รายงานวิจัยฉบับสมบูรณ์

โครงการ อันตรกิริยาระหว่างเซลล์กับวัสดุคอมโพสิต SMART สำหรับการซ่อมแซมกระดูกสันหลังหัก

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
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สารบัญ

Abstract

The aim of this project was to develop SMART, cheap and easy to manufacture materials that are: 1) Simple to mix and snap setting to prevent leakage from site of application; 2) Mechanically a match to surrounding bone to reduce adjacent fracture risk; 3) Antibacterial against resistant bacteria; 4) Release minerals to promote bone integration; 5) Therapeutic to enable surrounding bone repair. The OBJECTIVES were: 1) development of systematically varying materials and testing of setting and mechanical properties in addition to kinetics of antibacterial, remineralising and therapeutic component release (UK laboratory) 2) investigation of how mechanical properties and component releases affects cell

With 1:1 UDMA/PDGGMA ratio, adding polylysine (PLS) (2-4 wt%) in the experimental composites significantly reduced biaxial flexural strength (BFS) from 140 to 110 MPa, modulus of elasticity from 3.6 to 2.7 GPa and mean surface contact angles from 75 to 60 degrees. The effect of monocalcium phosphate monohydrate (MCPM) level on these properties was negligible, but composites containing 10 wt% of MCPM clearly promoted surface apatite precipitation. Both PLS and MCPM markedly increased protein adsorption capability of the experimental composites by 2-15 folds, which was up to 8 folds greater than that of the commercial cement. Experimental bone composites with high MCPM possessed better initial hydrophilicity compared with the others

Cytotoxicity of these formulations was initially tested using L929 cells following ISO 10993-5, and all the composite formulations were only mildly reactive which met the requirement of the ISO standard. Extracts of experimental bone composites differentially influenced proliferation of mesenchymal stem cells (MSCs) and monocyte/macrophage RAW 264.7 cells, with the extracts derived from each of the first 5 days (*i.e.,* elutions derived from incubating the experimental bone cements in culture medium for every 24 h consecutively until Day 5) highly suppressing the growth of these cells under the standard culture condition. However, MSCs proliferated normally in the extracts collected after Day 5, which in contrast inhibited the growth of RAW 264.7 cells. This may suggest an anti-inflammatory effect of the bone composites, possibly via a previously reported anti-inflammatory role of PLS. The number of viable MSCs on experimental bone composites with high MCPM surfaces at 2 h and 5 days were highest among all the experimental composites and were comparable to that of commercial sample Cortoss. All formulations allowed normal MSC mineralization under osteogenic stimulation. Moreover, following immersion in SBF for 28 days, only formulations containing MCPM had precipitation of apatite on the surface. This indicates the apatite precipitate-inducing property of MCPM contained in bone composites.

Since treatment of osteoporotic bone fracture involving the use of PMMA cement can lead to multiple complications, a range of non-cytotoxic new materials that have the potential

to help solve PMMA problems and possess enhanced apatite precipitation with antibacterial activity were tested in the present study. Currently tested composites possessed favorable properties, including snap setting, comparable mechanical properties to bone, apatite precipitation induction and improved cytocompatibility with allowing MSC mineralization, suggesting their potential application in the treatment of vertebral bone fracture *in vivo*.

Keywords
Osteoporosis; Vertebral fracture; Bone composite; Antibacterial; Apatite precipitation; Cytocompatibility; Bone repair

บทคัดย่อ

เป้าหมายของโครงการนี้คือการพัฒนาวัสดุ SMART ที่ราคาถูกและง่ายต่อการผลิตโดยมีคุณสมบัติ: 1) การผสมง่ายและการแข็งตัวเหมาะสมเพื่อป้องกันการรั่วไหลจากการใช้งาน 2) มีสมบัติเชิงกลใกล้เคียงกับกระดูกโดยรอบเพื่อลดความเสี่ยงต่อการแตกหักของชิ้นกระดูกที่อยู่ติดกัน 3) ต้านเชื้อแบคทีเรีย 4) ปล่อยแร่ธาตุเพื่อส่งเสริมการเชื่อมยึดของกระดูก และ 5) ส่งเสริมการซ่อมแซมกระดูกโดยรอบ โดยมีวัตถุประ สงค์คือ: 1) การพัฒนาสูตรวัสดุที่แตกต่างกันอย่างเป็นระบบและการทดสอบการแข็งตัวและคุณสมบัติทางกล รวมทั้ง จลนพลศาสตร์ของการปล่อยส่วนประกอบของสารต้านแบคทีเรีย (ห้องปฏิบัติการในสหราชอาณาจักร) และ 2) การทดสอบการตอบสนองของเซลล์ต่อสูตรวัสดุที่แตกต่างกัน

ในวัสดุทดลองคอมโพสิตที่มีสัดส่วน UDMA/PDGGMA เท่ากับ 1:1 นั้น โพลีไลซีนปริมาณ 2-4 wt% ลดความต้านทานแรงดัดโค้งแบบสองแกน (biaxial flexural strength) จาก 140 MPa เหลือ 110 MPa ลดโมดูลัสความยืดหยุ่น (modulus of elasticity) จาก 3.6 GPa เหลือ 2.7 GPa และ ค่าเฉลี่ยมุมสัมผัสพื้นผิวจาก 75 องศา เหลือ 60 องศา อย่างไรก็ตาม ปริมาณโมโนแคลเซียมฟอสเฟตโมโนไฮเดรต (monocalcium phosphate monohydrate) ไม่มีผลอย่างมีนัยสำคัญกับปัจจัยเหล่านี้ วัสดุทดลองคอมโพสิตที่มีปริมาณโมโนแคลเซียมฟอสเฟตโมโนไฮเดรต เท่ากับ 10 wt% ส่งเสริมการตกผลึกของอะพาไทต์ นอกจากนี้ยังพบว่าทั้งโพลีไลซีน และ โมโนแคลเซียมฟอสเฟตโมโนไฮเดรตช่วยเพิ่มการดูดซับโปรตีนของวัสดุทดลองคอมโพสิต ได้มากถึง 2-15 เท่า และมากกว่าวัสดุโบนซีเมนต์ในท้องตลาดถึง 8 เท่า นอกจากนี้ วัสดุทดลองคอมโพสิต ที่มีโมโนแคลเซียมฟอสเฟตโมโนไฮเดรตปริมาณสูงจะมีพื้นผิวที่มีความชอบน้ำ (surface hydrophilicity) สูงขึ้นเมื่อเทียบกับวัสดุทดลองคอมโพสิตสูตรอื่นๆ

ความเป็นพิษต่อเซลล์ของสูตรเหล่านี้ถูกทดสอบเบื้องต้นโดยใช้เซลล์ L929 ตามมาตรฐาน ISO 10993-5 และสูตรคอมโพสิตทั้งหมดมีปฏิกิริยาเพียงเล็กน้อยเท่านั้นซึ่งเป็นไปตามข้อกำหนดของมาตรฐาน ISO สารสกัดของวัสดุทดลองคอมโพสิตมีอิทธิพลต่อการเจริญเติบโตของเซลล์ต้นกำเนิดมีเซนไคม์ (mesenchymal stem cells) และ เซลล์ RAW 264.7 โดยมีสารสกัดที่ได้จากการบ่มวัสดุทดลองคอมโพสิตในอาหารเหลวเลี้ยงเซลล์นาน 24 ชั่วโมงติดต่อกันจนถึงวันที่ 5 ยับยั้งการเจริญเติบโตของเซลล์เหล่านี้ อย่างมาก อย่างไรก็ตาม เซลล์ต้นกำเนิดมีเซนไคม์สามารถเจริญเติบโตตามปกติในสารสกัดที่ได้จากการบ่ม วัสดุทดลองคอมโพสิต ในอาหารเหลวเลี้ยงเซลล์ที่รวบรวมหลังจากวันที่ 5 ในทางตรงกันข้าม สารสกัดที่ได้ จากการบ่มวัสดุทดลองคอมโพสิต ในอาหารเหลวเลี้ยงเซลล์เหล่านี้มีผลยับยั้งการเจริญเติบโตของเซลล์ RAW 264.7 ซึ่งชี้ให้เห็นว่า วัสดุทดลองคอมโพสิตเหล่านี้อาจมีฤทธิ์ต้านการอักเสบ ตามที่ได้มีการรายงานถึงความสามารถในการต้านการอักเสบของโพลีไลซีน จากการศึกษาการเกาะตัวของเซลล์ที่เวลา 2 ชั่วโมงและ 5 วัน พบว่า วัสดุทดลองคอมโพสิตที่มีองค์ประกอบของโมโนแคลเซียมฟอสเฟตโมโนไฮเดรตสูงส่งเสริมการยึด เกาะของเซลล์ต้นกำเนิดมีเซนไคม์เมื่อเทียบกับวัสดุทดลองคอมโพสิตสูตรอื่นๆ และเทียบเคียงได้กับวัสดุตัว อย่างเชิงพาณิชย์ยี่ห้อ Cortoss นอกจากนี้ยังพบว่า วัสดุทดลองคอมโพสิตสูตรต่างๆ ไม่ขัดขวางการสร้างตะ กอนแร่ธาตุคล้ายกระดูกจากเซลล์ต้นกำเนิดมีเซนไคม์ภายใต้การกระตุ้นให้เกิดการสร้างกระดูกในห้องปฏิบัติ การ และ วัสดุทดลองคอมโพสิตสูตรที่มีส่วนประกอบของโมโนแคลเซียมฟอสเฟตโมโนไฮเดรตสามารถ กระตุ้นการตกผลึกอะปาไทต์ที่ผิววัสดุหลังจากที่แช่ในสารที่จำลองของเหลวในร่างกายนาน 28 วัน ซึ่งบ่งชี้ถึงความสามารถในการกระตุ้นการตกผลึกอะพาไทต์ของวัสดุทดลองคอมโพสิตสูตรที่มีโมโนแคลเซียมฟอสเฟตโมโนไฮเดรตเป็นองค์ประกอบ

เนื่องจากการรักษากระดูกหักในผู้ป่วยที่มีภาวะกระดูกพรุนโดยใช้ซีเมนต์ชนิด PMMA สามารถนำไปสู่ภาวะแทรกซ้อนหลาย คณะผู้วิจัยจึงได้ทดสอบ วัสดุใหม่ที่ไม่เป็นพิษที่มีศักยภาพที่จะช่วยแก้ปัญหาต่างๆ

ของ PMMA และสามารถกระตุ้นการตกผลึกอะพาไทต์ และมีฤทธิ์ต้านเชื้อแบคทีเรีย โดยวัสดุคอมโพสิตที่ผ่านการทดสอบในการศึกษานี้มีคุณสมบัติเป็นที่น่าพึงพอใจ มีการแข็งตัวทันทีเมื่อถึงเวลาที่เหมาะสม มีคุณสมบัติเชิงกลที่เทียบเท่ากับกระดูก ชักนำให้เกิดอะพาไทต์ ไม่เป็นพิษต่อเซลล์ และไม่ขัดขวางเซลล์ต้น กำเนิดมีเซนไคม์ ในการสร้างเนื้อเยื่อคล้ายกระดูกในห้องปฏิบัติการ ซึ่งบ่งชี้ว่าวัสดุเหล่านี้มีศักยภาพในการใช้ รักษากระดูกสันหลังหักในผู้ป่วยต่อไป

คำสำคัญ

ภาวะกระดูกพรุน; กระดูกสันหลังหัก; วัสดุคอมโพสิตสำหรับกระดูก; ฤทธิ์ต้านเชื้อแบคทีเรีย; การเกิดผลึกอะพาไทต์; การเข้ากันได้ระดับเซลล์; การซ่อมแซมกระดูก

Executive summary

The aim of this project was to develop SMART, cheap and easy to manufacture materials that are: 1) Simple to mix and snap setting to prevent leakage from site of application; 2) Mechanically a match to surrounding bone to reduce adjacent fracture risk; 3) Antibacterial against resistant bacteria; 4) Release minerals to promote bone integration; 5) Therapeutic to enable surrounding bone repair. The OBJECTIVES were: 1) development of systematically varying materials and testing of setting and mechanical properties in addition to kinetics of antibacterial, remineralising and therapeutic component release (UK laboratory) 2) investigation of how mechanical properties and component releases affects cell

Addition of polylysine (PLS) in the experimental composites significantly reduced biaxial flexural strength (BFS), modulus of elasticity and mean surface contact angles. The effect of monocalcium phosphate monohydrate (MCPM) level on these properties was negligible. Both PLS and MCPM markedly increased protein adsorption capability of the experimental composites while experimental bone composites with high MCPM possessed better initial hydrophilicity compared with the others

All the experimental formulations were only mildly reactive which met the requirement of the ISO standard. Extracts of experimental bone composites differentially influenced proliferation of mesenchymal stem cells (MSCs) and monocyte/macrophage RAW 264.7 cells, with the extracts derived from each of the first 5 days highly suppressing the growth of these cells under the standard culture condition. However, MSCs proliferated normally in the extracts collected after Day 5, which in contrast inhibited the growth of RAW cells. This may suggest an anti-inflammatory effect of the bone composites, possibly via a previously reported anti-inflammatory role of PLS. The number of viable MSCs on experimental bone composites with high MCPM surfaces were highest among all the experimental composites and were comparable to that of commercial sample Cortoss. All formulations allowed normal MSC mineralization under osteogenic stimulation. Moreover, following immersion in SBF for 28 days, only formulations containing MCPM had precipitation of apatite on the surface. This indicates the apatite precipitate-inducing property of MCPM contained in bone composites.

Since treatment of osteoporotic bone fracture involves the use of PMMA cement, which can lead to multiple complications, a range of non-cytotoxic new materials that have the potential to help solve PMMA problems and possess enhanced apatite precipitation with antibacterial activity were tested in the present study. Currently tested composites possessed favorable properties, including snap setting, comparable mechanical properties to bone, apatite precipitation induction and improved cytocompatibility and biomineralization. This suggests their potential application in the treatment of vertebral bone fracture *in vivo.*

CHAPTER 1
Introduction

Vertebral fracture is the most common fracture associated with osteoporosis. It may occur in the absence of trauma or after only minimal trauma, such as bending, lifting or turning. Once people suffer a first vertebral fracture, this increases the risk of new vertebral fracture four to five-fold and the risk of other fragility fractures two- to four-fold. Vertebral fractures are associated with an increased mortality and lead to deficits in emotional and physical health. They can cause chronic pain, kyphosis, height loss, disability, and reduced quality of life. Across Europe, osteoporotic vertebral fracture prevalence is 12% of the population while in the USA, subsequent osteoporotic collapse of the vertebra occurs in over 700,000 patients annually. The prevalence of vertebral osteoporosis in Thai population is 19.8%. Incidence of osteoporotic vertebral fracture in women aged over 50 years, between 1997 and 2002, was 32.1/1000 person years and increased with advancing age. Additionally, it is common for cancer patients to develop spinal metastases that can lead to painful vertebral fractures and impinge on the spinal cord causing paralysis.

Vertebroplasty and cement problems

In both Thailand and UK, a common treatment for painful back fractures is vertebroplasty. This involves injection of polymethylmethacrylate (PMMA) cement into the fractured vertebra under fluoroscopic control. This cement is produced through mixing of PMMA beads containing a polymerization initiator with liquid methylmethacrylate containing a polymerization activator. PMMA cement problems, however, include:

1) Cement viscosity slowly changes after mixing. With too low viscosity, cement leaks from the site of application are commonplace. Subsequent complications include enhanced adjacent vertebral fracture risk, paraplegia and pulmonary embolism. Too high viscosity, however, limits bone bonding and repair;
2) Radiopacifier, required to enable monitoring, reduces cement strength;
3) Amine activator (DMPT), required to promote polymerisation, is highly cytotoxic;
4) Methylmethacrylate high heat of polymerisation, cytotoxicity, aqueous solubility and long term release due to incomplete conversion;
5) Low material strength may limit fracture stabilisation;
6) High stiffness can enhance adjacent vertebral fracture risk;
7) Poor integration with surrounding bone may prevent fracture stabilisation;
8) Limited antibacterial (gentamicin) release can promote antibiotic resistance enabling rare but life threatening infection;
9) A lack of localised therapeutic action will allow continuing osteoporosis or metastasis.

