using a forward primer (GGAATTCCATATGAGATTATCGTCACTGCAATG, containing an underlined *Nde*I site) and a reverse primer (CCCAAGCTTTTTCAGAGCGGCTTTC, containing an underlined *Hind*III site). The pET24 plasmid was purified from DH5 α . Both the vector and the DNA fragments were digested with *Nde*I and *Hind*III, and ligated to form the pET24/phoA plasmid.



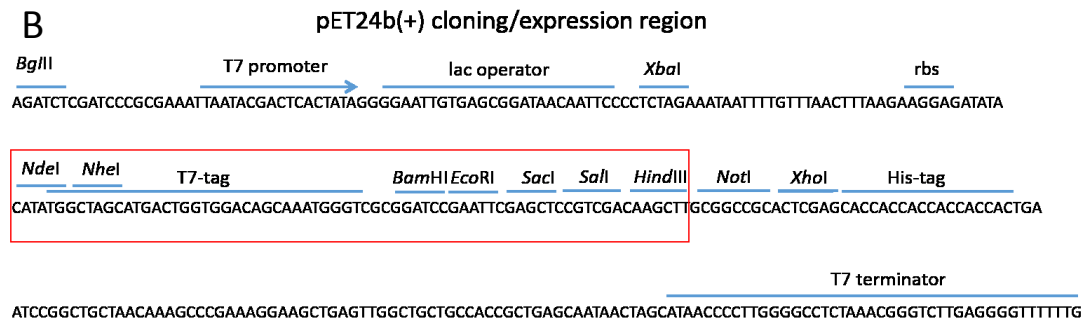


Figure 53. Construction process of plasmid pET24/*phoA* and the cloning/expression region of the pET24 vector. (A) Plasmids pEK48 and pET24 DNA were double digested with *Nde*I and *Hind*III, mixed, and ligated to form a new recombinant plasmid pET24/*phoA*, which contains the entire *phoA* gene with the signal sequence (1.68 kb) shown in green. A kanamycin resistance marker is shown in light blue, a *colE1* origin is shown in orange, a *lacI* gene is shown in red, and a pBR322 origin is shown in purple. The dark blue arrow represents the cloning/expression region of pET24. (B) The gene sequence of the cloning/expression region. It contains a T7 promoter, a *lac* operator, a ribosomal binding site (rbs), the multiple cloning sites, a T7-tag and a His-tag. The sequence shown in the red box between *Nde*I and *Hind*III restrictive cleavage sites was replaced by the *phoA* gene in the pEK48 plasmid.

Figure 54 shows the agarose gel electrophoresis results of PCR products. The DNA fragment containing the *phoA* and 4-186 gene, which encodes the wild-type and mutant *E. coli* ALP, was amplified from pEK48/*phoA* and pEK48/4-186, respectively. Each type of *E. coli* ALP has two different genes with (1.68 kb) and without (1.34 kb) the signal sequence. All groups showed dark and thick signal bands at the desired position. The results indicate that the PCR amplification of target DNA fragments was successfully performed. The concentration of the DNA fragments after PCR was further determined on Nanodrop spectrophotometer. The Nanodrop showed a yield of more than 200 ng/ μ l DNA in all groups. The results were in line with the agarose gel electrophoresis.

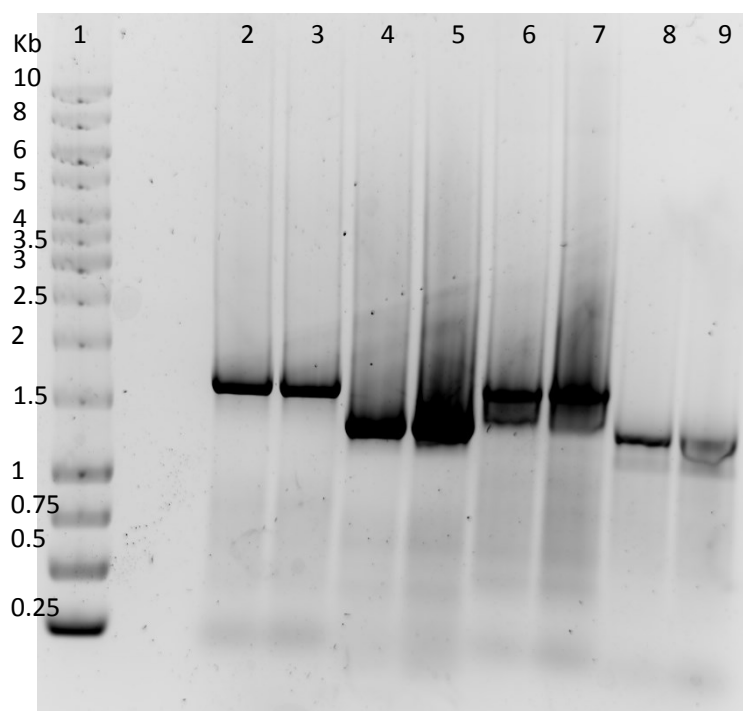


Figure 54. Agarose (1% w/v) gel electrophoresis of amplified target ALP genes after PCR from plasmids pEK48/4-186 and pEK48/phoA. Lane 1 is a 1 kb DNA ladder; lane 2 and 3 are 2 μ l and 4 μ l wild-type *phoA* gene with the signal sequence (1.68 kb), respectively; lane 4 and 5 are 2 μ l and 4 μ l wild-type *phoA* gene without the signal sequence (1.34 kb), respectively; lane 6 and 7 are 2 μ l and 4 μ l mutant 4-186 gene with the signal sequence (1.68b), respectively; and lane 8 and 9 are 2 μ l and 4 μ l mutant 4-186 gene without the signal sequence (1.34 kb), respectively.

Figure 55 shows the agarose gel electrophoresis results of the pET24 vector. The bacterial strain DH5 α containing plasmids pET24 was cultured in LB medium. The plasmids were isolated with a QIAprep Spin Miniprep Kit and verified by agarose gel electrophoresis. A single band of 5.3 kb was detected when the vector pET24 was digested with the restrictive enzymes *Nde*I or *Hind*III (Lane 3 and 4, respectively); only one sharp band (5.3 kb) was shown when the plasmids were double digested with *Nde*I and *Hind*III (Lane 5) because the other DNA fragment only have 70 bp, which is too small to show on the gel; a blurred band was shown when the undigested plasmids were loaded on the gel (Lane 2) due to their supercoiled structure. The results indicate the real size of the plasmid is consistent with the description, and demonstrate the digestion of the plasmids by restrictive enzymes is correct and efficient.

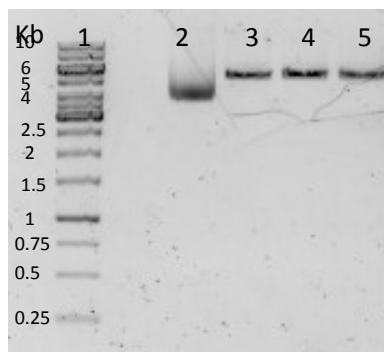


Figure 55. Agarose (1% w/v) gel electrophoresis of restriction enzymes digested plasmids pET24. Lane 1 is a 1 kb DNA ladder; lane 2, the undigested plasmid pET24; lane 3, the plasmid pET24 single digested with *NdeI*; lane 4, the plasmid pET24 single digested with *HindIII*; lane 5, the plasmid pET24 double digested with *NdeI* and *HindIII*.

Figure 56 shows the low melt agarose gel electrophoresis results of the pET24 vector and target gene fragments after double digestion with *NdeI* and *HindIII*. A clear single band of 5.3 kb was shown when the vector pET24 was double digested with restrictive enzyme *NdeI* and *HindIII* (Lane 2); Lane 3-6 were the double digested PCR DNA fragments containing the *phoA* and 4-186 genes with and without the signal sequence. These double digested bands were cut and purified using a Wizard PCR kit, and prepared for ligation.

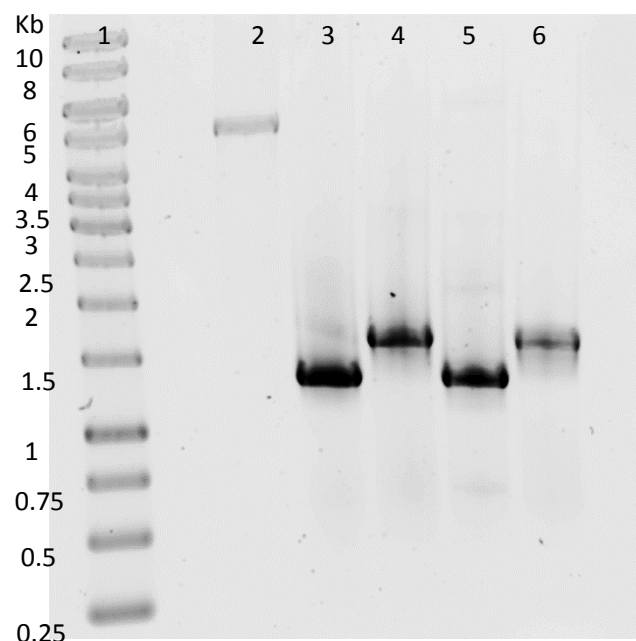


Figure 56. Low melt agarose (1% w/v) gel electrophoresis of restriction enzymes double digested plasmids pET24 and target genes. Lane 1 is a 1 kb DNA ladder; lane 2, 10 μ l

plasmid pET24 double digested with *Nde*I and *Hind*III; lane 3, wild-type *phoA* gene without the signal sequence double digested with *Nde*I and *Hind*III (1.34 kb); lane 4, wild-type *phoA* gene with the signal sequence double digested with *Nde*I and *Hind*III (1.68 kb); lane 5, mutant 4-186 gene without the signal sequence double digested with *Nde*I and *Hind*III (1.34 kb); lane 6, mutant 4-186 gene with the signal sequence double digested with *Nde*I and *Hind*III (1.68 kb).

4.3.2 Confirmation of pET24/*phoA* and pET24/4-186 plasmids

The ligation mixture containing the newly formed plasmids were transformed into the host cells for replication. The same transformation method was used as described in Section 3.2.7. pET24 recombinant plasmids containing wild-type and mutant alkaline phosphatase genes were transformed into DH5 α and BL21(DE3) competent cells.

As described in Section 1.5.2.2, during the molecular cloning, typically the target gene is first cloned into a cloning vector and then transferred to an expression vector [210] to ensure the accuracy of the cloned gene. A similar procedure is performed when transforming the recombinant plasmids into bacterial cells. The recombinant plasmids are transformed into bacterial host for cloning first to produce high copies of recombinant plasmids, then retransformed into a bacterial host for protein expression [215]. This strategy ensures the stability of the plasmids. In this study, DH5 α was chosen as the cloning host. As described in Section 3.3.7, DH5 α is a convenient host for cloning and maintaining recombinant plasmids due to the deletion of *recA* and *endA* gene [319], and high transformation efficiencies and good plasmid yields [324].

After transforming the plasmids pET24/*phoA* and pET24/4-186 into competent cells, the successful recombinants were screened on the LB Kan BCIP selective plate (Table 17). More than 30 white colonies were grown on each plate, but no blue colonies were found on plates with DH5 α containing plasmid pET24/*phoA* and pET24/4-186 after 48 hours of culture. This may due to a very low level of background protein expression in the DH5 α , which lacks the gene for T7 RNA polymerase in its chromosome to initiate protein expression.

Single white colonies on these plates were picked and cultured to isolate the plasmids. After restriction enzyme digestion, the plasmids in these colonies were verified by agarose gel electrophoresis. Figure 57 gives an example of some plasmids isolated from single colonies grown on the selective plates with DH5 α containing recombinant plasmids pET24/wild-type *phoA* gene with and without the signal sequence. Two out of five successful recombinants were found in the pET24/wild-type *phoA* gene without the signal sequence group (Lane 3 and 5, 6.64 kb), while one out of five successful recombinants was found in the pET24/wild-type *phoA* gene without the signal sequence group (Lane 11, 6.98 kb). A signal band of 5.3 kb was found in Lane 2, 4, 6, 8, 9, and 10.

Strain vector/target gene	Colony number
DH5 α pET24/ SS+ <i>phoA</i>	61
DH5 α pET24/ SS+ 4-186	67
DH5 α pET24/ <i>phoA</i>	35
DH5 α pET24/ 4-186	32
DH5 α pET24/His-tag + SS + <i>phoA</i>	59
DH5 α pET24/His-tag + SS + 4-186	53
DH5 α pET24/His-tag + <i>phoA</i>	38
DH5 α pET24/His-tag + 4-186	41
DH5 α pET24	70
DH5 α	3

Table 17. Single colony numbers on the LB Kan BCIP selective plate with transformed cells after 48 hours of culture. SS represents signal sequence.

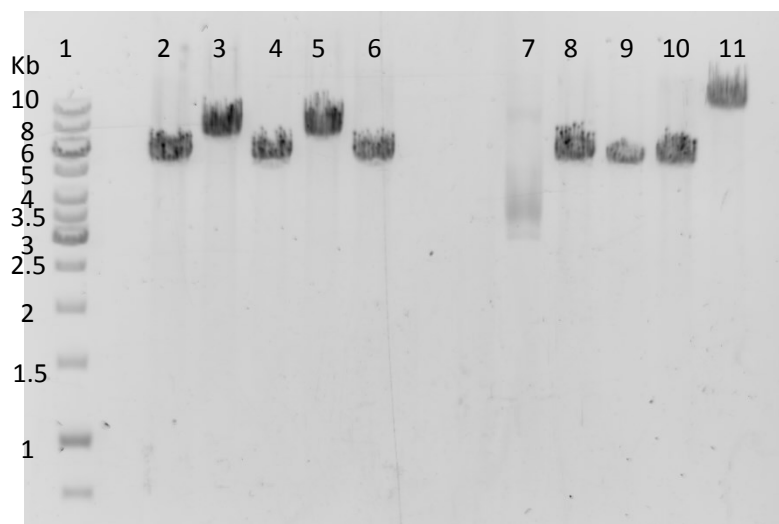


Figure 57. Agarose (1% w/v) gel electrophoresis of restriction-enzyme digested plasmids isolated from single colonies grown on the LB Kan BCIP selective plates. Lane 1 is a 1 kb DNA ladder; lane 2-6, single digested with *NdeI* of 5 plasmids isolated from 5 colonies on the plate with DH5 α pET24/wild-type *phoA* gene without the signal sequence; lane 7-11, single digested with *NdeI* of 5 plasmids isolated from 5 colonies on the plate with DH5 α pET24/wild-type *phoA* gene with the signal sequence.

There are several possible reasons for this low recombinant rate. First, it could be insufficient digestion performed before ligation so that some of the pET24 plasmids were recombined without the insertion of the target ALP gene. 70 colonies were found on the pET24 plasmid control plate, where no colonies should grow if the digestion was complete. The blurred band shown on Lane 7 was further proofed that the digestion was not completely effective. Because only one band (5.3 kb) can be seen when the pET24 was double digested with *NdeI* and *HindIII*, with the other DNA fragment (70 bp) being too small to detect on the gel, the digestion results were difficult to determine by agarose gel electrophoresis. A possible solution was to use new enzymes that show more digestion ability and can perform well in the same digestion conditions during double digestion. The second possible reason to explain the low recombinant rate was due to the ligation enzyme and conditions. The ligation was carried out with the Rapid DNA Ligation Kit and finished after 5 min of incubation. Although the procedure strictly followed the protocol of the kit, the optimised conditions of ligation for pET24 vector and the target DNA fragments were not achieved. To solve this problem, a new T4 DNA ligase and buffer were purchased, and new ligation condition of overnight incubation at 16°C was carried out. Third, the

transformation efficiency of the competent cells was not high because some colonies without plasmids were found by the agarose gel analysis. DH5 α competent cells used in this study were prepared by calcium chloride (CaCl₂) treatment in the laboratory. In order to achieve a high transformation efficiency of the competent cells, BL21(DE3) competent cells were purchased. Finally, the kanamycin concentration (10 μ g/ml) in the selective plate was not high enough to inhibit the growth of DH5 α bacterial cells without the plasmid. In the following studies, 30 μ g/ml kanamycin was used in the selective plates and LB Kan media.

4.3.3 Verifying the recombinant *E. coli* His-tag ALP expression

Successful recombinant plasmids containing the correct target ALP gene were isolated, and transformed again into the expression host BL21(DE3). The transformants were firstly screened by the LB Kan BCIP IPTG selective plate (Figure 58, Table 18). At the beginning, the LB Kan BCIP selective plates used for DH5 α recombinants screening were used in this study. However, no blue colonies were found after 48 hours of culture even though the agarose gel electrophoresis analysis of the plasmids from white-colonies showed a positive result. Considering the pET system is an IPTG inducible expression system, 0.5 mM IPTG was added into the agar plate to induce recombinant protein expression. Figure 58 shows the LB Kan BCIP IPTG plate with blue colonies of BL21(DE3) pET24/4-186 after culturing 24 hours in an incubator at 37°C. White colonies were found on the plate after 8 hours culture, after which they gradually turned blue. Within 24 hours of culture, the blue colour became darker with time. Similar to the process of screening *E. coli* pEK48 recombinants, blue colonies developed on the selective plate indicating the expression of His-tag ALP from pET24 recombinants.

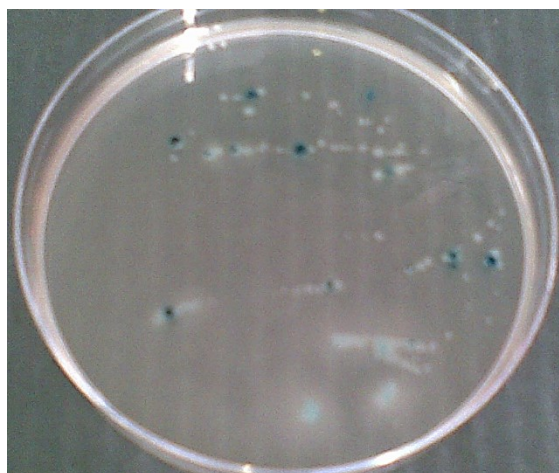


Figure 58. Image of LB Kan BCIP IPTG selective plates grew with BL21(DE3) pET24/4-186 after 24 hours incubating at 37°C. All colonies grew on the selective plate indicate they contain the correct pET24/4-186 plasmids. The blue colonies indicating they are expressing active *E. coli* His-tag ALP.

According to Table 18, fewer white colonies were found compared to Table 17. In addition, no colonies were grown in the control groups of BL21(DE3) pET24 and BL21(DE3). These indicate that the double digestion performed with the new enzymes was successful. The BL21(DE3) competent cells have higher transformation efficiency than DH5 α . The higher kanamycin concentration of 30 $\mu\text{g/ml}$ successfully inhibited the growth of non-plasmid bacterial cells. One important point was that all host cells with a non-signal sequence ALP gene in the plasmids were white-coloured colonies. That means that although the plasmids were successfully transformed in the protein expression hosts, no active ALP was expressed from the bacteria after 24 hours of culture. The result indicates that the signal sequence of *phoA* gene is essential for the expression of the protein. Therefore only the plasmids with the signal sequence were studied in the later research.

Strain vector/target gene	Blue/white colony number
BL21(DE3) pET24/ SS+ <i>phoA</i>	5/30+
BL21(DE3) pET24/ SS+ 4-186	3/30+
BL21(DE3) pET24/ <i>phoA</i>	0/20+
BL21(DE3) pET24/ 4-186	0/30+
BL21(DE3) pET24/His-tag + SS + <i>phoA</i>	2/20+
BL21(DE3) pET24/His-tag + SS + 4-186	2/20+
BL21(DE3) pET24/His-tag + <i>phoA</i>	0/20+
BL21(DE3) pET24/His-tag + 4-186	0/20+
BL21(DE3) pET24	0/0
BL21(DE3)	0/0

Table 18. Single colony numbers on the LB Kan BCIP IPTG selective plate with transformed cells after 48 hours of culture. SS represents signal sequence.

