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THERMOPLASTIC BIOACTIVE COMPOSITE

– With Special Reference to Dissolution Behaviour and Tissue Response

by

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To Julia and Alisa

ABSTRACT

Tiina Ranne (former Jaakkola)

Thermoplastic bioactive composite – with special reference to dissolution behaviour and tissue response

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Bioactive glasses are surface-active ceramic materials which support and accelerate bone growth in the body. During the healing of a bone fracture or a large bone defect, fixation is often needed. The aim of this thesis was to determine the dissolution behaviour and biocompatibility of a composite consisting of poly(ε-caprolactone-co-DL-lactide) and bioactive glass (S53P4). In addition the applicability as an injectable material straight to a bone defect was assessed.

In *in vitro* tests the dissolution behaviour of plain copolymer and composites containing bioactive glass granules was evaluated, as well as surface reactivity and the material's capability to form apatite in simulated body fluid (SBF). The human fibroblast proliferation was tested on materials in cell culture. In in vivo experiments, toxicological tests, material degradation and tissue reactions were tested both in subcutaneous space and in experimental bone defects.

The composites containing bioactive glass formed a unified layer of apatite on their surface in SBF. The size and amount of glass granules affected the degradation of polymer matrix, as well the material's surface reactivity. In cell culture on the test materials the human gingival fibroblasts proliferated and matured faster compared with control materials. In *in vitro* tests a connective tissue capsule was formed around the specimens, and became thinner in the course of time. Foreign body cell reactions in toxicological tests were mild. In experimental bone defects the specimens with a high concentration of small bioactive glass granules (<45 μm) formed a dense apatite surface layer that restricted the bone ingrowth to material. The range of large glass granules (90-315 μm) with high concentrations formed the best bonding with bone, but slow degradation on the copolymer restricted the bone growth only in the superficial layers.

In these studies, the handling properties of the material proved to be good and tissue reactions were mild. The reactivity of bioactive glass was retained inside the copolymer matrix, thus enabling bone conductivity with composites. However, the copolymer was noticed to degradate too slowly compared with the bone healing. Therefore, the porosity of the material should be increased in order to improve tissue healing.

Key words: Bioactive glass, hydroxyl apatite, composite, biocompatibility, poly(ε-caprolactone-co-DL-lactide)

TIIVISTELMÄ

Tiina Ranne (ent. Jaakkola)

Lämpömuokattava bioaktiivinen komposiitti – erityisesti hajoaminen ja kudosreaktiot

Hammasproteesioppi ja biomateriaalitutkimus, Hammaslääketieteen laitos, Turun yliopisto, Turku, Suomi. *Annales Univertitatis Turkuensis*, Turku 2008.

Bioaktiiviset lasit ovat pinta-aktiivisia keraamisia materiaaleja, joiden on todettu tukevan ja edistävän luun kasvua elimistössä. Luumurtumien ja laajojen luupuutosten yhteydessä paranemisen tueksi tarvitaan usein myös fiksaatiota. Väitöskirjatutkimuksen tavoitteena oli selvittää poly(ε-kaprolaktoni-co-DL-laktidi)sta ja bioaktiivisesta lasista (S53P4) valmistetun komposiitin hajoamista ja kudosyhteensopivuutta. Lisäksi tavoitteena oli selvittää materiaalin käyttökelpoisuus suoraan luuvaurioon ruiskuttettavana luukorvikemateriaalina.

Kokeellisissa töissä selvitettiin pelkän kopolymeerin ja lasigranuloita sisältävän komposiitin hajoamisnopeutta, pinta-aktiivisuutta ja kykyä muodostaa apatiittia simuloidussa kudosnesteessä. Laboratorio-olosuhteissa selvitettiin ihmisen sidekudossolujen käyttäytymistä materiaalin pinnalla. Prekliinisissä kokeissa tutkittiin materiaalin toksikologiaa, hajoamista ja kudosreaktioita sekä sidekudoksessa että kokeellisissa luuvaurioissa.

Lasigranuloita sisältäneet kopolymeerinäytteet muodostivat simuloidussa kudosnesteessä pinnalleen apatiittikerroksen. Lasigranuloiden koon ja määrän todettiin vaikuttavan komposiitissa käytetyn kopolymeerin hajoamisnopeuteen, samoin kuin materiaalin pinta-aktiivisuuteen. Soluviljelyissä ihmisen ikeneltä eristetyt sidekudossolut kasvoivat ja erilaistuivat testimateriaalien päällä nopeammin kuin kontrollimateriaalilla. Prekliinisissä kokeissa näytteiden todettiin muodostavan ympärilleen sidekudoskapselin, joka oheni merkittävästi ajan kuluessa. Vieraesinereaktiot sidekudoksessa ja toksikologisissa testeissä todettiin vähäisiksi. Kokeellisissa luudefekteissä pienet lasigranulat (<45 μm) suurina pitoisuuksina muodostivat komposiitin pintaan tiheän apatiittikerroksen, joka rajoitti luun kasvua pintaa syvemmälle. Isokokoiset lasigranulat (90-315 μm) suurina pitoisuuksina saivat aikaan parhaimman luusidoksen, mutta luun kasvu ulottui vain näytteiden pintakerroksiin polymeerin hitaan hajoamisen vuoksi.

Tässä tutkimussarjassa materiaalin käsiteltävyys todettiin hyväksi ja kudosreaktiot vähäisiksi. Bioaktiivisen lasin reaktiivisuus säilyi kopolymeeriin sekoitettaessa minkä vuoksi lasin luun paranemista edistävä ominaisuus säilyi myös komposiittimateriaalissa. Työssä käytetyn kopolymeerin todettiin kuitenkin hajoavan luun paranemiseen nähden liian hitaasti. Materiaalin huokoisuutta tulisi lisätä luun sisään kasvun edistämiseksi.

Avaisanat: bioaktiivinen lasi, hydroksiapatiitti, komposiitti, kudosyhteensopivuus, poly(ε-kaprolaktoni-co-DL-laktidi)

CONTENTS

ABBREVIATIONS

AAS	atomic absorption spectrophotometry
ATR	attenuated total reflectance (used with FTIR)
A-W	apatite-wollastonite (glass-ceramic)
BABC	bioactive bone cement
BAG	bioactive glass
BAG(40-70)S	composites containing glass granule (as wt%) size <45 μm
BAG(40-70)L	composites containing glass granule (as wt%) range 90-310 μm
BAG50L+	composite containing 50 wt% glass granules range 90-310 μm and 20 wt% sucrose
Bis-GMA	bisphenol-alpha-glycidyl methacrylate
CL	ε-caprolactone
Ca-P	calciumphosphate
COOH	carboxylate group
$-COO^-$	carboxylate ion
DPBS	Dulbecco´s phosphate buffered saline
D-MEM	Dulbecco´s minimal essential medium
FTIR	Fourier transform infra-red spectroscopy
LM	light microscope
M_n	number average molecular weight (g/mol)
M_w	weight average molecular weight (g/mol)
MMA	methylmethacrylate
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PCL	poly(ε-caprolactone)
P(CL/DL-LA)	poly(ε-caprolactone/DL-lactide)
PLA	polylactide
SBF	simulated body fluid
SEC	size exclusion chromatography

SEM	scanning electron microscopy
SEM-EDX	SEM coupled with energy dispersive x-ray
S53P4	BAG containing 53 wt% SiO_2 and 4 wt% P_2O_2
Si	silicon
T_g	glass transition temperature (˚C)
T_m	melting temperature (˚C)
TCP	tricalciumphosphate
TEGDMA	triethylene-glycol dimethacrylate
TRITC	tetramethylrhodamine isothiocyanate
WGA	wheat germ agglutinin
wt%	weight percent

DEFINITIONS

Bioactive material: Material designed to elicit or modulate biological activity (Williams, 1999)

Biocompatibility: Ability of a material used in a medical device to perform with an appropriate host response in a specific location (Williams, 1999)

Biomaterial: Material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body (Williams, 1999)

Bone-bonding: Establishment, by physico-chemical processes, of continuity between an implant and bone matrix (Williams, 1999)

Foreign body reaction: Variation in normal tissue behaviour caused by the presence of a foreign material (Williams, 1999)

LIST OF ORIGINAL PUBLICATIONS

I **T. Jaakkola**, J. Rich, T. Tirri, T. Närhi, A. Aho, J. Seppälä, A. Yli-Urpo. *In vitro* Ca-P formation on biodegradable thermoplastic composite of poly(ε-caprolactone-co-DL-lactide) and bioactive glass (S53P4). Biomaterials 25:575-581, 2004.

II J. Rich, **T. Jaakkola**, T. Tirri, T. Närhi, A. Yli-Urpo, J. Seppälä. *In vitro* evaluation of poly(ε-caprolactone-co-DL-lactide)/bioactive glass composites. Biomaterials 23:2143-2150, 2002.

III **T. Jaakkola**, T. Närhi, M. Hormia, J. Rich, J.Seppälä, A. Yli-Urpo. *In vitro* proliferation of the human fibroblasts on the p(ε-CL/DL-LA)/bioactive glass composites. Bioceramics 14, Key Engineering Materials, 218-220:261-264, 2001.

IV **Ranne T**, Tirri T, Laine VJO, Rich J, Seppälä J, Yli-Urpo A, Närhi T. *In vivo* behavior of poly(ε-caprolactone-co-DL-lactide)/bioactive glass composites in rat soft tissue. Journal of Bioactive and Compatible Polymers 22:249-264, 2007.

V T.O. Närhi, J.A. Jansen, **T. Jaakkola**, A. de Ruijter, J. Rich, J. Seppälä, A. Yli-Urpo. Bone response to degradable thermoplastic composite in rabbits. Biomaterials 24:1697-1704, 2003.

1. INTRODUCTION

In surgical disciplines where tissue has to be repaired, augmented or built up, tissue supplements are essential. Although tissue banks supply such substitutes, the natural tissue is not always an infallible material. Natural grafts may be resorbed after implantation and thus cause disruption in normal function (Enneking *et al.* 1980). Concern about the transmission of blood-borne diseases through the transplantation of allogenic tissue, has to some extent limited current indications for the use of these materials. Therefore, many attempts have been made to find and create biologically successful synthetic tissue-repairing materials.

In clinical and experimental studies, bioactive glasses and ceramic materials with HA coatings have been used, e.g. in dental, maxillofacial and orthopedic devices in order to enhance bone adaptation. Bioactive ceramics like bioactive glasses can provide mechanical strength for bone substitutes although they are currently mainly used in granule forms as bone fillers (Aitasalo *et al*. 2007, Peltola *et al.* 2006, Turunen *et al.* 2004). Novel surgical treatments using markedly improved technical equipment increase the demands on materials aimed to replace degenerated or injured structures of variable size and shape. Because of the complexity of graft structure and the need for regenerative osteosynthesis, the need for sophisticated artificial bone implants has increased.

During the past three decades, polymeric materials like biodegradable polyesters, polylactide (PLA) and polycaprolactone (PCL), have been investigated for medical purposes (Taylor *et al.* 1994). As a result, a wide range of applications from regenerative scaffolds to fracture fixation and drug delivery devices has been reported in the literature (Rokkanen *et al*. 1985, Pitt 1990, Multanen *et al.* 2000, den Dunnen *et al.* 1996, Domb 2002). An interesting character of polyesters is their thermal properties that can be beneficial in surgical applications. Depending on the co-monomer ratio, the melting and glass transition temperature can be tailored, for example, to near body temperature, thus offering a choice of direct application into a living tissue.

Therefore, a composite of polyesters and bioactive ceramic materials can offer better applicability for bioactive glasses without endangering the good properties of polymer, such as the possibility of embedding growth factors or other reactive agents into it when needed. This thesis is composed of studies concerning the basic biological behaviour of the composite of copolymer of poly(ε-caprolactone/DL-lactide) (monomer ratio 95.0/5.0) and bioactive glass S53P4. The purpose of this thesis work was to evaluate the bioactive properties of the thermoplastic composite in simulated body conditions, in a cell culture environment, and in living tissues.

2. REVIEW OF THE LITERATURE

2.1. Biology of bone healing

The classic stages of natural bone repair are inflammation, formation of soft and hard callus and remodelling. The mechanisms of bone repair were described by Hunter already in the 1830´s (reviewed in (Hunter 1837; Claiborne 1998)). He demonstrated that bone is a dynamic tissue involved in the equilibrium of bone deposition and resorption. In the 1980`s Brighton introduced the stages of impact and induction. The impact stage includes the interval from the first application of the force to the bone until the energy of force is completely dissipated, resulting in energy absorption by the bone until fracture occurs. The inflammation stage follows and usually lasts from one to three days clinically, and is evidenced by pain, swelling and heat. Inflammatory cells arrive at the injured site accompanied by vascular ingrowth and cellular proliferation. In the next stage, the cartilaginous elements appear. Brighton´s induction stage begins during the impact and inflammatory stages, and is believed to involve the formation of inducers and humoral factors that direct the regeneration of bone (Brighton 1980 and 1984, Friedenberg and Brighton 1981, Claiborne 1998).

The soft callus stage corresponds clinically to the time when clinical union occurs by fibrous or cartilaginous tissue. Histologically it is characterised by vascular ingrowth of capillaries into the fracture callus and the appearance of chondroblasts. In the hard callus stage the fibrocartilaginous union is replaced by a fibroosseous union. Clinically, this usually occurs at three to four months. The final stage of remodelling begins with clinical and radiographic union and persists until the bone has returned to normal, including restoration of the medullary canal. Histologically, the fibrous bone is replaced with lamellar bone, a process which may take from a few months to several years (Wheater *et al.* 1979, Claiborne 1998).