Composite cement solution

To solve the first problem, the UK Co-applicant's group helped a new start-up company (Ozics) produce a modified dental composite as a replacement for PMMA. This is now distributed in Europe as Comp06 bone cement. Her group has, however, now produced a range of systematically varying new materials that have the potential to help to solve all the above PMMA problems. The chemical composition of the new material is provided in Fig. 1 with a summary of the function of the different components. These composites help to solve the PMMA problems through:

1) Material supply as 2 pastes instead of powder and liquid. Unlike the PMMA cements, the composite viscosity remains unchanged after mixing allowing much greater control over placement. Lower material viscosity at placement would enable the composite to interdigitate with the bone. Snap set of the materials can then help to prevent flow from the site of application (leakage);

2) Radiopaque filler replacing both the polymethylmethacrylate and radiopacifer. This enables greater visualisation with X-rays. Silane treatment of this filler enables bonding to the polymer matrix thereby preventing reduction in strength upon its addition even at high volume fractions;

3) Use of a polymerisable amine (NTGGMA). Binding of this component within the set material would prevent amine toxicity problems;

4) Use of higher molecular weight and flexible dimethacrylate monomers instead of methylmethacrylate. Higher molecular weight reduces monomer toxicity and heat generation during setting. Use of flexible monomers can also enhance polymerisation to reduce probability of monomer diffusion from the set material;

5) Enhanced monomer conversion increasing mechanical strength (Fig. 2);

6) Flexible monomers reducing material stiffness;

7) Addition of monocalcium phosphate and low levels of a calcium binding monomer (4 META) promoting hydroxyapatite precipitation from body fluid (Fig. 3). and aiding bone attachment;

8) Release of high percentages of antibacterial polylysine (Fig. 4a). This would be more effective at killing gentamicin resistant bacteria than low level gentamicin release from a PMMA cement (Fig.4b and 4c);

9) Release of strontium and / or the bisphosphonate zoledronic acid. These could help to increase bone repair or prevent its resorption.

The UK group had undertaken extensive physical, chemical and mechanical testing of a wide range of formulations and some preliminary studies to confirm materials are not cytotoxic Collaboration with the Thai research group, however, would enable a much greater understanding of which materials are likely to be most beneficial in treating vertebral fractures and promoting surrounding bone repair. This collaboration would also enable tests that are

required before in vivo work which the Co-applicant wishes to undertake with collaborators at Stanmore Orthopaedic hospital.

Aim and objectives

The aim of this project was to combine the expertise of the UK applicant in biomaterials development with that of the Thai fellow in cell / biomaterial interactions to develop more optimal cements for bone fixation. This interaction helped improve our understanding of material cell interactions. This would aid gaining of regulatory approval for future manufacture, and translation into clinical use of new formulations that solve current major bone cementation problems. Although there are multiple potential medical applications for the materials under development, this proposal shall focus upon formulations for cementation of vertebral fractures via vertebroplasty.

The objectives were to:

1) Produce a systematically varying range of materials and test their physical, chemical and mechanical properties in the UK
2) Assess and provide understanding as to how changes in material chemistry and mechanical properties affect cell response in Thailand.

CHAPTER 2
Literature Review

Osteoporotic vertebral fractures

Epidemiology

The WHO considers osteoporosis a major health concern due to its high prevalence and severe complications. It affects approximately 200 million people worldwide (Lane, 2006). Worldwide, osteoporosis causes ~ 9 million fractures annually (Johnell and Kanis, 2006). It has been estimated that osteoporosis led to 1.5 million fractures per year in the US (Black and Rosen, 2016). Studies estimate the current number of osteoporotic vertebral fractures in the UK as 65,000 per year (Svedbom et al., 2013). Additionally, the incidence of vertebral fractures will increase by 23% by 2025 (Bouza et al., 2015). The five years survival rate after hip and vertebral fracture were approximately 80% (Harvey et al., 2010).

In the US, the estimated direct medical cost for osteoporosis and related fracture treatment is 20 billion dollars per year. In Europe, the economic burden of osteoporosis in 2010 was estimated to be 30.7 billion euro. This medical cost has been predicted to reach 76.7 billion euro in 2050 (Pisani et al., 2016). The estimated direct medical costs for treating osteoporotic fractures currently in the UK is ~ £1.8 billion per year but this could increase to £2.2 billion per year by 2025 (Burge et al., 2008).

Aetiology

Osteoporosis is a systemic skeletal disease resulting in a decrease of bone mineral density and profound changes in the bony micro architecture. The reduction of oestrogen either due to menopause or surgery leads to an increase in the production of the receptor activator of nuclear factor **κ**B (RANKL). The increase in this ligand and its reaction with the receptor initiates the proliferation and maturation of osteoclast precursors (Favus, 2010). This subsequently leads to an imbalance of bone remodelling.

The decrease of oestrogen production also leads to reduced intestinal calcium absorption and increase in calcium loss (Armas and Recker, 2012). Hence, the bone becomes weaker and susceptible to fracture from normal physiologic loads. Osteoporotic fractures are the most common complication found in osteoporotic patients. Frequently affected sites include hip, spine, and forearm (Rachner et al., 2011). The incidence of osteoporotic fractures varies by region. It has been estimated that up to 50 % of women older than 50 years will experience osteoporotic fractures during their lifetime (Eastell et al., 2016).

Vertebroplasty (VP) and kyphoplasty (KP)

An osteoporotic vertebral fracture causes pain, height loss, limited mobility, kyphosis, and reduced pulmonary function. The traditional non-operative treatments are bed rests, analgesics, and bracing. These non-operative managements failed to relieve severe pain in one-third of patients (Benzel, 2012; Lin et al., 2016). Furthermore, they also lead to the disease condition worsening and more complications.

Minimally invasive surgical treatments, vertebroplasty (VP) and balloon kyphoplasty (KP), have been employed to stabilise fractures, relieve pain, and increase mobility for patients who have failed to response from conservative treatments (McDonald et al., 2017). The currently accepted indications for VP and KP are painful osteoporotic vertebral compressive fracture, painful metastatic/malignant vertebral body lesions, and vertebral traumatic fracture (Wong and McGirt, 2013; Yimin et al., 2013).

Studies showed that patients treated surgically experienced rapid and significant pain reduction, improved pulmonary function, and had longer survival rates (Diamond et al., 2006; Lee et al., 2011; Blasco et al., 2012; Xu et al., 2012; Chen et al., 2013; Takura et al., 2017). Furthermore, a recent multicentre, randomised, double-blind, and placebo-controlled trial also revealed a superior pain reduction for patients that received vertebroplasty compared to patients that recieved simulated vertebroplasty (placebo intervention) (Clark et al., 2016). It has been estimated that the cost of VP and BKP is £800 and £2600 per procedure respectively (NICE, 2013). Both treatments, however, showed a comparable outcome in pain reduction and functional recovery (Ates et al., 2016).

Vertebroplasty (VP)

VP was introduced in 1984 by Gakibert and Deramond (Hulme et al., 2006). This treatment can be performed under local or general anaesthesia. Briefly, a cannula is inserted into the affected area. A bone cement is then injected into the collapsed vertebra under fluoroscopy control (Mukherjee and Lee, 2011). The treatment aims of VP, however, are not to restore the height of collapsed vertebra, but rather pain relief and the prevention of further spinal mal-alignment (Benneker and Hoppe, 2013; Yimin et al., 2013).

Kyphoplasty

Kyphoplasty or balloon kyphoplasty (KP) was introduced by Mark Reily in 1998 as an alternative to VP for restoring vertebral height and realigning the spine (Yimin et al., 2013). The inflatable bone tamp is inserted into the fracture site under fluoroscopy control (Fig 1-13 a). After creating the cavity and restoring the height of the collapsed vertebra (Fig 1-13 b), the balloon tamp is removed followed by the injection of bone cement (Taylor et al., 2006; Vallejo and Benyamin, 2010).

Current bone cements for VP and BKP

Currently there are no specific requirements for a bone cement used in VP and KP. (Lewis, 2006) has proposed several required properties for the cement. The currently used bone cements for VP and KP are described as follow.

Polymethyl methacrylate (PMMA)

PMMA, the polymer of methylmethacrylate (MMA), is a commonly used bone cement for various orthopaedic applications. A commercial example of PMMA cement is Simplex P® or Spineplex® (Stryker, Newbury, Berkshire, UK). The powder phase contains PMMA, MMA-styrene copolymer, radiopacifier (barium sulphate) and benzoyl peroxide (BP). The monomer phase contains methylmethcrylate and N-dimethyl-p-toluidine (DMPT). Advantages of PMMA bone cement include familiarity for the orthopaedic surgeons, high strength (flexural strength ~ 150 MPa), and cost-effectiveness.

Disadvantages of this cement include handling difficulty, rapid changing of viscosity after mixing, high heat generation (82 - 86 ^{o}C), high shrinkage, lack of bone bonding potential, and risk of toxic residual monomer release (Lewis, 2006; Boyd et al., 2008; Benzel, 2012; Vaishya et al., 2013; Khan et al., 2014). Another serious concern of PMMA cement is its mechanical mismatch with the vertebral bone. The elastic modulus of PMMA cement (1.7 - 3.7 GPa) (Boger et al., 2007) is much higher than that of cancellous bone (0.1 - 0.7 GPa)(Banse et al., 2002). The differences in modulus of elasticity between treated and untreated vertebra may then subsequently increase the risk of adjacent vertebral fracture (Hadley et al., 2010).

Calcium phosphate cements (CPCs)

The main advantage of CPCs is their osteoconductivity that may promote apposition at bone-cement interfaces (Tamimi et al., 2012). After the powder phase is mixed with the liquid phase, the paste then solidifies via a dissolution-precipitation process. The cements can be classified by their end product which is either apatite or brushite (Cama, 2014). In 2012, however, illegal testing of a CaP cement (Norian XR, Synthase, West Chester, Pa, USA) for vertebroplasty was performed on humans. This led to the death of five patients on the operating room table (Kimes, 2012). and put into serious question any use of CPCs in this application.

A common problem of the CPCs is separation of the liquid phase and powder phase during injection (O'Neill et al., 2016). Incomplete set can lead to pulmonary embolism (Bernards et al., 2004). The cements also tend to disintegrate upon exposure to fluids or blood (Wang et al., 2007). Furthermore, their excessive modulus of elasticity (8 -14 GPa) at early time may increase the risk of adjacent vertebral fracture (O'Hara et al., 2014). Moreover, inconsistency of resorption rate also led to rapid reduction in strength (Yang and Zou, 2011).

Bone composites

An example of a commercial bone composite used in vertebroplasty is Cortoss® (Stryker, Newbury, Berkshire, UK). Its monomer phase contains Bis-GMA, Bisphenol-A-ethoxy dimethacrylate (Bis-EMA), and TEGDMA. The powder phase contains bioactive glass (combeite, $Na_2O–CaO–P_2O_5–SiO_2$) to enhance bone bonding, and barium boroaluminosilicate glass to improve mechanical properties and radiopacity (He et al., 2015). This composite is supplied in a double-barrelled syringe with mixing gun which could facilitate the handling process.

This bone composite is believed to be bioactive as some *in vivo* studies showed new bone apposition at the bone-composite interface without fibrous interposition (Mehbod et al., 2003; Sanus et al., 2013). A randomised controlled clinical trial showed that patients treated with Cortoss exhibited early pain reduction but in addition better long-term preservation of function compared to that of the patient treated with PMMA (Bae et al., 2012).

Concerns of this composite cement include its high exothermic reaction (63 ^{o}C), high stiffness (~ 2 GPa), and lack of antibacterial or therapeutic properties (Boyd et al., 2008; Anselmetti et al., 2009). Additionally, an *in vitro* study found that Cortoss showed a cytotoxic effect on human cells (Becker et al., 2006)

Complications after VP and KP

Cement leakage

Cement leakage is the most common complication associated with VP and KP. The leakage depends on the fracture characteristic of the vertebra, injection method, and physicochemical properties of the bone cement (Xin et al., 2016). The incidence of this complication observed from normal radiograph of the patients was 31 % and 11 % for VP and KP respectively (Du et al., 2014). The incidence observed from normal radiograph was underestimated as it could increase up to 77 % when patients were assessed with CT scan (Tome-Bermejo et al., 2014).

Despite the fact that the majority of the leakage occurrences may not pose a clinical problem, some can have severe consequences (Hulme et al., 2006). The severity of the complication depends on the site that cement leaks to. For example, cement leaking into the neural foramen may result in neurologic complications (Boonen et al., 2011). Cement leaking into paravertebral veins may cause embolism at pulmonary or cardiovascular systems (Arnaiz-Garcia et al., 2014; Janssen et al., 2017).

Subsequent adjacent fractures

The second most common complication after VP and KP is an adjacent vertebral fracture. This complication was found in 12 – 50 % of patients (Li et al., 2012). The majority of those fractures were symptomatic and detected within one month (Takahara et al., 2016).

The risk of developing adjacent vertebral fracture is similar following both VP and KP procedures (Du et al., 2014).

Researchers have not yet been able to provide conclusive evidence that the high risk of adjacent fractures is caused by the treatments. Several studies have, however, suggested that the fracture risk may be exacerbated by the alteration of load transfer due to the increase of stiffness of the injected vertebra (Klazen et al., 2010; Fahim et al., 2011; Cho et al., 2015; Holub et al., 2015). The high stiffness of the injected cement may affect the load transferred to the adjacent bone. The lack of applied loads may lower the bone density and subsequently reduce the strength of the adjacent bone: a problem known as stress shielding (Papanastassiou et al., 2014). The fracture, however, could be caused by the natural progression of the disease, improper technique, and patient factors (Aquarius et al., 2013; Takahara et al., 2016).

Postoperative infection

Although postoperative infections are not common (< 1 %), they often require further invasive surgical intervention associated with high mortality rate (33 %) (Abdelrahman et al., 2013). The most common isolated organism from patients was *S. aureus* (Abdelrahman et al., 2013). Patient comorbidities such as multiple systemic diseases or immunosuppression may allow the low-virulence organism to colonise at the operation site. Hence, bone cement mixed with antibiotics such as gentamycin or vancomycin has been recommended for the medically compromised patient (Walker et al., 2004; Hashimoto, 2010). However, the use of antibiotic loaded bone cement as a prophylaxis is not yet widely accepted. Some clinicians routinely mix a bone cement with an antibiotic to prevent this serious complication (Lee et al., 2007).

Development of low stiffness, mineralising, and antibacterial bone cement

Due to above mentioned complications, several studies have been conducted to develop injectable bone cements for VP and KP that provide adequate mechanical matching to bone, bone-bonding ability, and antibacterial properties. Methods employed to improve those properties are described below.

Low stiffness bone cement

Studies have shown that the application of a low stiffness bone cement in *ex vivo* human spinal segments can reduce the risk of adjacent vertebral fracture. Pressure concentrations adjacent to the injected cement were smaller with low modulus cement compared with standard cement (Kinzl et al., 2012a; Kolb et al., 2013a). Several methods to lower the stiffness/modulus of elasticity have been proposed.

Boger et al. (2007) developed a low modulus bone cement by mixing a commercial PMMA with 35% sodium hyaluronate. This technique reduced the modulus of elasticity by

74% without causing any collapse of the injected vertebra. Lopez et al. (2011) mixed the PMMA cement with castor oil, reducing the modulus of elasticity by ~ 60 %. The castor oil however negatively affected polymerisation of the cement. Kolb et al. (2013b) managed to lower the modulus of elasticity of the PMMA cement by 51 % by mixing the cement with fetal bovine serum. The spinal segment injected with modified cement showed a higher fatigue fracture force than the group treated with standard PMMA. Schröder et al. (2016) modified a standard PMMA cement by addition of normal saline. Modulus of elasticity of the cement was reduced by almost three times upon adding 30 vol% of normal saline. This, however, was associated with an increase of setting time.

These modifications decreased the stiffness of bone cements but may interfere with the fluid structure and the setting mechanism of the cements. This may lead to other problems such as poor handling properties and injectability, phase separation, and cement toxicity, and cement extravasation.

Calcium phosphate containing bone composite

Due to the superior bioactivity of CPCs, calcium phosphate compounds (CaP) have been incorporated into bone cements. The addition of these compounds promoted additional benefits such as low setting temperature and improved bioactivity (Rodriguez et al., 2014; Wu et al., 2016a). When this composite is exposed to an aqueous environment, ions are exchanged and re-precipitate as a calcium phosphate apatite. The apatite composition depends mainly on the local pH and ion saturation. The apatite is believed to promote *in vivo* bone bonding (Kokubo et al., 2003; LeGeros, 2008).