Single blue colonies on these plate were picked and cultured to isolate the plasmids. After restriction enzyme digestion, the plasmids in these colonies were verified by agarose gel electrophoresis. Figure 59 gives an example of pET24/ SS+ *phoA* and pET24/ SS+ 4-186 plasmids isolated from single colonies grown on the selective plates with BL21(DE3). A clear single band of 6.98 kb was shown when the plasmids pET24/ SS+ *phoA* (Lane 2 and 4) and pET24/ SS+ 4-186 (Lane 6) were single digested with the restriction enzyme *Nde*I; two bands were shown when the plasmids pET24/ SS+ *phoA* (Lane 3 and 5) and pET24/ SS+ 4-186 (Lane 7) were double digested with the restriction enzymes *Nde*I and *Hind*III. The upper bands of 5.3 kb represent the pET24 vector gene, the lower bands of 1.68 kb were either the wild-type or the mutant ALP gene with the signal sequence. The agarose gel electrophoresis analysis indicates that all of blue colonies grown on the selective plates contain the correct recombinant plasmids. In order to further verify the accuracy of the recombinant plasmids, several rounds of DNA sequencing were performed by GATC Biotech Ltd. The wild-type and mutant ALP gene were exactly the same as shown in Section 3.3.1. The target gene was accurately inserted into the cloning/expression region in the pET24 vector as expected.

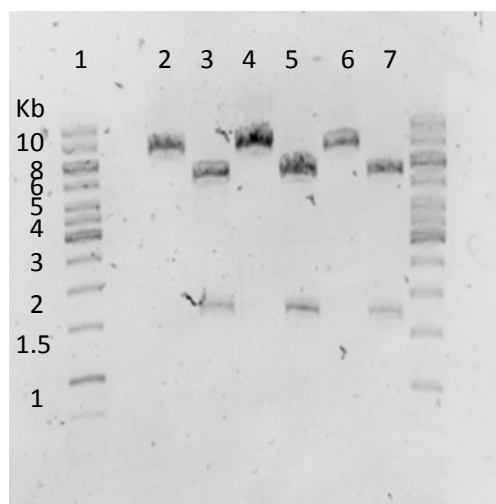


Figure 59. Agarose (1% w/v) gel electrophoresis of restriction enzyme-digested plasmids which were isolated from single colonies grown on the LB Kan BCIP IPTG selective plates. Lane 1 is a 1 kb DNA ladder; plasmid isolated from a blue colony containing the pET24/phoA gene with signal sequence either single digested with *Hind*III (lane 2), or double digested with *Nde*I and *Hind*III (lane 3); plasmid isolated from another blue colony containing the pET24/phoA gene with signal sequence either single digested with *Hind*III (lane 4), or double digested with *Nde*I and *Hind*III (lane 5); plasmid isolated from a blue colony containing the pET24/4-186 gene with signal sequence either single digested with *Hind*III (lane 6), or double digested with *Nde*I and *Hind*III (lane 7).

Cell lysates of BL21(DE3) pET24/phoA and BL21(DE3) pET24/4-186 with and without 5 hours IPTG induction in LB Kan media were analysed by SDS-PAGE and western blotting (Figure 60). On the SDS-PAGE gel (Lane 1-5), each sample shows many bands, which is similar as SM547 pEK48. The *E. coli* His-tag ALP showed a band at 53 kDa. The result indicates that *E. coli* His-tag ALP is not the main protein produced in BL21(DE3) even after 5 hours induction by 1 mM IPTG. The difference between the mutant (Lane 2 and 3) and wild-type (Lane 4 and 5) His-tag ALP produced from *E. coli* BL21(DE3) expression system was insignificant. However, for both types of the His-tag ALP, protein bands were visibly darker after IPTG induction (Lane 2 and 4) compare to the non-IPTG groups (Lane 3 and 5). This means the protein expression amounts in the IPTG induction groups were higher than the non-IPTG groups. However, when the SDS gel transferred to a nitrocellulose membrane and reacted with anti-alkaline phosphatase antibody, only one strong and clear band of *E. coli* His-tag ALP was observed in all groups (Lane 6-9). The western blotting result confirmed the expression of *E. coli* His-tag ALP from BL21(DE3). Unlike the SDS-PAGE result, similar bands were shown in the western

blotting among 4 groups. No difference was found between the IPTG induction groups and non-IPTG groups. This may be because of the improper induction conditions. More induction studies were carried out to optimise protein expression.

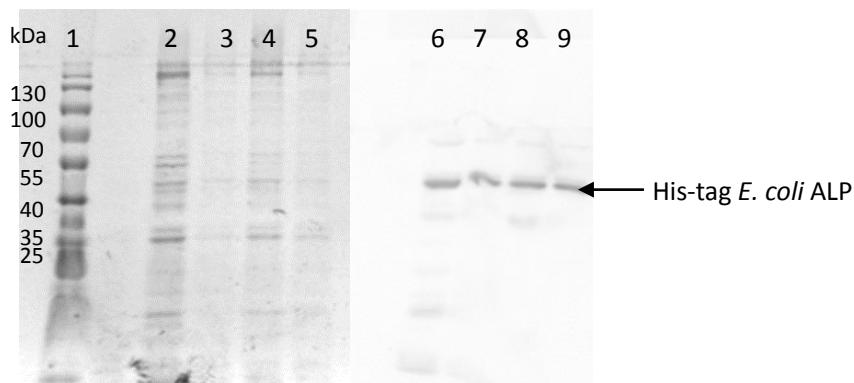


Figure 60. SDS-PAGE (8% w/v) gel electrophoresis (lane 1-5) and western blotting (lane 6-9) of cell lysates of BL21(DE3) pET24/4-186 and BL21(DE3) pET24/phoA with and without 5 hours 1 mM IPTG induction in LB Kan media at 37°C. Samples were electrophoresed in a SDS gel, then transferred to a nitrocellulose membrane and detected with an anti-alkaline phosphatase antibody. Lane 1, ladder; lane 2, cell lysates of BL21(DE3) pET24/4-186 after 5 hours IPTG induction on gel; lane 3, cell lysates of BL21(DE3) pET24/4-186 on gel; lane 4, cell lysates of BL21(DE3) pET24/phoA after 5 hours IPTG induction on gel; lane 5, cell lysates of BL21(DE3) pET24/phoA on gel; lane 6, western blotting analysis of cell lysates of BL21(DE3) pET24/4-186 after 5 hours IPTG induction; lane 7, western blotting analysis of cell lysates of BL21(DE3) pET24/4-186; lane 8, western blotting analysis of cell lysates of BL21(DE3) pET24/phoA after 5 hours IPTG induction; lane 9, western blotting analysis of cell lysates of BL21(DE3) pET24/phoA. SDS-PAGE gel was stained with Coomassie blue.

4.3.4 Optimising the expression of His-tag *E. coli* ALP

Protein expression from bacterial cells can be affected by many factors including the strength of the promoter, the stability of the vector in *E. coli* host, the properties of the foreign gene such as whether or not it contains rare codons, the location of the recombinant protein within the host cells, and the property of the recombinant protein such as location and the stability [341, 342]. In order to produce the wild-type and mutant His-tag ALP from the pET expression system in a soluble and active form, induction starting time, IPTG concentration, inducing time, glucose, and incubation temperature were optimised in this study. According to the pET System Manual, the

recommended protein expression conditions are: for pET24 plasmid system, 1 mM IPTG is added to the culture media when the absorbance at 600 nm (OD_{600}) of the culture medium reaches 0.6, followed by incubation at 37°C for another 3 hours before collecting the cell pellet [324]. Figure 61 shows SDS-PAGE gel electrophoresis result of the cell lysates of four different BL21(DE3) recombinants in this study with (Lane 9-15) and without (Lane 2-8) IPTG induction in LB Kan media under the recommended conditions. No difference was found among the groups with and without IPTG induction. Similar to Figure 60, many bands were shown on the gel, however, the His-tag ALP was not the main protein expressed by the host bacterial cells. The results indicate that the induction of the recombinant protein from the pET expression system under the recommended conditions was not successful. This may because of the 3 hours of induction time was too short for His-tag ALP expression, or the other induction conditions were not optimal.

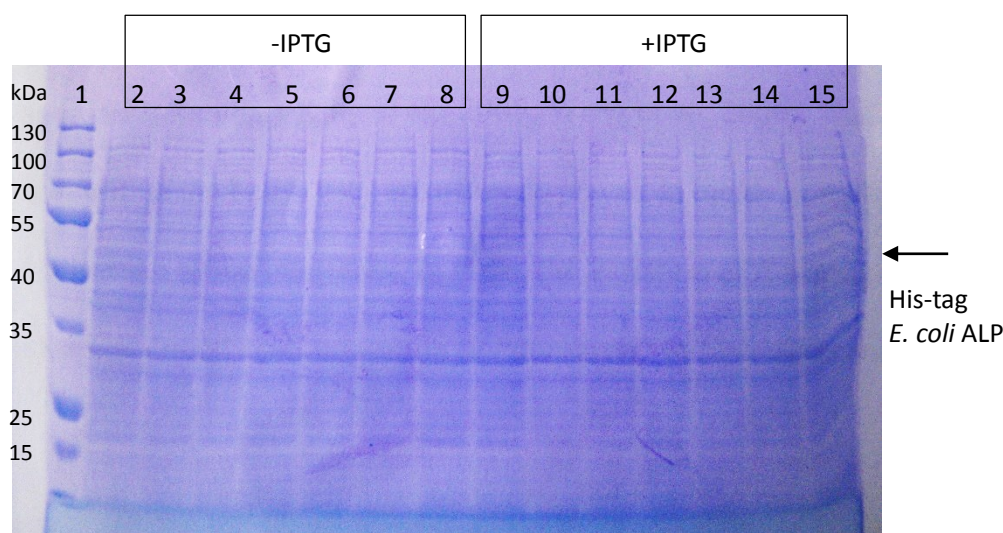


Figure 61. SDS-PAGE (8% w/v) gel electrophoresis of cell lysates of BL21(DE3) pET24/4-186 and BL21(DE3) pET24/phoA with (Lane 9-15) and without (Lane 2-8) 3 hours 1 mM IPTG induction in LB Kan media at 37°C. Lane 1, ladder; lane 2, 3, 9, and 10, cell lysates of BL21(DE3) pET24/phoA with signal sequence; lane 4, 5, 11, and 12, cell lysates of BL21(DE3) pET24/4-186 with signal sequence; lane 6, 7, 13, and 14, cell lysates of BL21(DE3) pET24/ His-tag phoA with signal sequence; lane 8 and 15, cell lysates of BL21(DE3) pET24/ His-tag 4-186 with signal sequence. SDS-PAGE gel was stained with Coomassie blue.

The bacterial cell growth curve was measured after both 30°C and 37°C incubation to determine the best induction starting time (Figure 62). The reason to lower the incubation temperature was to slow down the speed of protein expression so that the

protein can be folded correctly and produced in the soluble, active form. The best induction starting time should be in the exponential phase of growth [343, 344]. If the inducer is added too early in the culture medium, it may affect the growth of cells; but if too late, the protein expression cannot be induced anymore.

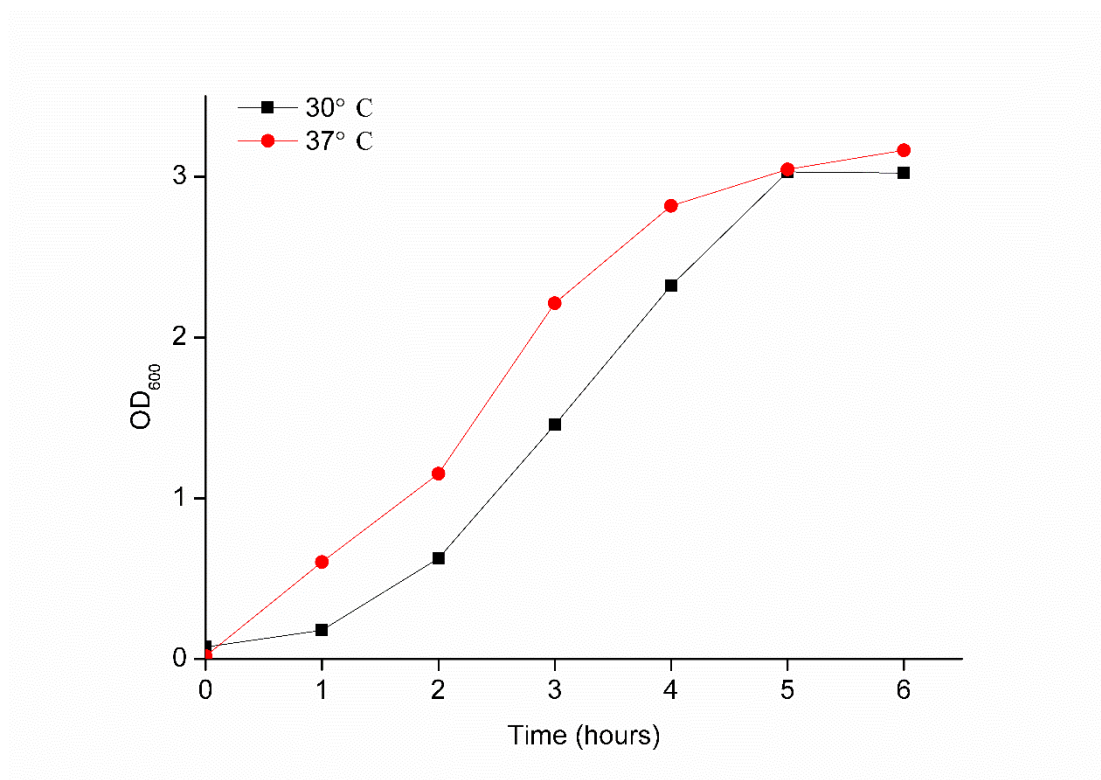


Figure 62. The growth curve of BL21(DE3) bacterial cells containing pET24 recombinant plasmids incubated in LB Kan media at 30°C and 37°C.

According to Figure 62, a higher temperature of 37°C promotes more cell growth compared to 30°C. When the BL21(DE3) cells were incubated at 37°C, the OD₆₀₀ increased quickly in the first 4 hours. The exponential phase started when OD₆₀₀ reached 1.1, and finished when the OD₆₀₀ reached 2.8. After that, the cell growth became slower and entered the stationary phase. For the cells grown at 30°C, a lag phase of 2 hours was observed. The exponential phase began when the OD₆₀₀ reached 0.7, and finished when the OD₆₀₀ reached 3.0, which was similar to the cells incubated at 37°C. The growth curve result indicates that the recommend induction starting time of OD₆₀₀ 0.6 is not suitable for the BL21(DE3) bacterial cells containing pET24 recombinant plasmids. In the following studies, the IPTG was added when the absorbance at 600 nm of the culture medium reached 1.0.

A time course induction study was carried out to determine the best inducing time [345]. 0.05% glucose was added into the culture media to reduce the non-induction protein expression. Figure 63A shows the OD₆₀₀ of BL21(DE3) pET24/His-tag 4-185 with the signal sequence incubated at 30°C in LB Kan medium after IPTG induction. Figure 63B shows SDS-PAGE gel electrophoresis of the cell lysates of BL21(DE3) pET24/His-tag 4-185 with the signal sequence after 0.5 hours to overnight induction by 1 mM IPTG.

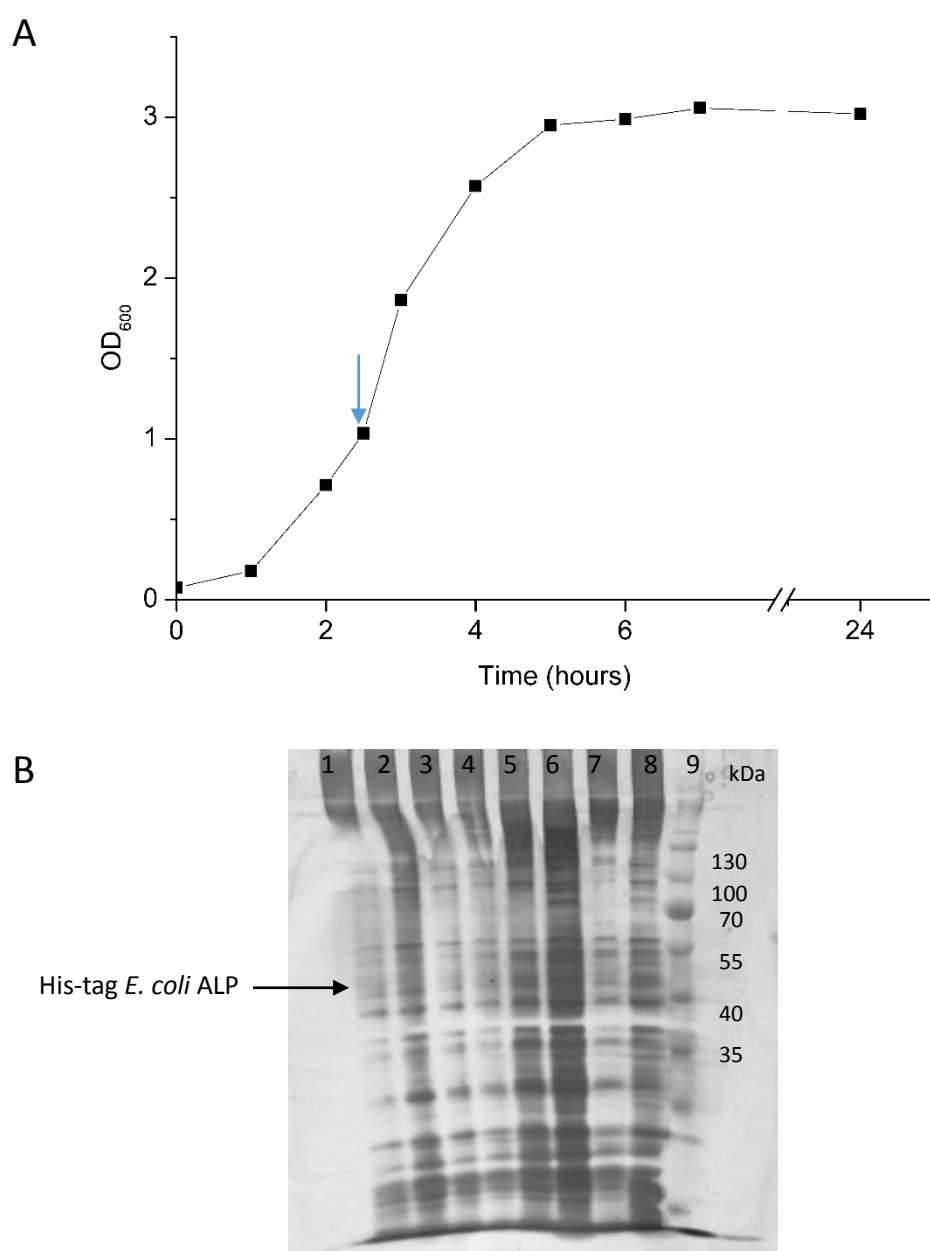


Figure 63. The time course induction study of BL21(DE3) pET24/His-tag 4-185 with the

signal sequence incubated at 30°C in LB Kan medium after 1 mM IPTG induction. (A) The absorbance at 600 nm of the culture medium changes with incubation time. The blue arrow indicates the induction starting time. (B) SDS-PAGE (8% w/v) of cell lysates of samples during the time course of induction. Lane 1, 0.5 hours after induction; lane 2, 1.5 hours after induction; lane 3, 2.5 hours after induction; lane 4, 3.5 hours after induction; lane 5, 4.5 hours after induction; lane 6, overnight induction; lane 7, 4.5 hours without induction; lane 8, overnight culture without induction; lane 9, ladder. The gel was stained by silver stain kit.