The healing of bone defects follows basically the same route. The surrounding soft tissue provides the supply of new vessels, which bring into a wound site a source of undifferentiared mesenchymal cells, i.e. pluripotential cells with chondrogenic potential. These osteoprogenitor cells mature towards secreting osteoblasts that initiate woven-bone formation. Woven bone is an immature form and is characterised by random organisation of its fibrous elements. This newly formed bone is then remodelled and replaced by a lamellar bone structure. Lamellar bone is composed of successive layers, each of which has a highly organised infrastructure. Lamellar bone may be formed as a solid mass when it is described as compact bone, or may be formed as a spongy mass, described as cancellous bone (Hulth 1989, Wheater *et al.* 1979).

2.2. Bone-filling procedures

The most frequent indications for bone grafting are to fill cavities or defects resulting from infections, cysts, tumours or trauma. Bone grafts may also be used for nontraumatic disorders, like congenital, degenerative or developmental bone loss.

The principles, indications and techniques for bone grafting procedures were well established already before "the metallurgic age" or modern surgery. Cortical bone grafts, with a dense structure, are used primarily for structural support. As an onlay graft, they may be used in single or dual form, when treating nonunions in bone or bridging massive bone defects. The fresh segments of the cancellous bone are the best osteogenic material available, and thus multiple cancellous chip grafts are widely used in grafting. These two fundamentally differently acting bone types may be combined when selecting the graft (Ceruso *et al.* 2001). Thereby, the selected graft may provide structural support, bridge major defects, establish a union or promote bone healing (Crenshaw 1998).

Due to the necessity of using autogenic materials the fixation of the graft was rather crude. The two principles, fixation and osteogenesis, were efficiently combined by Venable and Stuck (1941), who initiated fixation by inert metal screws. In 1987, Lane and Sandhu introduced internal bone fixation. The adjacent bone harvesting and banking with its obvious advantages was introduced. (Gunby 1978, Friedlaender and Mankin 1981).

2.2.1. Autografts

When the bone grafts are taken from the patient himself, the grafts are usually harvested from the tibia (Jakse *et al.* 2001), fibula (Bähr 1999) or ilium (Kurtz *et al.* 1989, Sjöström et al. 2007). These three bones provide cortical grafts, whole bone transplants, and cancellous bone, respectively (Enneking *et al*.1980). In maxillofacial surgery small grafts can also be harvested from intraoral sites (Hunt and Jovanovic 1999, Capelli 2003). The benefits of autogenous bone grafts for bone healing have been recognized for over 100 years. However, there are disadvantages in using autogenic grafts: removal of the graft adds to the duration and magnitude of the surgery, convalescence is prolonged, and at times it is not possible to obtain enough autogenous bone to meet the needs of the operation. The use of autogenous bone marrow injections, started in the beginning of the 1970's (Congdon 1971), is a more recent technique that has been reported to be an effective osteogenic graft (Good *et al.* 1983, Kinsel and Turbow 2004, Smiler and Soltan 2006).

2.2.2. Allografts

Allograftic bone, human bone harvested from another individual, has been in clinical use since 1880, when Macewen successfully used a fresh allograft in surgery (dicussed in (Macewen 1881, Heikkilä 1996)), but through the work of Lexer (1908) allografts were

introduced and led to wider clinical use (Gitelis *et al.* 1988, Trumble and Friedlaender 1987, Jofe *et al.* 1988). The modern bone-banking method, storing bone by freezing, was developed by Bush in the 1940s´ (Bush 1947). With lipid extraction from allografts, the immune response has been reduced (Thoren *et al.* 1994). Tissue typing and the use of immunosuppressants are currently being examined in order to improve safety and results (Ward *et al.* 2005 and 2008, Liu *et al.* 2008).

Bone can be stored and sterilized in several forms; it can be harvested in a clean nonsterile environment, sterilized by irradiation, strong acid or ethylene oxide, then freeze-dried for storage. Bone under sterile conditions can be deep frozen for storage. The method with lowered temperature is widely used when storing massive osteoarticular grafts, as well as allograft chips (Marx *et al.* 1981, Goldberg and Stevenson 1987, Aho *et al.* 1994). Fresh frozen bone is stronger, and therefore better as structural allograft material. Bone can also be obtained in a demineralised form, which increases its osteogenic potential but greatly decreases its strength (Rosenthal *et al.* 1999, Lewandrowski *et al.* 2002). Autogenic cancellous bone can be mixed with allograft bone to provide osteogenic potential and ensure more rapid incorporation (Crenshaw 1998).

2.2.3. Xenografts

Xenograft is a bone graft origin of another species. Deproteinized bovine bone, the mineralized part of bone matrix, has been clinically used with various preparation techniques (Richardson *et al.* 1999, Noumbissi *et al.* 2005). Lipid extraction of grafts has been shown to decrease the antigenic response (Urist *et al*. 1975), but the osteogenic response to these materials has been reported to vary or be lacking.

Hislop and co-workers (1993) reported that the use of anorganic bovine bone in oral and maxillofacial surgery can be successful as an interpositional graft in osteotomy sites with supporting internal fixation. Altough, its benefit in post-traumatic deformity or hypoplastic correction is limited. As an augmenting graft material it may be used when combined with particulate marrow and cancellous bone, but as such the anorganic bovine bone has no osteocondustive or osteoinductive properties (Mandelkow *et al.* 1990).

2.3. Synthetic bone-replacing materials

Biomaterials, synthetic materials used for biomedical applications, can be classified as metals, ceramics, polymers and composites. They are generally classified according to the tissue responses they elicit in a living body. Success has been achieved as a direct result of progress in understanding the cellular and molecular biology of the immune system. It is impossible to make artificial materials which equate completely with living tissues, and attempts to raise the quality of implanted biomaterials to the level of the

recipient's tissue are likely to be in vain; but better substitutes must continue to be sought (Rokkanen 2002, Middleton *et al.* 2000).

2.3.1. Metals

In both dental and orthopedic surgery, metals are connected to bone, e.g. when fixed to a dental implant or total artificial joint. Metals create an electrical potential when bathed with the saline environment of the tissues, hence metals with the lowest electrolytic coefficient have been evaluated and tested (Venable *et al.*1937). The components made of metals and alloys can be passivated to resist corrosion, but with the fretting wear caused by motion between metal components, corrosion may be significantly increased (Claiborne 1998).

Most of the metallic orthopedic or dental fixation devices and implants are constructed of stainless steel (composed of iron, chromium, and nickel), titanium aluminium vanadium alloy, or commercially pure titanium (titanium and oxygen). Of these, titanium (alloys) have the highest corrosion resistance and the mechanical properties closest to bone (Becker and Bolton 1997).

New low modulus metal bone grafts and implants, made of tantalum are currently available. Porous tantalum is characteristic of cancellous bone (Levine *et al.* 2006). Like titanium it is considered to be a biocompatible material for bone contact applications. The disadvantage of both these metals is that the passive oxide layers formed on these metals do not allow direct bone bonding, but only mechanical interlocking. To increase the bioactivity of the titanium and tantalum implants, much effort is being made to produce a surface-reactive coating from the bone-conductive material on to the metal surface. Ceramic coatings have been made from various calcium phosphates, such as tricalcium phosphates (TCP), hydroxyapatite (HA) and bioactive glasses (BAG) (Meirelles *et al.* 2008, Gomez-Vega *et al.* 2000, Kokubo *et al.* 2004, Kitsugi *et al.* 1996), e.g. by dipping and sintering or by plasma-spraying. In the case of nanostructures, coatings have also been made with sol-gel-derived titania (Areva *et al.* 2004 and 2007), which with local densification can be made with CO_2-laser (Moritz *et al.* 2003). The CO_2-laser treatment provides an advantage for the surface treating of materials that do not tolerate high temperatures and furnace-firing.

2.3.2. Bone cements

In modern total joint reconstructions, the implants may be fixed in the living bone with cold-curing polymethylmethacrylate (PMMA) cement. Aseptic loosening is the most serious long-term drawback, caused by biochemical factors and mechanical overloading (Jasty *et al.* 1992, Tomita *et al.* 2002). Fatigue cracking in the cement mantle has been considered to be dependent on the mixing method; variations in the relative portion

of solid particles (powder) and monomer (liquid) will affect total setting time, the amount of heat generated, expansion and shrinkage during the curing, and the flow characteristics or the degree of polymerization and amount of residual monomer that may be present after the setting reaction has ended. Since the first introduction of PMMA bone cement (Charnley 1960 and 1970), it is considered to have become one of the most effective fixation methods of total joint prostheses. To improve the mechanical properties of PMMA bone cement, centrifugation and pressurization of the cement, as well as fillers are nowadays used (Topoleski *et al.* 1995, Klein *et al.* 2004). However, according to recent studies, the use of artificial hip prosthesis without cement is justified by the biological and adaptive characteristics of the implant. Cementless fixation should be favoured especially when placed in good quality bone, in young and physically active patients (Callaghan *et al.* 1997, Engh and Hopper 1998).

2.3.3. Synthetic bioabsorbable polymers

Polymers are widely used in regenerative medicine. Based on their behaviour in living tissues, polymeric biomaterials can be divided into biologically inactive (biostable), bioabsorbable (biodegradable or bioresorbable), and partially bioabsorbable materials.

Biodegradation has been accomplished by synthesizing polymers that have hydrolytically unstable linkages in the backbone. These most common chemical functional groups are esters, anhydrides, orthoesters, and amides (Middleton and Tipton 2000).

The most important bioabsorbable polymers in surgical use are aliphatic polyesters, their polymers and copolymers. Most of them are thermoplastic, partially crystalline, or totally amorphous. Their mechanical properties and speed of degradation can be tailored according to the clinical requirements (Rokkanen 2002, Domb *et al.* 2002).

Poly(L-lactide) (PLA), poly(glycolide) (PGA) and poly(DL-lactide-co-glycolide) (PLGA) belonging to the poly(α-hydroxy esters) are commonly used in trauma surgery as bone fixation and sutures (Nakamura *et al.* 1992, Molea *et al.* 2000). The advantage of bioabsorbable material is that they do not require a second surgical event for removal (Böstman *et al.* 1990, Edwards *et al.* 2001).

Lactide is a cyclic dimer of lactid acid, which exists in three stereoisometric forms; L-lactide, D-lactide and mesolactide, which contains L-lactic and D-lactic acidic units in the ring. Additionally, DL-lactide is an equimolar mixture of L- and D-lactides. Poly(DL-lactide) is an amorphous polymer melting in the range of 45-55 ˚C. P(DL-LA) has a random distribution of both isometric forms of lactic acid, lacking the ability to arrange into a crystalline organized structure (Pitt 1981).

Polycaprolactone (PCL) is a semicrystalline aliphatic polyester, melting in the range of 54-64 ˚C. The glass transition temperature of –60 ˚C can be increased by copolymerization

with DL-lactide, and the biodegradation time will be enhanced. Poly(DL-lactide) degrades much faster than PCL (Pitt 1981).

The potential of the polymeric biomaterials is based on the drug delivery. PCL has good permeability to many therapeutic agents and has therefore been investigated for long-term contraceptive drug delivery (Pitt 1990, Ozawa *et al.* 2002). For example, delivery of bone morphogenic protein (Wang *et al.* 1990, Bessho *et al.* 2002) and antibiotic (Ramchandani and Robinson 1998) has been studied in orthopedic applications. Nevertheless, for devices where diffusion is important, such as biosensors and sustained-release systems (Rathbone *et al.* 2002), encapsulation can be a considerable impediment to device function (Anderson *et al.* 1981).

2.3.4. Copolymers

Copolymer is a polymer derived from two or more monomer units, and copolymerization refers to methods used to chemically synthesize a copolymer. In copolymerization, the properties of man-made copolymers can be modified to specific needs. The factor that affects mechanical performance are monomer selection, polymerization and process conditions, and the presence of additives (e.g. fillers). These factors influence the crystallinity, hydrophilicity, molecular weight and molecular weight distribution, and modify melt and glass transition temperature. Further, all these then influence the thermal and mechanical properties, and degradation time of the copolymers can be varied greatly (Middleton and Tipton 2000).

Aliphatic polyesters can be synthesized by polycondensation from a mixture of a diol and diacid, from a hydroxy acid, or by ring-opening polymerization of lactones. Polycondensation requires high temperatures with long reaction times to produce high molecular weight chains. But, with ring–opening polymerization, high molecular weight molecules can be prepared in a short time under relatively mild conditions (Löfgren *et al.*1995)

The copolymers of ε-CL and DL-lactide are produced by ring-opening polymerization (Pitt 1990). Degradation of the semicrystalline CL takes 1-2 years *in vivo,* whereas amorphous poly(DL-lactide) degrades much faster, in 12 to 16 months. Copolymers of these polyesters are reported to degrade faster than in either of the homopolymers (Pitt 1981).

In the case of internal bone fracture fixation the fixation device needs to be in place only temporarily, and in such cases bioabsorbable or partially bioabsorbable materials are more appropriate than the biostable ones. These biodegradating bone devices can slowly transfer the load to healing bone (Athanasiou 1998). The thermal properties can be tailored to enhance the processability of the material to be shaped into fibres, films,

rods, screws, plates, clamps etc. Fibre-reinforced PGA/PLA copolymer implants have been successfully used in fracture treatment (Törmälä *et al.* 1987, Peltoniemi 2002).

2.3.5. Bioceramics and bioactive glasses

Ceramic biomaterials are solid inorganic and non-metallic materials. These materials include amorphous and crystalline glass, and glass-ceramics (containing both amorphous and crystalline phase). These special design ceramics are used in reconstructive surgery, due to their bioactivity, the ability to form the firm fixation of the implant in host tissue (Cao and Hench 1996, Kokubo *et al*. 1992, Andersson *et al*. 1990a). The bioceramics and glasses are brittle materials, which are usually not sufficient alone for load-bearing applications.