Bone bonding ability of the cement is required to promote new bone apposition at the bone-cement interface. The formation of the biomimetic bone substrate or an adsorption of bone specific proteins by this layer may promote osteoblast adhesion and its activity (Alves et al., 2010). Hence, the addition of calcium phosphate compounds is a simple method to encourage bioactivity of the bone cement.

An *in vivo* study showed that incorporation of β-TCP to PMMA promoted osseointegration with no obvious local toxicity signs (Dall'Oca et al., 2014). The addition of CaP compounds unfortunately caused a reduction of mechanical properties, increased cement viscosity, and poor handling properties of the cement. It has been shown that addition of 40 wt% brushite into PMMA cement led to poor handling properties and injectability, and reduced strength (Rodriguez et al., 2014).

Antibiotic-loaded bone cement

Postoperative infection after VP or KP is a serious complication. Systemic antibiotic prophylaxis has been recommended for high-risk patients. Antibiotics, such as gentamycin, have been incorporated into bone cements to minimise the occurrence of infection. Bone cement with added antibiotics may provide local release of antibiotic. Antibiotic

concentration may then exceed that obtained with systemic administration and reduce systemic adverse reactions (Anagnostakos, 2017). The addition of this agent has reduced the rate of infection after total joint arthroplasty (Chang et al., 2013). The addition of antibiotic however reduced mechanical properties of the cement (Wang et al., 2013). Moreover, the slow release of sub inhibitory amount of antibiotics over time may also increase the risk of developing antibiotic resistance (Walker et al., 2016). For patients who need revision surgery an alternative antibiotic to that used in any original bone cement is therefore needed (Jiranek et al., 2006).

CHAPTER 3
Materials and Methods

Preparation of bone cement (manufactured in UK)

Monomer phase (wt%)

UDMA	47.625
PPGDMA	47.625
4-META	3
BP	1
NTGGMA	0.75

Powder phase (wt%)

7 um glass	44
0.7 um glass	44
MCPM	8 or 6
PLS	4 or 2

Formulations	F5	F1	F2	F3	F4
MCPM (wt%)	0	8	8	6	6
PLS (wt%)	0	4	2	4	2

Curing and Cured bone cement discs (5 mm in diameter and 1 mm thick) were prepared as follows:

- Curing samples were prepared by preparing the bone cement discs and storing in dry storage at 4°C.
- Cured samples were prepared by pre-incubating the samples in sterile water (one 5 mm-diameter disc in 1ml of sterile water) and incubated in a humidified incubator (37°C and 5% CO_2) for the time indicated before use.

Both types of bone cement discs were cleaned by disinfectant towelettes and then sterilised by UV for 30 minutes on each side prior to each experiment.

Preparation of bone cement extract

Cured bone cement discs were prepared as mentioned above. Following an indicated time of pre-treatment in water, the bone cements were sterilised and placed into a 96 well plate by sterile forceps, and an amount of 120 ul **α**-MEM was added into each well. The samples were then incubated in the humidified incubator (37°C and 5% CO_2) for 24 h and the supernatant was collected and stored at -20°C for subsequent experiments.

Protein adsorption

Bone cement sample is placed into fresh Eppendorf tube and incubate with protein solutions for 1 h in 37°C incubator. The tested solutions included: 1) 5% w/v bovine serum albumin (BSA) in PBS: representing the most abundant protein in serum, 2) Fetal bovine serum (FBS): representing total serum protein and 3) Distilled water: used as Blank. The protein solution was then removed and the bone cement was washed gently with deionised water (1 ml) for 3 times. The bone cement was dried in tissue papers and then placed into a fresh 48-well plate. An amount of 400 μL of BCA solution (Reagent A + B) was added into the sample, shaken gently (very gently) and incubated in 37°C incubator for 30 minutes. The incubated BCA solution was removed from the sample and transferred into a 96-well plate for protein detection using a BCA assay kit.

Cell culture

Human mesenchymal stem cells (MSCs) and RAW cells were used. MSCs are undifferentiated cells that have an ability to differentiate along with various lineages, including the osteoblastic lineage under proper stimulation, and RAW cells are murine monocytic cells which can be induced into either inflammatory macrophages or osteoclastic cells under certain induction. Cells were cultured in α-minimum essential medium (α-MEM) (Gibco Life Technologies Ltd, Paisley, UK) containing 10% fetal calf serum (FCS) (PAA Laboratories, Yeovil, UK) supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 2 mM L-glutamine (all from Gibco) at 37°C in a humidified atmosphere of 5% CO2 in air.

SEM analysis of cells on bone cement discs

The samples were fixed in 3% glutaraldehyde with 0.14 M sodium cacodylate buffer (pH 7.3) at 4°C for 18 h. The samples were then dehydrated in a graded series of alcohols (50%, 70%, 90% and 100%), washed with hexamethyldisilazane (TAAB Laboratories;Berkshire, UK) for 5 min and stored in a desiccator. The dehydrated samples were placed onto stubs using a conductive carbon tap and then sputter-coated with gold. Cell morphology and cell attachment were observed at 10 kV using a JEOL JSM 5410LV SEM (JEOL UK, Welwyn Garden City, UK).

Alamar blue assay and cell morphology study

The sterilised bone cement discs were placed into a 96 well plate by sterile forceps. 200 µl of 4,000 MSCs or 5,000 RAW cells was seeded on each well and cultured in the humidified incubator (37°C and 5% CO_2). Cell viability was detected by Alamar blue assay at 1, 3 and 7 days of culture. In some experiments, the cell morphology was determined under Scanning Electron microscope (SEM). For assessment of bone cement extract, MSCs 2000 cells/well or RAW cells 5000 cells/well was seeded into the 96 well plate and incubated of 24 h. After the incubation, 100 µl of the bone media extraction was added to the cells and further incubated in the humidified incubator for indicated times. Cell viability was examined

by using the Alamar blue assay at 1 and 3 days of incubation and then stained by crystal violet for morphology observation under a light microscope at day 3.

In some experiments, L929 cells were used for cytotoxicity testing following ISO 10993-5, and the cytotoxicity was categorized following USP (United States Pharmacopeia)*, as shown below.

Grade	Reactivity	Condition of Cultures
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen and up to 4 mm
3	Moderate	Zone extends 5-10 mm beyond specimen
4	Severe	Zone extends greater than 10 mm beyond specimen

*The sample meets the requirement of the test if the response to the sample preparation is not greater than grade 2 (mildly reactive)

Physical and mechanical property testing

Inhibition times and final monomer conversions following two-paste mixing were assessed using FTIR (n=3). Biaxial flexural strength (BFS) and elastic modulus were tested using ball-on-ring testing jig (n=5) after 1-day simulated body fluid immersion. Water contact angle on cured composites was measured using a goniometer (n=3). Surface apatite precipitation was assessed using Raman microscopy and scanning electron microscopy (SEM) after 7 days. Factorial analysis was used to quantify effects of low versus high additive contents and a formulation with no MCPM or PLS employed as control.

Experimental SMART cement formulations (manufactured in Thailand)

Currently adjusted formulations of the SMART bone composites are shown in Table 1. Amounts of each of the compositions, for manufacturing 10 g of initiator paste and 10 g of activator paste, are shown in Table 2.

Manufacturing process

1. Prepared stock monomer solution for the initiator and activator by mixing UDMA, PPGDMA and HEMA, as displayed in the above tables, by stirring at room temperature for 30 minutes.

2. Added BPO into the stock initiator phase and NTGGMA into the stock activator phase, and stirred at room temperature for 2 hours followed by sonicating the samples at 40°C for another 2 hours.

3. Prepared the glass phase by mixing the compositions, as shown in the table

4. Mixed the glass phase and the monomer phase with the glass:monomer ratio of 3:1 using the planetary mixer deaerator at rotation speed 4285 rpm for 1 minute for the total mixing of 4 cycles

5. Mixed the initiator phase and the activator phase at 1:1 ratio by weight by hand mixing on a paper pad for 1 minute

6. Added the well mixed paste into a metal ring (diameter 10 mm for mechanical testings; 4 mm and 9 mm for biological testings), covered the samples with an acetate sheet and incubated at 37°C for 1 hour to reach optimal polymerization.

Table 1. Compositions of the currently adjusted formulations **(manufactured in Thailand)**

Code	Monomer phase		Glass phase	
	Initiator	*Activator*	*Initiator*	*Activator*
UCL	1. UDMA:PPGDMA (2:1) 2. BPO 3%wt 3. HEMA 2.5%wt of UDMA+PPGDMA	1. UDMA:PPGDMA (2:1) 2. NTGGMA 2%wt 3. HEMA 2.5%wt of UDMA+PPGDMA	Glass*	Glass
UCL+6.6P			Glass + PLS 6.6%wt	Glass + PLS 6.6%wt
UCL+10.5M			Glass + MCPM 21%wt	Glass
UCL+6.6P +10.5M			Glass + PLS 6.6%wt + MCPM 21%wt	Glass + PLS 6.6%wt

Glass phase : monomer phase = 3:1 *Glass 0.7 um : 7 um = 1:1

Table 2. Amount of each of the compositions for manufacturing 10 g of initiator paste and 10 g of activator paste

Code	Monomer phase		Glass phase	
	Initiator	Activator	Initiator	Activator
UCL	Stock initiator phase (2.5 g) 1. UDMA = 1.578 g 2. PPGDMA = 0.789 g 3. HEMA = 0.059 g 4. BPO = 0.075 g	Stock activator phase (2.5 g) 1. UDMA = 1.594 g 2. PPGDMA = 0.797 g 3. HEMA = 0.060 g 4. NTGGMA = 0.050 g	1. Glass 0.7μm = 3.75 g 2. Glass 7μm = 3.75 g	1. Glass 0.7μm = 3.75 g 2. Glass 7μm = 3.75 g
UCL+6.6P			1. Glass 0.7μm = 3.5 g 2. Glass 7μm = 3.5 g 3. PLS = 0.5 g	1. Glass 0.7μm = 3.5 g 2. Glass 7μm = 3.5 g 3. PLS = 0.5 g
UCL+10.5M			1. Glass 0.7μm = 2.95 g 2. Glass 7μm = 2.95 g 3. MCPM = 1.6 g	1. Glass 0.7μm = 3.75 g 2. Glass 7μm = 3.75 g
UCL+6.6P +10.5M			1. Glass 0.7μm = 2.7 g 2. Glass 7μm = 2.7 g 3. MCPM = 1.6 g 3. PLS = 0.5 g	1. Glass 0.7μm = 3.5 g 2. Glass 7μm = 3.5 g 3. PLS = 0.5 g

CHAPTER 4
Results and Discussion

Differential protein adsorption capacity of bone cements

The results in Fig. 1 show that the cured (3 days pre-soaked in water) bone cements had higher BSA adsorption ability than the curing (non pre-soaked bone cement). F5 had the lowest BSA adsorption. F3 had the highest BSA adsorption. F1, F3, F4 adsorbed BSA at comparable levels to the Kyphon (KY; a commercially available PMMA cement). In marked contrast, the curing samples appeared to adsorb more FBS than the cured samples (Fig. 2). F1 and F5 had significant higher level of adsorbed FBS proteins than the other formulations.

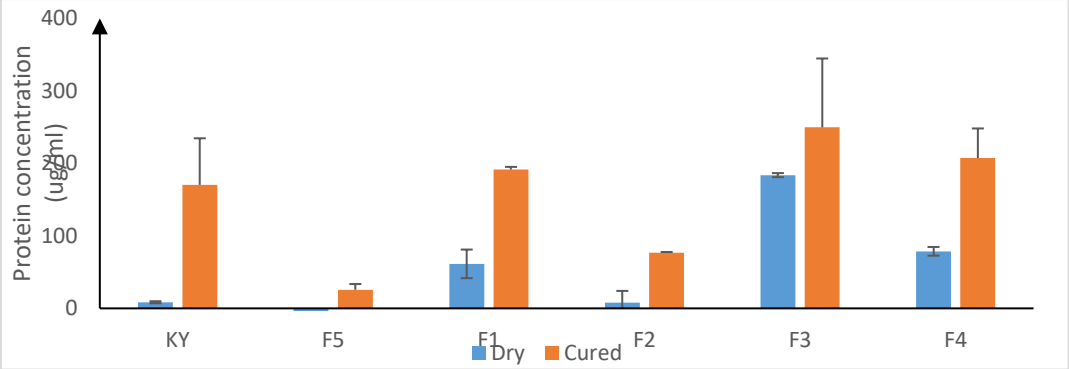


Fig. 1. Adsorption of BSA on bone cements (F1-F5). A commercially available bone cement used in vertebral fracture treatment (KY) was used for comparison, while F5 was used as a control formulation (without adding MCPM or PLS).

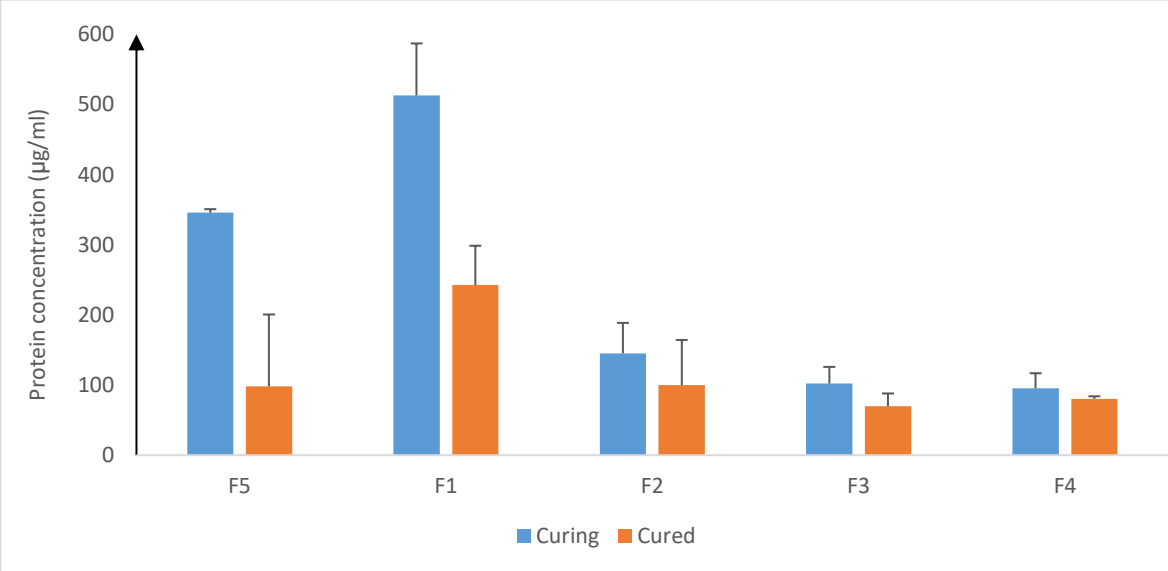


Fig. 2 Adsorption of FBS on bone cements (F1-F5). A commercially available bone cement used in vertebral fracture treatment (KY) was used for comparison, while F5 was used as a control formulation (without adding MCPM or PLS).

Effect of bone cements on cell viability of MSCs

The fluorescence signal (RFU) from alamar blue assay was measured to determine the cell viability of MSCs cultured on curing (dry bone cement) samples (Figure 1) and cured (bone cement pre-incubated in sterile water for 3 days) samples (Figure 2). In general, the cell viability of MSCs on both types of bone cements and all formulation including the KY cement was decreased as time progresses, suggesting their cytotoxicity. F5 curing show the highest RFU among bone cements at day 7. However, MSCs were not observed in all bone cement sample at day 7 under SEM (Table 3 and 4). In conclusion, all bone cements and the KY bone cement were cytotoxic to MSCs under conditions tested here.