The results of the time course induction study indicate that the BL21(DE3) cells with the pET24 recombinant plasmids show a similar growth curve with and without IPTG induction. After 4.5 hours of induction, the His-tag ALP started to accumulate when the cell entered into the stationary phase. A higher level of protein expression of the His-tag ALP was achieved after overnight incubation at 30°C in LB Kan medium compare to a shorter incubation time and non-incubation groups.

4.3.5 ÄKTA explorer purification of *E. coli* His-tag ALP

Similar to *E. coli* ALP, His-tag *E. coli* ALP was purified on the ÄKTA explorer purification system with a HiTrap Ni Sepharose High Performance chromatography column. However, fewer purification steps were needed because of the strong and specific binding between the His-tag and the column. Figure 64 shows one example of ÄKTA explorer purification analysis of *E. coli* His-tag ALP expressed from BL21(DE3) pET24/4-186. Similar to Figure 41, temperature, pH, conductivity, and UV₂₈₀ were shown on the figure. The OD₂₈₀ detector shown that exclusive proteins were washed off from the column. However, only a small peak was detected in the linear gradient step, which should be the His-tag ALP. The corresponding fractions of pure His-tag *E. coli* ALP were collected and identified by electrophoresis and western blotting (Figure 65), and characterised by biochemical assays.

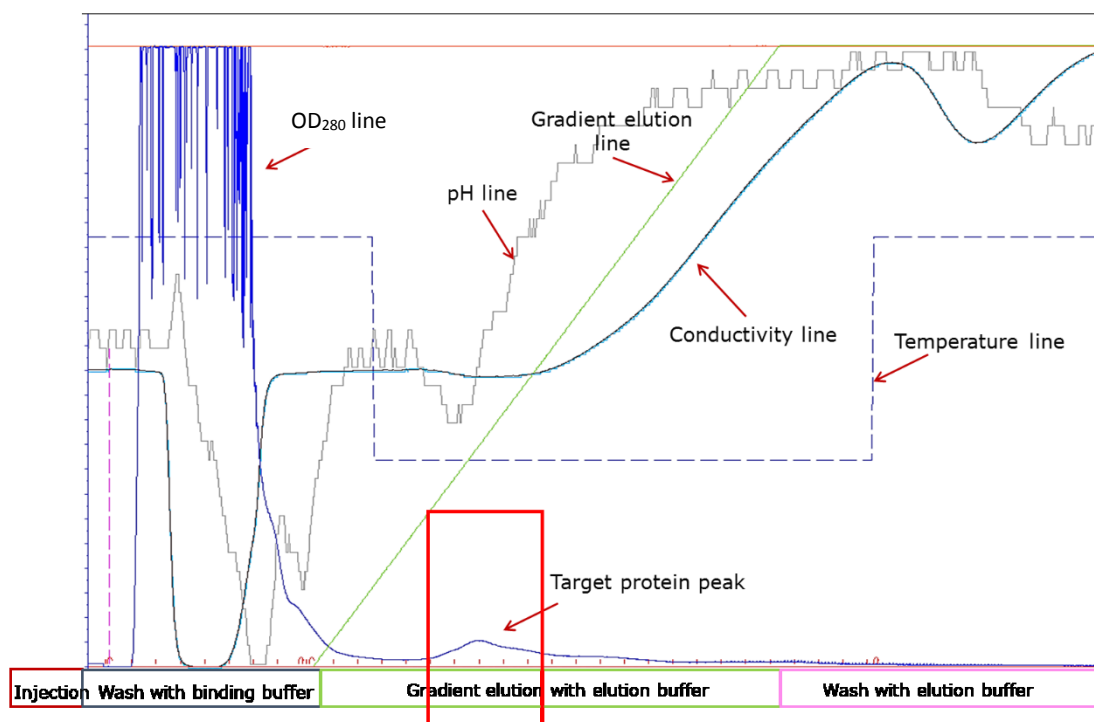


Figure 64. ÄKTA explorer purification analysis of *E. coli* His-tag ALP expressed from BL21(DE3) pET24/4-186. A peak was shown on the OD₂₈₀ line (solid blue line) in the linear gradient step (shown in the red box).

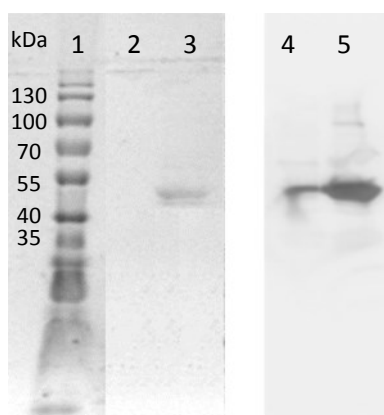


Figure 65. SDS-PAGE (8% w/v) gel electrophoresis (lane 2 and 3) and western blotting (lane 4 and 5) of purified *E. coli* His-tag ALP after ÄKTA explorer purification system. Samples were electrophoresed in an SDS gel, then transferred to a nitrocellulose membrane and reacted with anti-alkaline phosphatase antibody. Lane 1, ladder; lane 2, *E. coli* His-tag ALP expressed from BL21(DE3) pET24/phoA; lane 3, *E. coli* His-tag ALP expressed from BL21(DE3) pET24/4-186; lane 4, western blotting analysis of *E. coli* His-tag ALP expressed from BL21(DE3) pET24/phoA; lane 5, western blotting analysis of *E. coli* His-tag ALP expressed from BL21(DE3) pET24/4-186. SDS-PAGE gel was stained with Coomassie blue.

Because the BL21(DE3) cell pellet after induction was disrupted by sonication, massive cell proteins were released in the cell lysate. These non-His-tag fusion proteins were

washed away from the column and showed high protein peaks. Only the target His-tag ALP was bound on the HiTrap Ni Sepharose High Performance column and eluted by imidazole in the elution step. The peak of the target His-tag protein was significantly lower compared to the non-His-tag fusion proteins produced in the cells. These results were in accordance with the SDS-PAGE electrophoresis analysis. The His-tag ALP concentration in the target peak fraction was too low to show on the gel (under 10 µg/ml) so that several batches of peak fractions were combined and loaded on the SDS-PAGE gel in Figure 65. The *E. coli* His-tag ALP showed a weak band at 53 kDa. The result indicates the purification was successful. The western blotting result confirmed the expression of *E. coli* His-tag ALP from BL21(DE3).

The ALP activity was measured during the purification process. Figure 66 compares the ALP specific activity of both wild-type and mutant His-tag ALP before and after purification. All groups show ALP specific activities in the supernatant of cell lysates before purification. There is no significant difference between the wild-type and mutant His-tag ALP before or after IPTG induction. The results indicate that the outstanding high ALP specific activity of the mutant ALP is lost after engineering. Brunel and Cathala found that histidine inhibits the activity of mammalian ALPs [346]. 56% of enzyme activity of brain ALP was inhibited by 5 mM histidine in the presence of Mg^{2+} [347]. Therefore the reduction of the activity may relate to the His-tag added on the protein. In addition, after the purification through ÄKTA explorer purification system, no ALP specific activity was found in the His-tag ALP fractions, neither in the flow-through nor other fractions. This means the His-tag ALP was either stuck in the column or denatured during the purification process. Imidazole in the elution buffer was reported to strongly inhibit the activity of mammalian ALP isolated from brain, kidney, liver, bone, lung and spleen, and slightly inhibit the placental and intestinal ALPs [346, 347]. 36% and 50% of the enzyme activities of ALP purified from rat uterine deciduomata and brain were inhibited by 5 mM imidazole, respectively [347, 348]. The mechanism of the inhibition may relate to the chelation of Mg^{2+} in the system [347]. Mg^{2+} is believed to play essential role to maintain the activity of ALPs. A mutant *E. coli* ALP containing a substitution of aspartic acid into histidine at the 153 position, which is an indirect ligand of the Mg^{2+} , was shown to be remarkably similar to mammalian

ALP properties such as the activity depending on the presence of Mg^{2+} [349]. Due to similarity of the structures of the *E. coli* and the mammalian ALPs in the active site region [168], the activity of *E. coli* His-tag ALP produced in this study may be inhibited in the same manner as the mammalian ALPs.

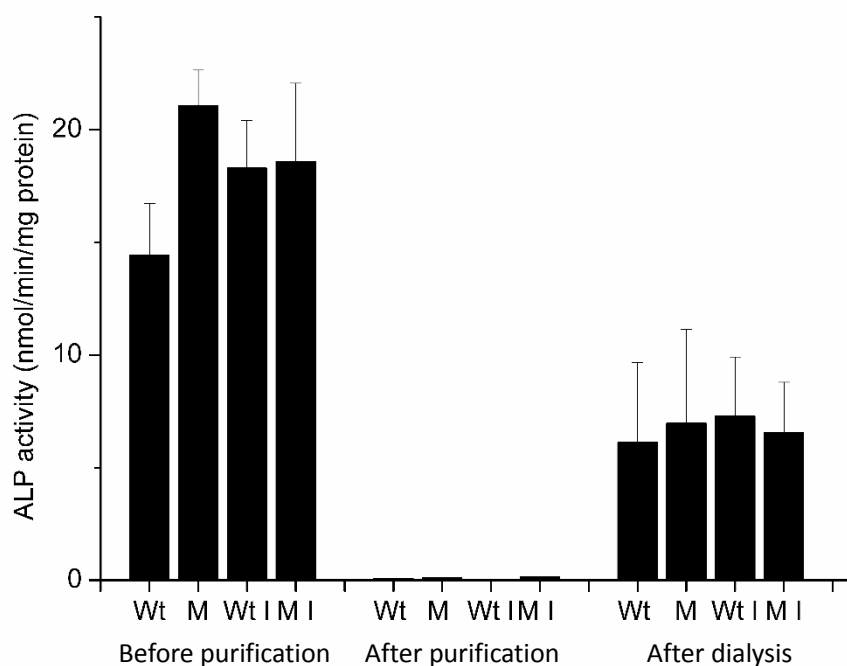


Figure 66. ALP specific activity of mutant and wild-type His-tag ALP before and after purification by the ÄKTA explorer purification system, and after dialysis against TMZP buffer (see Appendices) for 12 hours. Wt represents wild-type His-tag ALP; M represents mutant His-tag ALP; I represents 1mM IPTG inducer.

4.3.6 Troubleshooting of *E. coli* His-tag ALP

Table 19 summaries all troubleshooting and some attempts to construct and produce *E. coli* His-tag ALPs. Most of the solutions were described in the chapter. The heat pre-treatment of the supernatant at either 65°C or 80°C for 30 min before column purification was attempted to improve the protein recovery rate.

Problem	Reason	Solution
Unknown products after PCR	Improper PCR conditions	Using a touch-down program instead of standard PCR
Low PCR recovery rate from agarose gel	Incomplete dissolution of the gel	Low melt agarose gel
Low ligation rate	• Incomplete digestion • Improper ligation conditions	• New restriction enzymes • New ligation enzyme and conditions
Low transformation rate	Low efficiency of competent cells	Purchasing competent cells instead of laboratory-made
Unexpected colonies on the control plates	Low antibiotic concentration	Increasing Kan from 10 µg/ml to 30 µg/ml
No blue colonies on the selective plates	No recombinant protein expression	Adding 0.5 mM IPTG to induce protein expression
Low protein induction level after IPTG treatment	Improper induction conditions	• Optimising the IPTG concentration from 0.2 mM to 1 mM • Incubation temperature at 30°C or 37°C • Optimising the induction starting time from OD₆₀₀ 0.6 to 1.0 • Inducing time from 0.5 hour to overnight
Low purification recovery	Massive unwanted bacterial proteins in the cell lysates	Heating the supernatant at either 65°C or 80°C for 30 min before column purification

Table 19. Main problems, possible reasons, and solutions in the construction, production and purification of recombinant *E. coli* His-tag ALP in bacterial cells.

This purification pre-treatment is based on the fact that ALPs are thermostable enzymes which can maintain their biological activity at 50 °C for 60 min and retain more than 90% of their original activity for 120 min [350]. *Thermus caldophilus* GK24 ALP was purified from *E. coli* after the heat treatment of 80°C for 30 min [323]. A hyperthermophilic alkaline phosphatase was purified from *Thermotoga neapolitana* by incubating at 100°C [351].

4.4 Conclusion

The wild-type and mutated recombinant *E. coli* His-tag ALP were successfully constructed with genetic cloning methods, produced through bacterial *E. coli* cells, and purified using ÄKTA explorer purification system to improve the yield, purification efficiency, and immobilisation properties. The induction conditions were optimised to enhance the protein expression. However, the mutated recombinant *E. coli* His-tag ALP lost its outstanding activity after the incorporation of a His-tag on either the N-terminal or C-terminal. In addition, the activities of both the wild-type and mutated recombinant *E. coli* His-tag ALP were inhibited by the imidazole in the elution buffer. Although the inhibitions were weaker after dialysis, the activities of the mutated recombinant *E. coli* His-tag ALP were still significantly lower than the mutated *E. coli* ALP described in Chapter 3. Without the high activity, the His-tag ALP is not worth studying further to combine with scaffolds for bone tissue engineering applications. That is why the protein induction conditions have not been completely optimised to achieve a mass production. Future studies may consider the mechanism of the effect of His-tag on *E. coli* ALP since the majority of the research was focused on the mammalian ALPs. The mutant *E. coli* ALP with other fusion tags such as T7 tag may be designed to improve the production, purification, and immobilisation, as well as to maintain the outstanding activity.

CHAPTER 5

Engineered ALP with improved functionality for bone tissue engineering applications

5.1 Introduction

ALPs play an important role in bone mineralisation and enhance bone formation by increasing the local concentration of inorganic phosphate, while decreasing the concentration of extracellular pyrophosphate. The mutated *E. coli* ALP with outstanding biological activity produced from the *E. coli* recombinant protein expression system in this study is potentially a superior candidate for bone tissue engineering. In order to use the ALPs, two bone tissue engineering strategies, one being a Ti implant with recombinant *E. coli* ALP coating, and the other being an ALP encapsulated PCL nanofibrous composite (ALP/PCL) scaffold, are described in this chapter. The motivation to develop ALP coated implants and ALP/PCL scaffolds is to accelerate mineral deposition and enhance implant fixation with the surrounding tissue by using the catalytic ability of ALPs (Figure 67). This chapter demonstrates the main concepts and design principles of the combination of ALPs with supporting biomaterials for bone tissue engineering applications.

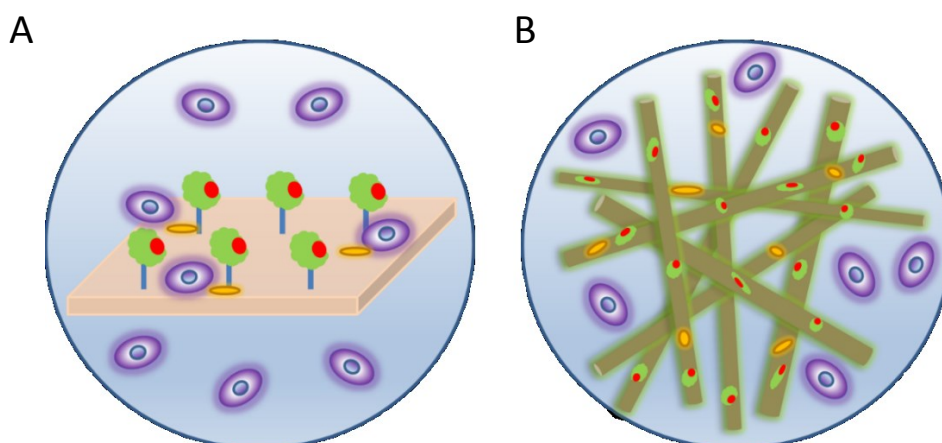


Figure 67. Concept of the development of bioactive metal implants (A) and polymer scaffolds (B) with ALPs for bone tissue engineering applications. Bioactive enzyme ALPs

(green with red active site) are either immobilised by covalent binding on implant surfaces (A) or co-electrospun with polymers (B). Immobilised ALPs collaborate with bone cells (purple) to produce mineral deposits (orange) on implant surfaces or electrospun fibres.

5.1.1 Bone substituting implants with ALP covalent coatings

As described in Section 1.3 and 2.1, Ti implants with bioactive molecule coatings facilitate the attachment of proteins and cells, maintain the native conformation and biology activity of proteins, and induce a specific cellular response to promote bone formation. In this study, ALPs were chosen as the bioactive molecule. Ti implants with ALPs coatings increase the local concentration of inorganic phosphate and accelerate mineral deposition to enhance implant fixation with the surrounding tissue.

A variety of immobilisation methods have been explored for binding ALPs onto Ti implants, such as adsorption, covalent binding, cross linking and chelation (Section 1.6.2). Cross linking immobilisation methods may cause significant changes to the active site and affect the biological activity of ALPs, which go against the purpose of using the superior activity of mutant *E. coli* ALP in bone tissue engineering. Similar to cross linking, chelation, which needs the His-tag ALP, was demonstrated to reduce the activity of the mutant. Therefore, covalent binding was chosen to immobilise ALPs onto Ti implants. Covalent binding provides high surface loading [352], low protein leakage [353], and most importantly, a site-specific binding to control the orientation of the ALPs to cells [354]. Although a loss of protein activity was detected after covalent binding, the loss was dependent on the protein types [352] and reagents used during covalent binding [355]. For example, the enzyme ALP shows higher activity and enzyme loading in organic solvents such as toluene and acetone than aqueous solvents during silanisation; coupling chemistries glutaraldehyde and arylamine yield the highest active loading by using the aqueous silanisation method [353]. Several coupling techniques are available for attaching biomolecules to biomaterial surfaces, such as the use of silanes to modify the surface of Ti implants, and the deposition of thiols as self-assembled monolayers onto stable metals such as gold [354, 356]. In this

study, the Ti implant surface was first treated by silanisation to add amino groups to titanium oxide, and then covalently bound with ALPs (Figure 68).