Synthetic hydroxyapatite (HA) ceramics are biomaterials which form a chemical bond to bone (Jarcho 1981, Denisse and de Groot 1979). Synthetic HA is chemically and crystallographically closely related to the natural bone mineral, hydroxylapatite. Hydroxylapatite is a calcium phospate including hydroxide with a Ca/P ratio of 1.67, and the chemical formula $Ca_{10}(PO_4)_6(OH)$. Synthetic HA contributes not only to the strong direct bone-bonding but also to the early phase of bone formation on the implant surface. However, due to its low strengthening effect, it is often used as a thin coating layer on a metal implant surface to enhance the biological fixation (Thomas 1994).

Tricalcium phosphate (TCP), formula $Ca_3(PO_4)_2$, is a resorptive biocompatible ceramic material used in bone substitutes. Due to its crystalline structure the biodegradation rate of TCP has been shown to be much greater than that of HA (Klein *et al.* 1990).

Hench and colleagues discovered, in the early 1970's, that bone can bond chemically to certain glass compositions. Since the discovery of the first bioactive glass (Bioglass®), various types of glasses and glass-ceramics have been found to bond to living bone. One of them is the apatite- and wollastonite-containing, highly strong and bioactive, glass-ceramic A-W (Kokubo *et al.* 1990, 1994). Bioactive ceramics are known to bond to bone through an apatite layer, which is formed on their surface in contact with body environment (interstitial fluids) (Hench 1988, Gatti *et al.* 1994, Li *et al.* 1997). The series of complex reactions inducing this bond is described by Rawlings (1993).

The composition of Bioglass® is based on a SiO_2-Na_2O-CaO-P_2O_5 system (Hench 1990). The special characteristics of bioactive glass versus other surface-active bioceramics is the wide possibility of controlling their range of chemical properties and their rate of bonding (Andersson *et al.* 1990b). Even a minor modification in molecular composition may change its properties significantly.

2.3.6. Composite materials

The composite material is a product which consists of at least two distinct phases normally formed by blending together components with different structures and properties. The purpose is to produce a material with properties, which could not be achieved from any of the individual components alone (McCabe 1990). To improve the biological properties of PMMA bone cement, bioactive glass or bioceramic particles have been added, and the mechanical properties further developed by using cross-linked polymer chains bisphenol-alpha-glycidyl methacrylate (bis-GMA). The polymerization shrinkage for bis-GMA is considerably lower compared to PMMA (McCabe 1990).

The formation of *in situ* porosity in the acrylic polymer matrix is believed to improve the fixation between the bone and cement. In the studies of Puska and co-workers (2005), the polyamide of trans-4-hydroxy-L-proline serves as porogen filler in nondegradable acrylic bone cement.

Fujimura and coworkers (2003) investigated the bioactive bone cements (BABC) with a matrix consisting of Bis-GMA and triethylene-glycol dimethacrylate (TEGDMA), with fillers of silane-treated CaO-SiO_2-P_2O_5-MgO-CaF_2 glass (A-W- glass-ceramic) powder. The results suggested that this BABC has good handling properties, a high bonding strength to bone and good biocompatibility. After a 12- and 48-week implantation period, they concluded that the material had potential for clinical application as a substitute material for autogenous bone transplantation.

The suitability of composites of P(ε-CL/DL-LA) (with minor CL contents) and alpha tricalcium phosphate (TCP) as a bone-filling material was studied by Ekholm and co-workers (Ekholm *et al.* 2006). The TCP filler made the composite excellent to handle, but because of its too long degradation it was decided not to be recommend this composite as bone-filling material.

The bioabsorbable, self-reinforced (SR), polymeric rods, screws and plates have been reported to ease bone fracture and osteotomy fixations (Ashammakhi *et al.* 2001, Suuronen *et al.* 2004). The implanted SR-PGA and SR-PLLA have been shown to initiate new bone formation from periosteal grafts. The approach used to enhance the osteoconductivity of these polymers is to combine bioactive glass with the polymeric matrix (Kellomäki *et al.* 2000, Maquet *et al.* 2003, Blaker *et al.* 2003, Verrier *et al.* 2004).

2.4. Bioactivity

Bioactivity is defined as a spontaneous communication of a material in a biological environment, resulting in strong adhesion between tissue and the material. Bioactive (or surface active) materials are able to bond with host tissue, forming a strong chemical bond. Bioactive materials can be divided into bioactive glasses, bioactive glass ceramics,

bioactive calcium phosphate ceramics and bioactive composites and coatings (Cao and Hench 2006, Hench *et al.* 1971, Kokubo 1990a).

In *in vitro* studies, the term bioactivity is defined as the ability of the material to release cationic ions to promote Ca-P formation on the material itself or on the materials brought into close contact with it. Most of these bioactivity tests are done in a laboratory environment using simulated body fluid resembling human blood plasma (Kokubo *et al.* 1990b). The bioactivity of the composite, indicated *in vitro* by the apatite formation rate, can be adjusted by varying glass quantity (Jaakkola *et al.* 2004, Niemelä *et al*. 2005, Brink 1997). It has been suggested that the *in vitro* Ca-P formation test reflects the *in vivo* Ca-P formation ability (Kokubo *et al.* 1990b).

Bioactive glasses are able to form a chemical bond with bone through the silica rich layer. In aqueous media, simulating extracellular interstitial fluids, bioactive glasses exchange alkali ions (Ca^{2+}, Na^{+}, K^{+}) and PO_4^{3-} from the glass with hydrogen or hydronium ion (H^{+}, H_3O^{+}) from the body environment. The silica network of bioactive glass starts to dissolve along with the attack of hydroxyl ions and the soluble silica released from glass forms Si-OH or $Si(OH)_4$ groups on the surface of the glass; as the reaction proceeds, a SiO_2 –rich layer is formed by condensation and repolymerisation. The Ca^{2+} and PO_4^{3-} groups migrate through the SiO_2 –rich layer and an amorphous $CaO\text{-}P_2O_5$ –rich film forms on top of the SiO_2 –rich layer. This stage is called calcium phosphate precipitation. The SiO_2 –rich layer continues to grow by diffusion-controlled alkali ion exchange. Soluble calcium phosphates incorporated from the solution (interstitial fluids) promote the growth of the $CaO\text{-}P_2O_5$ –rich layer, which is amorphous, but in favourable conditions, crystallized to apatite. Organic components are incorporated and bone minerals are able to bond to BAG (Hench *et al.* 1971, Rawlings 1993).

2.5. Biocompatibility

In experimental studies, the biocompatibility of implanted materials is determined by local tissue responses. In general, the wound-healing process can be subdivided into two phases, i.e. the inflammatory phase and the repair phase. The presence of an implant can provide a continuous inflammatory stimulus, and as a result, the inflammatory phase can be prolonged. This is associated with increased cellular activity and a delayed repair phase. The changes in time scale and extensiveness of wound healing are determined by the biocompatibility of the implanted material (Jansen 1999).

2.5.1. Cell behaviour

A biomaterial that is intended to be used for tissue reconstruction must have an appropriate shape and sufficient bulk mechanical properties on the macroscopic level. On the microscopic scale, however, the biomaterial must have structure and surface

characteristics which promote cell growth. The porosity and pore size are key factors for tissue-engineering scaffolds and meshes. Besides the surface chemical characteristics, soluble agents like ions or molecules, as well as insoluble agents, like wear debris or material fragments released from the implanted material can interfere with healing (Cao and Hench 1996).

Inflammation is defined as a local response of vascularized tissue to injury, for example, that created by the implantation process. Implanted materials elicit an inflammatory response in the body in which the monocytes and macrophages play a significant role (Anderson and Miller 1984, Anderson 1984). The compact, rounded monocytes may develop to macrophages. This development is indicated by a distinguishable increase in cytoplasmic area. Further, the macrophages can fuse to form giant cells (Collier *et al.* 1999).

Phagocytosis of small particles, in which cells ingest and attempt to digest extraneous matter, is accompanied by a set of biochemical events. The macrophage cells release pro-inflammatory kinins, like fibroblast growth factor and platelet-derived growth factor, which influence fibroblast behaviour. These kinins can also induce thickening of the fibrous capsule (Ratner *et al.* 1996).

In 1994, Ali and coworkers demonstrated in an *in vivo* -chamber designed experiment, that the presence of free radicals generated by the inflammatory cells, such as magrophages, had an accelerating influence on the rate of degradation of PCL and P(DL-LA). The degradation of the PLA and PCL occurs in the first stage as hydrolytic chain scission followed by nonspecific entzymatic action of the responding host tissue. The final elimination occurs via respiration (Pitt *et al.* 1981a and b).

2.5.2. Tissue reactions

Biologically inactive materials, like biostable polymers, cause minimal response in the surrounding tissue, and they retain their mechanical properties for years, while a clear inflammatory reaction is usually seen when biodegradable polymer implants are placed in a tissue environment. However, it should be emphasized that tissue tolerance of an implant is determined not only by its chemical composition but also by its size, surface texture, and shape, while implantation site also plays a significant role (Taylor *et al.* 1994).

Soon after the clinical introduction of biodegradable polymeric fracture fixation devices, it became obvious that these materials provoked an adverse tissue response that had the characteristics of an inflammatory, abacterial foreign-body reaction (Woodward *et al.* 1985). The fast-degradating polymers, such as PGA, in host tissue are usually associated with a higher incidence of adverse tissue responses than the slower ones (Böstman and Pihlajamäki 2000, Peltoniemi *et al.* 2002).

Although the biocompatibility of PLLA is reported to be good (Suuronen *et al.* 1998), problems occurred when pure PLLA plates and screws were used. Bergsma and coworker (1993) reported the PLA particles to be encapsulated in thick fibrous tissue with intracellular PLA crystals. Two years later they conducted a study on patients that had received poly(L-LA) plates and srews for zygomatic fractures. They removed and analyzed the remaining fixation material after 3.3 and 5.7 years. The particles were not irritable and did not cause injury to the cells, but did induce a reaction in the form of detectable swelling (Bergsma *et al.*1995).

The biocompatibility and toxicity of PCL have mostly been tested in long-term drug delivery systems (Vandamme and Mukendi 1996, Pitt 1990) and as nerve guide constructions (den Dunnen *et al.* 1997). During degradation, only very mild foreign-body reactions were reported.

Several studies on degradation of the different poly(α-hydroxy acids) as such have been carried out and reported (Li 1999, Middleton and Tipton 2000). Degradation is dependent on so many factors that when modified by adding compounds as well as by mixing different material combinations, prediction of material behaviour and properties is often difficult to estimate.

3. AIMS OF THE STUDY

The purpose of this study was to investigate the potential of copolymer poly(ε-caprolactone/DL-lactide) to function as a carrier for bioactive glass S53P4 granules in degradable bone substitute material.

The study was based on the working hypothesis that bioactive glass S53P4 can be used as a bioactive component for biodegradable composite to promote the bone tissue healing process.

Special attention was paid to:

1. Determining ion release and apatite formation as a function of time on composite and copolymer surfaces in simulated body conditions.

2. Determining the degradation rate and water absorption of the copolymer matrix *in vitro*.

3. Investigating the human fibroblast cell attachment, proliferation and morphology on material surfaces in a cell culture environment.

4. Evaluating local soft tissue response to copolymer and composite materials and possible abnormalities in vital organs after subcutaneous implantation.

5. Assessing the material behaviour and the effect on the healing of experimental bone defects.

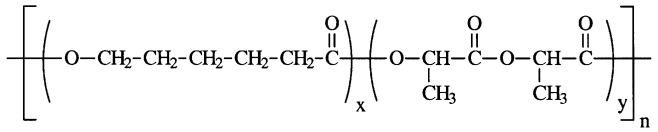
4. MATERIALS

4.1. Thermoplastic bioactive composite

The experimental composites consisted of thermoplastic biodegradable copolymer and granules of bioactive glass (BAG).

4.1.1. Copolymer poly(ε-caprolactone/DL-lactide)

The copolymer of ε-caprolactone (Fluka, Buchs, Switzerland) and DL-lactide (Purac, Gorinchem, The Netherlands) was used in experimental studies. Copolymer was polymerized in bulk using stannous(II)octoate as the catalyst. Glycerol was used as a co-initiator in ring-opening polymerization. The chemical structure of the copolymer of ε-caprolactone and DL-lactide is presented in Scheme 1. The polymerization was carried out at 160 ˚C for 4.5 h. The polymer was stored in dry conditions and further dried under reduced pressure for 24 h before sample preparation. The comonomer ratio of CL and DLLA in feed was 95 and 5 weight percent, respectively. And weight average molecular weight was 110 000 g mol^{-1}. The melting temperature of the copolymer was 50 ˚C.



P(CL/DL-LA)

Scheme 1: P(CL/DL-LA)

4.1.2. Bioactive glass S53P4

The bioactive glass used is a commercially available S53P4 (Abmin Technologies Ltd, Turku, Finland). The chemical composition of the S53P4 in weight percentages (wt%) was SiO_2 53.0; Na_2O 23.0; CaO 20.0 and P_2O_5 4.0. Two ranges of glass granule size were used. Granule size range <45 μm was chosen to produce composites with a high surface area/volume ratio of the BAG, whereas the composites with a granule size range of 90-315 μm had a lower glass surface area/volume ratio. The density of bioactive glass was 2.658 g/cm^3.

4.1.3. Experimental composite

Composites were produced by blending the BAG particles homogenously with copolymer matrix in a batch mixer (Brabender W50EH) at 100 ˚C for 5 min at 60-75 rpm. Five different composites containing 40 to 70 wt% of BAG were prepared for the experimental tests in order to obtain a range of composites with varying bioactivity, i.e.

slower or faster formation of a silica-rich layer on the surface of the composite (Table 1). For studies I-III, the test composites were compression-molded (Fontijne TP400) into discoid specimens with a diameter of 10 mm and a thickness of 2 mm. In study IV, the specimens with diameter of 4 mm and thickness of 2 mm were punched out of the compression-molded composite plates, whereas in study V, the composites were packed into 5 ml syringes. The test composites and the copolymer specimen were sterilized with γ-radiation (Gammaster, Wageningen, The Netherlands), except in study IV when the dipping in ethanol was used as a sterilization method immediately before the implantation procedure in the implantation site. Discoid specimens were tested as such without further processing (polishing). Because no surface treatment would be possible for injectable composite material, when heated immediately before application.