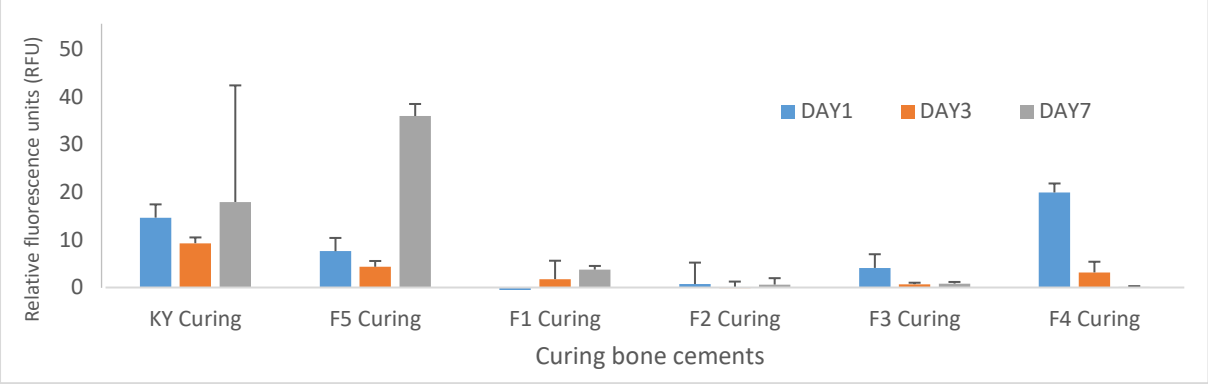


Fig. 3. Cell viability of MSCs on bone cement (Dry) at time points 1, 3 and 7 days.

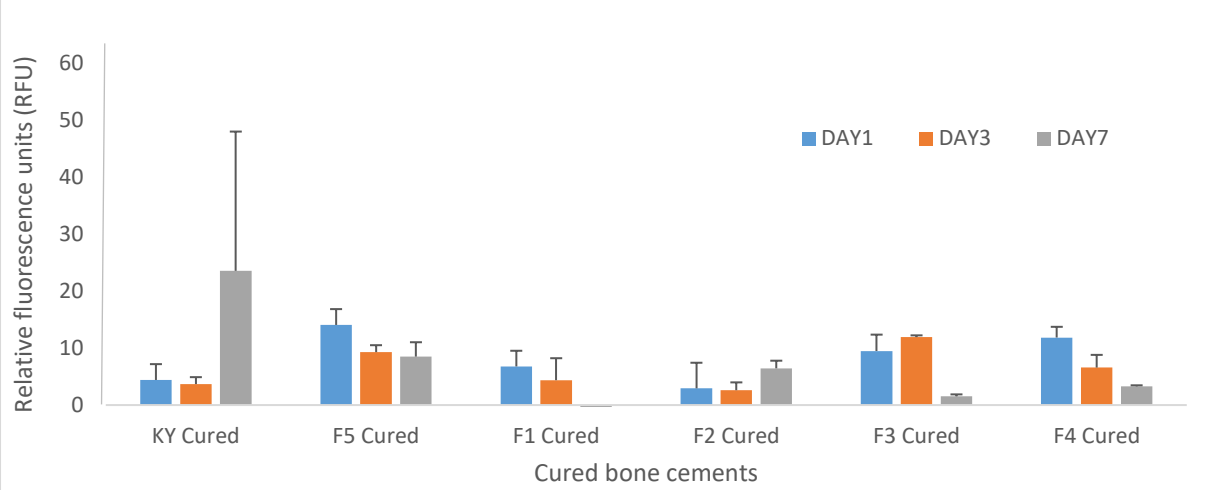


Fig. 4. Cell viability of MSCs on bone cement (soaked in sterile water for 3 days) at time points 1, 3 and 7 days.

Table 3 Cell morphology of MSCs at day 7on curing bone cements observed under SEM

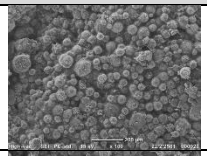
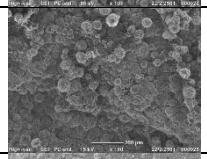
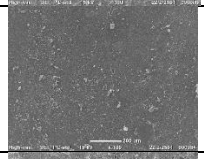
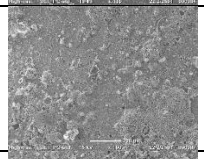
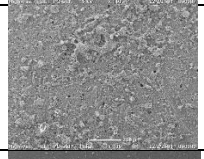
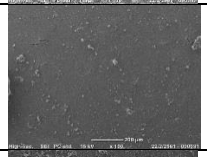
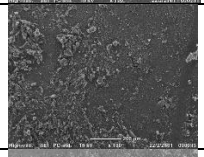
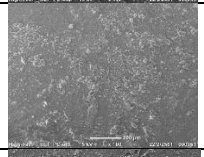
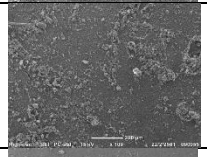
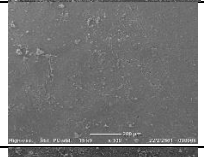
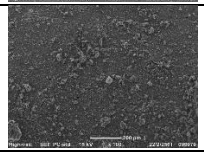
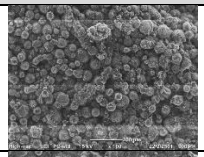
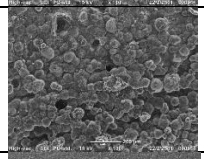
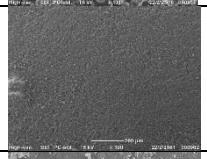
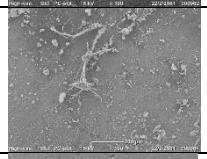
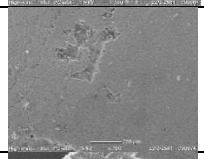
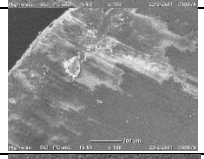
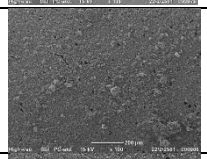
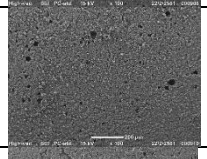
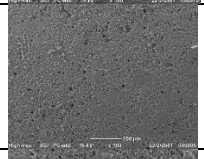
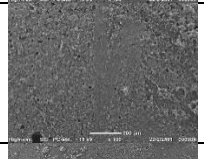
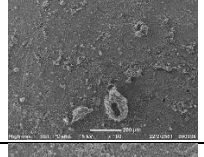
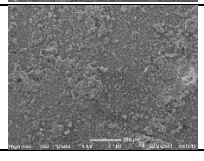
Bone cements curing	100X
KY	
KY + MSCs	
F5	
F5 + MSCs	
F1	
F1 + MSCs	
F2	
F2 + MSCs	
F3	
F3 + MSCs	
F4	
F4 + MSCs	

Table 4 Cell morphology of MSCs at day 7on cured bone cements observed under SEM

Bone cements cured	100X
KY	
KY + MSCs	
F5	
F5 + MSCs	
F1	
F1 + MSCs	
F2	
F2 + MSCs	
F3	
F3 + MSCs	
F4	
F4 + MSCs	

Effect of bone cements on cell viability of RAW cells

The fluorescence signal (RFU) from alamar blue assay was used to determine the cell viability of RAW cells culture on curing (dry bone cement) in Fig. 5 and cured (bone cement soaked in sterile water) in Fig. 6. The RAW cell viability was decreased in all bone cements. Curing KY, F5 and cured KY showed the highest RFU among bone cements at day 7. However, RAW cells were not presented on all bone cement sample surfaces at day 7 under SEM (Table 5 and 6). In conclusion, all bone cements had cytotoxicity effects on RAW cells.

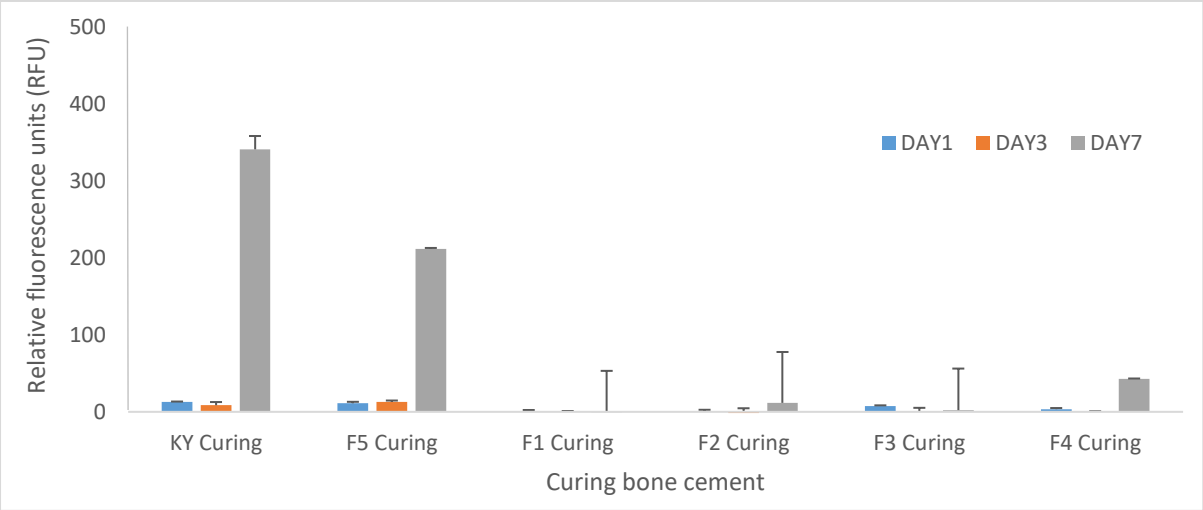


Fig. 5 Cell viability of Raw cells on curing (dry) bone cement at time points 1, 3 and 7 days.

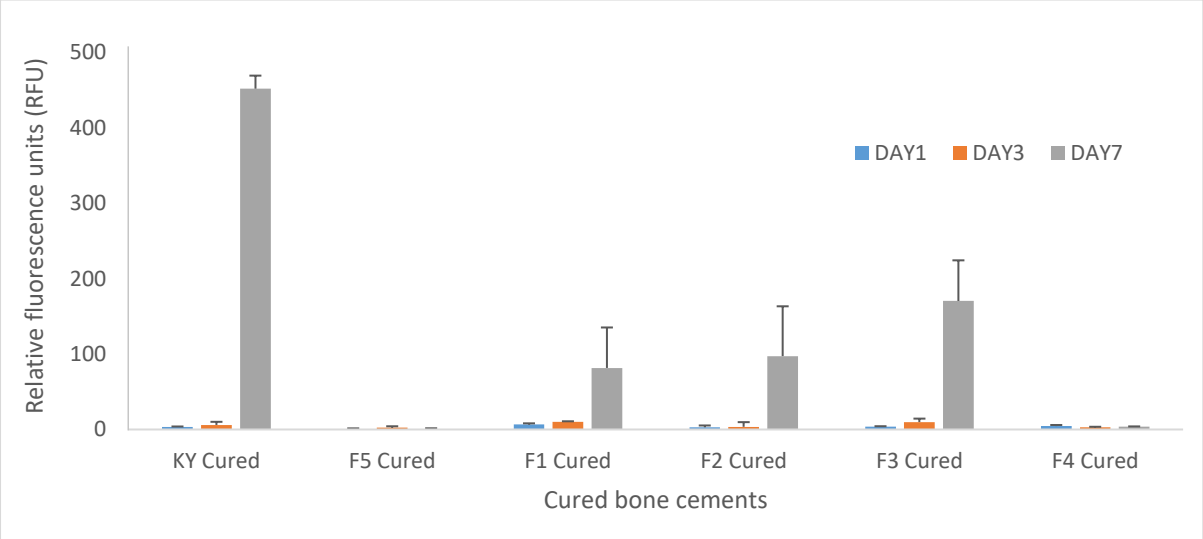


Fig. 6. Cell viability of Raw cells on cured bone cement (soaked in sterile water for 3 days) at time points 1, 3 and 7 days.

Table 5 Cell morphology of RAW cells on bone cement curing observed by SEM at day 7

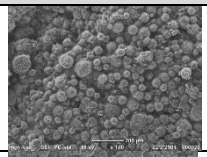
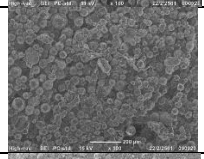
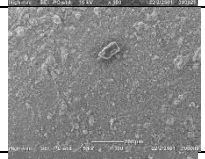
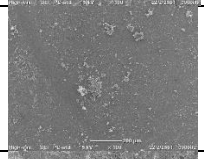
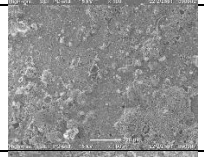
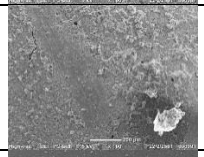
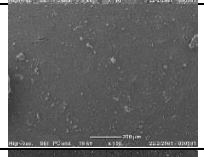
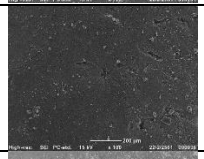
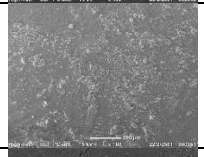
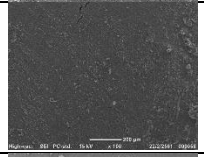
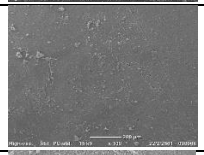
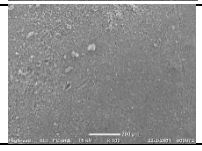
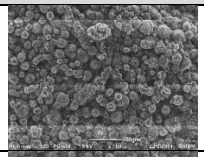
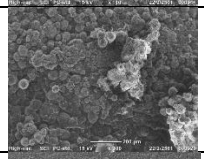
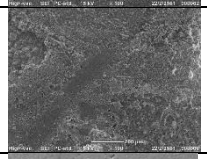
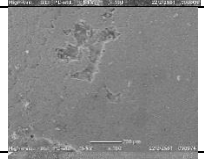
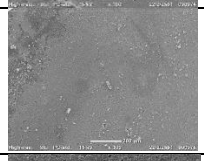
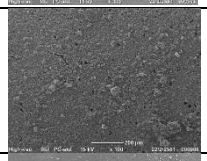
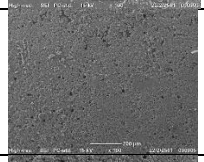
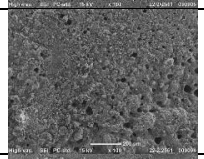
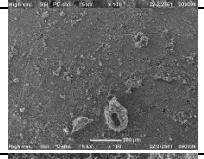
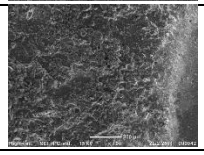
Bone cements curing	100X
KY	
KY + Raw cells	
F5	
F5 + Raw cells	
F1	
F1 + Raw cells	
F2	
F2 + Raw cells	
F3	
F3 + Raw cells	
F4	
F4 + Raw cells	

Table 6 Cell morphology of RAW cells on bone cement cured observed by SEM at day 7

Bone cements cured	100X
KY	
KY + Raw cells	
F5	
F5 + Raw cells	
F1	
F1 + MSCs	
F2	
F2 + Raw cells	
F3	
F3 + Raw cells	
F4	
F4 + Raw cells	

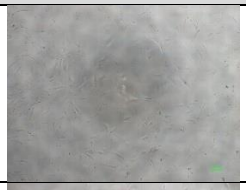
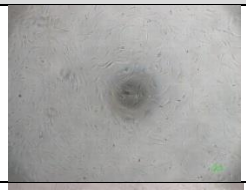
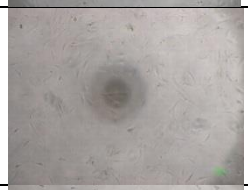
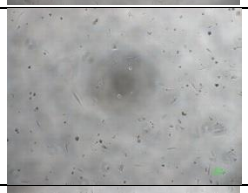
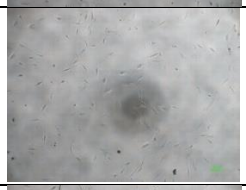
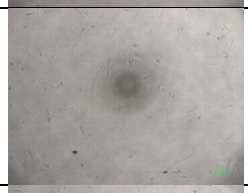
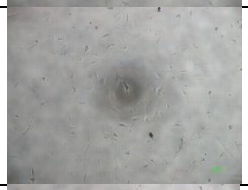
Effect of bone cement extracts on cell viability of MSCs

Next, we tested the effect of bone cement extract (after the bone cement disc was pre-incubated in water for 3 days) on cell viability and proliferation of MSCs. The results in Table 7 show that MSCs exposed to the F5 bone cement extract was the lowest from each time point 1 and 3 days. Cell proliferation of MSCs was increased from day 1 to Day 3 after exposure to the bone cement extract of KY and F1-4. However, the F5 extract showed the lowest cell proliferation among the others bone cement extracts (Fig. 7). In conclusion, all the bone cement extracts tested, except that of F5, showed no suppressive effect on MSC proliferation compared to the control MSCs.