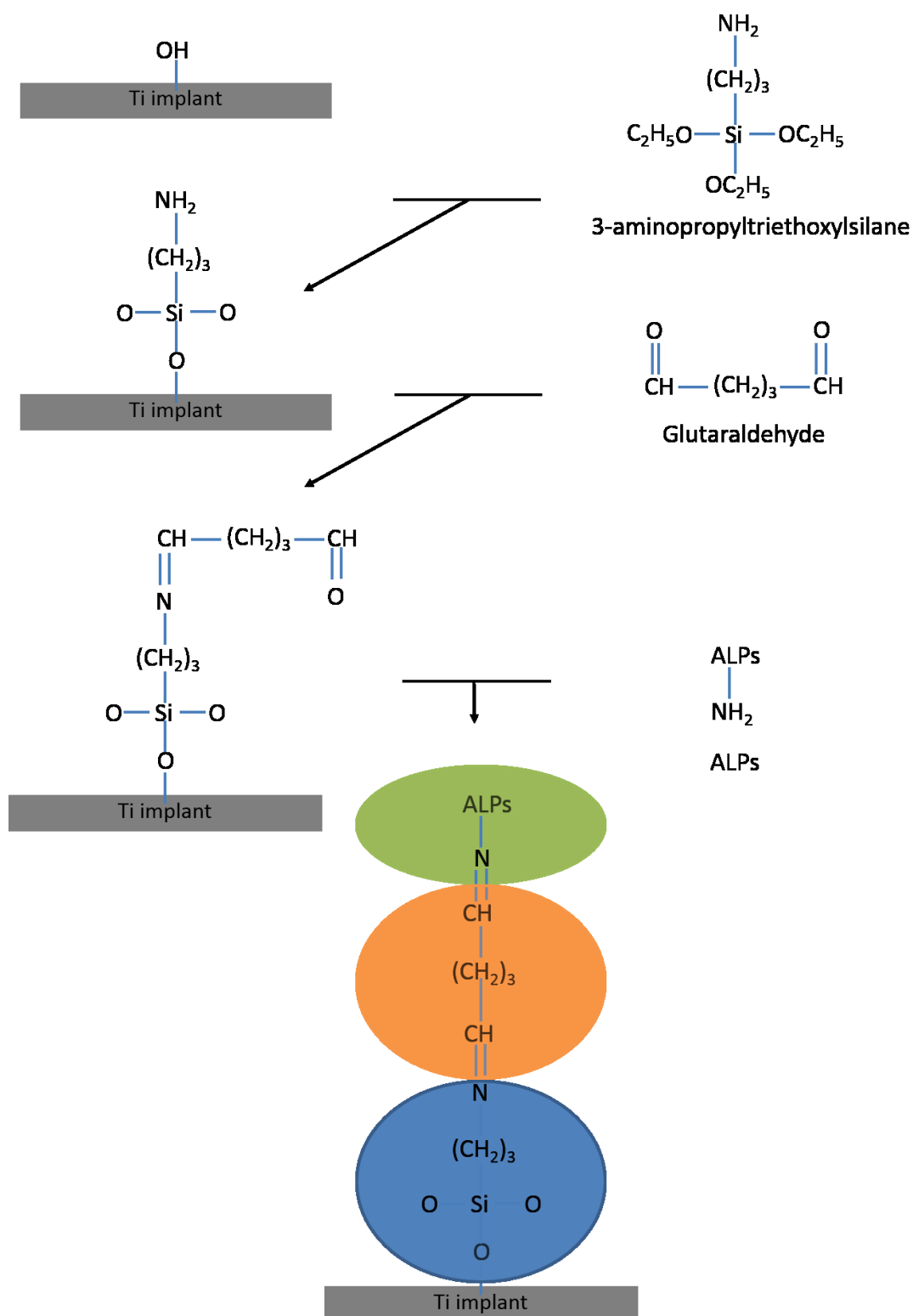


Figure 68. Schematic representation of immobilised ALPs on Ti implants by silanisation. Ti implant (grey) surface is modified by 3-aminopropyltriethoxysilane (APS, blue) to

add amino groups. A coupling agent glutaraldehyde (pink) is used to attach the terminal amino groups of ALPs (green).

There are several advantages of covalently binding ALPs to Ti implants with a silane complex [357]. First, this immobilisation method strengthens the cell adhesion *in vitro* [358]. Second, the activities of enzymes immobilised by this method were well-preserved, such as the enzymes trypsin [359] and ALPs [356, 360]. Third, the silane complex acts as a spacer between the biomolecules and the supporting materials. The spacer provides a flexible environment and enhance cell growth [354, 361]. ALPs immobilised by physical adsorption were used as a control to compare the immobilisation results.

5.1.2 Electrospinning PCL fibres with encapsulated ALPs

In addition to immobilisation of enzymes on biomaterial surfaces by adsorption and covalent binding, encapsulation of enzymes in the supporting nanofibre matrix has increasingly gained interest. Nanofibre matrices, which mimic the native structure of the extracellular matrix, range in diameter from 50 to 500 nm [362] and have great potential in bone tissue engineering. Nanoscaled structure plays important roles in cell behaviour. For example, osteoblast adhesion was enhanced by nanoscaled surfaces [363, 364]; in addition to osteoblast adhesion, cell proliferation, alkaline phosphatase activity, and ECM secretion were increased with decreasing diameter of nanofibres [365-367]. Various techniques have been developed to fabricate nanofibres including drawing, template synthesis, self-assembly, phase separation, and electrospinning (Table 20, [368, 369]). Among them the electrospinning technique is the most popular method because of its superior properties such as being simple, efficient, and its ability to encapsulate biomolecules by co-electrospinning.

	Advantage	Disadvantages	Fibre diameter
Drawing	Easy operation	Discontinues process	2-100 nm
Template synthesis	Repeatable and controllable	Need templates or moulds	100 nm
Self-assembly	Good for smaller nanofibres	Complex process, time-consuming	7-100 nm
Phase separation	Easy operation, controllable	Limited polymers, time-consuming	50-500 nm
Electrospinning	Cost effective, easy operation	Jet instability	3-1000 nm

Table 20. Main features of techniques to fabricate nanofibres.

This enzyme immobilisation method has several advantages [98, 99, 370]: 1) rather than the complex chemical modification process of covalent binding, electrospinning provides a simple and straightforward way to encapsulate enzymes; 2) the enzyme loading in the matrix can be further increased compared to covalent binding (up to 50% of the fibres) [371]; 3) because of the adjustable nanofibre-porosity structure, a huge surface area can be easily achieved to maximise the protein and cell binding sites; nanostructure also decreases the mass transfer limitation and retains the catalytic activity of the enzymes; 4) more than one kind of biomolecule can be encapsulated simultaneously in the same nanofibres; 5) different from the Ti implants, biodegradable polymers such as poly(lactic acid) (PLA), poly(ethylene oxide) (PEO), and poly(ϵ -caprolactone)(PCL) are used to fabricate the scaffold matrix, which is more desirable than the non-degradable metal implants in bone tissue engineering applications [369, 370, 372].

In this study, PCL is chosen as the active matrix material to encapsulate the enzyme ALPs. As described in Section 1.3.3, PCL is a FDA-approved candidate and has great potential for bone tissue engineering applications due to its superior properties, such as its stability, affordability, biocompatibility, and biodegradability [373, 374]. PCL

nanofibres encapsulated with proteins by electrospinning has gained increasing attention as a promising approach to fabricate bone tissue engineering scaffolds [98, 99, 370]. High protein loading and high biological activity from the protein/PCL scaffold have been achieved. In this study, bovine ALP as a model ALP was co-electrospun with PCL to fabricate a bioactive ALP/PCL scaffold for bone tissue engineering.

5.1.3 Scope of chapter

The objective of this chapter is to incorporate the biomolecules that have been studied in this thesis with different biomaterials to determine their functionality and biological efficiency. In this chapter, two applications of engineered ALP for bone tissue engineering are explored, including a Ti implant with recombinant ALP coating and an ALP/PCL scaffold.

5.1.4 Hypotheses

The hypotheses tested was that ALP can incorporate with different supporting materials, such as Ti implants and PCL scaffolds, and improve the functionality and biological efficiency in bone tissue engineering applications. In order to test these hypotheses, the following questions have been addressed:

- (1) How to prepare ALP coatings on Ti implants?
- (2) What is the biological activity of Ti implants with ALP coating?
- (3) What is the effect of ALP coating on soaking experiments?
- (4) How to prepare ALP/PCL scaffolds?
- (5) What is the biological activity of ALP/PCL scaffolds?
- (6) What is the effect of ALP/PCL scaffolds on soaking experiments?
- (7) What is the effect of ALP/PCL scaffolds on MC3T3-E1 pre-osteoblasts?

5.2 Materials and methods

5.2.1 ALP coated on Ti implants using silanisation method

5.2.1.1 Materials

Unless stated otherwise, all chemicals including 3-aminopropyltriethoxysilane (APS), acetone, glutaraldehyde, and bovine intestinal ALP were purchased from Sigma-Aldrich (Gillingham, UK). *E. coli* mutant and wild-type ALP were produced and purified from *E. coli* bacterial cells in this studies. Phosphate-buffered saline (PBS) and other soaking media were purchased from Gibco/Life Technologies, (Paisley, UK). A number of commercially available Ti alloy (grade 5, Ti 6AL/4V) discs (dimension 10 mm, height 3 mm) were purchased from Ti-Tek Ltd (Birmingham, UK)

5.2.1.2 Substrate preparation

In order to obtain a consistent and reproducible titanium oxide layer, Ti discs were first soaked in a 5.0 M NaOH solution at 60°C for 24 hours, and then rinsed with abundant distilled water. This step ensures all contaminants were removed from the Ti surfaces. In addition to the alkali oxidation treatment, a heat treatment of incubation Ti discs at 800°C for 6 hours was used to form an oxide layer on the Ti surface. Then the cleaned discs with uniform oxide layers were silanised by the following the procedure: 1) place the discs in acetone containing 10% (w/v) APS (pH 7.0) at ambient temperature for 4 hours; 2) dry the discs at 37°C for 2 hours; 3) immerse the discs in 1% (w/v) glutaraldehyde in PBS (pH 7.0) at ambient temperature for 4 hours; 4) wash the discs with abundant PBS to remove glutaraldehyde.

The used Ti discs were cleaned by the following procedure: 1) the discs were soaked in 2% HCl solution in the ultrasonic bath for 15 min, and rinsed twice with distilled water; 2) the discs were soaked in acetone in the ultrasonic bath for 15 min, and rinsed twice with distilled water; 3) the discs were soaked in isopropanol in the ultrasonic bath for 15 min, and rinsed twice with distilled water. Repeat step one once if

necessary.

5.2.1.3 ALP addition

Bovine intestinal ALP was used as a model enzyme to optimise the immobilisation conditions. Once the best immobilisation conditions were achieved, *E. coli* mutant and wild-type ALP were immobilised onto the Ti implants. The process of fabricating ALP coatings on Ti implants by silanisation is shown in Figure 69. Three bovine ALP solutions with different concentrations were prepared (10 µg/ml, 100 µg/ml, and 1000 µg/ml). After preparation, the Ti discs were placed in ALP solutions at 4°C on a shaker (50 rpm) overnight. The samples were rinsed 3 times with PBS, and transferred to a new 24-well plate for the ALP activity assay.

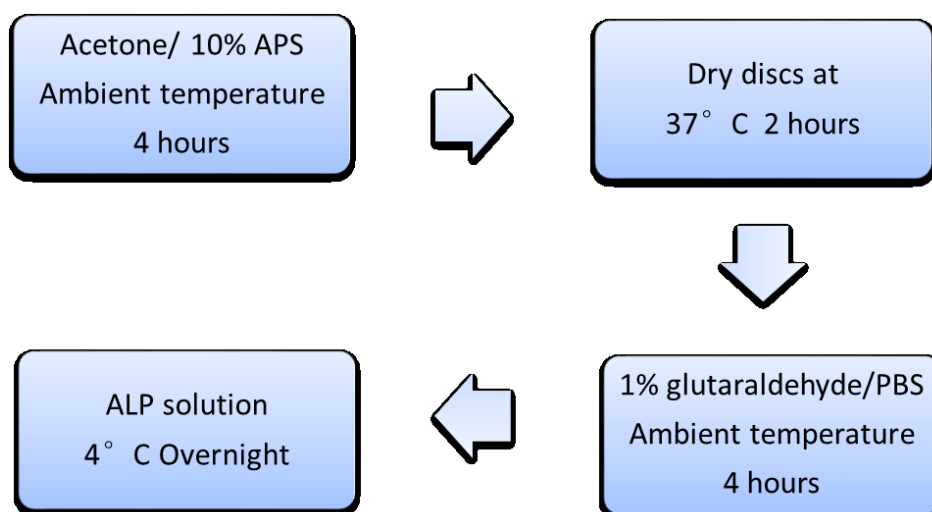


Figure 69. Treatment process of ALP coating on Ti implant by silanisation.

5.2.1.4 ALP specific activity measurement of ALP silane coating

The ALP activity assay depends on the hydrolysis of pNPP by the enzyme ALP to generate p-nitrophenol and inorganic phosphate. p-nitrophenol is converted to a yellow product under alkaline conditions and exhibits a high absorbance at 400-420 nm. The Ti discs with immobilised ALPs in 24-well plates were immersed in pNPP buffer solution and incubated at 37°C. The reaction was stopped by adding 1 M NaOH. The

reaction time was recorded. Light absorbance of these samples was measured at 405 nm on a 96-well plate reader. A standard curve was constructed using p-nitrophenol as a standard. Experiments were performed independently in triplicate, and the ALP activity of each sample was normalised to sample surface area and expressed as mean $\pm$ standard deviation.

5.2.1.5 Soaking experiment of ALP silane coating

The biological activity of the Ti implants with immobilised ALPs was determined *in vitro* by soaking experiments. *In vitro* soaking experiments in simulated body fluid (SBF) are commonly used as a standard procedure to predict the *in vivo* bone bioactivity of the bioactive implants [375]. The amount of calcium phosphate (CaP) deposition produced under *in vitro* conditions after soaking experiments indicates the potential bone formation ability *in vivo*. However, SBF does not contain any organic phosphates, which are the substrate of ALPs in the human blood plasma [53]. In order to better mimic the *in vivo* tissue conditions and take the advantage of the immobilised ALP coatings on Ti implants, a modified cell culture medium containing 10 mM organic phosphate β -glycerophosphate (β -GP) was used in the soaking experiments [53].

The discs with immobilised ALPs were placed in cell culture medium (α -Minimum Essential Medium (α -MEM) supplemented with 10% (v/v) FBS, 50 μ g/ml gentamycin, 50 μ g/ml ascorbic acid, 100 nM dexamethasone, and 10 mM β -GP), and incubated at 37°C. Non-coated Ti discs served as controls. The cell culture media were changed every 2-3 days. After 3, 7 and 14 days, the samples were removed from cell culture media, washed thoroughly first with PBS, then with distilled water, and dried at 40°C for 5 hours, and stored at -80°C until the deposited calcium was measured.

5.2.1.6 Deposited calcium measurement of ALP coating after soaking experiments

The deposited calcium on the Ti implants after soaking experiments was measured by a calcium assay using the o-cresolphthalein complexone (OCPC) procedure. This procedure is based on the reaction between the Ca^{2+} and the OCPC in an alkaline solution to form an intense violet coloured complex and show a strong absorbance at 577 nm [376-378]. The Ti implants with ALP coatings after soaking experiments were immersed in 0.5 N acetic acid overnight at ambient temperature on a shaker (50 rpm). The calcium deposition should dissolve in the acid solution, and react with working buffer containing OCPC at ambient temperature for 10 min. Light absorbance of these samples was measured at 577 nm on a 96-well plate reader. A standard curve was constructed using CaCl_2 as a standard. Experiments were performed independently in triplicate, and the calcium concentration of each sample was normalised to sample surface area and expressed as mean $\pm$ standard deviation.

5.2.2 ALP/PCL scaffold prepared by electrospinning

5.2.2.1 Materials

Unless stated otherwise, all chemicals including PCL (M_w 80000), acetone, dimethylformamide (DMF), and bovine intestinal ALP were purchased from Sigma-Aldrich (Gillingham, UK). Chloroform (99%) and Tris buffer were purchased from VWR (Poole, UK). All cell culture reagents, phosphate-buffered saline (PBS) and other soaking media were purchased from Gibco/Life Technologies, UK.

5.2.2.2 Electrospinning

Electrospinning is a polymer-processing technology known for more than 70 years to create fibres [367]. The principle of electrospinning is to use high voltage (10–20 kV) electrical charge to form solid fibres from liquid polymers [379]. The liquid polymers

are obtained either by melting the solid form, or dissolving in a solvent. Usually a two-dimensional non-woven film is produced from electrospinning, however, three-dimensional non-woven fibrous meshes can be obtained by increasing the processing time [367].

The setup of electrospinning is shown in Figure 70 [380]. There are mainly three parts: a syringe containing the liquid polymer, a high voltage supplier, and a cylinder collector covered with a grounded aluminum sheet. The nozzle of the syringe is connected to the high voltage supplier, and a high electric field is formed between the nozzle and the collector. A 10 cm distance between the nozzle and the collector was used in this study. This collector can move horizontally while rotating so that a homogenous film of nanofibres can be formed on the aluminum sheet.

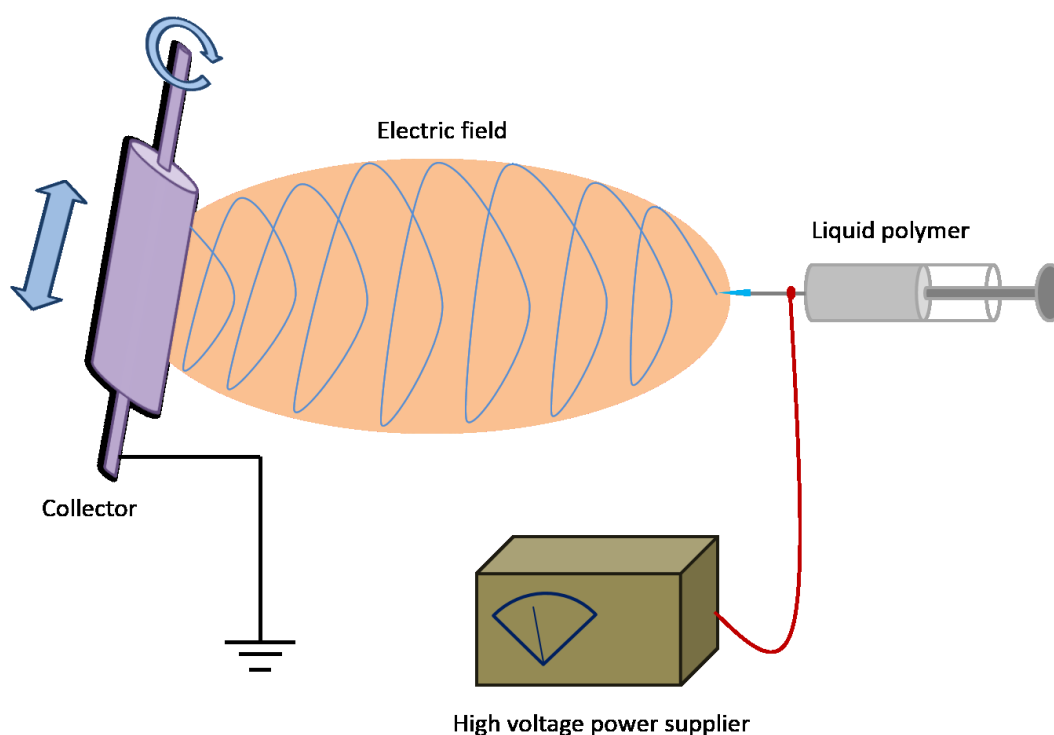


Figure 70. Schematic diagram to produce polymer nanofibres by electrospinning [380].

5.2.2.3 Preparation of ALP/PCL scaffolds

In this study, the ALP/PCL scaffolds were prepared by the following procedure (Figure

71). 12% (w/v) PCL was dissolved in solvent solution consisting of DMF and chloroform (v:v=2:3). At the same time, an ALP solution was prepared by dispersing different amount of ALP (10, 20, and 30% (w/w)) in 2 ml ethanol. The two solutions were mixed in a sonication bath for 15 min before poured into the syringe. To break up any aggregation, ALP was passed through the needle several times to make sure the ALP was well-dispersed. A flow rate of 2 ml/hour, and a voltage of 18 kV was used to fabricate ALP/PCL nanofibres. The electrospun ALP/PCL scaffolds were stored at 4°C before being cut into small pieces (diameter 10 mm) for further studies.