Table 1. Two different BAG granule ranges used in experimental composites, and the glass contens quantified by ashing of the original specimen weights (presented as weight percents).

Composite	Bioactive glass Granule size range (μm)	In feed (wt%)	Measured (wt%)
P(CL/DL-LA)	–	–	–
BAG40S	<45	40	39.0 ± 0.8
BAG60S	<45	60	58.7 ± 1.0
BAG40L	90-315	40	39.9 ± 0.3
BAG60L	90-315	60	60.8 ± 1.3
BAG70L	90-315	70	71.0 ± 1.9

In study IV, sucrose (granule size 300-1000 μm) was added as a porogen agent to one of the experimental composites. Copolymer P(ε-CL/DL-LA) without BAG granules was used as control material throughout the studies (I-V).

4.2. Human fibroblastic cells

Tissue samples were taken from periodontally healthy sites by carefully separating the gingiva from the tooth surface. Fibroblast cultures were initiated from the tissue explants taken during tooth extractions. The cells were grown in D-MEM medium with 10 % fetal calf serum (FCS) and antibiotics (Invitrogen Life Technologies, Carlsbad, CA) at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. The cells were subcultured by standard trypsinization and the culture medium was changed every three days. Cells from passage 9 were used for the experiments. The human experimental protocols were approved by the Ethical Committee of the Institute of Dentistry, University of Turku, Finland.

4.3. Experimental animals

For study IV, 20 Long-Evans rats were used, 10 males and 10 females weighing an average of 280 g. In study V, 20 adult female New Zealand white rabbits weighing approximately 5 kg were used. During the study, the animals were allowed to move freely while kept in cages under standardized conditions and fed with standard hard diet pellets. National guidelines for the care and use of laboratory animals were followed. The experimental procedures were first reviewed by the local Ethics Committee on Animal Experimentation at the University of Turku and then approved by the local Provincial State Office of Western Finland.

5. METHODS

5.1. Polymerisation and quantification of bioactive glass content

The ash content of the various compression moulded composites was measured to quantify the amount of BAG in composites. Organic material was burned off the composites at 700°C for 4 h. After ashing, the specimens were allowed to cool to room temperature before weight measurements. BAG content was calculated as percentage of the original specimen weight.

5.2. Dissolution test (I and II)

5.2.1. Immersion in simulated body fluid (I, II)

The discs were placed individually into falcon tubes filled with SBF with a pH of 7.4 at 37°C. The SBF was prepared by dissolving reagent chemicals of NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_20$ and Na_2SO_4 in deionized water (Kokubo *et al*. 1990). The ion concentrations of the used SBF resemble human blood plasma. The fluid was buffered at physiological pH 7.40 at 37 ºC with tris(hydroxymethyl)aminomethane and hydrochloric acid. The specimens were immersed in 10 ml of SBF for 6 h up to 8 weeks, and in 40 ml for 3 to 6 months. Three copolymer samples without BAG and three plain SBF buffer samples enclosed in tubes served as controls. All the test tubes were placed in a water bath (Heto Lab Equipment SBD-50 type BIO, 160 strokes per minute, amplitude 36 mm) at a constant temperature (37 °C). The specimens were present in triplicate and buffer samples were taken and replaced with fresh SBF after 6, 24, and 120 hours, and thereafter weekly up to 8 weeks. After 8 weeks, the immersion fluid was completely replaced and total fluid volume was increased from 10 ml to 40 ml. After 8 weeks, samples were taken monthly and immersion was continued for 6 months.

5.2.2. Ion concentration analysis (I)

Sample solutions were monitored for calcium (Ca^{2+}) and silica (Si^{-}) concentrations as a function of immersion time. Si-concentrations were determined by the Molybdenum blue method with a UV-visible spectrophotometre (Shimadzu UV-1601, Tokyo, Japan). Si-analysis was based on reduction with 1-amino-2-phenol-4-sulfonic acid (Koch and Koch-Dedic 1974). Ca-concentrations were determined with an atomic absorption spectrophotometre (Perkin-Elmer Model 460, Perkin-Elmer corporation, Eden Praire, USA). All the samples were measured three times.

5.2.3. Surface characterization (I)

At each time point, three specimens from each test group were removed from the SBF. The specimens were rinsed with deionized water, dried, and stored in a dessicator. Material surfaces were analyzed with scanning electron microscopy (SEM) (coupled with energy dispersive X-ray (SEM-EDX)) (Stereoscan 360, Cambridge, UK), and with Fourier transform infra-red spectroscopy (FT-IR) using the attenuated total reflectance (ATR) technique (Spectrum One, Perkin Elmer, USA). The IR spectra were obtained at 4 cm^{-1} resolution, averaging 32 scans at 0.20 cm/s in speed, air being used as a background.

5.2.4. Water absorption measurements (II)

Water absorption was calculated as the difference between the weigh of the wet specimen after hydrolysis, divided by the weight of the dried specimen.

5.2.5. Weight average molecular weights (II)

Molecular weights were determined by room temperature size exclusion chromatography (SEC) (Waters System Interface module, Waters 510 HPLC Pump, Waters 700 Satellite Wisp, and four linear PL gel columns: 10^4 Å, 10^5 Å, 10^3 Å and 1000 Å connected in series). Chloroform was used as solvent and eluent for polymers.

5.3. Fibroblastic cell culture (III)

5.3.1. Cell proliferation assay

For the determination of cell proliferation, cells were grown in 24-well cell culture plates. Before seeding of the cells the test material discs, precorroded 10 days in D-MEM, or glass coverslips, used as a negative control material, were placed in the bottom of the wells. Each material was studied in quadruplicate. 25 000 cells were seeded to each well in 1 ml volume (12 500 /cm^2). The number of viable cells was determined at 1, 2, 3, 7, 10 and 14 days of culture by using a non-radioactive cell proliferation assay (CellTiter96®, Promega, Madison, USA). The reaction mixture volumes were adjusted so as to compensate for dimensions of the test material discs vs glass coverslips. Absorbances were recorded in a plate reader at 570 nm. For comparison, in some experiments, cell counts were determined by trypsinizing the cells attached to the specimens and by counting them in a hemocytometer.

5.3.2. Lectin labelling and fluorescence microscopy

Tetramethylrhodamine isothiocyanate (TRITC) coupled lectin from Triticum vulgaris (Wheat germ agglutinin, WGA) was obtained from Sigma Chemical Company (St. Louis, MO). WGA binds to N-acetylglucosamine (glcNac) and N-acetylneuramininc

acid (NeuNac) residues expressed on cell membranes (Damjanov 1987, Bampton *et al.* 1991) and can therefore be used as molecular label for human fibroblasts. The specimens were fixed in 3.5 % paraformaldehyde for 10 min and washed 3 times for 10 min in Dulbeccos's phosphate buffered saline (DPBS). After this they were exposed for 1 h to 50 µg/ml of TRITC-WGA diluted in DPBS. After washes in buffer, the samples were embedded in Mowiol® (Calbiochem Novabiochem, La Jolla, CA, USA) mounting medium. Saccharide inhibition tests were carried out by preincubating the lectin conjugate for one hour with 0.5 M N-acetylglucosamine. The specimens were studied in a Leitz microscope equipped with an epifluorescence unit and a filter module for TRITC-fluorescence, and photographed with a Wild MPS 46 automatic camera system on Kodak Tmax 400 film at EI 800.

5.3.3. Scanning electron microscopy

For SEM the cells were fixed for 45 min in 3 % glutaraldehyde made in phosphate-buffered saline (PBS). After washing in PBS, the samples were dehydrated in graded ethanol and dried with a Balzers CPD 020 critical point dryer (Bal-Tec, Liechtenstein, Switzerland). The samples were then coated with gold using a Jeol Fine Coat-1100 sputtering device (JEOL Ltd, Tokyo Japan) for 6 min and examined in a Zeiss DSM 962 scanning electron microscope (Carl Zeiss, Oberkochen, Germany) at standard conditions of 5 kV acceleration voltage and 0° tilt.

5.4. In vivo tissue reactions (IV and V)

5.4.1. Implantation in rat subcutaneous tissue (IV)

Surgery was performed under general anesthesia, induced by fluanison 10 mg/ml + fentanyl 0.2 mg/ml (Hypnorm® inject, Veterinary drug co. PLC). For the operation the animals were immobilized and the operation area was shaved, washed and disinfected with providone-ionide (Betadine®, Oy Leiras Finland Ab, Helsinki, Finland). After 0.3 ml infiltration of articainhydrocloride 40 mg/ml + adrenaline 5 µg/ml (Ultracain®, Aventis Pharma AG, Zürich, Switzerland) into the operation area, a transcutaneous incision was made into the midline on the dorsal area of the rat. The specimens were implanted to the distended pockets made with blunt-point scissors in subcutaneous tissue. In the 6-month group, surgical sutures (Prolene®, Ethicon, Johnson & Johnson Gateway, New Jersey; USA) were made to the cranial and caudal sides of the implantation area to aid the location of the implants.

5.4.1.1. Histological preparations

After periods of 3, 7 and 28 days, 3 and 6 months, four rats were stunned and sacrificed with carbondioxide gas. The specimens were removed with a 5 mm margin and fixed in

70 % ethanol, dehydrated in a rising alcohol series, and embedded in methylmetacrylate. Correspondingly, the intestinal organs were resected and fixed in 4 % phosphate-buffered formalin before being dehydrated in the alcohol series and embedded in paraffin. All the blocks were sectioned longitudinally in two halves through the middle plane of the implant. Then, 20 µm thick histological sections from methylmethacrylate and 15 µm thick sections from paraffin blocks were made using a cutting and grinding method and stained with toluidine blue and van Gieson.

5.4.1.2. Histological evaluation

The histological sections were evaluated and examined using a light microscope. The connective tissue capsule formation was analyzed and the thickness of the capsule surrounding the specimens was measured from four different standardized areas, one on each side of an implant. The final value was recorded as an average of these measurements. The fibroblast proliferation and inflammatory reactions were analyzed in general for the tissues seen beside the implant and scored according to 4-point evaluation scales (Table 2.). The presence of mast cells and other inflammatory cells was determined from the sections stained with toluidine blue, while the connective tissue encapsulation surrounded by muscles was more accurately defined by van Gieson staining.

Table 2. The fibroblast proliferation scores (FS) and the inflammation scores (IS) used based to the s cene from the capsule and the surrounding connective tissue in the histological section.

score	*FS*	*IS*
0	No reaction	No inflammation
1	Some connective tissue formation	A few mononuclear inflammatory cells
2	A moderate fibroblast proliferation and/or loose soft tissue proliferation	Some inflammatory cells
3	Plenty of proliferative firbroblasts around the implant	Numerous inflammatory cells infiltrated

For the toxicological observation, the histological sections of each animal were examined for abnormalities or to find any possible exceptional phenomena in the intestinal organs; superficial cervical lymph nodes, spleen, liver, kidney, thymus, heart and lungs. The histological sections were stained with hematoxylin-eosin and toluidine blue.

5.4.2. Implantation in rabbit femoral bone (V)

The rabbits were assigned to two groups (ten rabbits in each group): the 8- and 16-week groups. Surgery was performed under general anesthesia, induced by intravenous injection (50 mg/kg/animal) of a cocktail consisting of ketamine hydrochloride (Ketalar®, Parke-Davis, France), xylazine and 0.9 % physiologic saline. To reduce the perioperative infection risk, an antibiotic (Terramycine®) was administered postoperatively by subcutaneous injection.

For the operation the animals were immobilized, and the bilateral knees and tibias were shaved, washed and disinfected with povidone-iodine. Longitudinal incisions were made on the medial side of the left and right knee and the left and right tibia. The periosteum was elevated, and the medial side of left and right condyles and tibia were exposed. Thereafter, two holes of 4 mm diameter and 6 mm depth were made into the femoral condyles. In addition, two 4 mm holes were made in the tibiae. The holes penetrated through the medial cortex into the medullar cavity. The holes were made with drills that gradually increased in diameter. The bone preparation was performed using low rotational drill speed (450 rpm) with continuous cooling. The bone cavities were washed with saline during and after the drilling. Thermoplastic composite was applied to bone defects at 46 °C (Figure 1). Three different composites were used: a) composite with 60-wt% of bioactive glass granules (granule size <45 μm; BAG60S), b) composite with 40-wt% of bioactive glass (granule size 90-310 μm; BAG40L) and c) composite with 60-wt% of bioactive glass (granule size 90-310 μm; BAG60L). One cavity was filled with the copolymer without bioactive glass granules. Each cavity was filled with one of the four experimental materials based on the balanced split plot technique. A small titanium pin (Stabilok, Fairfax Dental Inc., Miami, FL) was placed between the filled bone holes to aid in locating the experimental defects after the healing period.

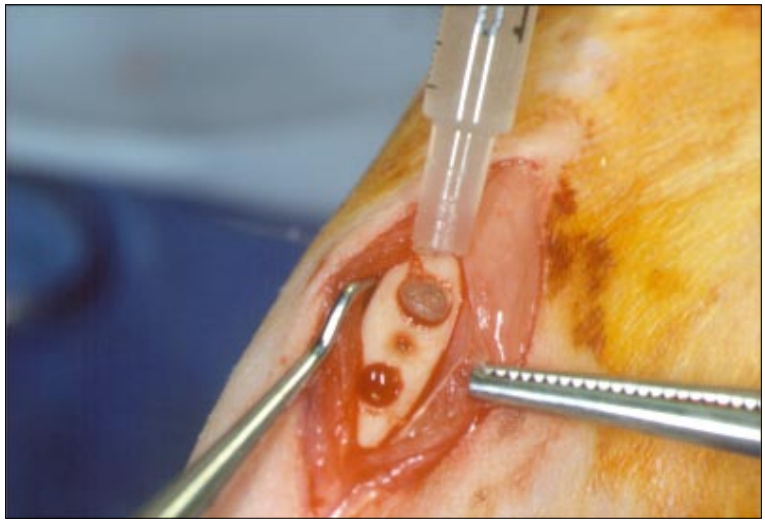


Figure 1. Photograph of material application into the drilling hole in tibia.