Effect of bone media extraction on cell viability in RAW cells

The suppressive effect of bone cement extract (after the bone cement disc was pre-incubated in water for 3 days) on cell viability and proliferation of Raw cells was observed for all type of samples (Table 8 and Fig. 8).

Table 7 Cell morphology of MSCs exposed to bone cement extracts observed under a light microscope

Bone media extract	DAY 1	DAY 3
control		
KY		
F5		
F1		
F2		
F3		
F4		

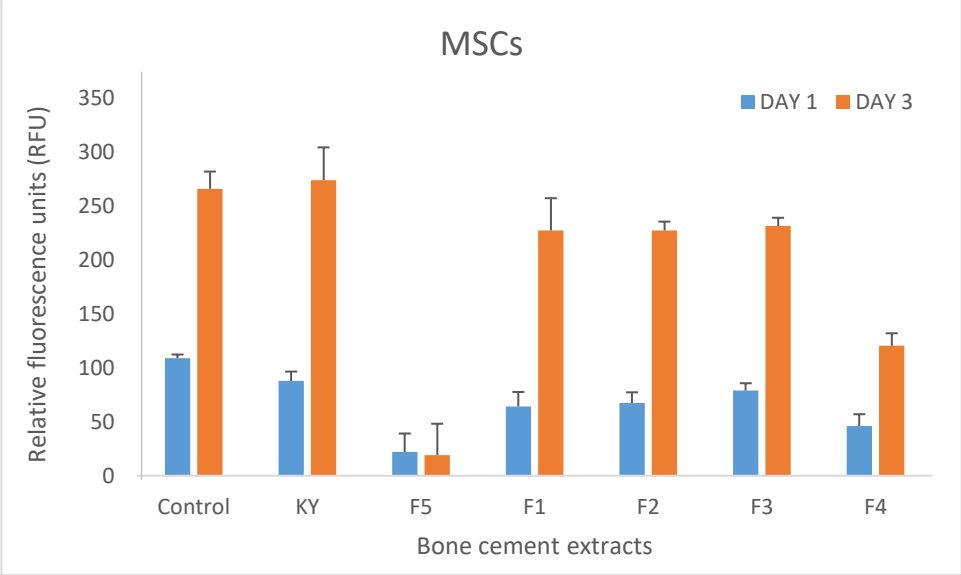
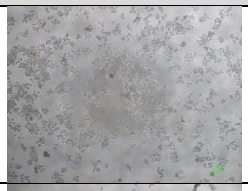
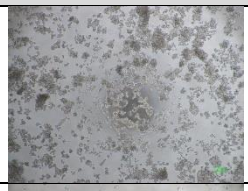
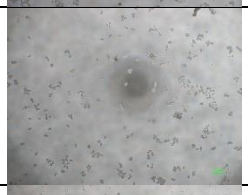
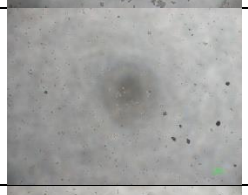
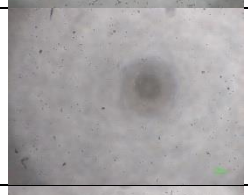
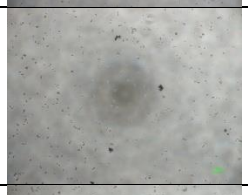
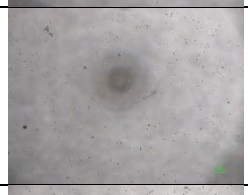
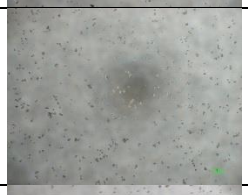
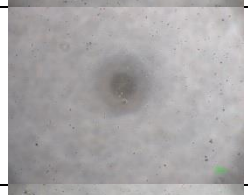
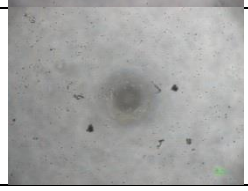


Fig. 7. Cell viability of MSCs exposed to bone cement extracts at time point 1 and 3 days.

Table 8 Cell morphology of RAW cells exposed to bone cement extracts observed under a light microscope

Bone media extract	DAY 1	DAY 3
control		
KY		
F5		
F1		
F2		
F3		
F4		

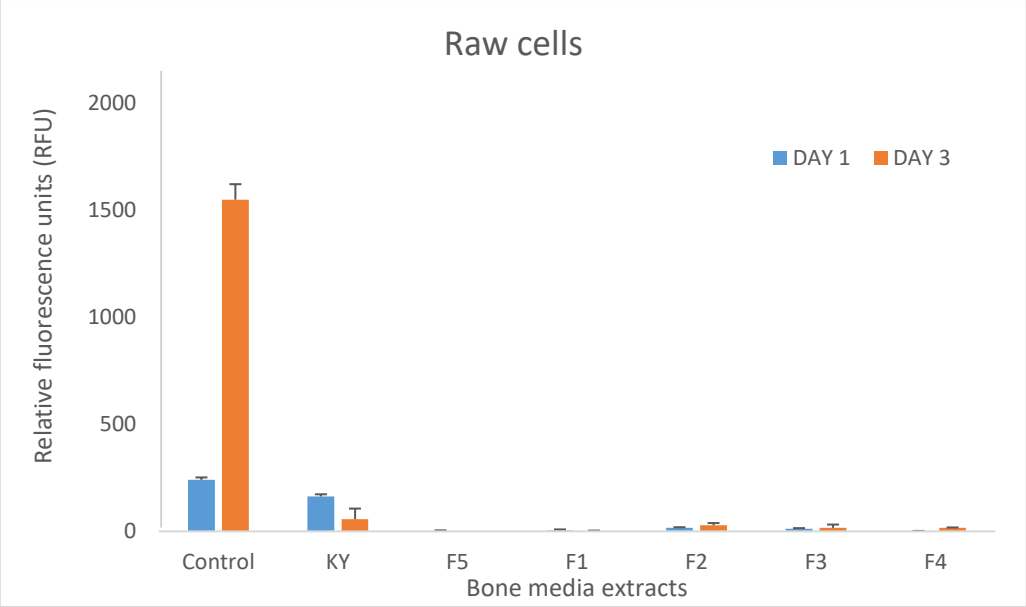


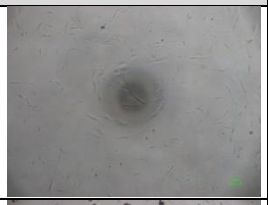
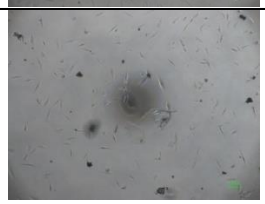
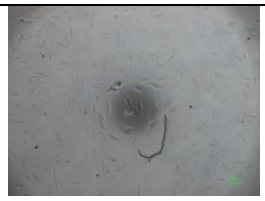
Fig. 8 Cell viability of RAW cells in bone cement extracts at time point 1 and 3 days.

Effect of F5 extract collected after 4- and 5-day pre-incubated in water (4dCuredF5 and 5dCuredF5) on cell viability of MSCs and RAW cells

At day 1, MSCs, which exposed to 4dCuredF5, had lower cell viability than control and 5dCuredF5 (Table 9). Cells viability of MSCs exposed to 4dCuredF5 was decreased from Day 1 to Day 3. However, 5dCuredF5 showed similar cell viability on Day 1, but appeared to have lower viability on Day3, compared with cells in the control group. Cell morphology of MSCs observe under the light microscopy in Fig. 9 confirmed the findings observed under the light microscope. In conclusion, 5dCuredF5 had no cytotoxicity effects on MSCs.

In contrast to the effect on MSCs, both 4dCuredF5 and 5dCuredF5 remained cytotoxic to RAW cells (Table 10).

Table 9 Cell morphology of MSCs in F5 bone media extract observed by light microscope

F5 Bone media extract	DAY 1	DAY 3
Control		
Soaked in sterile water for 4 days		
Soaked in sterile water for 5 days		

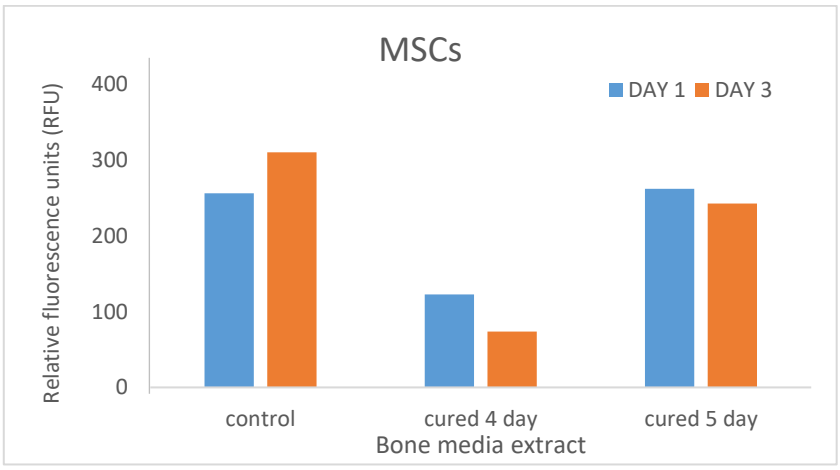
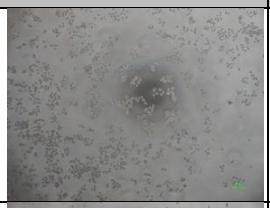
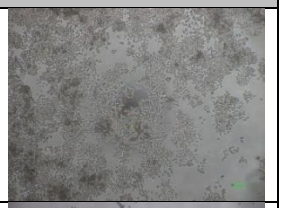
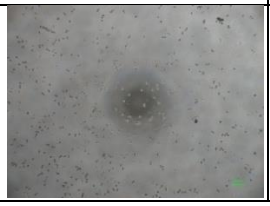
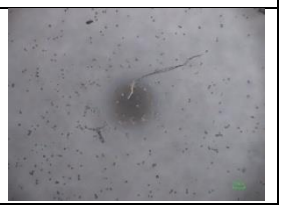


Fig. 9. Cell viability of MSCs in 4dCuredF5 and 5dCuredF5 at time point 1 and 3 days.

Table 10 Cell morphology of RAW cells in F5 bone media extract observed by light microscope

F5 Bone media extract	DAY 1	DAY 3
control		
Soaked in sterile water for 4 days		
Soaked in sterile water for 5 days		

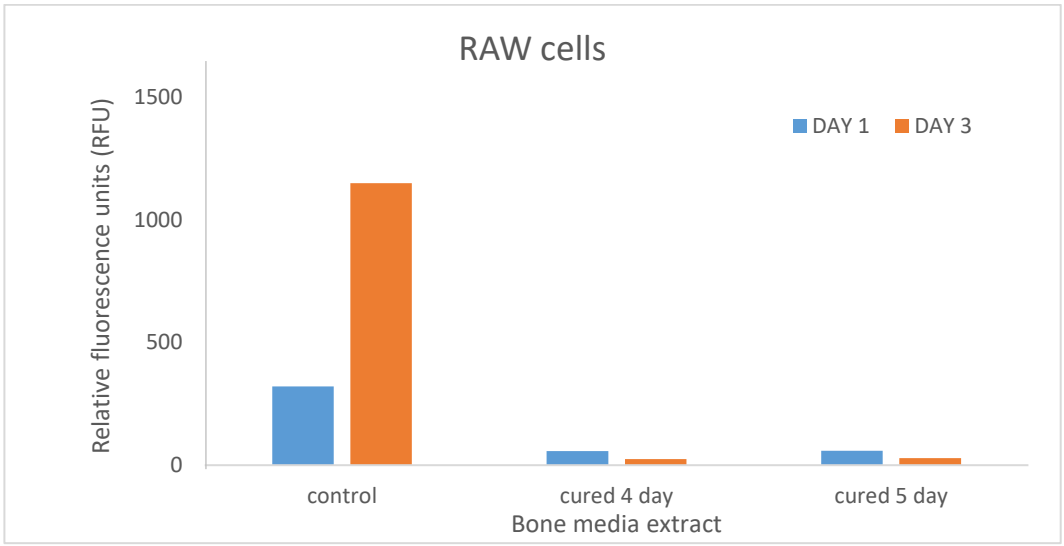


Fig. 10. Cell viability of RAW cells in 4dCuredF5 and 5dCuredF5 at time point 1 and 3 days.

Physical and mechanical properties

1). All composites exhibited an inhibition time of 2-3 minutes followed by rapid polymerisation reaching final monomer conversion greater than 80 % (Fig. 11).

2). Hydrophilic additives reduced BFS from 136 to 108 MPa and modulus from 3.6 to 2.7 GPa, and mean surface contact angles from 76 to 62 degrees (Figs. 12 and 13). High versus low level of PLS decreased BFS and contact angle by 12 ± 6% and 19 ± 13 % respectively, whilst the effect of MCPM level was negligible

3). A surface Raman phosphate peak (960 cm^{-1}) attributed to apatite precipitation appeared over time with MCPM present (Fig. 14).

4). Apatite precipitation was observed at the interface between bone composites containing MCPM and 3D collagen gel (Fig. 15).

All formulations had setting and mechanical properties suitable for bone repair. High monomer conversions should reduce potential toxic monomers release, whilst increased hydrophilicity and apatite precipitation could enhance cement adaptation to water-containing bone, and promote bone bonding, respectively.

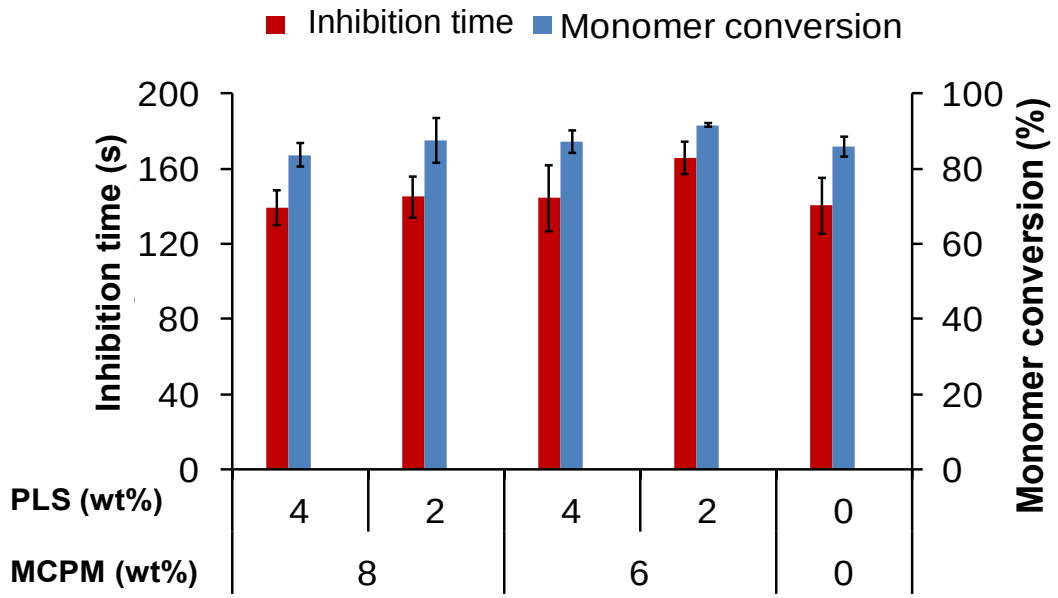


Fig. 11. Inhibition time and final monomer conversion of experimental bone composites tested at 37 °C. Error bars are 95% CI (n=3).

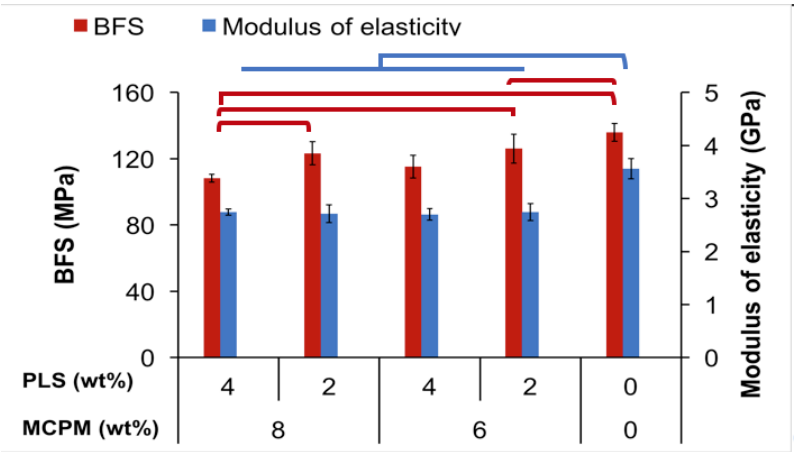


Fig. 12. Biaxial flexural strength (BFS) and modulus of elasticity of the experimental bone composites after immersion in SBF for 24 hr. Error bars are 95% CI (n=3). Lines indicate significant differences ($p < 0.05$).