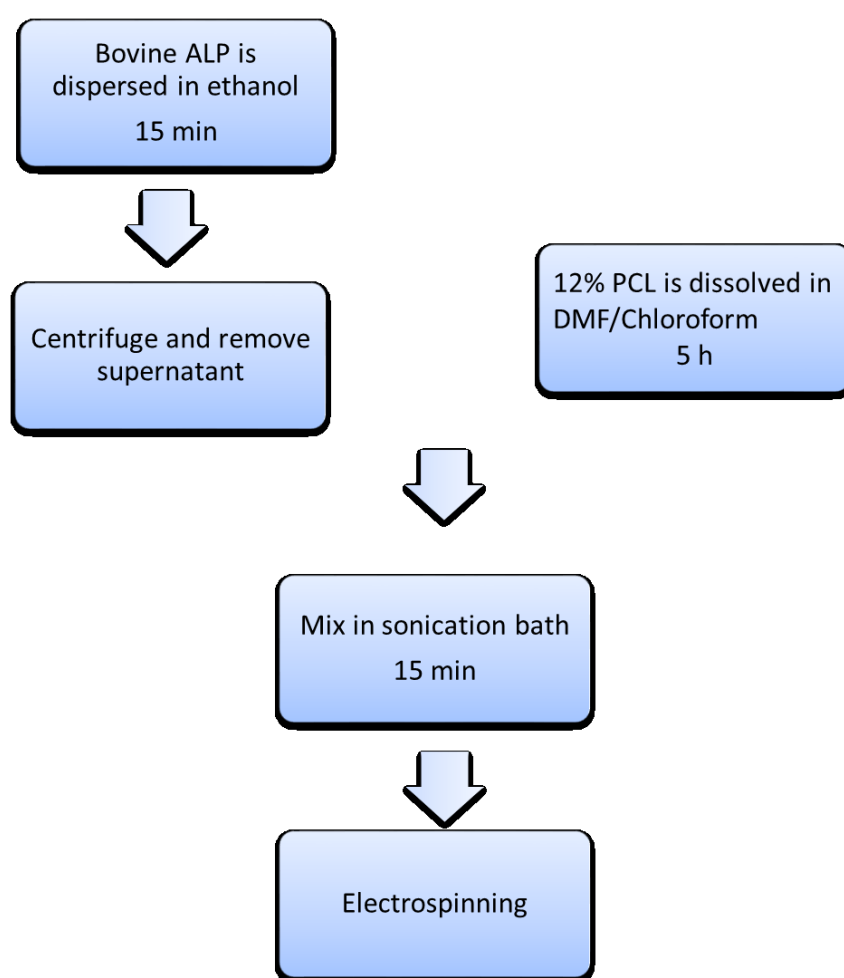


Figure 71. The precedence of producing the electrospun ALP/PCL scaffolds.

5.2.2.4 ALP specific activity measurement of ALP/PCL scaffolds

A similar method to measure the ALP coating activity on Ti implants was used to

determine the ALP specific activity of ALP/PCL electrospun scaffolds. In brief, the small pieces of ALP/PCL scaffolds were immersed in pNPP buffer solution in 24-well plates and incubated at 37°C, and the reaction was stopped by adding 1 M NaOH. The reaction time was recorded. Light absorbance of these samples was measured at 405 nm on a 96-well plate reader. A standard curve was constructed using p-nitrophenol as a standard. Experiments were performed independently in triplicate, and the ALP activity of each sample was normalised to the weight of nanofibres and expressed as mean $\pm$ standard deviation.

5.2.2.5 Soaking experiment of ALP/PCL scaffolds

In order to determine the potential bone formation ability of the ALP/PCL scaffolds *in vivo*, *in vitro* soaking experiments were performed. A similar procedure was used as described in Section 5.2.1.5. In brief, the ALP/PCL nanofibres were placed in cell culture medium (α -Minimum Essential Medium (α -MEM) supplemented with 10% (v/v) FBS, 50 μ g/ml gentamycin, 50 μ g/ml ascorbic acid, 100 nM dexamethasone, and 10 mM β -GP), and incubated at 37°C. The cell culture media were changed every 2-3 days. After 1, 3, 7, 14, and 28 days, the samples were removed from cell culture media, washed thoroughly first with PBS, then with distilled water, and stored at -80°C until the deposited calcium was measured.

5.2.2.6 Calcium assay of ALP/PCL scaffolds after soaking experiments

A similar calcium assay of the measurement of deposited calcium on ALP coatings of Ti implants after soaking experiments was used to determine the calcium deposition amount on ALP/PCL electrospun scaffolds. In brief, the ALP/PCL nanofibres after soaking experiments were immersed in 0.5 N acetic acid overnight at ambient temperature on a shaker (50 rpm). Then the samples were reacted with working buffer containing OCPC at ambient temperature for 10 min. Light absorbance of these samples was measured at 577 nm on a 96-well plate reader. A standard curve was constructed using CaCl_2 as a standard. Experiments were performed independently in

triplicate, and the calcium concentration of each sample was normalised to sample surface area and expressed as mean $\pm$ standard deviation.

5.2.2.7 *In vitro* cell culture study of ALP/PCL scaffolds

In vitro cell culture studies were carried out to determine the effect of the ALP/PCL nanofibre scaffolds on MC3T3-E1 cells. MC3T3-E1 cells, a mouse osteoblast cell line, were seeded on ALP/PCL pieces in 24 well plates in the growth medium (α -Minimum Essential Medium (α -MEM) supplemented with 10% (v/v) FBS, 50 μ g/ml gentamycin, 50 μ g/ml ascorbic acid, 100 nM dexamethasone, and 10 mM β -GP), in humidified 5% CO₂ at 37°C. The cell culture media were changed every 2-3 days. After 1, 3, 7, 14, and 28 days, the samples were removed from cell culture media, washed thoroughly first with PBS, then with distilled water, and stored at -80°C until the ALP activity assay, and SEM were performed.

ALP activity assay for cells and SEM were performed according to the procedures described in Section 2.2.2.

5.3 Results and discussion

5.3.1 ALP coated on Ti implants using silanisation method

5.3.1.1 Surface analyses of Ti implants

Ti surfaces are spontaneously covered by a 3-10 nm stable oxide layer when the implants are exposed to oxygen [53]. This layer is responsible for the biocompatibility of Ti implants [68]. In addition, the oxide is necessary for coupling to silanes as shown in Figure 65 [381]. However, this native formed oxide layer is irregular in thickness and composition, and may contain contaminants [356, 382]. In order to form a reproducible and clean implant surfaces, oxidation pre-treatments of Ti were carried

out. Various oxidation methods are available, such as electrochemical treatment, acid treatment, alkali treatment, and heat treatment. In this study, alkali treatment and heat treatment were chosen.

Figure 72 shows the SEM analysis of Ti implants surfaces with and without oxidation treatments. Untreated Ti disc with a native oxide layer show a relatively smooth surface (Figure 72A). Some contaminants can be found on the surface as well. After heat treatment (Figure 72B) and alkali treatment (Figure 72C), the surface roughness is significantly increased. High surface roughness is a desired property for implants in bone tissue engineering because more microtexture on the implant surface offers increasing surface binding sites for bioactive proteins and cells, therefore enhancing bone formation [383-385]. Although both oxidation methods produce microtexture on the implant surfaces, different morphologies were formed. When the alkali pre-treated Ti implants were modified with silanes, the microtexture on the implants was further increased as shown in Figure 72D.

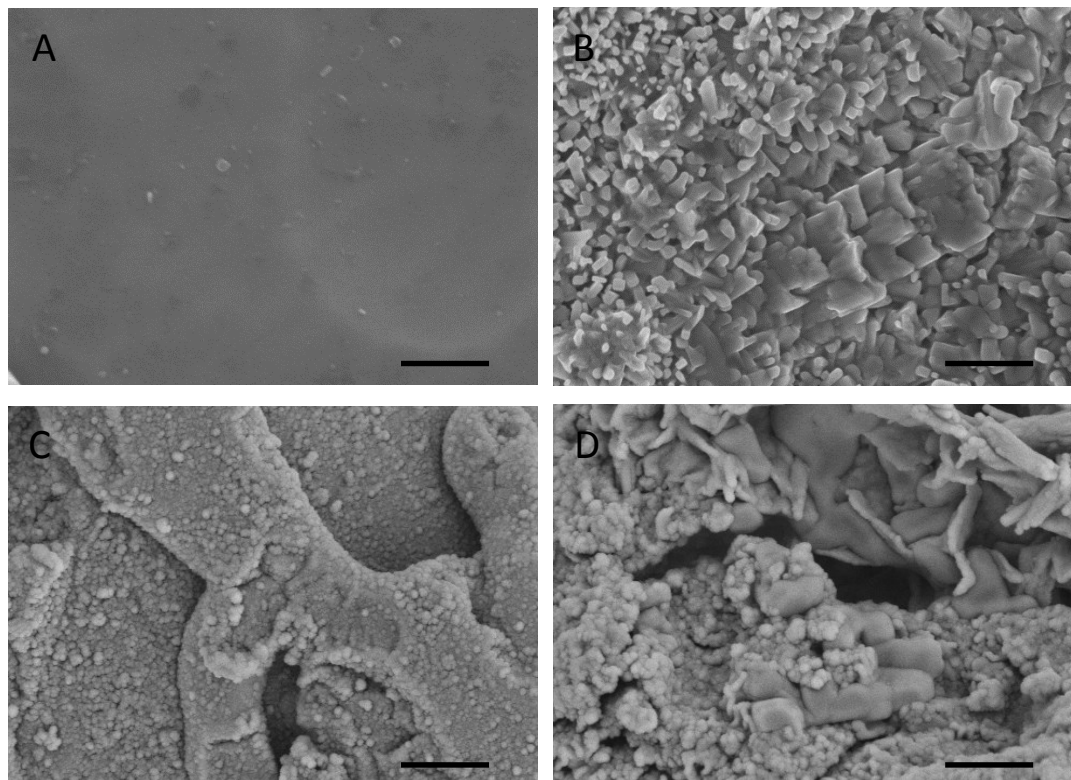


Figure 72. Scanning electron micrographs of (A) untreated Ti disc received from the manufacturer, (B) Ti disc after heat treatment at 800°C for 6 hours, (C) Ti disc after alkali treatment in 5.0 M NaOH solution at 60°C for 24 hours, and (D) Ti disc silanised

by 10% (w/v) APS and 1% (w/v) glutaraldehyde as described in Section 5.2.1.2. Scale bars represent 1 μm .

5.3.1.2 Optimising the Ti implants with ALPs coatings

In order to optimise the Ti preparation and immobilisation methods, the ALP activity of the ALPs coatings on Ti implants by different treatments was determined. Bovine ALP was used in this study. In Figure 73A, all Ti discs were pre-treated with 5.0 M NaOH solution at 60°C for 24 hours before immersing in different concentrations of bovine ALPs (10 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, and 1000 $\mu\text{g/ml}$). Bovine ALP immobilised on alkali treated Ti discs by silanisation showed significantly higher ALP activity compared to the physically adsorbed ALP coatings. The results indicate that immobilisation of the enzyme ALP on Ti surface by covalent binding is a preferred method to adsorption. ALP activity of the coating increased with the concentration of bovine ALP solutions in which the silanised Ti discs immersed. The ALP coating immobilised from the 1000 $\mu\text{g/ml}$ ALP solution showed the highest ALP activity compared to less concentrated solutions. All Ti discs pre-treated by incubating at 800°C for 6 hours before immersing in different concentrations of bovine ALPs showed a similar trend to alkali treatment (Figure 73B), in that silanised ALP coatings display significantly increased ALP activity compare to the adsorbed ALP coating. In addition, the ALP activity of the coating increased with the concentration of the bovine ALP solutions in which the silanised Ti discs were immersed. Higher ALP activity was shown from the ALP coating, indicating that more ALP was immobilised on the Ti implants. The results indicated that silanised Ti discs immersed in 1000 $\mu\text{g/ml}$ ALP solution have the highest enzyme loading. Therefore, 1000 $\mu\text{g/ml}$ ALP solution was prepared for enzyme immobilisation in the following studies.

There was no significant differences of ALP activity of ALP coatings on Ti implants between the two oxidation methods. The alkali treatment was chosen as the oxidation method for the later experiments because it was easier to perform.

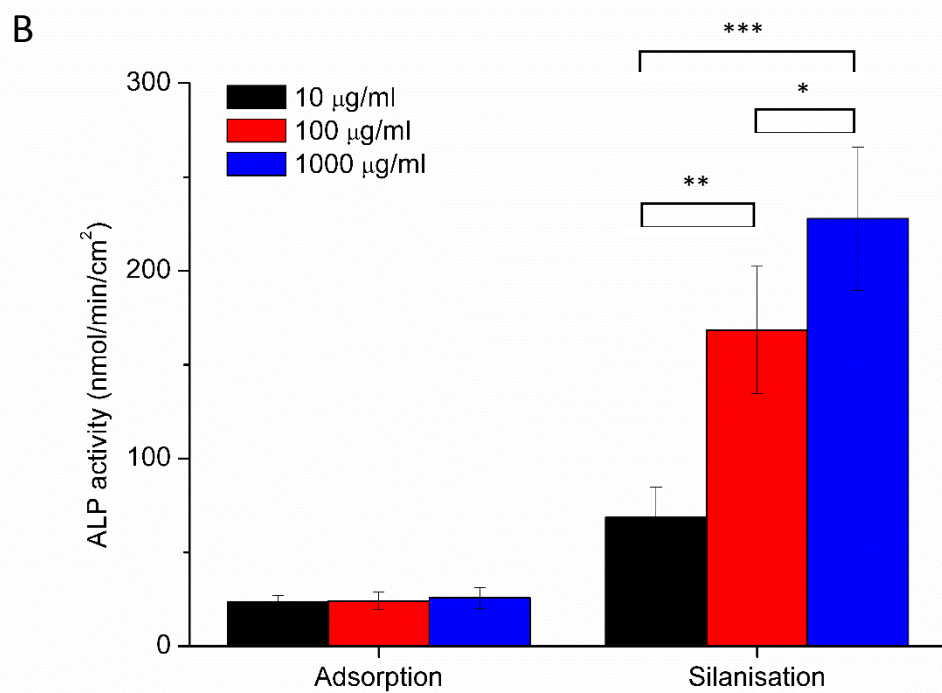
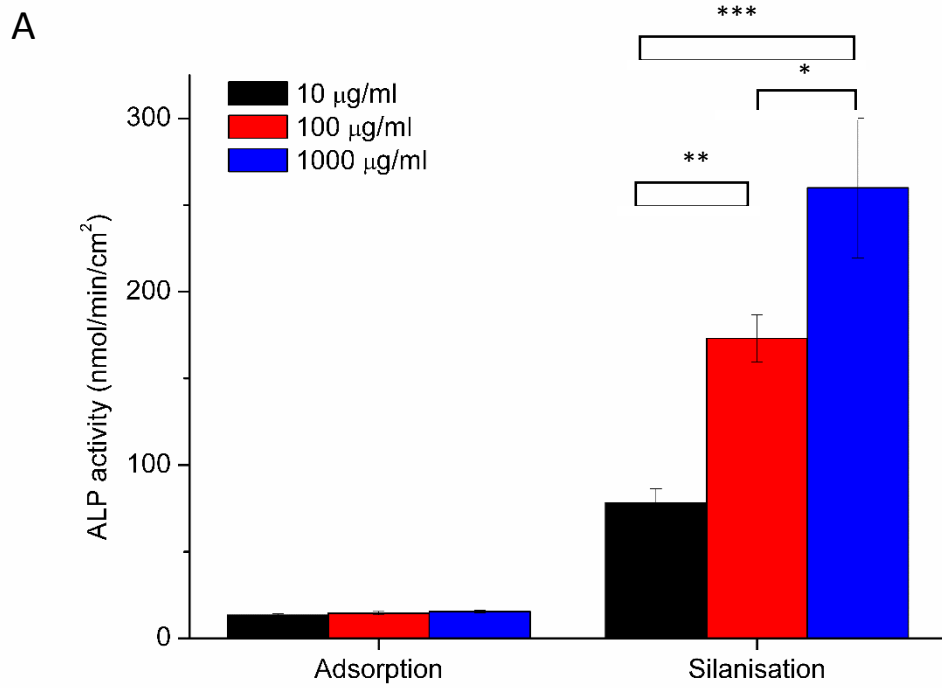


Figure 73. ALP activity of Ti implants with bovine ALP coatings was measured using pNPP as a substrate. (A), the Ti discs were oxidised by alkali treatment; (B) the Ti discs were oxidised by heat treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant differences.

E. coli mutant and wild-type ALP were immobilised on Ti implants by the optimised conditions. In Figure 74, ALP activity of ALP coatings on Ti implants with different types of ALPs were compared. *E. coli* mutant ALP coating showed significantly higher ALP activity compared to the bovine ALP coating ($p < 0.01$) and wild-type ALP ($p < 0.001$). Assuming that same amount of ALPs were immobilised on the Ti discs, the mutant ALP shows more than 17 times higher activity than the wild-type ALP, which is in line with the results shown in Figure 44. However, despite the significant difference in ALP activity that was found between the *E. coli* mutant and the bovine ALP coating, the difference is not as big as the activity difference between the mutant ALP and the bovine ALP. This may be due to the different amount of enzyme being loaded, since the structure and conformation of the mutant ALP and the bovine ALP are different.

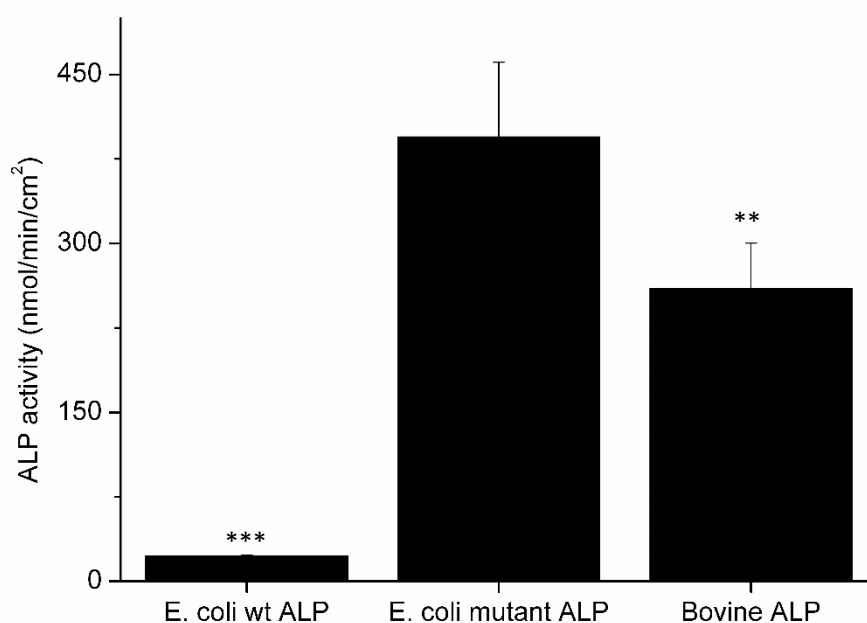


Figure 74. ALP activity of Ti implants with *E. coli* mutant and wild-type ALP coatings was measured using pNPP as a substrate. Bovine ALP was used as a control. The Ti discs were oxidised by alkali treatment, and immersed in 1000 µg/ml ALP solution. ** $p < 0.01$, *** $p < 0.001$ indicate statistically significant differences between the respective group and the mutant ALP group.