5.4.2.1. Histological procedure

At the end of both implantation periods, all animals were sacrificed by injecting an overdose of pentobarbitalsodium (Nembutal®, Abbott Laboratories, Chigaco Il, USA). After sacrificing the animals, the tibias and femoral condyles were removed and fixed in 10 % buffered formalin solution. Subsequently, the blocks were reduced in size and separated in specimens that each contained one implant material. These small samples

were dehydrated in a series of alcohol and embedded in polymethylmetacrylate (PMMA). After polymerization, non-decalcified thin sections (10 μm) were prepared with a modified diamond blade sawing microtome technique (Van der Lubbe *et al.* 1988). For the tibial specimens the plane of sectioning was along the long axis of the implanted material, whereas the femoral specimens were sectioned sagitally. The sections were stained with methylene blue and basic fuchsin and evaluated with a light microscope.

5.4.2.2. Histomorphometrical evaluation

A Leitz light microscope was used for histological evaluation. Image analysis was performed with a computerized analysis system (Micro-Scale TC, Digithurst, Royston, UK). Measurements were performed along the outermost surface of the composite or copolymer samples. The amount of bone contact was defined as the percentage of total surface area where there was no contact with intervening soft tissue layers. The measurements were performed separately at the interface implant-femoral trabecular bone, the implant-tibial cortical bone, and the implant tibial medullary cavity.

5.5. Statistical methods

The differences in ion concentrations (I) and fibroblast cell count (III) among different composite materials as a function of time were statistically analysed by analysis of variance (ANOVA) for repeated measurements. The statistical computation was performed with the SAS (version 8.01; SAS Institute, Cary, NC, USA) statistical program package. Because of the multiple comparison, Bonferroni correction was used in study III.

In study IV, the data were analyzed using analysis of variance for repeated measures. Inflammation score, proliferation score and capsule thickness were dependent variables in the models. Implant group (control, BAG50L+ and BAG70L) was used as the repeated factor and time (3, 7 and 28 days, 3 and 6 months) as the fixed factor. The differences in changes during the time among the groups were tested by group interaction term. Further multiple comparisons between other days compared to day 3 were done using Dunnett's test. Analyses were done with SAS System for Windows, release 8.02 (SAS Institute, Cary, NC). P-values less than 0.05 were considered statistically significant.

In study V, the statistical analysis was performed with StatView 5.0.1. program (SAS Institute Inc., Cary, NC, USA). The Mann Whitney U-test was used for pairwise comparisons. Comparison among several means was performed with the Kruskall-Wallis test.

The statistical analysis in study II was performed in the Department of Chemical Technology in Helsinki University of Technology, Espoo, Finland.

6. RESULTS

6.1. Copolymer composition and quantification of bioactive glass content (II)

6.1.1. Preparation of the composites

Total monomer conversion in bulk polymerisation was nearly complete since no monomer peaks were observed in ^{1}H-NMR spectrographs. The monomer composition of the copolymer determined by ^{13}C-NMR analysis was P(CL/LA) 97.7/2.3 (mol/mol) compared with the composition of monomers in the feed which was 96.0/4.0. The molecular weight and the melting temperature of the copolymer matrix were adjusted to enable the application of the composite material by injection below 50 ˚C. Use of the initiator ensured faster polymerisation and also allowed the molecular weight of the copolymer to be adjusted to the right level. As the aim was to produce an injectable composite material, the molecular weight had to be such that the melt viscosity of the copolymer combined with the bioactive glass would not be too high. The weight average molecular weight of the copolymer was 110 000 g/mol, number average molecular weight was 70 000 g/mol and molecular distribution was thus 1.6. Choosing the right comonomer ratio of ε-caprolactone and DL-lactide and using glycerol to adjust the molecular weight adjusted the melting temperature of the matrix polymer. The melting temperature of the copolymer was 50 ˚C and the glass transition temperature -58 ˚C measured by DSC. Addition of the BAG did not alter the shape or place of the melting peak of the copolymer matrix. As expected, addition of DL-lactide lowered the melting temperature, and increased the glass transition temperature of the copolymer compared to poly(ε-caprolactone) homopolymer (Rich 2002).

6.1.2. BAG content in composites

The true weight fractions of the BAG in the different composites were confirmed by ash test and were found to be in good agreement with the theoretical amounts of glass particles blended (Table 1). With SEM, uniform distribution of the irregular-shaped granules of BAG was seen on the fracture surface of the composite materials (publication II, Rich *et al.* 2002, Fig 1 p.2146).

6.1.3. Dynamic mechanical thermal analysis

BAG does not chemically interact with the P(CL/DL-LA) copolymer matrix. The stiffening effect can be observed in the storage modulus values but the glass transition is not affected. The stiffening effect was more noticeable with the smaller granule range, especially above the glass transition temperature.

6.2. In Vitro Dissolution test (I,II)

6.2.1. Ion concentration analysis

6.2.1.1. Cation release (Si^- and Ca^{2+}) from the composites

Ca-P precipitations on the surfaces of the composites were evaluated by measuring the Ca and Si ions' release rates and profiles of the immersion media. On the basis of Si ion release, all composite materials suggested bioactivity. Increases in Si concentrations were noted already after 6 hours of immersion, the concentration being highest in BAG60L material. The release pattern of Si ions describes the influence of the size and amount of BAG granules within the composite (Figure 2). With the specimens BAG40S and BAG60S, the Si concentrations start to increase more slowly compared to groups containing larger glass granules. In the samples containing small glass granules, the total reacting glass surface area is greater and, as expected, the Si releasing rate increases in time with the increasing volume of glass granules. However, the Si release from composites containing the same amounts of larger glass granules was faster over the first two months. This can be explained by the faster Ca-P precipitation seen on the materials containing smaller glass granules. The precipitation layer forms a mechanical barrier that restricts contact with SBF and thus

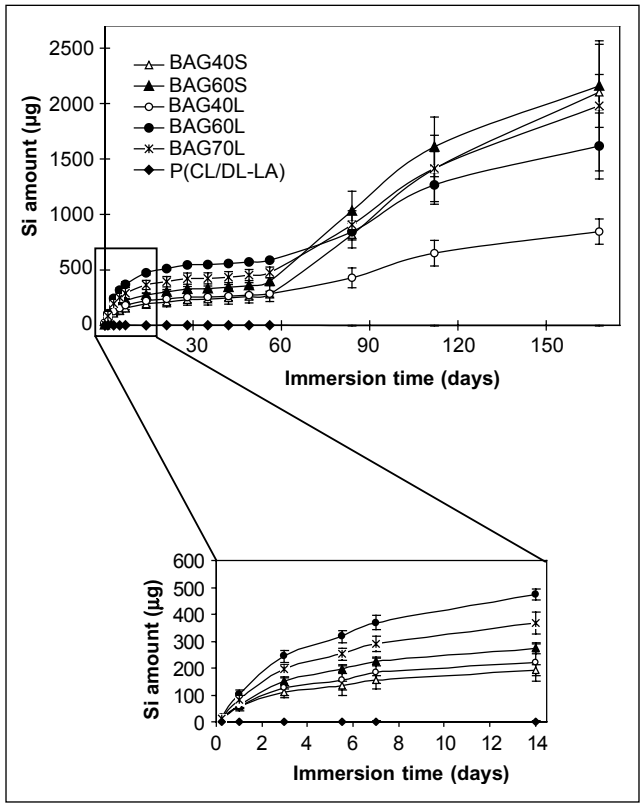


Figure 2. Cumulative changes in Si^- amounts of the SBF of different composite materials P(CL/DL-LA) (♦) and composites BAG70L (×), BAG60L (●), BAG40L (○), BAG60S (▲) and BAG40S (Δ) as a function of immersion time.

effectively slows down the ion release. The copolymer matrix is hydrophobic and hence the total release of Si from the material BAG40L stayed significantly lower compared to other tested materials ($p<0.03$). No statistically significant difference in total Si release was found between the BAG40S and BAG60S, nor between the BAG60S, BAG60L and BAG70L samples during the experiment. Among the cumulative Si ion releasing rates, approximately 1.6 to 4.4 wt% of total Si amounts were released in six months. The plain copolymer P(CL/D,L-LA) did not contain Si and, therefore, no changes in Si concentrations were observed during the immersion period.

The Ca concentrations of the immersion fluid increased at the beginning of the immersion, after which they started to decrease gradually as Ca-P deposition occurs. The tendency is comparable to the reaction seen in bioactive glass ceramics (Andersson and Kangasniemi 1991). The measured Ca concentrations in dissolution media of the different composites over the six-month period are illustrated in Figure 3. The Ca concentration of plain

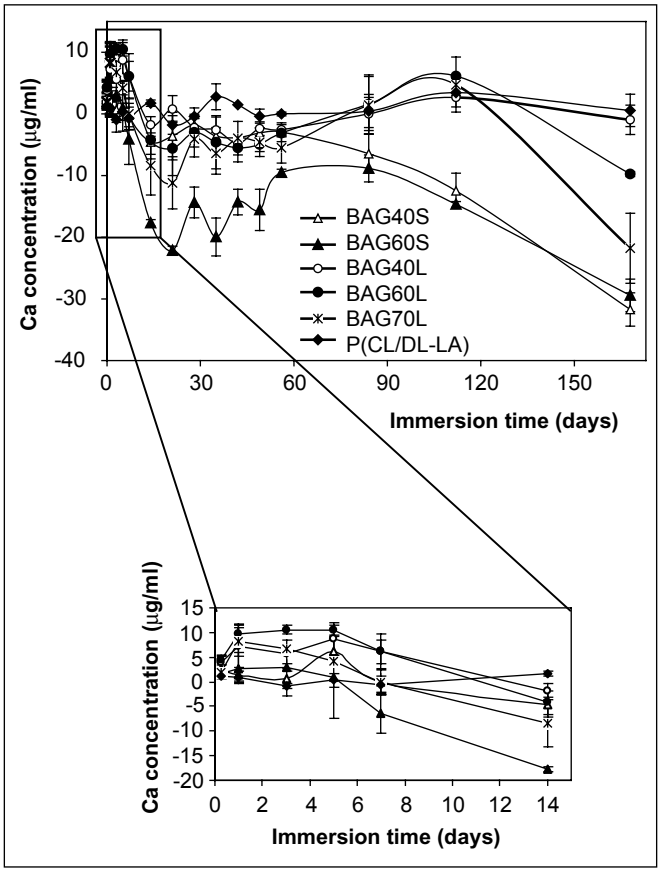


Figure 3. Changes in Ca^{2+} concentrations affected by each tested material as a function of immersion time. The concentration of the immersion fluid of different composite materials declined with the concentration of the plain SBF. P(CL/DL-LA) (♦) and composites BAG70L (×), BAG60L (●), BAG40L (○), BAG60S (▲) and BAG40S (Δ)

immersion fluid is subtracted from each curve to clarify the deposition formation. The negative curve values illustrate the precipitated Ca. Because of the long immersion period, the SBF was replaced several times. After each renewal, the Ca saturation changed and caused a temporary increase in Ca concentration, seen as waves in dissolution curves. The SBF does not contain Si and, therefore no similar sway is seen in Si dissolution profiles.

6.2.2. Surface characterization

6.2.2.1. SEM and FTIR analysis

In the polymer surface, the ester pendent chains are cleaved via hydrolysis, and carboxylate groups (-COOH) are formed. These reactive groups have the potential to complex Si and Ca ions, which in turn may act as nucleation sites for the forming hydroxyapatite layer (James *et al.* 1999). IR shows the peak of $-COO^-$ at 1550-1650 cm^{-1} seen in Figures 4a and 4d on day 3. Due to the hydrophobic nature of polycaprolactone, the hydrolysis was slow and these nucleation sites evidently did not lead to nucleation on the copolymer surface, as was observed in SEM and EDXA, whereas, in the case of the subsurface presence of BAG granules, precipitation formation was seen. When comparing the SEM images and

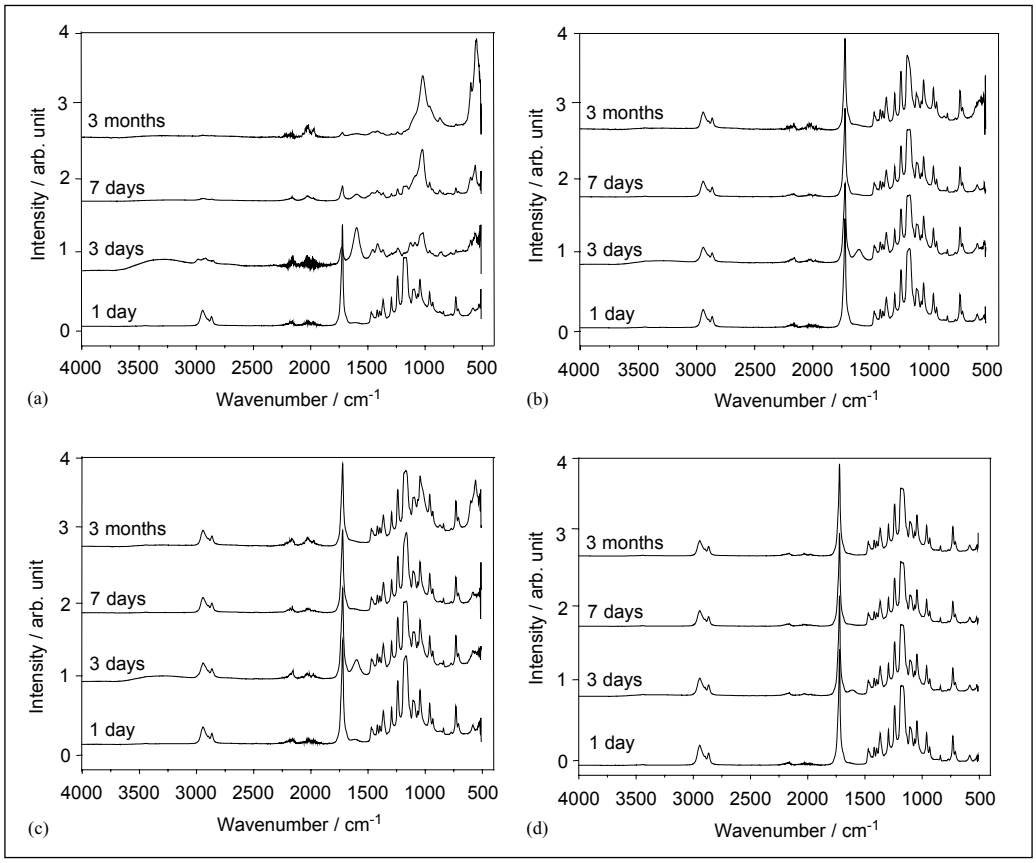


Figure 4. The ATR-FTIR spectrum of the composites BAG60S (a), BAG40L (b), BAG70L (c) and the P(CL/DL-LA) polymer (d) immersion in SBF for 1, 3, and 7 days and 3 months at 37 °C.