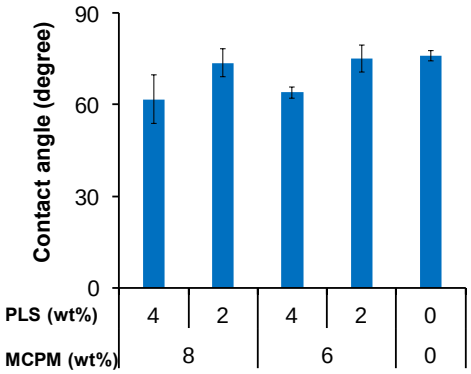


Fig. 13. Water contact angle.

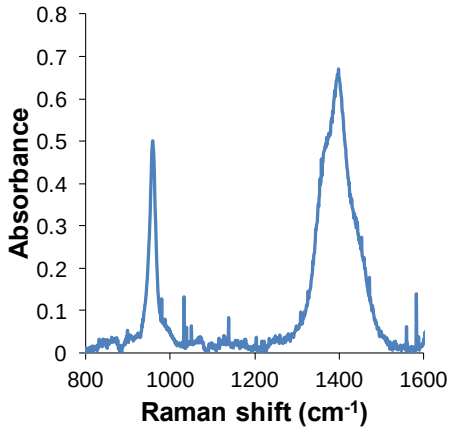
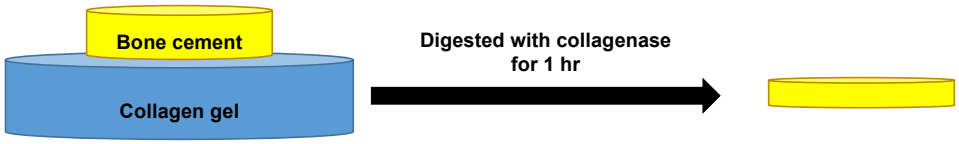


Fig. 14. Representative Raman spectrum indicating apatite precipitation (phosphate peak at 960 cm^{-1}) on the surface of specimens containing MCPM after immersion in SBF for 1 week.

(A)



(B)

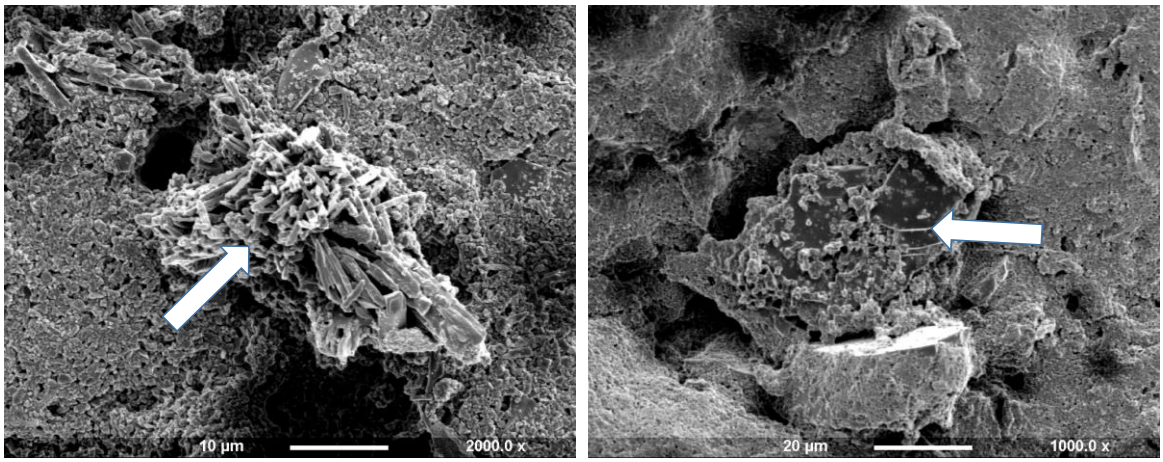


Fig. 15. Apatite precipitation at the interface of bone composites containing MCPM and 3D collagen gel. (A) Schematic representation of a bone composite-3D gel model. (B) Representative SEM images indicating apatite precipitation on the surface of specimens containing MCPM after the collagen gel was digested.

Results performed on bone cement formulations manufactured in Thailand

The UK manufactured formulations possessed favorable strength, monomer conversion and apatite precipitation induction. However, certain properties can be improved to obtain sufficient inhibition time/working time of approximately 10 min with snap set, low elastic modulus of less than 700 MPa with sufficient BFS of more than 50 MPa and lower cytotoxicity. The following sections (shown below) summarize the results derived from optimization of bone cements manufactured in Thailand.

Table 11 shows details of compositions of each of the 9 formulations initially manufactured in Thailand and one commercial bone cement for vertebroplasty. A number of physical and polymerization characteristics were investigated and are summarized in Table 12. It is noted that higher % monomer conversion resulted in shorter working time and setting time. Formulations 3 and 4 seemed to possess favorable working/setting times and % monomer conversion although the formulation 3 was more cytotoxic than the formulation 4 (Table 13).

Table 11. Thailand manufactured bone cement formulations and a commercial bone cement used in Thailand

Samples	Composition				Formulations
	Initiator	Activator	Glass/Monomer	Additive (included in glass)	
1	1. UDMA:PPGDMA (1:1) 2. BPO 6%wt 3. 4-META 6%wt	1. UDMA:PPGDMA (1:1) 2. NTGGMA 0.75%wt	75/25	-	Formulation 1
2	1. UDMA:PPGDMA (1:1) 2. BPO 6%wt 3. 4-META 6%wt	1. UDMA:PPGDMA (1:1) 2. NTGGMA 1.1%wt	75/25	-	Formulation 2
3	1. UDMA:PPGDMA (1:1) 2. BPO 6%wt 3. 4-META 6%wt	1. UDMA:PPGDMA (1:1) 2. NTGGMA 0.75%wt	65/35	-	Formulation 3
4				MCPM6%wt	Formulation 3 + MCPM6%wt
5				PLS 2%wt	Formulation 3 + PLS 2%wt
6				Sr 10%wt	Formulation 3 + Sr 10%wt
7				MCPM6%wt + PLS2%wt + Sr10%wt	Formulation 3 + MCPM6%wt + PLS2%wt + Sr10%wt
8	1. UDMA:PPGDMA (2:1) 2. BPO 6%wt 3. 4-META 6%wt	1. UDMA:PPGDMA (2:1) 2. NTGGMA 1.5%wt	75/25	-	Formulation 4
9	1. UDMA:PPGDMA (3:1) 2. BPO 6%wt 3. 4-META 6%wt	1. UDMA:PPGDMA (3:1) 2. NTGGMA 1.8%wt	75/25	-	Formulation 5
10	Kyphon (commercial bone cement)				Kyphon

Table 12. Analysis of the physical and chemical properties of the cement (working time, setting time, inhibition time and % monomer conversion)

Samples	Formulations	Working time (min)	Setting time (min)	Inhibition time* (min)	Monomer conversion (%) (at 40 min)
1	Formulation 1	มากกว่า 15	10	7	68.33
2	Formulation 2	13	5	3	79.56
3	Formulation 3	มากกว่า 15	10	4	77.85
4	Formulation 3 + MCPM6%wt	มากกว่า 15	15	5	75.87
5	Formulation 3 + PLS 2%wt	มากกว่า 15	15	7	67.08
6	Formulation 3 + Sr 10%wt	มากกว่า 15	15	6	74.31
7	Formulation 3 + MCPM6%wt + PLS2%wt + Sr10%wt	มากกว่า 15	15	N/A**	N/A**
8	Formulation 4	8	5	1	69.98
9	Formulation 5	3	3	0.40	73.23
10	Kyphon	3	5	0.40	85.77

Note * Inhibition time is the time in which the polymerization started

** This was because adding 3 additional additives caused colloid in nature of samples causing polymerization inhibition and therefore the formulation adjustment is planned.

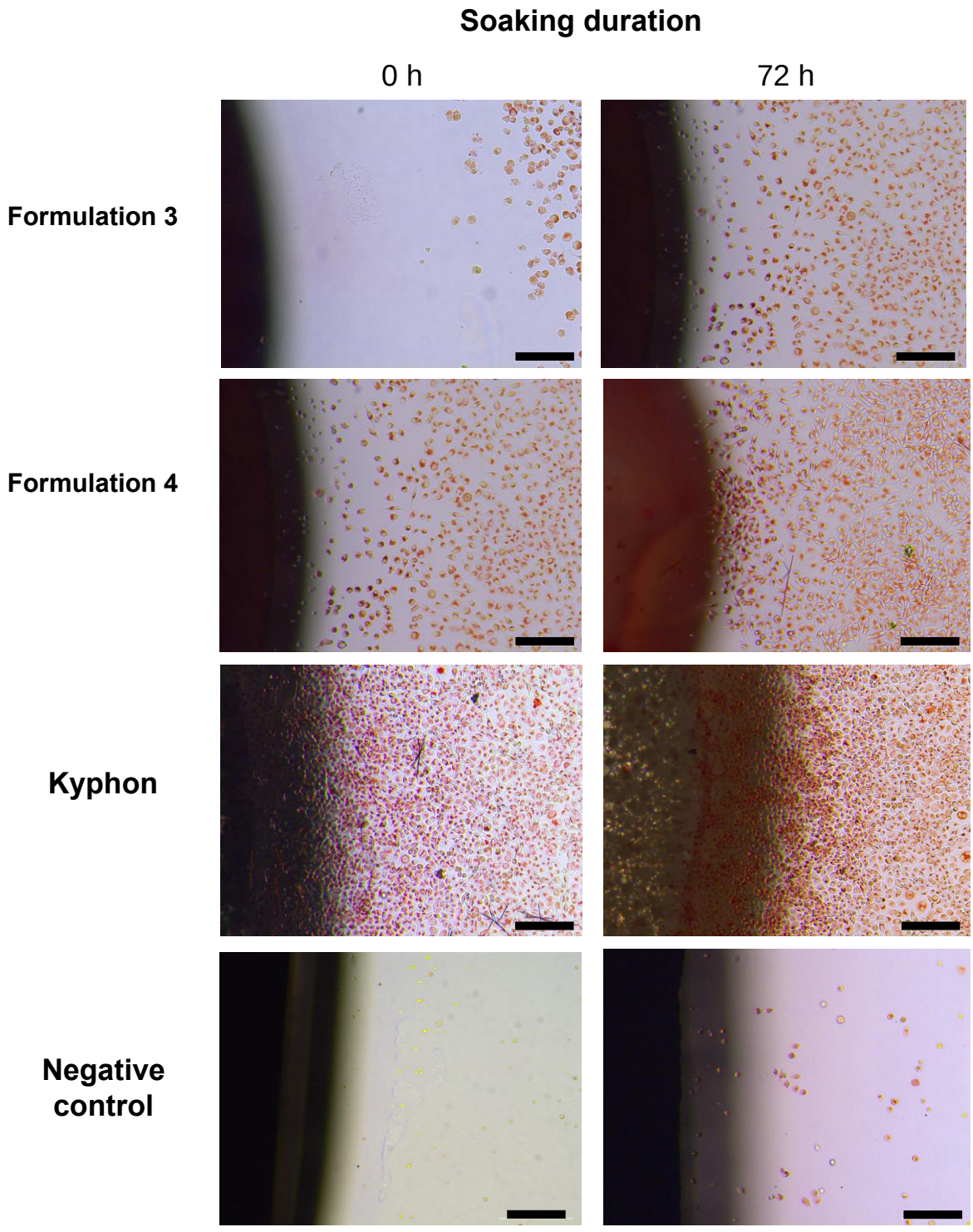


Fig. 16. Cytotoxicity of selected formulations of bone cements manufactured in Thailand and a commercial bone cement widely used in Thailand. Carbon black was used as a negative control sample. The method followed cytotoxicity testing using L929, as mentioned in the ISO standard. Scale bar = 200 um.

Table 13. Summarized cytotoxicity of selected formulations of bone cements manufactured in Thailand and a commercial bone cement widely used in Thailand

Formulations	Grade	Reactivity
Formulation 3	2	Mild
Formulation 4	2*	Mild
Kyphon	1	Slight
Negative control	4	Severe

Note: *Formulation 4's reactivity was milder than Formulation 3 despite the same cytotoxicity grade

Monomer conversion

The results in Table 14 show that % monomer conversions of all the experimental composites are higher than that of the Cortoss. Final monomer conversion of the SMART, SMART+ 8% MCPM, SMART+ 5% PLS, SMART+ 8% MCPM + 5% PLS and and Cortoss at 40 minutes after mixing were approximately 75%, 77%, 76%, 78% and 72% at room temperature, respectively. The inhibition times of these formulations were less than 1 minute, with the % monomer conversion at 1 minute after mixing being 4-11%. The results suggest that these formulations may have final monomer conversion of approximately 80% or higher under body temperature (37 °C) and the addition of MCPM and PLS had little influence on the monomer conversion. However, the inhibition time of less than 1 minute suggests the need for adding the polymerization inhibitor to increase working time. This is being tested.

Table 14 Monomer conversion profile of the adjusted formulations manufactured in Thailand

Time (minute)	% Monomer conversion				
	SMART	SMART-5%PLS	SMART-8%MCPM	SMART-8%MCPM-5%PLS	Cortoss
0	0	0	0	0	0
0.4	0	0	0	0	0
1	4	11	7	9	1
2	31	31	31	28	4
3	43	45	46	41	4
4	50	52	54	49	4
5	54	55	57	55	14
6	57	60	60	59	33
7	61	63	64	62	50
8	61	63	64	62	54
9	64	63	70	66	58
10	64	66	70	66	61
15	68	70	70	69	65
20	72	73	74	72	69
25	75	73	77	75	69
30	75	76	77	75	70
35	75	76	77	78	72
40	75	76	77	78	72

Surface hydrophilicity of bone composites

Surface hydrophilicity of the bone cements were determined by water contact angle at 0-10 min using the static sessile drop method (Fig. 17). SMART-8%MCPM and Cortoss possessed better initial hydrophilicity compared with the others. However, within 4-5 min the experimental bone composites which contain PLS, i.e., SMART-5%PLS and SMART-8%MCPM-5%PLS, had improved hydrophilicity with the water contact angle of approximately 50 degree, comparable to the commercial product Cortoss.