5.3.1.3 Effect of silanisation coating on mineralisation after soaking experiments

Surface morphology of the ALP coatings on Ti implants after soaking experiments was determined by SEM on days 3 and 14 (Figure 75). Ti implants with different ALP coatings show a similar morphology. Figure 72 only shows the bovine ALP coatings on day 3 (A), and *E. coli* wild-type ALP on day 14 (B). Organic matrix depositions covered the coating after 3 days soaking in cell culture media. A few mineral depositions were observed, which had a Ca/P ratio of between 1.6 and 1.8 as determined by EDS. On day 14, the implants were fully covered by a deposition layer. Abundant spheres were found in all groups, including the uncoated Ti implants on both day 3 and day 14. No mineral components were detected from the spheres, which may come from the cell culture media. In the future soaking experiments, the cell culture media were filtered through a 0.22 μm filter before adding it to the substrates.

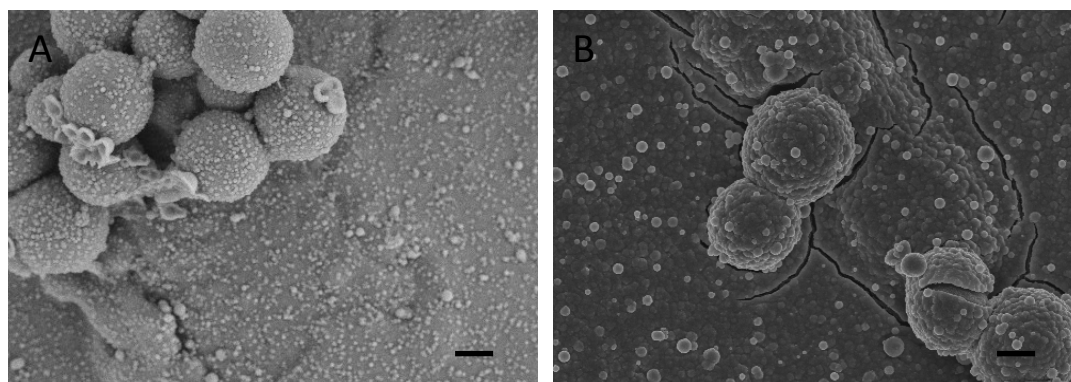


Figure 75. Scanning electron micrographs of (A) bovine ALP coating on Ti implants after a 3 day soaking experiment, (B) wild-type ALP coating on Ti implants after a 14 day soaking experiment. Scale bars represent 1 μm .

The amount of deposited calcium on the surface of Ti implants with ALP coatings after soaking experiments was measured by a calcium assay (Figure 76). The calcium content from three ALP coatings was increased with soaking time. The control group without any coatings showed the lowest calcium content at every time point. No significant difference in the calcium content was observed between the bovine ALP coating and the *E. coli* mutant ALP coating at each time point. The *E. coli* wild-type ALP coating produced a significant decrease in calcium content on day 7 and day 14 of

soaking, compared to the bovine ALP coating and mutant ALP coating. However, compared to uncoated Ti implants, significant increases of calcium content were detected from the wild-type ALP coating. The results indicate that Ti implants with ALP coatings improve calcium deposition after 7 days soaking in cell culture media. The *E. coli* wild-type ALP coating on Ti implants with lower biological activity shows limited mineral producing ability compared to the *E. coli* mutant ALP coating and bovine ALP coating. Although the *E. coli* mutant ALP coating displays significantly high ALP activity than the bovine ALP coating as shown in Figure 74, no significant difference between the two groups was observed for mineral deposition ability. The possible reason is due to a limited source of organic phosphate and calcium ions in the cell culture media.

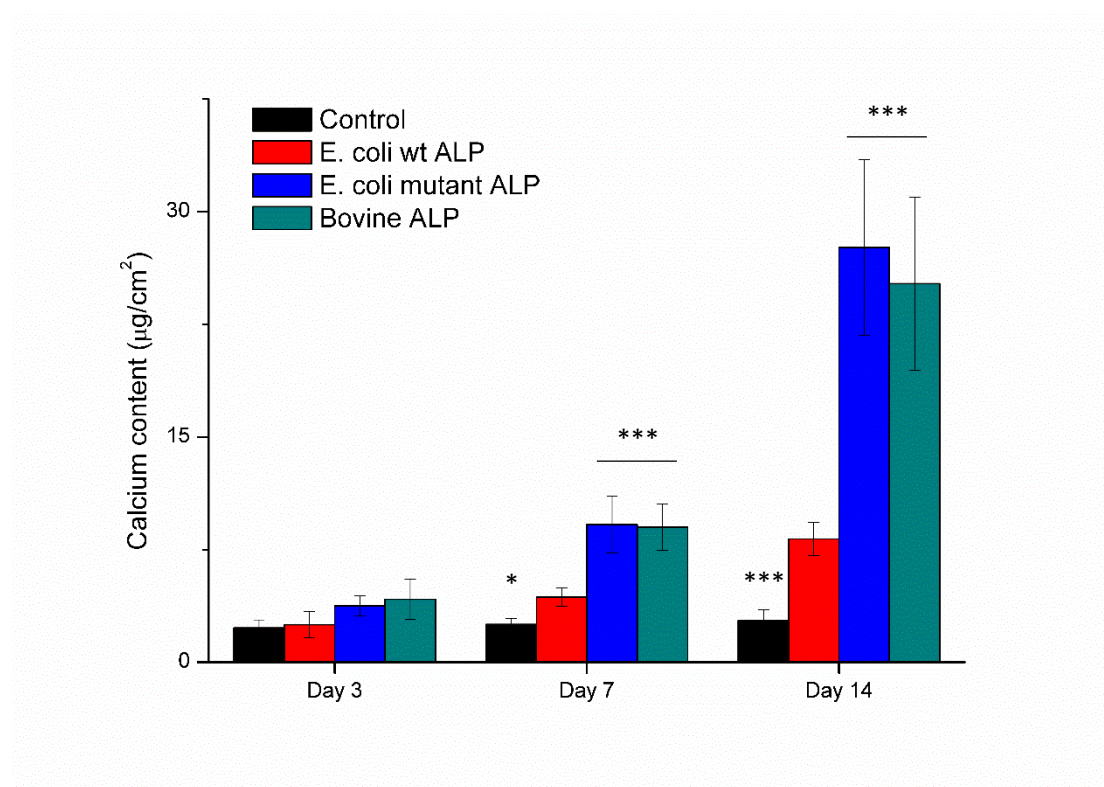


Figure 76. Calcium deposited on the surface of Ti implants with ALP coatings after soaking in cell culture media was measured by a calcium assay. * $p < 0.05$, *** $p < 0.001$ indicate statistically significant differences between the respective groups and the wild-type ALP coatings.

5.3.2 ALP/PCL scaffold prepared by electrospinning

5.3.2.1 Surface analyses of ALP/PCL scaffolds

The properties of electrospun nanofibres such as the fibre diameter, porosity, pore size, and mechanical properties are adjustable by changing the percentage of PCL, the solvent, and the electrospinning parameters. In this study, 12% (w/v) PCL was dissolved in DMF/chloroform, and electrospun with and without bovine ALP under 18 kV voltage.

Figure 77 shows the SEM images of PCL electrospun nanofibres with and without bovine ALP. High porosity networks with uniformed nanofibres were fabricated from 12% PCL. The fibre diameter of pure PCL nanofibres ranged from 100 to 500 nm, and the pore size ranged from 500 nm up to several micrometers (Figure 77A and B). When the ALP was co-electrospun with PCL (Figure 77C and D), a similar porosity and pore size of the network was achieved as the pure PCL network. However, the ALP/PCL fibres were rougher and thicker compared to pure PCL. In addition, some nodules with a diameter of up to 10 μm were formed in the fibres. These nodules are believed to be encapsulated ALP aggregates. Although several strategies were carried out to reduce the ALP aggregates, such as dispersing the ALP lyophilised powder in ethanol before mixing with liquid polymers, sonication of the ALP/PCL solution, and passing the aggregates through a needle several times, some ALP aggregates still existed especially in the highly concentrated samples. Among them, the sonication treatment of the ALP/PCL solution was the most effective method to reduce aggregation.

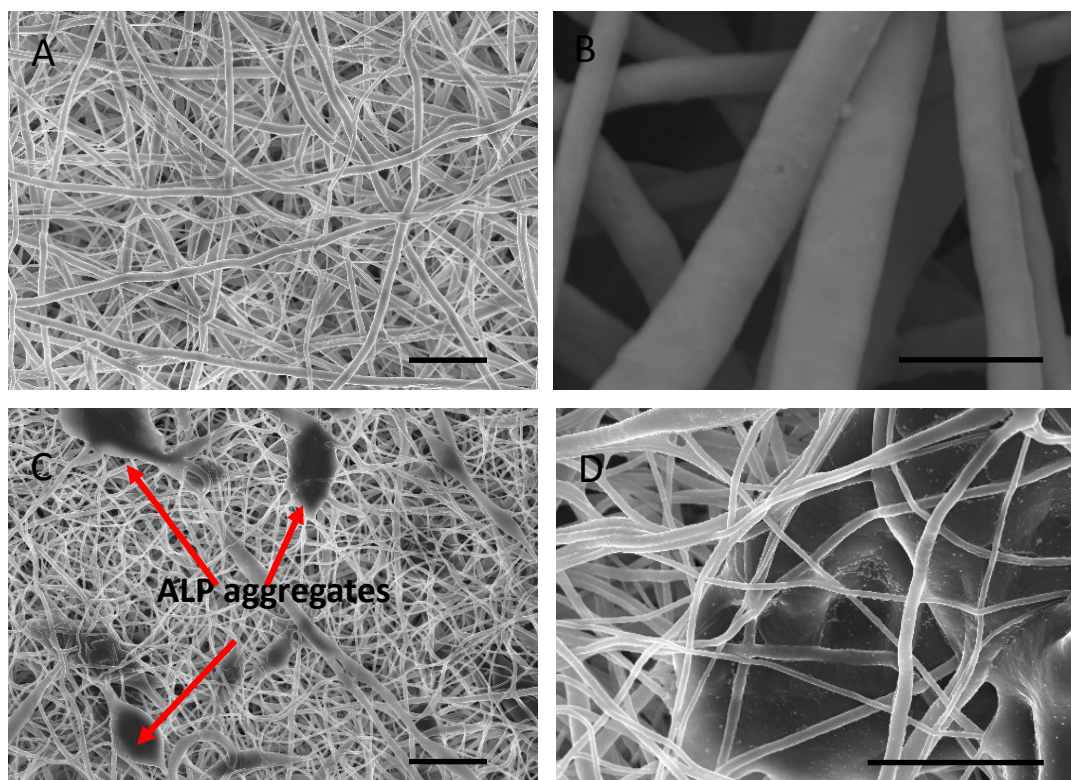


Figure 77. Scanning electron micrographs of (A, B) pure PCL nanofibres, (C, D) 20% (w/v) ALP/PCL nanofibres. Uniform fibres were formed from pure PCL, rough fibres with some ALP nodules were formed from ALP/PCL mixture. Scale bars represent 10 μm in A, C, and D; scale bar in B represents 1 μm .

5.3.2.2 Determining the ALP specific activity of ALP/PCL electrospun scaffolds

Figure 78 shows the ALP specific activity of ALP/PCL scaffolds after electrospinning. Three different amounts of ALP (10, 20, and 30% (w/w)) were mixed and co-electrospun with liquid PCL polymers. There was no significant difference of ALP activity between the pure PCL fibres and the 10% ALP/PCL fibres. ALP activity of the electrospun scaffold was significantly higher, following the increasing ALP amount in the fibres from 10 to 30% ($p < 0.001$). The results indicate that ALP/PCL scaffolds fabricated by electrospinning preserved the ALP activity. The more ALP encapsulated in the fibres, the higher the ALP activity detected from the scaffolds. However, due to the operating difficulties of dispersing a large amount of ALP powder in PCL liquid polymer, 30% (w/w) of ALP is a highest percentage can be achieved.

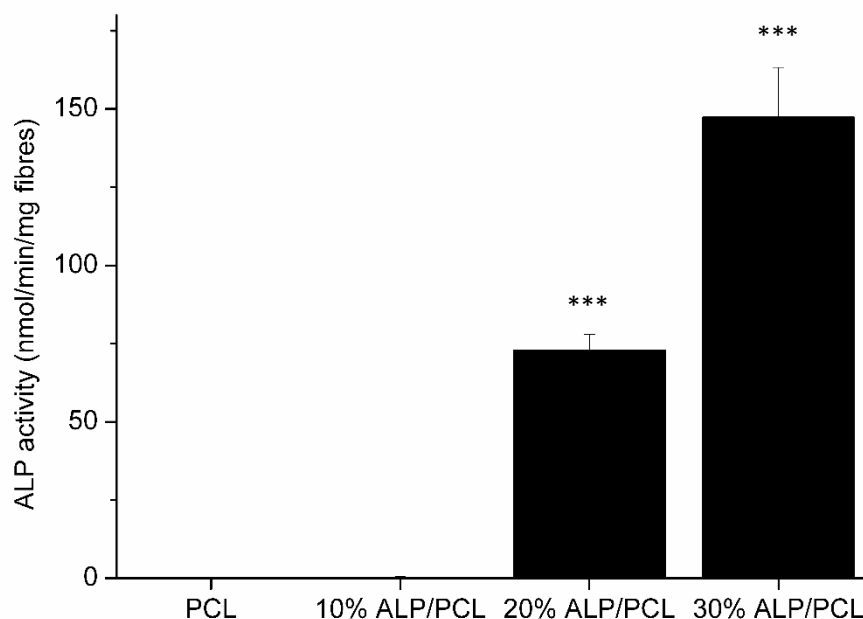


Figure 78. ALP activity of ALP/PCL scaffolds was measured using pNPP as a substrate. Pure PCL scaffold was used as a control. *** $p < 0.001$ indicates statistically significant differences between the respective group and the other groups.

5.3.2.3 Effect of ALP/PCL scaffolds on mineralisation after soaking experiments

Surface morphology of ALP/PCL scaffolds after soaking experiments was determined by SEM on day 3, 7, 14, and 28 (Figure 79). Figure 79 are pure PCL scaffolds after soaking in cell culture media after 3, 7, 14 and 28 days, respectively. A few mineral depositions on the surface of the PCL nanofibres were found starting at day 3. The number of mineral depositions was increased in line with the culture time. Similar to pure PCL scaffolds, mineral depositions on the surface of the ALP/PCL nanofibres can be found since day 3, and more depositions were observed with the increasing culture time. At each time point, more mineral depositions were found on ALP/PCL fibres than the PCL fibres. Especially on day 28, a layer of mineral deposition completely covered the 30% ALP/PCL scaffold, and no fibrous structure could be seen, while the fibrous PCL scaffold were still observed in other groups.

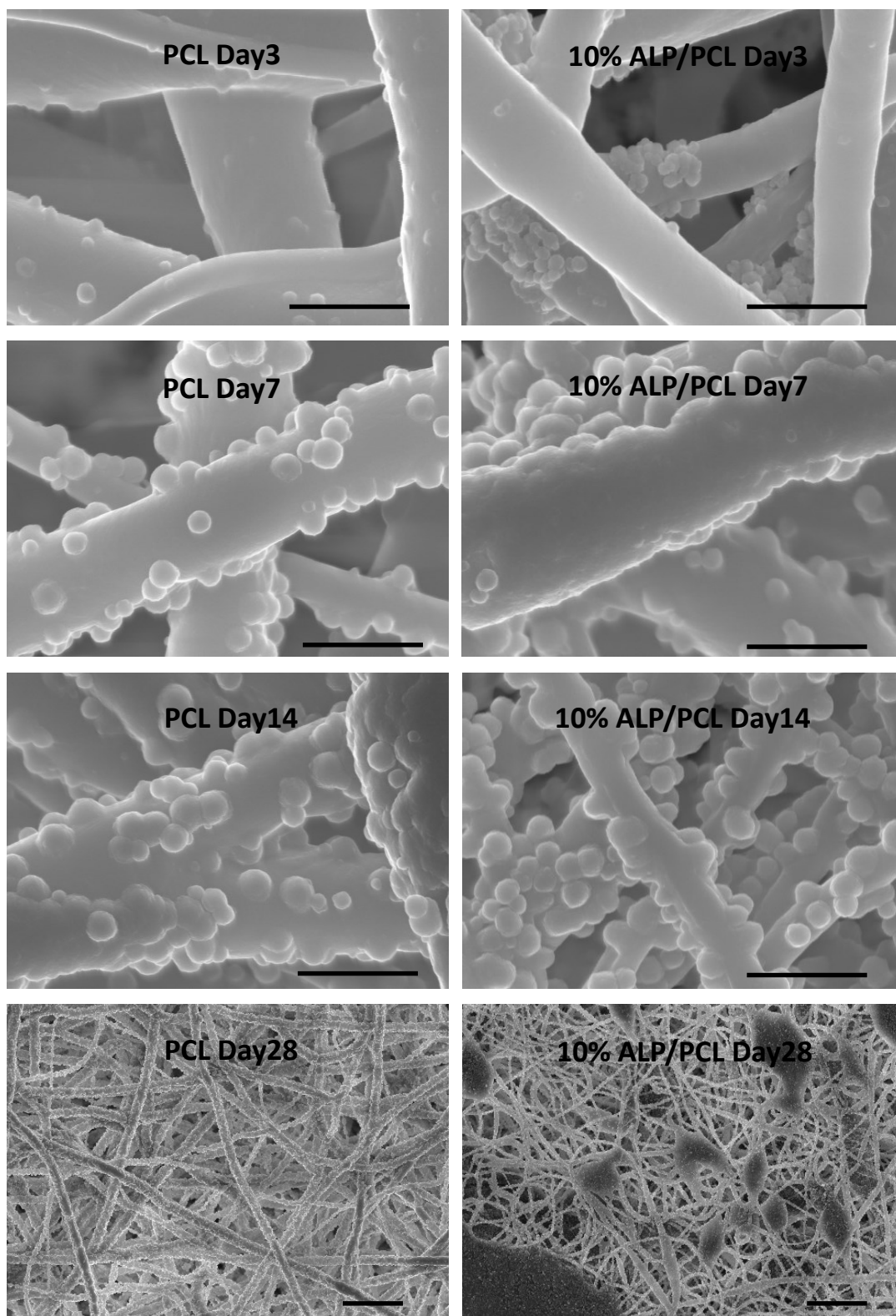


Figure 79. Scanning electron micrographs of pure PCL scaffolds and ALP/PCL scaffolds after soaking in cell culture media for 3, 7, 14 and 28 days, respectively. Scale bars represent 1 μm in Day 3, 7, and 14; scale bars in Day 28 represent 10 μm .