ion dissolution profiles, the decrease in Ca concentration may be related to the apatite formation on the composite surface (Figure 5a-i). The Ca concentration curve of the material BAG40L indicated only minimal Ca nucleation on the material surface during the experiment. The slow degradation rate delays the BAG granules making contact with the immersion fluid. The more glass granules the composite contained (BAG60S, BAG60L, BAG70L), the sooner Ca concentrations of the immersion fluid started to decrease. After 6 hours, Ca concentration was lowest in the composite BAG60S and highest in the BAG60L. The samples with small glass granules (BAG60S and BAG40S) showed significantly different Ca concentrations at all measurements compared to BAG40L, BAG60L and polymer (p-values from $p<0.006$ to $p<0.0001$). The nucleation was noticed earlier and Ca-P formation was faster on the glass containing composite specimens, with a tendency for increasing glass content. In the long run, however, the total content of larger glass granules did not make a significant difference among the three composites containing larger glass granules (BAG40L, BAG60L and BAG70L). It seems that dense Ca-P precipitation, formed on the material surface during the first half of the experiment, impaired any further precipitation. The Ca concentration increased after two months in the samples with higher contents of larger BAG granules. This may be due to increasing water absorption as described above.

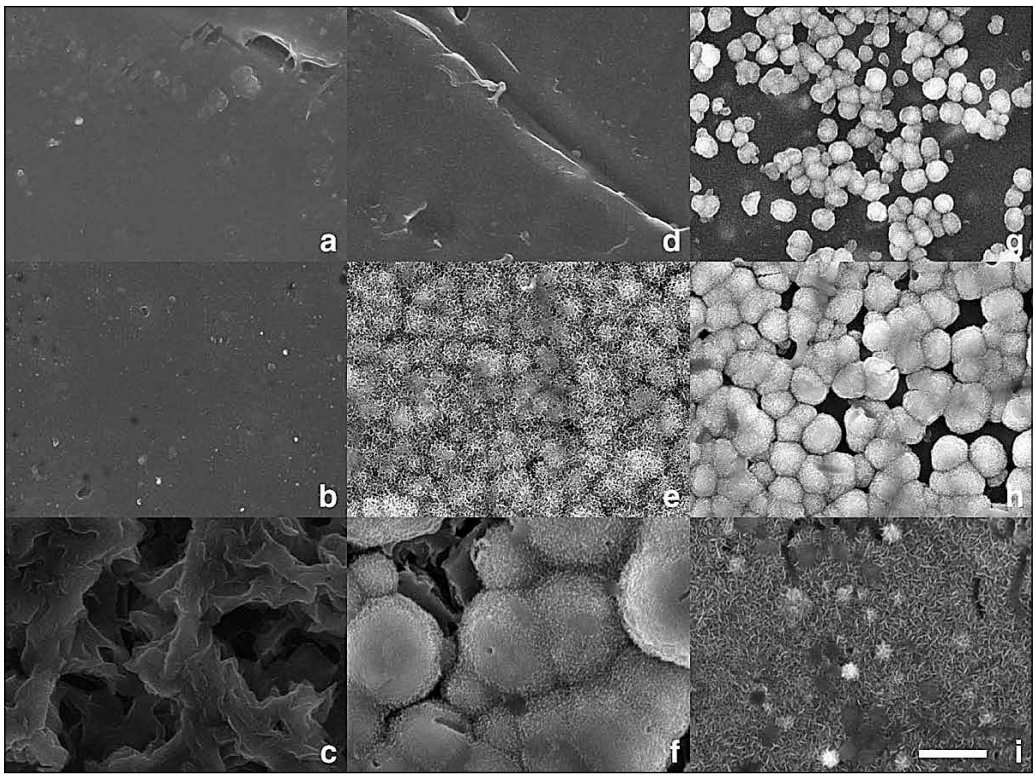


Figure 5. SEM pictures of composite surfaces BAG40L (a-c), BAG70L (d-f) and BAG60S (g-i) after 3 days, 7 days and 3 months immersion in SBF (original magnification x 5000, scale bar = 5 μm).

In the scanning electron micrographs in Figure 5, the Ca-P deposition is seen to form on the surfaces of specimens containing BAG, except for the BAG40L specimens and the plain copolymer. The Ca-P precipitation is noticed on composite surfaces during the first week of immersion and it is in line with the changes seen in Si and Ca concentration curves. The layer was noticed in SEM photographs and identified as apatite on the BAG60S materials by the IR spectra measurement shown in Figure 4 (Cohn and Younes 1988, Rehman and Bonfield1997). The measurements were done after 24 hours, 3 and 7 days, and 3 months of immersion. Two vibrational modes of carbonate ions, v_2 and v_3, are seen in the IR spectrum. In our specimens, the v_2 vibrational mode is seen as a peak in the region at 871 cm^{-1}, and the v_3 at 1418 cm^{-1}. These carbonated ion peaks are weak but clearly seen in BAG60S specimens. Especially in Figure 4a, the peak of the ester carbonyl compound seen at 7 days stretching to 1722 cm^{-1} is almost absent at three months, while the carbonate ion v_3 is seen as a wider band region, 1500-1350, and v_2 more clearly at 871 cm^{-1}. All four vibrational modes of phosphate ions, v_1, v_2, v_3 and v_4, are infrared active. The strongest bands with clear peaks of phosphate ions are seen in Figure 4a at the regions 1023-1040 cm^{-1} (v_3) and 601-560 cm^{-1} (v_4). The sharp peaks at 602 and 562 cm^{-1} indicate the apatite formation. The phosphate bands are also seen with smaller intensity in Figures 4b and c, at 3 months. The phosphate v_1 mode is seen as a peak at 960 cm^{-1}, and is clearly seen only in curves in Figure 4a. The peak is observable in all the spectra of the specimens but is interfered with by the polymer bands. In the literature, the phosphate v_2 band is observable in the region at 470 cm^{-1} (Rehman and Bonfield 1997), but below 500 cm^{-1} the signal was too weak to detect in our samples.

The Ca-P layer was seen to detach easily from the specimen surfaces containing larger glass granules. This might be related to the smoother surface compared to the roughness formed in dissolution of smaller glass-containing specimens. The SEM shows Ca-P formation on the BAG70L samples and this is verified as apatite by the EDX results (not shown) beginning on day 7. Apatite was not found from the BAG40L specimens, which showed only minor changes in Ca concentrations, although their bioactivity was suggested by increasing Si dissolution. This indicates that Ca and P from the SBF stores were insufficient for hydroxyapatite (HA) maturation on these samples. A cracking surface related to fluid uptake and degradation of the copolymer matrix is also seen on the surface of the BAG40L specimen in Figure 5c.

In the SBF, the concentration of HCO_3^- ions is lower than in blood plasma. According to this concentration difference between the environmental systems, a lower Ca/P ratio of the apatite formed in SBF may be found when compared with the *in vivo* situation (Kim *et al.* 1999). Furthermore, the presence of proteins affects HA formation *in vivo*, so the results of this study cannot be directly extrapolated to clinical conditions.

6.3. Degradation of the composite (I, II)

The degradation of the composite samples was determined as a function of time in SBF at 37 ˚C. Degradation was monitored by water absorption and molecular weight changes. Biodegradable polyesters degrade with random chain scission by ester hydrolysis in a process autocatalysed by a generation of carboxylic acid end groups (Kulkarni *et al.* 1971, Jaakkola *et al.* 2004).

6.3.1. Water absorption measurements

The copolymer was rather stable during the 6-month hydrolysis period due to the hydrophobic nature of the polycaprolactone blocks. The water absorption into the neat copolymer was <1 % (Rich *et al.* 2002, Fig 3. p.2147). The presence of BAG increases the water absorption compared to the absorption in the plain copolymer sample, and the higher area/volume ratio of the smaller granule size range clearly enhanced the water absorption compared to larger granules in the matrix even at 70 wt% load.

6.3.2. Weight average molecular weights

Higher water absorptions and faster loss of molecular weight were observed in the composites containing the smaller granule size range of BAG, namely, <45 μm. As random chain scission proceeds in the copolymer, smaller chain fragments are formed. The change in average chain length can be observed as a growing difference between the number average and weight average molecular weight. Degradation is clearly enhanced from the moment the BAG is introduced into the matrix.

6.4. Fibroblastic cell culture (III)

6.4.1. Cell proliferation assay

After 24 hours of culture on the substrates the fibroblasts seemed to proliferate better on all test materials compared to cell-culture glass, the inert silica glass cover slips. The highest absorbances were measured in the group of copolymer without BAG. No significant differences were found in cell proliferation among the three tested specimens. On the other hand, more cells appeared to grow on plain copolymer than on control glass. This difference was not significant at 168 h ($p=0.05$) but increased and remained significant until the end of the experiment (at 336 h $p<0.0045$). At 240 h the absorbances were significantly higher in the groups of BG40S and BG60L than in the glass group ($p<0.0001$). However, the difference was not significant at 336 h, indicating that a final maximum cell density was obtained on these surfaces.

6.4.2. Lectin labelling and fluorescence microscopy

Fluorescence microscopy showed that cells had attached and spread on the various substrata, and that they had a very flattened appearance and a spindle-like shape. In the 24-hour samples, only a few cells were seen growing on all the tested surfaces. In the 72-hour samples, the number of cells had increased dramatically and they formed an even network of spindle-shaped cells covering the whole surface area of all samples. In later samples the fibroblasts formed a confluent, uninterrupted sheet of elongated cells. In these late samples the underlying material surface could no longer be seen in fluorescence micrographs (Figure 6). The highest cell numbers were measured in the specimen group of plain copolymer P(ε-CL/DL-LA).

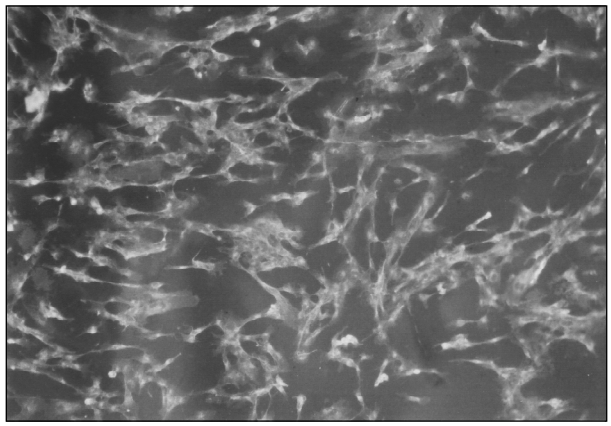


Figure 6. The TRITC-labeled human fibroblasts on the specimen of BAG60L incubated 72 hours in cell medium in body temperature.

6.4.3. Scanning electron microscopy

In SEM, the fibroblast cells appeared well attached and spread out on all the substrata. Observation with a SEM confirmed the flattened appearance and spindle-like shape of the cells. The SEM analysis revealed that the corrosion process resulted in an apatite-formation-like surface on all composite disks. No apatite formation was observed on copolymer or coverslips. In precorroded samples the cells did not appear entrapped within the hydroxyapatite deposits but grew as an organized and even layer on top of the newely formed calcium phosphate layer.

6.5. Implantation in rat subcutaneous tissue (IV)

6.5.1. Histological analysis

6.5.1.1. Inflammatory reactions

A moderately acute inflammatory reaction related to the surgical procedure was characteristic on day 3 in all specimen sections, inflammation score average being 2. On day 7 the inflammation seemed to subside and occasional mast cells were seen in the pericapsular area of all tested materials. At further time points, in the majority of sections, only minimal inflammatory cell reactions were seen, whereas the number of mast cells seemed to increase, being at its highest on day 28.

The inflammation score was smallest in the group of BAG70L throughout the experiment. Even though the changes in the inflammation reaction were noticeable and the inflammation scores diminished significantly (day 3 vs 3 months, $P<0.05$) during the period, the tendency of these changes was so similar that no significant differences were seen between the implant groups.

When evaluating the host foreign-body cell reactions to composite material, the specimens with bioactive glass appeared to have more foreign-body giant cells compared to the plain copolymer specimens. These multinucleated giant cells were seen in close proximity to the surrounding fibrous tissue capsule.