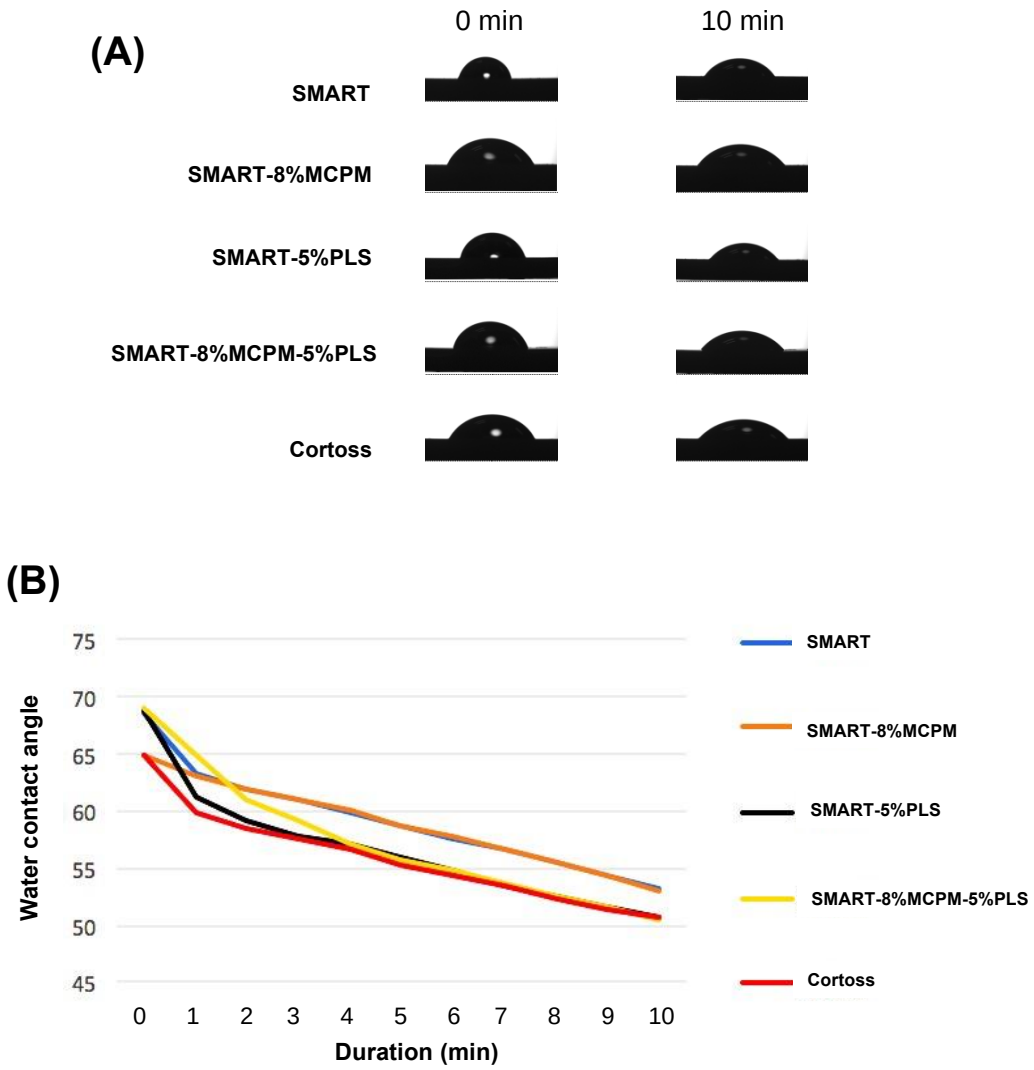


Fig. 17. Surface hydrophilicity of the adjusted formulations manufactured in Thailand and a commercially available bone cement used in vertebral fracture treatment (Cortoss). Water contact angle values were used to determine the hydrophilicity of the composite surfaces. Representative images of water droplets on the material surfaces are shown in (A), and a summary of water contact angle with in the first 10 min is shown in (B).

Protein adsorption capacity of bone composites

The results in Fig. 18 show that all the formulations had higher protein adsorption compared with the commercially available bone composite used in vertebral fracture treatment Cortoss. The addition of MCPM and PLS increased protein adsorption on the composite surface for more than 4 and 2 folds, respectively. However, combination of both MCPM and PLS did not further increase protein adsorption of the SMART-8MCPM-5PLS.

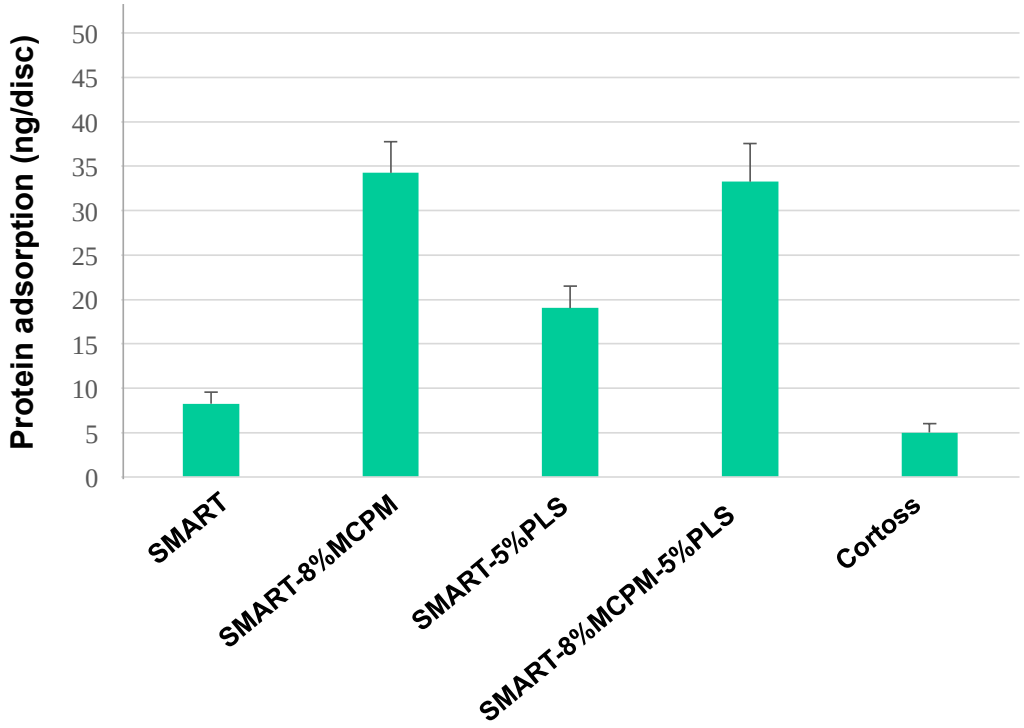


Fig. 18. Adsorption of FBS on bone composites. A commercially available bone cement used in vertebral fracture treatment (Cortoss) was used for comparison

Determination of cytotoxicity of SMART and Cortoss to hMSCs

The cytotoxicity of the adjusted formulation SMART was tested following ISO 10993-5 using hMSCs. The commercial product Cortoss was used for comparision. The results in Fig. 19 show that our adjusted formulation SMART was not toxic to hMSCs, and this was comparable to Cortoss.

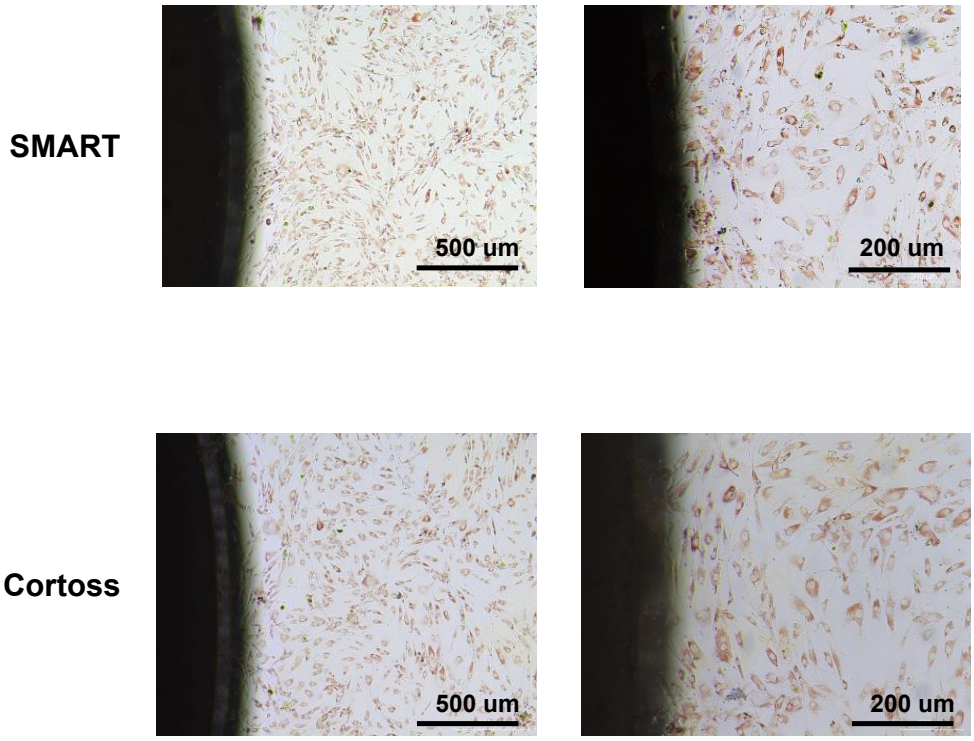


Fig. 19. Cytotoxicity of the adjusted formulations following ISO 10993-5 using hMSCs. The cells were culture for 48 h with the composites. The cells were then stained with neutral red and observed under a light microscope at low and high magnifications.

MSC adhesion on bone composites

Representative SEM images of hMSCs seeded on bone composites for 2 h are shown in Fig. 20. Cells on SMART, SMART-8%MCPM-5%PLS and Cortoss showed various cell shape, including polyhedral, elongated and round shapes. Elongated cells were a majority of cells found on SMART-8%MCPM while more round and flat cells were specifically observed on SMART-5%PLS. At 2 h, pore like structures (red arrow) were observed on the cell membrane of hMSCs seeded on SMART-5%PLS. This may cause cell death in long-term culture observed after cells were cultured in osteogenic medium for 28 days. The experiment is now being repeated to confirm the cytotoxicity to hMSC of 5% PLS in long-term culture although normal cell morphology remained normal after 5 days in culture (Fig. 21). The number of viable cells on SMART-8%MCPM composite surfaces at 2 h and 5 days were highest among all the experimental composites and were comparable to that of Cortoss (Fig. 22).

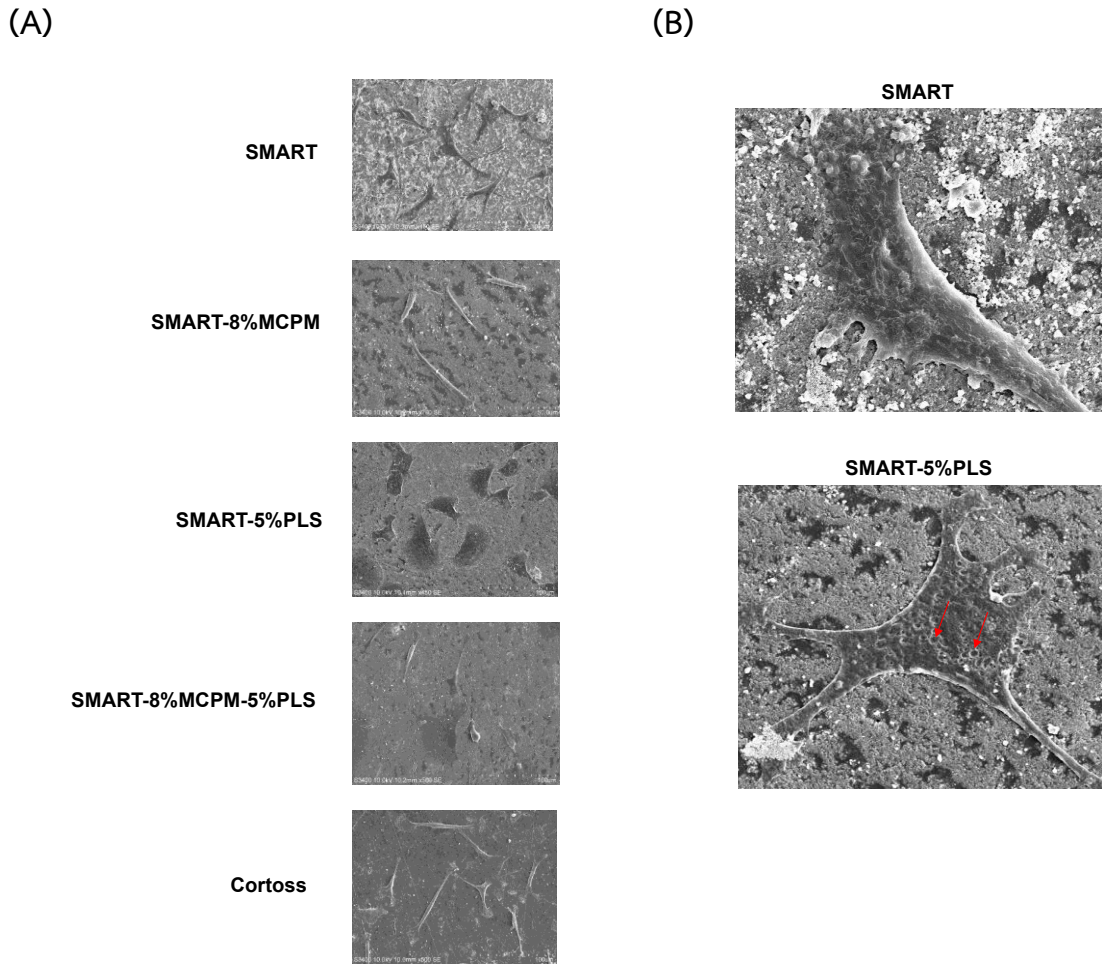


Fig. 20. Cell morphology of MSCs cultured on bone composites for 2 h observed under SEM. pore like structures (red arrow) were observed on the cell membrane of hMSCs seeded on SMART-5%PLS

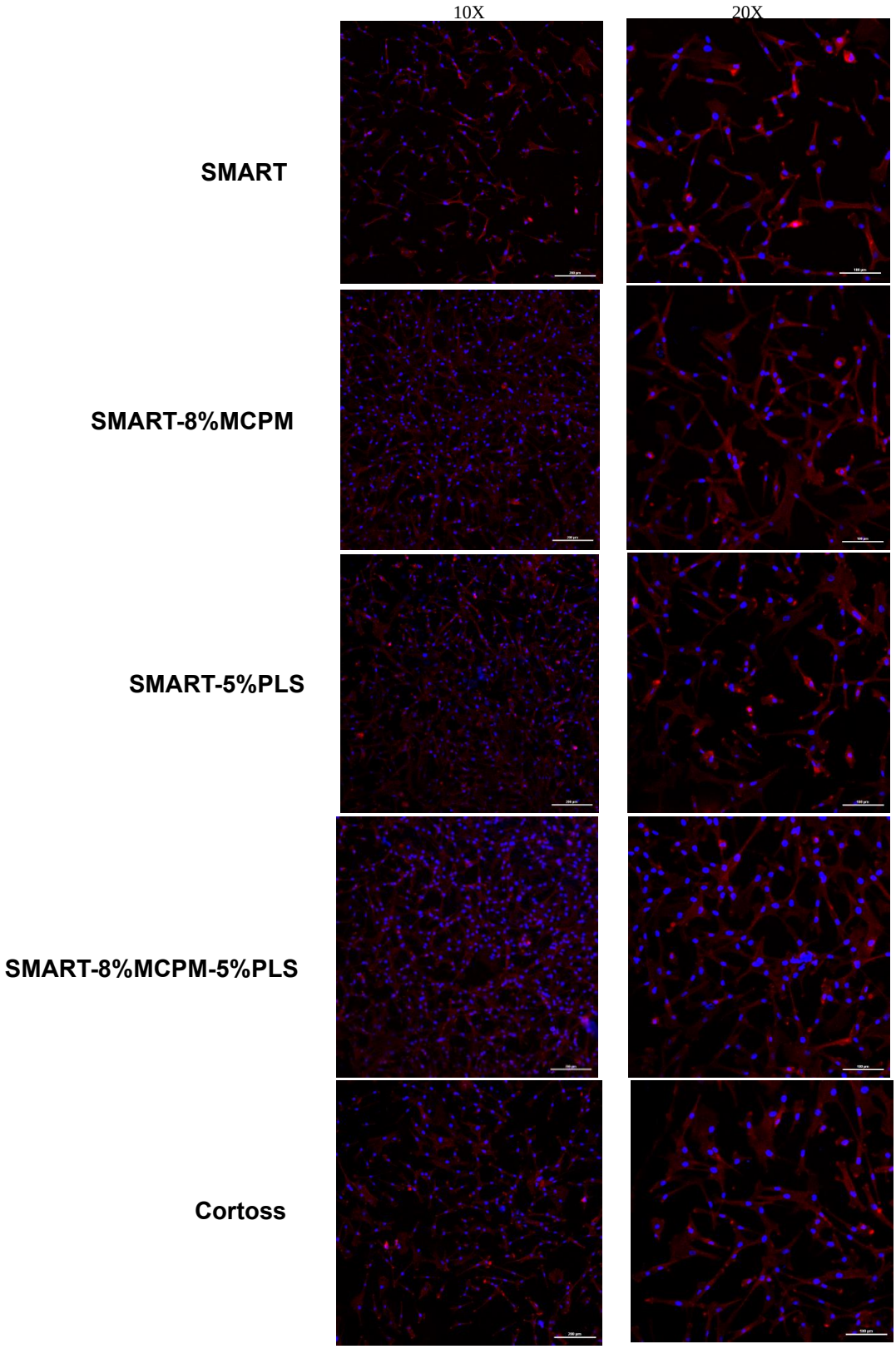


Fig. 21. Cell morphology of MSCs cultured on bone composites for 5 days observed under a confocal fluorescence microscope. The cells were stained for actin filament (red) and nucleus (blue).