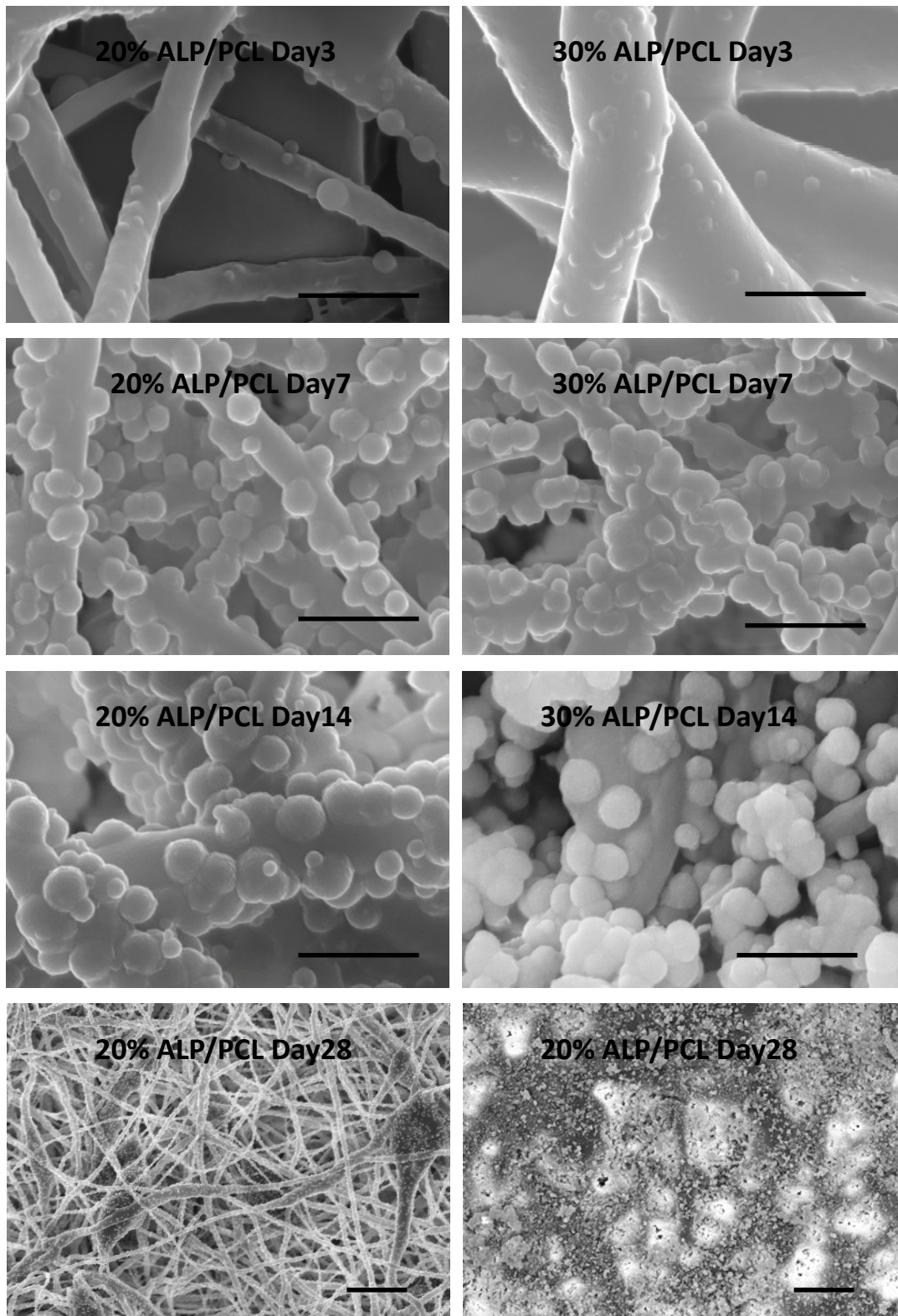
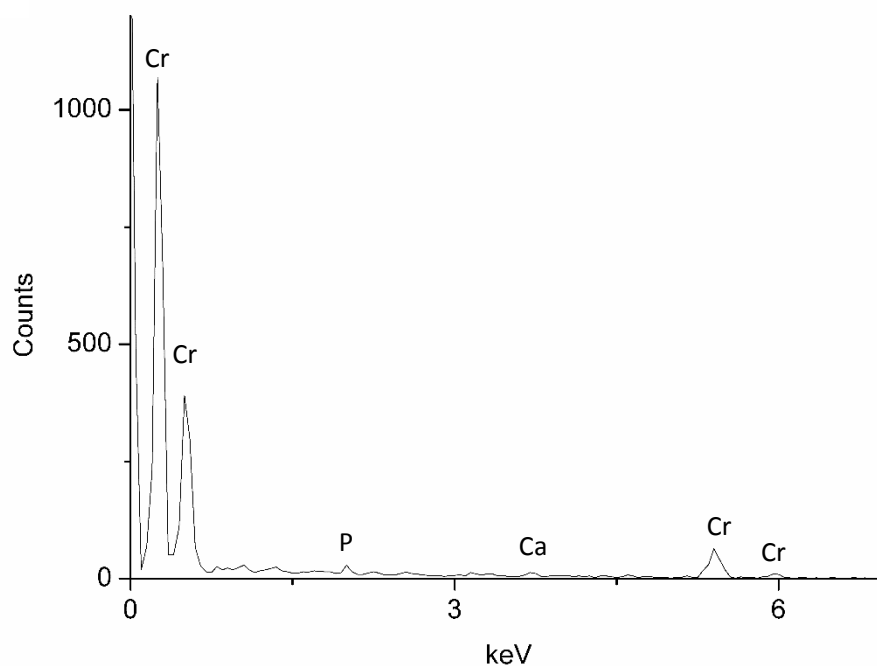


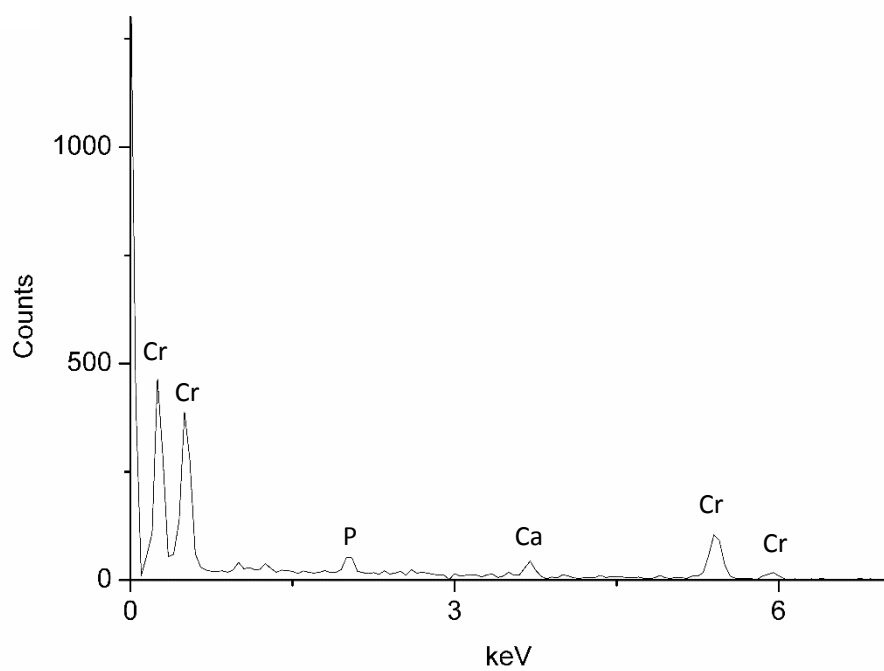
Figure 79. (Continued) Scanning electron micrographs of pure PCL scaffolds and ALP/PCL scaffolds after soaking in cell culture media for 3, 7, 14 and 28 days, respectively. Scale bars represent 1 μm in Day 3, 7, and 14; scale bars in Day 28 represent 10 μm .

The SEM results indicate that PCL nanofibres support mineral deposition after soaking in cell culture media. The ALP/PCL scaffolds with higher ALP activity enhance the amount of mineral deposition and accelerate the mineralisation process. In order to identify the mineral deposition formed during the soaking experiment, EDS analysis was used (Figure 80). It revealed spherical structures on the nanofibres show a Ca/P ratio between 1.6 and 1.8 (Figure 80A, B, C), while no mineral signal was observed from the nanofibres alone (Figure 80D). The EDS signals were higher with a longer culture time (Figure 80A, B, C). The EDS result indicates that the spherical structures are mineral depositions.

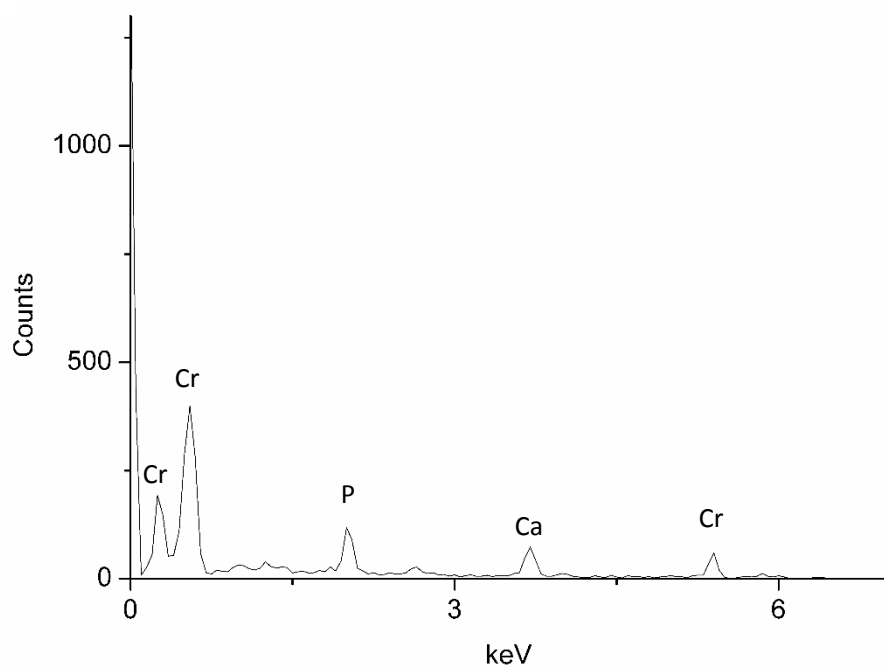
A



B



C



D

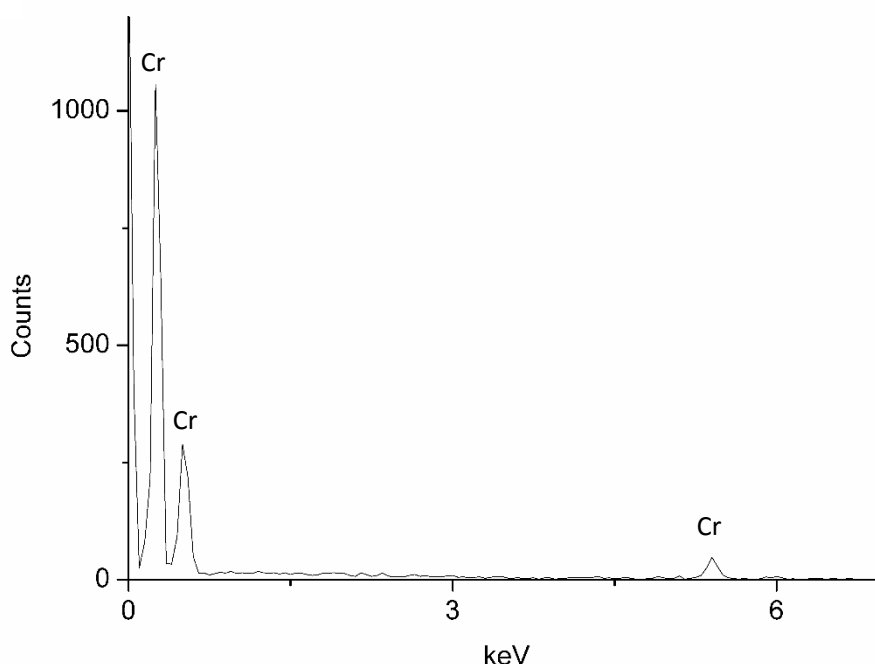


Figure 80. EDS analysis of mineral depositions on ALP/PCL scaffolds after 3 days (A), 7 days (B) and 28 days (C and D) soaking in cell culture media. A, B, and C analyse the spherical structures on the surface of the nanofibres, while D analyses the nanofibre.

The amount of deposited calcium on the ALP/PCL scaffolds after soaking as measured by calcium assay (Figure 81). The calcium deposited on the scaffolds was higher with a longer soaking time in every groups. Before day 7 of the soaking experiment, there was little calcium deposition accumulated on the ALP/PCL scaffolds. After day 7, the calcium content increased significantly. In contrast to the ALP activity results shown in Figure 73, PCL scaffolds containing 10% and 20% ALP produced similar amounts of calcium deposition compared to pure PCL scaffolds at every time point. The results indicate that a similar mineral producing ability is provided by the PCL scaffold and PCL fibres electrospun with a lower amount of ALP. PCL scaffolds containing 30% ALP exhibit significantly higher calcium content compared to other groups on day 14 and 28 ($p < 0.001$). Therefore PCL scaffolds containing 30% ALP were prepared for the following cell culture studies.

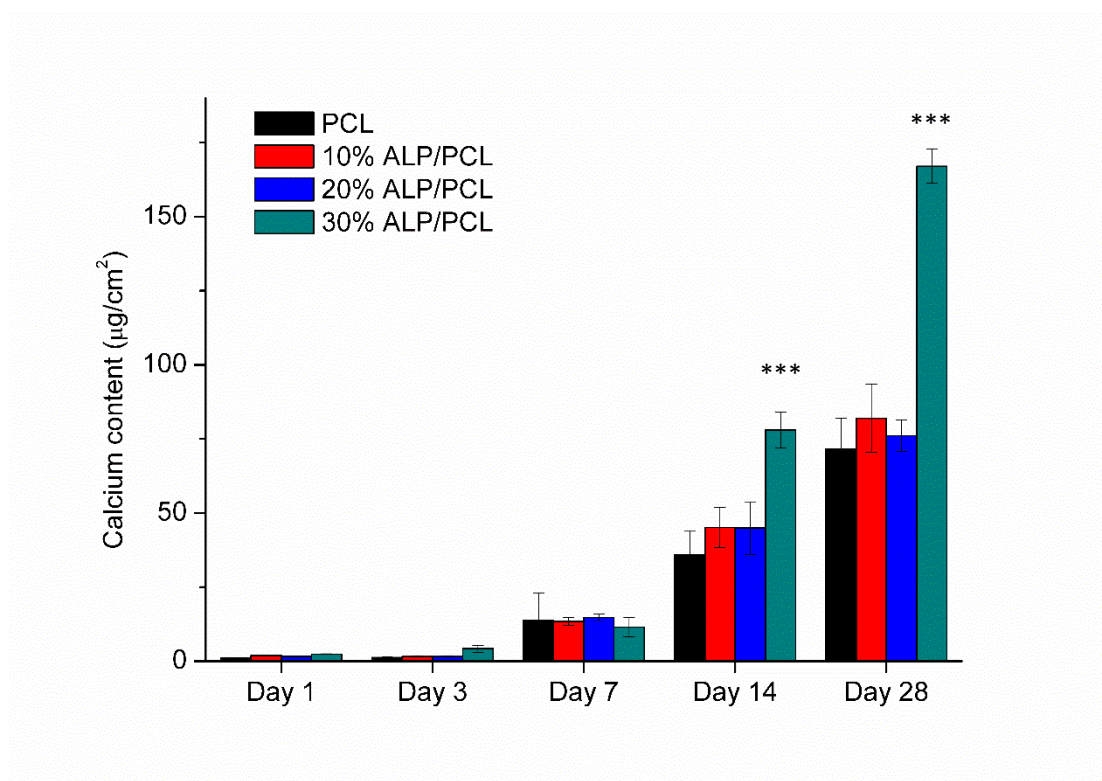


Figure 81. Calcium deposited on the ALP/PCL scaffolds after soaking in cell culture media was measured by calcium assay. *** $p < 0.001$ indicates statistically significant differences between the respective groups and other groups on the respective day.

5.3.2.4 Determining the effect of the ALP/PCL scaffolds on MC3T3-E1 cells

In order to determine the effect of the ALP/PCL scaffolds on cell behaviours, MC3T3-E1 cells were seeded on the ALP/PCL scaffolds. A PCL scaffold was used as a control. Cell morphology and differentiation were verified with SEM, ALP activity and calcium content assay, respectively.

Cell morphology of MC3T3-E1 cells cultured on ALP/PCL scaffolds was evaluated by SEM (Figure 82). No significant difference was observed before 7 days of cell culture between the ALP/PCL scaffold and the PCL scaffold. After day 7, extracellular matrix was gradually deposited on the nanofibres. Mineral signal was observed by EDS. On day 14, a layer of extracellular matrix and mineral depositions almost covered the ALP/PCL nanofibres (B). In contrast, less extracellular matrix and mineral deposition were observed on the PCL fibres (A).

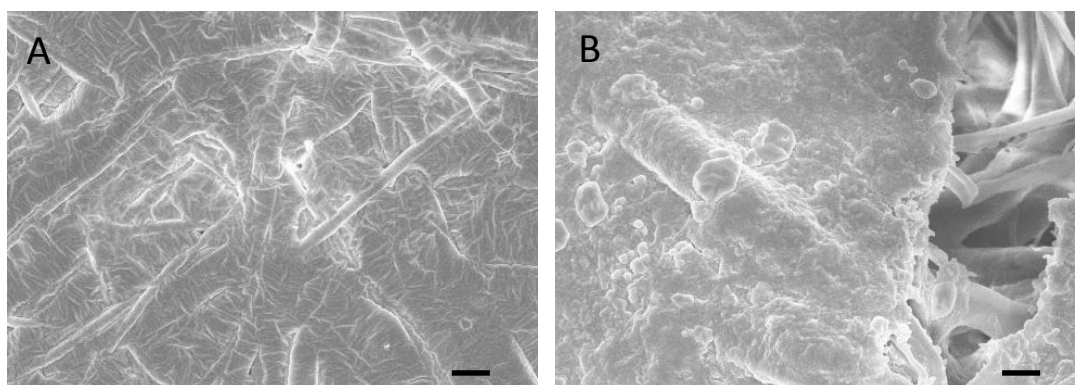


Figure 82. Gross changes in cell morphology of MC3T3-E1 cells cultured on PCL scaffold (A) and ALP/PCL scaffold (B) for 14 days. Scale bars represent 1 μm .

Cell differentiation of MC3T3-E1 cells cultured ALP/PCL scaffolds was determined by measuring the ALP activity (Figure 83), which indicates early osteogenic differentiation, and calcium content (Figure 84), which indicates the late differentiation of the cells. In Figure 83, the ALP activity of cells on ALP/PCL scaffolds was significantly increased compared to PCL groups at each time point ($p < 0.001$). A peak value of ALP activity was achieved after 14 days of culture for the ALP/PCL scaffold. The results indicate 30% ALP/PCL scaffold enhances cell differentiation compared to the PCL scaffold. However, the ALP activity of the cells was extraordinary high compared to other experiments. The possible reason is that the ALP/PCL scaffold contributes a part of the ALP activity. According to Figure 78, high ALP activity was displayed in the 30% ALP/PCL scaffold group. Due to the operating challenges of complete removing the thin electrospinning fibre film from the cells, ALP/PCL scaffolds were reacted with pNPP as well as MC3T3-E1 cells.