6.5.1.2. Connective tissue reactions

A connective tissue capsules surrounded the specimens at all time points. In the P(CL/DL-LA) group a clear boundary connective tissue capsule was formed and only few reactive areas where fibroblasts were able to grow in the material, were seen in the implantation sites. The capsule thickness averages around these specimens after day 3 and day 28 and 6 months were respectively, 0.04, 0.15 and 0.10 mm. These capsules appeared completely mature, thin and dense in the proceeding of the experiment. In the composite materials, i.e. in the presence of BAG, the capsules were thicker, inaccurate and clearly integrated with the implants (Figure 7). The measured capsule thicknesses after day 3, day 28 and 6 months in group BG50L+ were 0.07, 0.19 and 0.13, and in the group BG70L, 0.06, 0.20 and 0.09 mm, respectively. The changes between the implant groups were similar. Although the capsule thickness changed during the period, the increase was significant between day 3 and day 28 ($P<0.001$). After day 28 the capsule thickness decreased again.

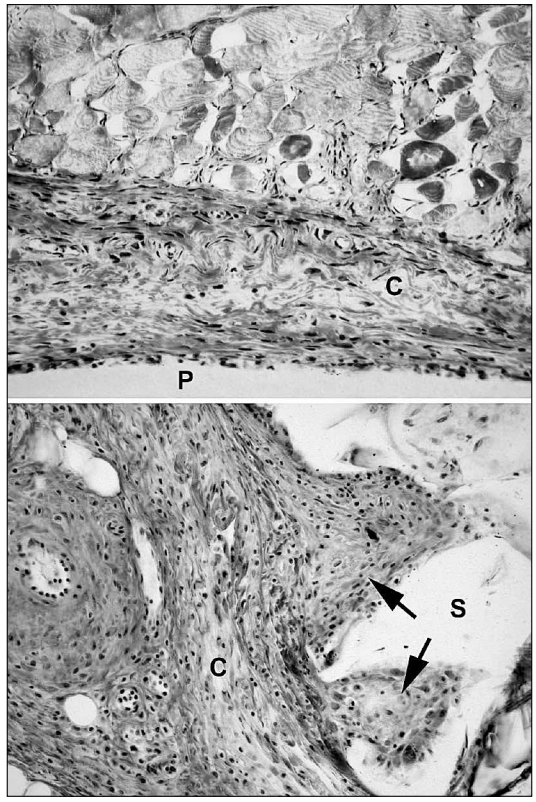


Figure 7. Upper: Typically to the plain copolymer implants, the fibroblastic cells of the formed capsule (C) are lining the outer copolymer (P(CL/DL-LA)) specimen (P) surface. Lower: In BAG containing (BAG70L) specimen (S) the cells are invasively spreading in to the implant material (→). Histological pictures taken after 7 days (Original magnifications x 10).

6.5.1.3. Material degradation and tissue ingrowth

The macroscopic changes in specimen dimensions were not recognizable when the implants with their surrounding tissues were excised. The microscopic evaluation confirmed that the copolymer was relatively slowly degrading, but the presence of bioactive glass seemed to increase the degradation rate of the polymer matrix. Degradation of the polymer, as well as the ingrowth of fibrous tissue, was clearly observed in the composite samples in the areas where bioactive glass granules were in close contact with the surrounding tissue (Figure 8). The glass dissolution process was seen to start from the granule surfaces near the soft tissue capsule when coming in contact with extracellular fluids. The reaction layer spread and proceeded in to composite materials and as a result, at 6 months, the glass granules even in the middle of the composite displayed a thick reactive surface layer typical for the bioactive glasses. Accelerated and induced by dissolving glass granules, fibroblastic cells grew into the bulk of implants.

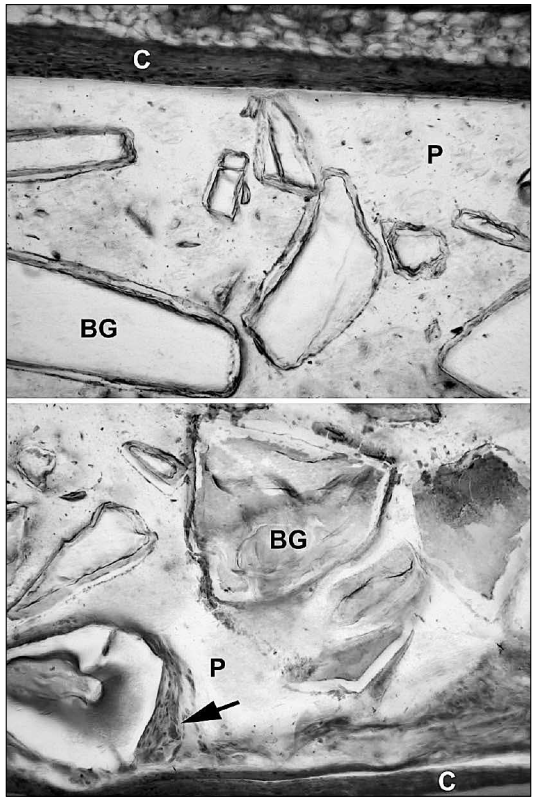


Figure 8. Histological pictures taken of the BAG70L-implants after 6 months. Upper: Bioactive glass granules (BG) are still covered by the copolymer matrix (P). Lower: Only when the bioactive glass granules are reacting with the extracellular fluid and transforming to silica-gel, the polymer degradation is further accelerated, and fibroblastic cells from the surrounding capsule (C) are able to grow into the material (→) (Original magnifications x 10).

Even though the proliferation of fibroblasts around the BAG50L+ specimen was mainly scored as highest during the study, the changes between the material groups were again similar. In all material groups, on day 3, the fibroblast proliferation was most active and differed significantly from that seen after 3 ($P<0.01$) and 6 months ($P<0.01$).

6.5.2. Histopathological analysis

In intestinal histological examination, some exceptional reactive changes were found in two rats. Both of these were in the 6 months group. One had a reactive cyst at the site of the BG50L+ implant, pneumonia, and some reactive changes in the liver. The other

animal had changes in the spleen and peritoneum, which were diagnosed as a foreign body granulomatosis. Further abdominal examination of the same animal revealed a massive suppurative inflammation in the peritoneal area, as well as scraps outside the gastrointestinal tract which were found to be connected to an intestinal obstruction. This animal suffered continuously from gastrointestinal problems, but still the possible influence of implanted specimens on the granulomatosis can not be overruled. The other two rats in this 6-month group were healthy, and no pathological cell reactions were seen. Only irritation of the nonresorbable sutures when hit in the microscopic section, was found.

The other 18 rats in the groups from day three up to six months appeared to be healthy. In their intestinal histological sections, organs seemed to be viable and they showed no pathological changes.

6.6. Implantation in rabbit femur and tibia (V)

6.6.1. Histology and histomorphometrical evaluation

6.6.1.1. Femoral implants

After the implantation period of 8 weeks no signs of polymer degradation were seen. Irrespective of the type of material, filling of the experimental bone cavities was excellent. All materials were partly surrounded by a connective tissue capsule with a mild inflammatory reaction. The thickness of the capsule ranged between 2-10 cell layers. The capsule appeared to be somewhat thicker around the copolymer without bioactive glass granules compared to the samples with glass granules. In the bone surrounding all the materials, a thin reaction layer was observed with more or less mature bone between the old bone and implanted material. New bone formation was seen evident in the samples with large glass granules that had direct glass-to-bone contact (Figure 9). Occasionally, these samples displayed bone ingrowth, but this was restricted to the superficial layers of the material.

At 16 weeks, all materials revealed a reduction in capsule thickness. Bone ingrowth was frequently seen in the BAG60L (Figure 10). Nevertheless, this was limited to the superficial layers of the composite or it took place when a void was present in the polymer matrix. Neither the plain copolymer nor the composite with small bioactive glass granules displayed bone ingrowth.

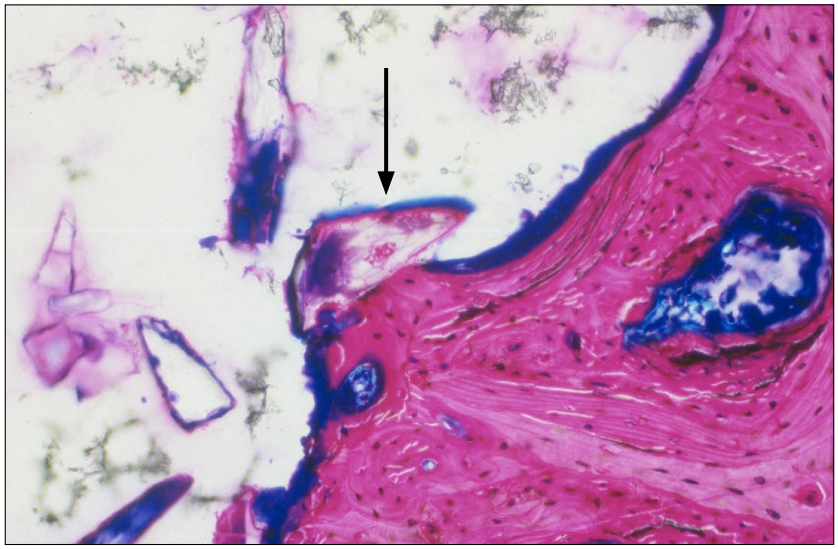


Figure 9. BAG40L 8 weeks after implantation in femur. New bone formation is clearly visible in the areas that displayed direct glass-to-bone contact (black arrow).

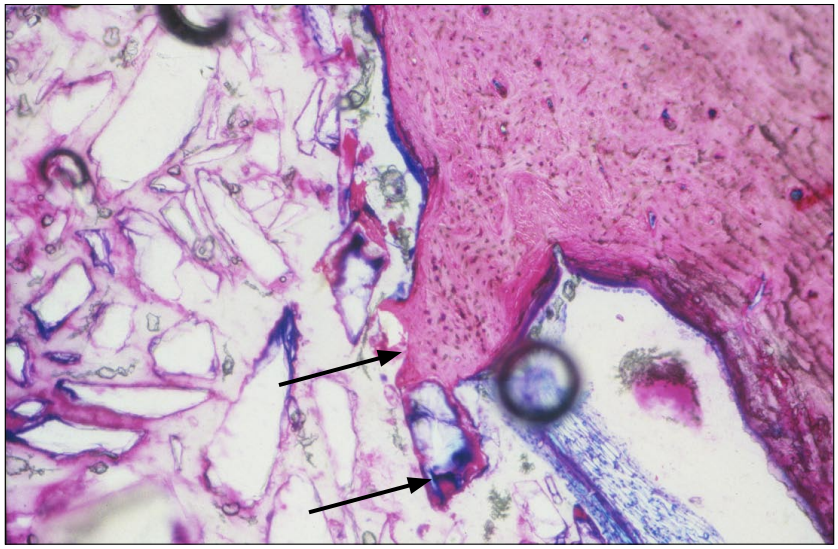


Figure 10. Bone ingrowth in the composites BAG60L 16 weeks after implantation in femur (black arrows).

6.6.1.2. Tibial implants

All implanted materials penetrated into the medullar cavity. At 8 weeks, all samples showed fibrous encapsulation, but the capsule was thinner than around the femoral implants (Figure 11). In all samples, new bone formation was found on top of the materials at the level of the cortical passage (Figure 11). On the other hand, bone overgrowth was not always complete. Bone ingrowth was minimal and mainly limited to the superficial layers of the BAG40L and BAG60L samples, which allowed direct glass-to-bone contact. All materials revealed moderate cellular reaction in the bone marrow, but all composite materials supported bone formation in the medulla.

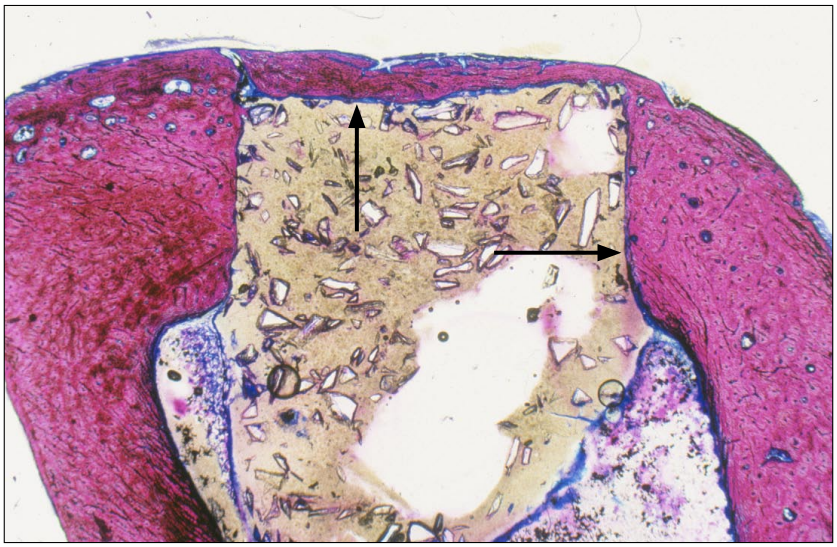


Figure 11. Fibrous encapsulation between the composite BAG40L and the surrounding bone 8 weeks after the implantation into the tibia. New bone formation can be observed on the top of the composite in the level of cortical passage (black arrows).

At 16 weeks, bone ingrowth was frequently seen in the superficial layers of the BAG40L and BAG60L composites, or it occurred when a void was present in the polymer matrix (Figure 12). The fibrous capsule became thinner with time, and in most of the composite samples it had even disappeared completely in the transcortical area. Further, in most specimens, bone deposition was observed in the medullar cavity and even around the separate composite particles that had no contact with the surrounding bone structure. Only minimal signs of polymer degradation were noticed.