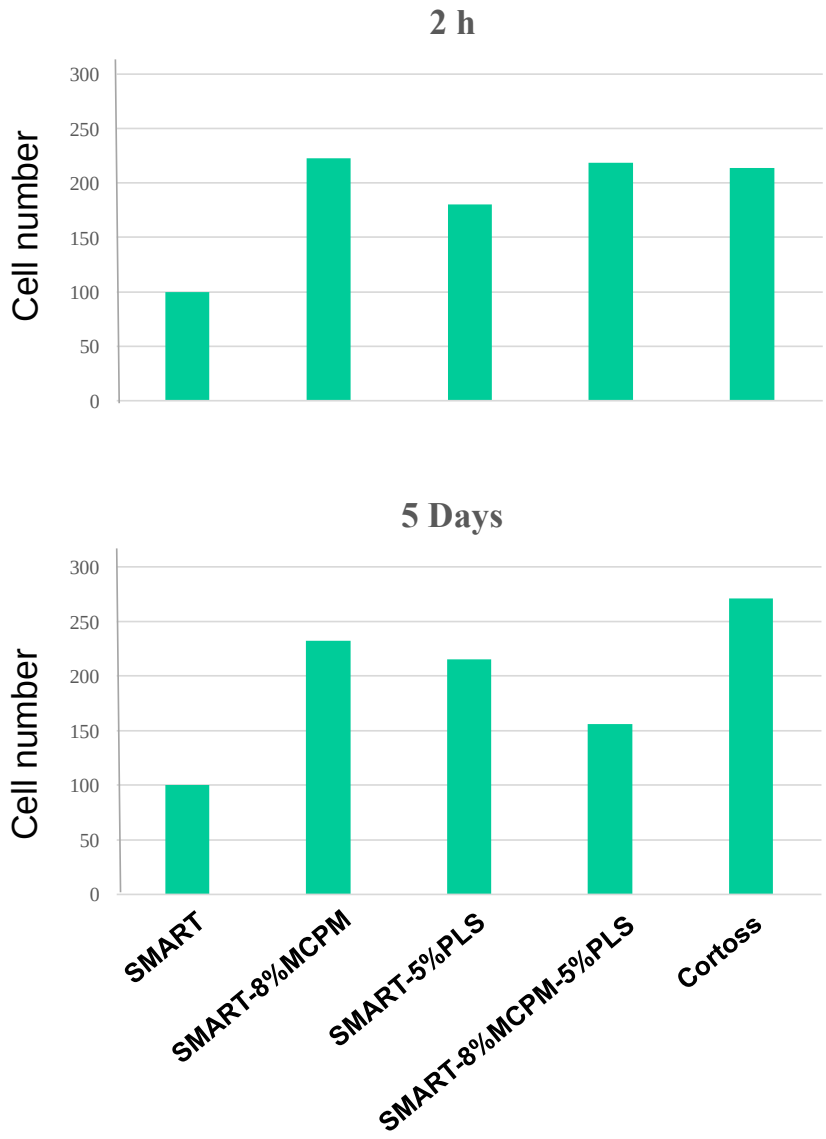


Fig. 22. Effect of bone composites on hMSC adhesion and growth. Viable cells were determined using tryphan blue exclusion test and the number of viable cells on composite surfaces were recorded at 2 h and 5 days.

Apatite precipitates on bone composites

Following immersion in SBF for 28 days, only SMART-8% MCPM and SMART-8% MCPM-5% PLS had precipitation of apatite on the surface (Fig. 23). This indicates the apatite precipitate-inducing property of MCPM contained in bone composites.

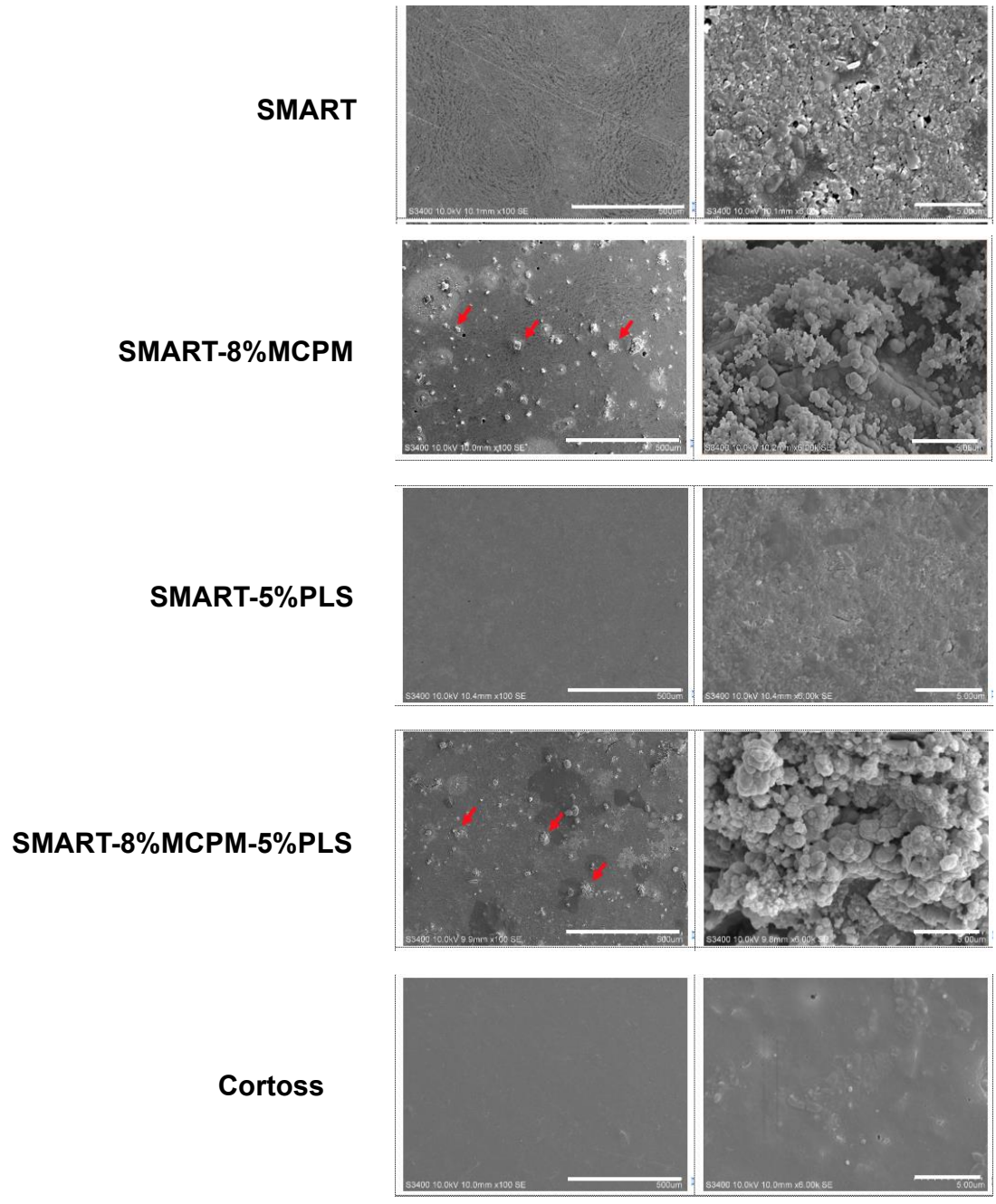


Fig. 23. Effect of bone composites on apatite precipitation. After 28 days in SBF, the surface of experimental composites and Cortoss were investigated un SEM at 100x (left), 6000X (right). Apatite precipitate loci were indicated by red arrows.

Mechanical properties of bone composites

Biaxial flexural strength (BFS) and modulus of elasticity of the SMART composite was 125 MPa and 2.6 GPa, respectively (Fig. 24). MCPM and PLS reduced BFS from 125 to 118 and 110 MPa, respectively, and modulus of elasticity from 2.6 to 2.3 and 2.3 GPa, respectively (Fig. 24). Combination of both additives further reduced BFS and modulus of elasticity to the levels of 104 MPa and 2.1 GPa. These are higher than the standards recommended by ISO.

All formulations had mechanical properties suitable for bone repair. High monomer conversions should reduce potential toxic monomers release, whilst increased hydrophilicity and apatite precipitation could enhance cement adaptation to water-containing bone, and promote bone bonding, respectively.

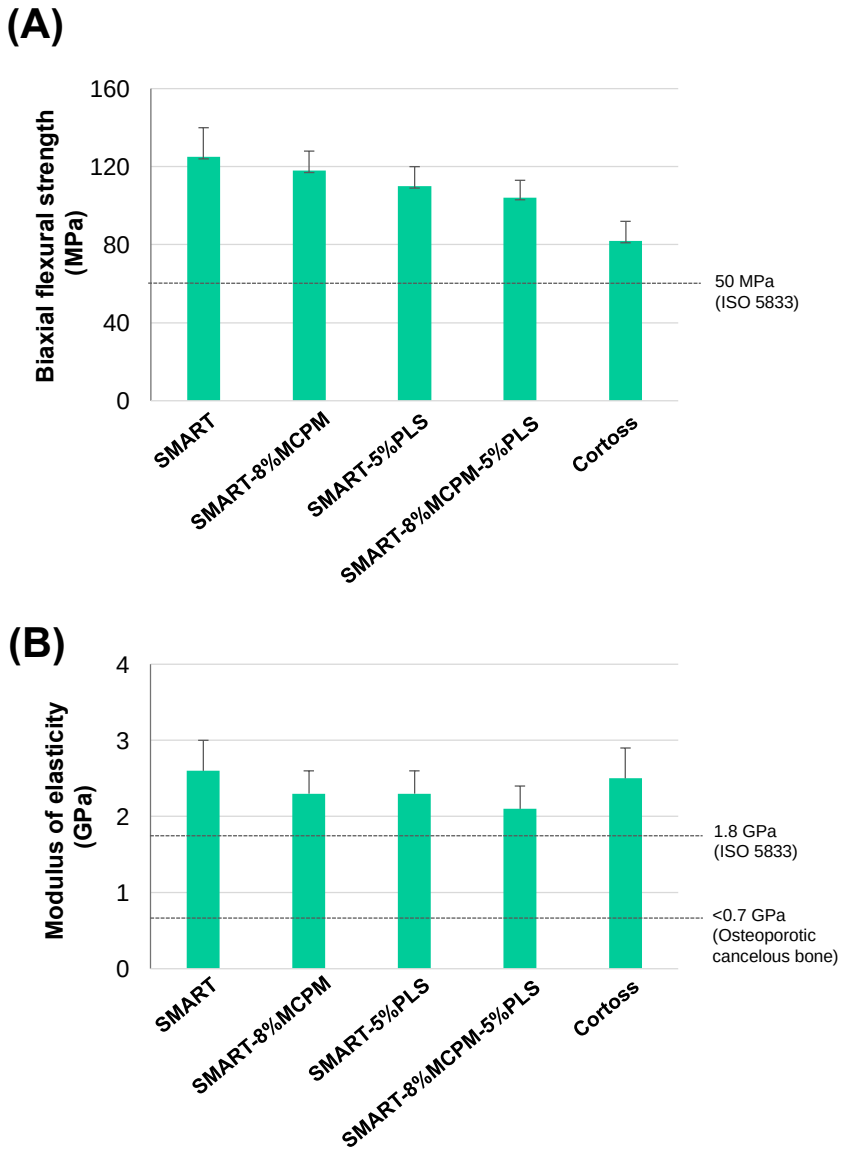


Fig. 24. Biaxial flexural strength (BFS) and modulus of elasticity of the experimental bone composites (n=15).

CHAPTER 4
Skill development

Skills missing of the Thai research group include biomaterial production / characterisation and the associated manufacturing issues to enable translational research. These missing skills were gained through the training programme provided by the Eastman Dental Institute. Details are as follows:

1. Setting and flow of materials
    - Use of multiple variable factorial design in composite development
    - Monomer/polymer structures of methacrylates
    - Polymerisation kinetics
    - Heat and shrinkage calculation
    - Rheological and colloidal properties
    - Spreading and surface hydrophilicity
2. Mechanical properties and testing o Gravimetric study
    - Biaxial flexural strength
    - Modulus of elasticity
    - Three-point bending strength o Compressive strength
    - Fatigue testing
    - Shear bond strength
3. Antibacterial polylysine release
    - Concept of chromatography
    - Theory of drug releasing kinetics o Controlled release mechanisms
4. Remineralising properties
    - Chemistry of calcium phosphates
5. Regulatory pathway of medical devices in European Union
6. Writing multidisciplinary grant proposal for future in vivo test with multi group collaboration
7. Coordinating and working with larger research group

The following additional experiences were also gained from the collaborative programme:

- Organising and running larger research group;
- Collaborating with other departments at Eastman Dental Institute and throughout UCL

such as Department of Materials and Tissue at Royal National Orthopaedic Hospital NHS Trust (RNOH) which is the centre of clinical translational research in orthopaedics;

- Communicating with a wide range of experterts in Bone biology at Bloomsbury Skeletal research group;
- Writing and applying larger multi institutional and international grant applications in future study

CHAPTER 5
Conclusion and Recommendation

The aim of this project was to develop SMART, cheap and easy to manufacture materials that are: 1) Simple to mix and snap setting to prevent leakage from site of application; 2) Mechanically a match to surrounding bone to reduce adjacent fracture risk; 3) Antibacterial against resistant bacteria; 4) Release minerals to promote bone integration; 5) Therapeutic to enable surrounding bone repair. The OBJECTIVES were: 1) development of systematically varying materials and testing of setting and mechanical properties in addition to kinetics of antibacterial, remineralising and therapeutic component release (UK laboratory) 2) investigation of how mechanical properties and component releases affects cell

With 1:1 UDMA/PDGGMA ratio, adding polylysine (PLS) (2-4 wt%) in the experimental composites significantly reduced biaxial flexural strength (BFS) from 140 to 110 MPa, modulus of elasticity from 3.6 to 2.7 GPa and mean surface contact angles from 75 to 60 degrees. The effect of monocalcium phosphate monohydrate (MCPM) level on these properties was negligible, but composites containing 10 wt% of MCPM clearly promoted surface apatite precipitation. Both PLS and MCPM markedly increased protein adsorption capability of the experimental composites by 2-15 folds, which was up to 8 folds greater than that of the commercial cement. Experimental bone composites with high MCPM possessed better initial hydrophilicity compared with the others

Cytotoxicity of these formulations was initially tested using L929 cells following ISO 10993-5, and all the composite formulations were only mildly reactive which met the requirement of the ISO standard. Extracts of experimental bone composites differentially influenced proliferation of mesenchymal stem cells (MSCs) and monocyte/macrophage RAW 264.7 cells, with the extracts derived from each of the first 5 days (*i.e.,* elutions derived from incubating the experimental bone cements in culture medium for every 24 h consecutively until Day 5) highly suppressing the growth of these cells under the standard culture condition. However, MSCs proliferated normally in the extracts collected after Day 5, which in contrast inhibited the growth of RAW cells. This may suggest an anti-inflammatory effect of the bone composites, possibly via a previously reported anti-inflammatory role of PLS. The number of viable MSCs on experimental bone composites with high MCPM surfaces at 2 h and 5 days were highest among all the experimental composites and were comparable to that of commercial sample Cortoss. All formulations allowed normal MSC mineralization under osteogenic stimulation. Moreover, following immersion in SBF for 28 days, only formulations containing MCPM had precipitation of apatite on the surface. This indicates the apatite precipitate-inducing property of MCPM contained in bone composites.

Since treatment of osteoporotic bone fracture involving the use of PMMA cement can lead to multiple complications, a range of non-cytotoxic new materials that have the potential

to help solve PMMA problems and possess enhanced apatite precipitation with antibacterial activity were tested in the present study. Currently tested composites possessed favorable properties, including snap setting, comparable mechanical properties to bone, apatite precipitation induction and improved cytocompatibility with allowing MSC mineralization, suggesting their potential application in the treatment of vertebral bone fracture *in vivo.*

This in vitro work will undoubtedly enable the continuing collaboration between Thailand and UK regarding the contribution in the *in vivo* study and subsequently clinical trials. This would be pivotal for the company spin out or patent licensing. Additionally, I also wish to continue the collaboration with the UK co-applicant in order to develop and extend the usage of the wide range of material formulations to other applications, such as dental composites, craniofacial reconstruction, bone infections (osteomyelitis), and hip and knee implant stabilization by replacement of PMMA cements.

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