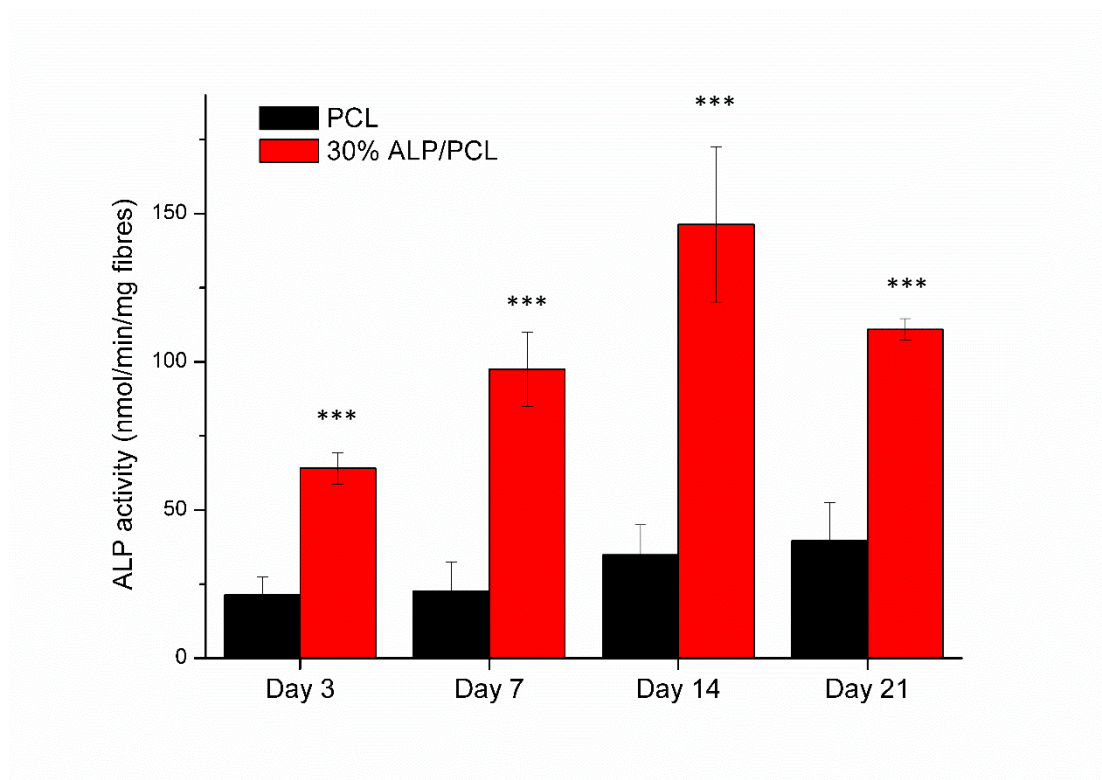


Figure 83. The ALP activity of EC3T3-E1 cell lysate grown on ALP/PCL scaffolds for 21 days was measured using pNPP as a substrate. *** $p < 0.001$ indicates statistically significant differences between the ALP/PCL scaffolds and the PCL scaffolds on the respective day.

Figure 84 shows the calcium content deposited on the electrospun nanofibres after 21 days of cell culture. The deposited calcium amounts increased along with the culture time. Before day 7, no difference of calcium content was observed between the two scaffolds. The calcium deposited on the scaffolds was at a low level. After day 7, calcium depositions increased sharply in both groups. However, the ALP/PCL scaffolds accumulated more calcium depositions than the PCL scaffold ($p < 0.01$) at day 14 and day 21. This result was in agreement with the result of the soaking experiments. PCL nanofibres containing 30% of ALP showed the best ability to enhance mineralisation.

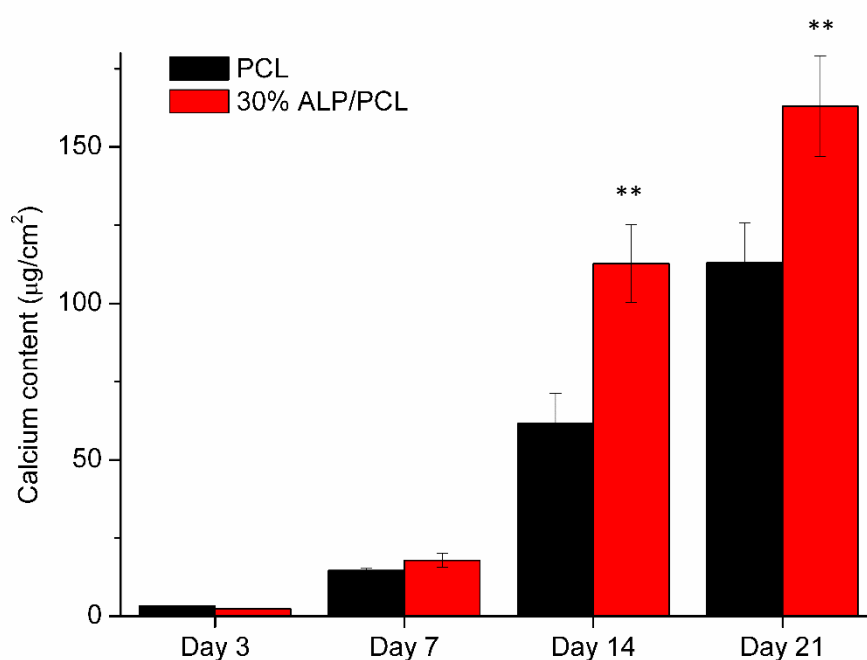


Figure 84. Calcium content of EC3T3-E1 cell lysate grown on ALP/PCL scaffolds for 21 days was measured by a calcium assay. ** $p < 0.01$ indicates statistically significant differences between the respective groups and PCL scaffolds on the respective day.

5.4 Conclusion

Two applications of ALPs in bone tissue engineering, one being a Ti implant with recombinant *E. coli* ALP coating, and the other being an ALP/PCL scaffold, were successfully fabricated in this chapter. The surface roughness of the Ti implants was significantly increased after oxidation treatment. In addition, silanised ALP coatings displayed significantly increased ALP activity compared to the adsorbed ALP coating. Regards to recombinant *E. coli* ALPs, mutant ALP coatings showed a significantly higher ALP activity compared to bovine ALP coatings ($p < 0.01$) and wild-type ALP coatings ($p < 0.001$). After a soaking experiment in cell culture media containing organic phosphates, *E. coli* mutant ALP coatings on Ti implants with high biological activity show outstanding mineral producing ability compared to the wild-type ALP coatings. However, no significant difference in calcium deposition on the implants after soaking experiment was observed when comparing the *E. coli* mutant ALP coatings and bovine

ALP coatings.

Electrospun ALP/PCL nanofibres showed significantly higher ALP activity than the pure PCL nanofibres. ALP activity of the ALP/PCL was significantly increased along with the increasing ALP amount in the fibres ($p < 0.001$). After soaking in cell culture media for 28 days, a layer of mineral deposition completely covered the 30% ALP/PCL scaffold. Both EDS analysis and calcium assay after soaking indicated that the ALP/PCL scaffolds with a high ALP activity enhanced the mineral deposition ability and accelerated the mineralisation process. Similar results were obtained from the MC3T3-E1 cell culture experiments, where the ALP/PCL scaffold enhanced cell differentiation and mineralisation compared to the PCL scaffold. In conclusion, both ALP coated implants and ALP/PCL scaffolds show outstanding mineral-promoting ability, and can be considered promising candidates for bone tissue engineering applications.

CHAPTER 6

Conclusions

Bone tissue engineering has been considered as the alternative strategy to the current therapies for bone regeneration or substitution. Bioactive factors, being an important element in bone tissue engineering, have been thoroughly investigated over the decades to support bone formation. In particular, studies have been focused on well-known bone growth factors and cellular matrix proteins, such as BMPs and collagen. In fact, biomolecules such as bioactive factor-derived peptides and mineralisation-promoting proteins also have the ability to enhance bone formation and regeneration. This study concerns these biomolecules and reveals a novel strategy of engineering biomolecules with improved functionalities for bone tissue engineering applications.

This thesis has explored the development of two biomolecules with improved functionality to accelerate and improve bone mineralisation, and therefore enhance bone formation. First, were short peptides derived from the bone growth factor BMP-7, which are reported to enhance mineral deposition [136]. Second, was an engineered recombinant metalloenzyme *E. coli* ALP, which generates inorganic phosphate required for mineralisation [180]. Ti implants and PCL scaffolds have been used to incorporate these engineered biomolecules to verify the improvement of the functionality and biological efficiency for tissue engineering applications. In this chapter, the main results and findings are presented and concluded.

Research in bone tissue engineering over the past decades has been focused on biomolecules to enhance bone formation and develop bone substitutes. In the Introduction chapter, a number of widely used biomolecules were reviewed, such as bone growth factors, cellular matrix proteins, bioactive factor-derived peptides, and other mineralisation-promoting proteins. In Chapter 2, the BMP-7-derived peptide KAISVLYFDDS, and the other two peptides with His-tag on both sides,

KAISVLYFDDSHHHHHH and HHHHHHKAISVLYFDDS, which strongly interacts with the nickel-Ti implant surface *via* metal-affinity coordination and allows a controlled immobilisation, were successfully synthesised, purified, and characterised. Their biological activities were determined by the mouse myoblast cell line C2C12, and the mouse pre-osteoblast cell line MC3T3-E1.

The other biomolecule of recombinant *E. coli* ALP was described in Chapter 3, 4 and 5. In Chapter 3, plasmids pEK48/phoA and pEK48/4-186, encoding the wild-type and 4-186 mutant *E. coli* ALP were isolated and purified. Both the wild-type and mutant recombinant *E. coli* ALP with improved biological activity were successfully expressed from bacterial cells and verified by BCIP selective plates, SDS-PAGE and western blotting analysis. After purification from the ÄKTA explorer purification system, the mutant ALP showed a significantly higher activity compared to the wild-type ALP (nearly 25 times) and bovine ALP (more than 9 times) ($p < 0.001$). However, the wild-type and mutant recombinant *E. coli* ALP have a low protein yield, difficulties in protein purification, and limited immobilization property. In order to further improve the recombinant *E. coli* ALP and solve the problems, the mutant and wild-type alkaline phosphatase genes were recloned into a pET24 expression vector which contains His-tag to produce the engineered recombinant His-tag *E. coli* ALP in the pET expression system in Chapter 4. In chapter 5, two applications of bone tissue engineering with ALPs, one being a Ti implant with recombinant *E. coli* ALP coating, and the other being an ALP/PCL scaffold, were described to accelerate mineral deposition and enhance implant fixation with the surrounding tissue by using the catalytic ability of ALPs.

The main findings draw in the study are listed below:

(1) BMP-7-derived peptides support the proliferation of C2C12 cells, however, 10 µg/ml BMP-7-derived peptides failed to convert the differentiation pathway of C2C12 myoblast to osteoblast.

This is the first report that BMP-7-derived peptides showed no burst of ALP activity when exposed to C2C12 cells. The possible reason is that the peptide KAISVLYFDDS is not derived from the related active site of the BMP. The regulation of C2C12

differentiation by BMPs may be related to the DNA binding and/or transcriptional activities of MyoD [275]. However, the peptide KAISVLYFDDS is derived from the Finger 2 domain of BMP-7 that is responsible for ligand-receptor binding interaction.

(2) BMP-7-derived peptides support the proliferation of MC3T3-E1 cells, but do not possess the same ability as BMP-2 to induce osteoblast differentiation.

These results were different from the results in literatures. According to Chen and Webster, 100 µg/ml peptide KAISVLYFDDS in culture media increased alkaline phosphatase activity in human fetal osteoblasts after 14 days compared to negative controls and BMP-7 [136]. The possible reason is due to the different cell line. MC3T3-E1 may fail to respond to the short peptides used in this study. Another possible reason is the concentration of peptides (100 µg/ml) was too low to induce cell differentiation in MC3T3-E1.

(3) The *E. coli* mutant ALP showed a significantly higher activity compared to the wild-type ALP (nearly 25 times) and bovine ALP (more than 9 times) ($p < 0.001$). The wild-type ALP showed a significantly lower activity than bovine ALP (more than twice) ($p < 0.01$).

(4) The activity of *E. coli* ALP can be affected by NaCl and lyophilisation treatment.

In order to avoid the activity reduction, a dialysis is necessary after ÄKTA explorer purification analysis. And the protein was directly frozen at -80°C instead of lyophilising.

(5) BL21 strain produces the highest protein amount and highest ALP activity of wild-type *E. coli* ALP. For mutant ALP, BL21 produces a higher protein amount, but SM547 expresses the highest ALP activity.

(6) A higher level of protein expression of the His-tag ALP was achieved after overnight incubation at 30°C in LB Kan medium compare to a shorter incubation time and non-incubation groups.

(7) The mutated recombinant *E. coli* His-tag ALP lost its activity after incorporating a His-tag on either the N-terminal or C-terminal.

According to the literature, histidine and imidazole in the elution buffer were reported to inhibit the activity of mammalian ALP [346-348]. The mechanism of the inhibition may relate to the chelation of Mg^{2+} in the system. Due to the similarity of the structures of the *E. coli* and the mammalian ALPs in the active site region [168], the activity of *E. coli* His-tag ALP produced in this study may be inhibited in the same manner as the mammalian ALPs.

(8) The *E. coli* mutant ALP coating on Ti implants showed significantly higher ALP activities compared to bovine ALP coating ($p < 0.01$) and wild-type ALP ($p < 0.001$).

(9) The *E. coli* mutant ALP coating on Ti implants with high biological activity showed outstanding mineral producing ability compared to the wild-type ALP coating after soaking in cell culture media containing organic phosphates.

(10) PCL scaffolds containing 30% ALP exhibit significantly higher calcium contents compared to other groups on day 14 and 28 ($p < 0.001$), and the ALP/PCL scaffolds with higher ALP activity enhance the mineral deposition ability and accelerate the mineralisation process.

(11) The ALP/PCL scaffold enhanced cell differentiation and mineralisation compared to the PCL scaffold.

This study has therefore explored a novel strategy of engineering biomolecules with improved functionalities, increased the understanding of the mechanism of action of these engineered biomolecules, and demonstrated the potential applications of these engineered biomolecules in bone tissue engineering. At the same time, many challenges remain to be overcome in this study including the limited function of BMP-7-derived short peptides, the difficulties in the *E. coli* ALP production, and the activity

loss of the *E. coli* His-tag ALP. These challenges expand the scope of the current research areas, and could be designed to follow up for the future research.

The future research strategies could be designed are listed below:

(1) Bioactive factor-derived peptides with biological function

Despite previous studies had shown that BMP-7-derived short peptide KAISVLYFDDS enhanced mineral deposition, the results in this study demonstrate that it only shows limited function and is not promising candidate for bone tissue engineering applications. However, the findings in this research do not go against the benefits of using peptides, which include easier production, more precise control of the chemical components, and a higher using efficiency compared to whole proteins while preserving the entire or a certain part of the protein function. It would be an interesting approach for the future study that to verify the biological function of bioactive factor-derived peptides, especially from the bioactive regions.

(2) High level of recombinant protein expression

The mutant *E. coli* ALP possess outstanding biological activity, and could be considered promising candidate for bone tissue engineering applications. Although previous studies had shown the benefits of using the mutant *E. coli* ALP, few research had focused on bone tissue engineering applications. In this study, ALP coated implants and ALP/PCL scaffolds are two successful examples in bone tissue engineering applications, which show outstanding mineral-promoting ability. However, more bone tissue engineering strategies of the combination of ALPs with other supporting biomaterials are available and with a high possibility of success. One possible challenge of this approach would be the difficulties in protein production. The current expression system and purification method only satisfy a laboratory-scaled process. If the *E. coli* ALP-incorporated scaffolds are used in bone tissue engineering, a more efficiency protein production system, which produces large scales of *E. coli* ALP, is needed to meet the demands of manufacturing process.

(3) Fusion tag ALP with improved functionalities

One motivation behind the study of the *E. coli* His-tag ALP was to overcome the protein

production problem of *E. coli* ALP. However, the results demonstrate that the *E. coli* His-tag ALP lost its outstanding activity after incorporating a His-tag. Without the high activity, the His-tag ALP is not worth studying further to combine with scaffold for bone tissue engineering applications. However, adding fusion tags and engineering recombinant protein is still a feasible way to solve the problem. Future studies may use other expression systems and consider other fusion tags for purification. Regards to the feature of controlled immobilisation of the *E. coli* His-tag ALP, which was brought from His-tag, future study may achieve the controlled immobilisation by choosing specific functional groups through covalent binding. In addition, it is interesting for the future studies to consider the mechanism of the effect of His-tag on *E. coli* ALP since the majority of the research was focused on the mammalian ALPs.

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Appendices

1. Buffers and media used in the thesis

1) Luria-Bertani (LB) Broth 100 ml

Trypton enzymatic digest from casein	1 g
Yeast extract	0.5 g
NaCl	1 g
Distilled water	90 ml

Mix all components, adjust to pH 7.2, and then make up to 100 ml. Seal the bottle and autoclave at 121°C for 30 min. Store at 4°C.

2) Luria-Bertani (LB) BCIP/IPTG Kan Plate 100 ml

Trypton enzymatic digest from casein	1 g
Yeast extract	0.5 g
NaCl	1 g
Agar	1.5 g
BCIP	4 mg
10 mg/ml kanamycin stock	0.3 ml
100 mM IPTG stock	1 ml
Distilled water	90 ml

Mix all components, adjust to pH 7.2, and then make up to 100 ml. Seal the bottle and autoclave at 121°C for 30 min. When the solution is cooling down to 50°C, pour 15 ml solution to plate and place at room temperature until polymerised. Store at 4°C.

3) Washing buffer 1000 ml

Trizma base	1.2114 g
NaCl	1.755 g
Distilled water	900 ml

Mix all components, adjust to pH 7.3, and then make up to 1000 ml. Store at room temperature.

4) Suspension buffer 50 ml

Trizma base	0.19988 g
Distilled water	40 ml

Mix all components, adjust to pH 7.3, and then make up to 50 ml. Store at room temperature.

5) Agarose gel

Agarose powder	0.8 g
SYBR Safe DNA gel stain	8 µl
TBE running buffer	80 ml

Mix all components, and heat the mixture to boiling in microwave. When the solution is cooling down to 50°C, pour all solution to gel mold and place at room temperature until polymerised.

6) SDS gel

A 4X Running gel buffer 500 ml

Trizma base	90.83 g
10% SDS	20 ml
Distilled water	400 ml

Mix all components, adjust to pH 8.8, and then make up to 500 ml. Store at room temperature.

B 4X Stacking gel buffer 250 ml

Trizma base	15.14 g
10% SDS	10 ml
Distilled water	200 ml

Mix all components, adjust to pH 6.8, and then make up to 250 ml. Store at room temperature.

C 10X Stacking gel buffer 1000 ml

Trizma base	30.20 g
SDS	10 g
Glycine	144 g
Distilled water	1000 ml

Mix all components, and then make up to 1000 ml. Store at room temperature.

7) Transformation buffer

A Treatment buffer 1 500 ml

RbCl	6 g
MnCl ₂ ·4H ₂ O	5 g
1 M KOAc	15 ml
CaCl ₂ ·2H ₂ O	0.75 g
Glycerol	75 ml
Distilled water	400 ml

Mix all components, adjust to pH 5.8, and then make up to 500 ml. Sterilised by filtration through 0.22 µm filter. Store at 4°C.

B Treatment buffer 2 500 ml

RbCl	0.6 g
CaCl ₂ ·2H ₂ O	5.5 g
Glycerol	75 ml
0.5 M MOPS	10 ml
Distilled water	400 ml

Mix all components, adjust to pH 6.5, and then make up to 500 ml. Sterilised by filtration through 0.22 µm filter. Store at 4°C.

C SOC medium

Trypton enzymatic digest from casein	1 g
Yeast extract	0.5 g
NaCl	0.05 g
MgCl ₂	0.0952 g
KCl	0.0186 g
1M Glucose	2 ml
Distilled water	90 ml

Mix all components, adjust to pH 7.2, and then make up to 100 ml. Seal the bottle and autoclave at 121°C for 30 min. Store at 4°C.

8) TMZP buffer 1000 ml

Trizma base	1.2114 g
MgCl ₂	0.0952 g
NaH ₂ PO ₄	0.01379 g
ZnSO ₄ ·7H ₂ O	0.0028 g
NaN ₃	0.2015 g
Distilled water	900 ml

Mix all components, adjust to pH 7.45, and then make up to 1000 ml. Store at room temperature.

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