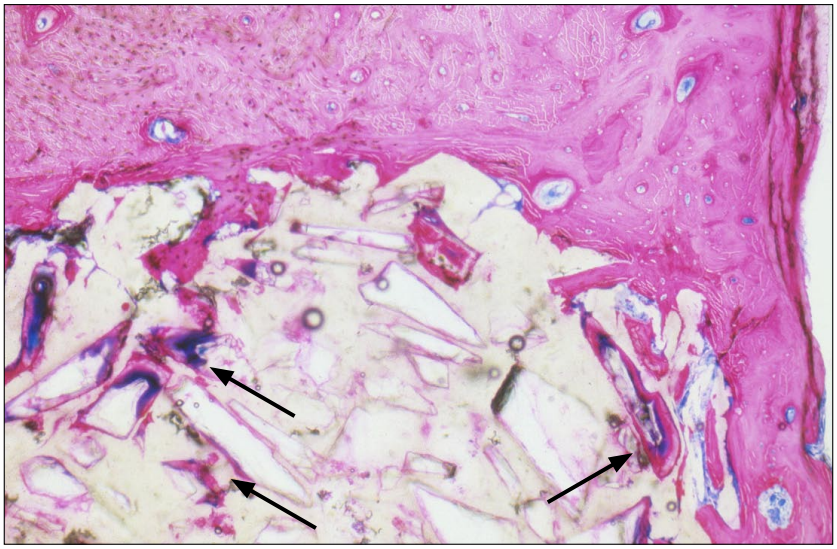


Figure 12: Ingrowth of bone in the superficial layer of the composite (BAG60L). Bone ingrowth was frequently seen when a void was present within the polymer matrix (black arrows). 16 weeks after implantation in tibia.

6.6.2. Histomorphometry

Table 3 shows the results of histomorphometrical analysis of the femoral implants. The area of direct bone contact was larger in composites with small glass granules (BAG60S) than in any other material after 8 weeks of implantation. The amount of direct bone contact decreased significantly in all composite materials from week 8 to 16 ($p<0.05$). The differences among the four materials were not statistically significant.

Table 3. Percentage of direct bone contact in the trabecular bone of rabbit femur evaluated by histomorphometry.

Sample	8 weeks	16 weeks
BAG60S	76±9	31±21
BAG40L	64±29	50±25
BAG60L	57±14	45±30
Copolymer	59±23	49±43

The results of histomorphometrical analysis in tibia are shown in Table 4. At 8 weeks, the amount of bone contact for all materials was about the same as for the 8-week femoral implants. Further, no changes were observed in the amount of direct bone contact in the transcortical area between 8 and 16 weeks. On the other hand, a tendency towards increased bone formation in the medullar cavity with time was noticed in all composite materials.

Table 4. Percentage of direct bone contact in the cortical bone and percentage of new bone formation in the medullary cavity of the rabbit tibia evaluated by histomorphometry.

Direct bone contact in cortical bone		
Sample	8 weeks	16 weeks
BAG60S	74±16	77±9
BAG40L	58±25	64±14
BAG60L	59±2	51±23
Copolymer	44±30	67±41
New bone formation in medullary cavity		
Sample	8 weeks	16 weeks
BAG60S	10±6	16±13
BAG40L	6±4	12±11
BAG60L	4±4	18±16
Copolymer	10±3	5±4

7. DISCUSSION

Autogenous bone grafts are preferential and thus the most commonly used treatment method in bone repair. However, in surgical disciplines where large defects of bone tissue have to be repaired, augmented or built up, tissue supplements are essential.

The aims of the studies were to evaluate the basic biological behaviour of the thermoplastic composite of P(ε-CL/DL-LA) (95.0/5.0 wt%) and bioactive glass S53P4 in simulated body conditions, in a cell culture environment, and in living tissue. Using this composite as a biodegradable and injectable material, it has good potential to solve problems associated with donor site morbidity, or the need for additional operation associated with graft harvesting. The capacity for osteoconductivity and of offering mechanical support during the healing process were also kept in mind when evaluating the bioactivity and biological response of the composite.

In vitro bioactivity and degradation

The hydrolytic degradation of the copolymer of poly(ε-caprolactone-co-DL-lactide) was relatively slow as has also been shown in other investigations with similar copolymers (Woodward *et al.* 1985, Ekholm *et al.* 1999). However, the presence of BAG granules increases the degradation rate, as indicated by the increased water absorption at two months (Rich *et al.* 2002). The increase in water absorption is associated with a boost in the dissolution of Si ions from the embedded glass granules observed in study I. In the composite specimens, the outermost granule surfaces first make contact with the immersion fluid. When exposed to fluid, the bulk of the glass granules will dissolve further, and reactivity will proceed deeper into the composite material via the granules embedded in the copolymer. This may explain the stable phase seen in Si dissolution curves between days 30 and 60, as well as the increase in Si concentration noticed after two months. The higher ion release is evident with larger volumes, and another explanation might be that with larger glass granules when positioned in wide contact with the material surface, the Si supplies may be released more rapidly.

In *in vitro* analysis (dissolution test, SEM and EDXA), it was obvious that dense Ca-P precipitation was formed on the material surface during the first half of the experiment, which impaired any further precipitation. The Ca concentration was increased after two months in the samples with higher contents of larger BAG granules. This may be due to increasing water absorption as described earlier. However, the Ca-P depositions might also have dissolved to some extent during days 60 to 90 due to the changes in ion saturation. At the beginning of this period, the immersion fluid was completely renewed and total fluid volume was changed.

Biocompatibility (fibroblast proliferation and soft tissue reactions)

Besides the foreign-body cells (such as macrophages and giant cells), eosinophilic leucocytes have been found in the implantation sites of ε-caprolactone-lactide copolymer samples also in other studies (Pitt 1990, Ekholm *et al.* 1999, Nakamura *et al.* 1992). The increase in eosinophilic leucocytes is related to many allergic and hypersensitive reactions in humans. Better vascularization may be one reason for more pronounced cell reactions in soft tissue, like muscle, when compared to bone. Severe inflammation elicited by this material (copolymer of ε-caprolactone and lactide) has been discussed, in part, to be the result of high concentrations of degradation products and the poor transport potential of muscle tissue. The giant cells' phagocytosing of a piece of test material has been reported by several investigators (Dunnen *et al.* 1993 a and b, Nakamura *et al.* 1992, Ekholm *et al.* 2006b). Ali and coworkers (1994) made an *in vivo* chamber test with rats and demonstrated that the phagocytic inflammatorial cells are involved in the biodegradation of the implanted polymers by releasing biologically active free radicals into the area surrounding the implant. Their results suggested that the hydroxyl radicals produced by inflammatory cells are likely to be one of the main causes of hydrolytic degradation of PCL and P(DL-LA).

Bone response

Ekholm and coworkers (1999) studied the cell reactions to copolymer of (ε-CL/DL-LA) (40:60 w/w) in bone and muscle. They concluded that the inflammatory reaction in bone was less pronounced compared to reactions in muscle. During the total one-year follow-up period, they noticed foreign-body reactions with macrophages and giant cells in all test samples. In a few samples placed in bone the connective tissue capsule was found to surround the empty material spaces.

In study II, the water absorption of the composites increased clearly after two months (Rich *et al.* 2002), which obviously boosted the dissolution of cationic ions from the embedded glass granules. This most likely increases the pH of the surrounding bone, and can temporarily hamper the bone healing process. On the contrary, in the medullar cavity, the amount of new bone seemed to increase around the composite materials after 8 weeks. In the medullar cavity, fluid flow can eliminate the degradation products and maintain neutral pH, whereas in the trabecular bone, fluid flow is limited.

Slow degradation of the copolymer of poly(ε-caprolactone-co-DL-lactide) has previously been found in muscle and bone tissues in rats (Ekholm *et al.* 1999). The presence of bioactive granules has been found to increase the polymer degradation rate in vitro (Rich *et al.* 2002). However, no visible signs of polymer degradation were noticed in histological slides after 16 weeks, although it is known that the molecular weight of composites had markedly decreased by that time. Signs of polymer degradation become visible only after the resorption of degradation products begins. No clear signs of resorption were

noticed in any of the samples during our 16-week study period (Närhi *et al.* 2003). In some applications, slow degradation of a material can be favourable as it prevents the degradation products from accumulating in the surrounding tissues (Ali *et al.* 1993). On the other hand, a material that intended to initiate bone formation in trabecular bone should preferably degrade and be replaced with (pre)osseous tissue within six weeks (Urist 1965). Longer degradation prevents the spontaneous healing process.

Bioactive glasses can induce bone formation provided the glass surface is in direct contact with the surrounding bone (Beckham *et al.* 1971). In study V, during the material application, the polymer matrix frequently came in contact with the bone surface and prevented direct contact between glass granules and bone. Small glass granules even became totally embedded within the polymer, whereas only large granules occasionally formed close contact with the bone surface. This may explain why bone ingrowth was mainly seen in the superficial layers of the composites with a high concentration of large glass granules.

Under favourable conditions, implant materials do not get encapsulated or else the capsule disappears quickly. In the case of biodegradable materials, the degradation products may accumulate in the surrounding tissues that can initiate fibrous capsule formation (Taylor *et al.* 1994). In study IV, all the implanted materials revealed fibrous capsules after eight weeks. Although the capsules became thinner with time, they remained relatively thick around the copolymer samples without glass granules. The bone bonding ability of bioactive glasses is based on the release of Si and Ca -ions that initiates an apatite formation on the glass surface (Clark *et al.* 1976; Andersson *et al.* 1990). Dissolution appears to be fastest from the composites with BAG60S glass granules (<45 μm). As indicated above and before the dissolution of cationic ions increases the pH in the surrounding environment, whereas degradation of polymer matrix decreases it (Qiu 2000). The occurrence of such differences in pH around our implanted materials may explain the differences in the capsule thickness around the composites and copolymer samples without bioactive glass.

Clinical considerations

As a result of the filler particles, the mechanical properties of the composite materials are changed when compared to plain polymers or copolymers. Impact and tensile strengths are usually decreased, while hardness and stiffness are increased.

BAG does not chemically interact with the P(ε-CL/DL-LA) copolymer matrix. The stiffening effect can be observed in the storage modulus values, but the glass transition is not affected. Strong interactions between the filler and matrix would affect the glass transition temperature. The stiffening effect of the filler in modulus values is more noticeable in the smaller granule range (Rich *et al.* 2002). The damping peak decreases with increasing glass content. The lowering of the damping energy suggests a restraining

effect of the filler on the polymer segment mobility, demonstrating the increasing trend in composite rigidity. Despite the stiffening effect of the BAG, the melting temperature of the matrix is of prime importance in determining the applicability of the material.

The handling properties of the composite are good, and the defect filling ability and thermoplasticity are excellent. However, considering the practical use for surgical purposes, the composite is not as good as intended. An interconnective porous structure is needed for bone ingrowth, and the pores must be large enough, i.e. 100-500 μm (Karageorgiou and Kaplan 2005). Surface erosion might be another promising approach. No matter what the degradation procedure is, the degradation rate should support the bone healing process and not prevent it.

8. SUMMARY AND CONCLUSIONS

The series of studies aimed to evaluate the biocompatibility and suitability of the thermoplastic biodegradable composite of poly(ε-caprolactone-co-DL-lactide) and BAG (S53P4) as a filling material in bone defects. The results of this study confirmed the working hypothesis: BAG is a suitable bioactive component in degradable composite material. The findings of the study demonstrate that the composite of poly(ε-caprolactone-co-DL-lactide) and BAG (S53P4) forms an apatite-like surface layer after a few days of immersion in SBF. The higher the glass content and the glass surface/volume ratio in the matrix the faster is the Ca-P formation. The ester pendent chains offer, via hydrolysis, reactive groups that are potential nucleation sites. Chemical changes in composite and polymer surfaces and the formation of -COOH were observed in IR. However, on the plain copolymer surface, the precipitation was not formed. The capability of BAG to form a confluent Ca-P on the copolymer surface and precipitation plays an important role in the final tissue behaviour of the material. The Ca-P formation of the composite can be adjusted by changing the size range and amount of BAG granules within the composite. A homogenous distribution of the BAG granules in the composite and the desired loading of the bioactive glass can be achieved for the composites with the processing techniques used in this study. The thermal properties of the polymer matrix enable the in situ application of the material. In in vitro proliferation of the human fibroblasts on the composites the highest fibroblast numbers were measured on plain copolymer P(ε-CL/DL-LA). This might indicate that the high bioactivity and alkali dissolution products of the glass granules restrict cell proliferation to some extent, even though the highest ion leach occurs during the preincubation. In in vivo studies made on the subcutaneous space of rat and in femoral and tibial experimental bone defects of rabbit; no adverse tissue reactions were seen in most tested specimens during the test periods. During surgery, the handling properties of the composite appeared to be excellent. The material was well injectable and moldable. All experimental bone defects were complitely filled with the material and the composites remained in place during the whole follow-up period. However, the percentage of direct bone contact decreased significantly after eight weeks in all composite materials. Based on this study, it may be concluded that although the composite is compatible and initiated new bone formation on its superficial layers, as such it is not yet an optimal bone filler material. Glass granules can only conduct bone growth efficiently provided that a direct glass-to-bone contact can be achieved. The biocompatibility of the composite must be further improved by enhancing the contact of glass granules with the surrounding bone by creating interconnected porous structures within the composite or accelerating the degradation rate of the copolymer itself.

Within the limitations of this study the following conclusions can be drawn:

1. The composite of poly(ε-caprolactone-co-DL-lactide) and BAG (S53P4) forms an apatite-like surface layer in SBF. BAG has the ability to form a confluent layer of Ca-P on the copolymer surface that can be adjusted by changing the size range and amount of BAG granules within the composite.

2. The presence of BAG affects the rate of polymer degradation. The greater the amount of and the smaller the granule size range of BAG present, the more rapid is the deterioration in molecular weight of the composite specimen.

3. Human fibroblasts attach and proliferate exponentially on the composite of poly(ε-caprolactone-co-DL-lactide) and BAG (S53P4).

4. Soft tissue reactions of the copolymer of P(ε-CL/DL-LA) are more pronounced in the presence of BAG granules. In healing and degradation processes in the subcutaneous environment of rat, a more significant factor than the glass content was the course of time.

5. In experimental bone defects no visible signs of polymer degradation were noticed during 16 weeks' implantation. Bone ingrowth was restricted and seen only in superficial layers; thus slow degradation of the polymer matrix and lack of interconnective porosity prevented complete ossification of the defect.

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