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DEVELOPMENT OF CRANIOFACIAL BONE DEFECT RECONSTRUCTION IMPLANT BASED ON FIBRE-REINFORCED COMPOSITE WITH PHOTOPOLYMERISABLE RESIN SYSTEMS

Experimental studies *in vitro* and *in vivo*

by

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Kerran aloitettuasi uneksimisen

älä hetkeksikään lopeta.

Uneksi vain mahdottomia, sillä huomista eivät

järkevät latteudet kiinnosta.

Ole hyvä unelmiasi kohtaan,

ja anna niiden toteutua.

Älä hämmästele ihmeitä, iloitse niistä.

-Tommy Tabermann-

Artwork of cover : *Sari Tuusa, Petra Saraste-Savimäki, Tero Kylä-Junnila, Jouko Sandholm.*

ABSTRACT

Sari Tuusa: Development of Craniofacial Bone Defect Reconstruction Implant Based on Fibre-Reinforced Composite with Photopolymerisable Resin Systems. Experimental studies *in vitro* and *in vivo*. Department of Prosthetic Dentistry and Biomaterials Science, Institute of Dentistry, University of Turku. *Annales Universitatis Turkuensis*, Turku, Finland, 2007.

Reconstruction of defects in the craniomaxillofacial (CMF) area has mainly been based on bone grafts or metallic fixing plates and screws. Particularly in the case of large calvarial and/or craniofacial defects caused by trauma, tumours or congenital malformations, there is a need for reliable reconstruction biomaterials, because bone grafts or metallic fixing systems do not completely fulfill the criteria for the best possible reconstruction methods in these complicated cases. In this series of studies, the usability of fibre-reinforced composite (FRC) was studied as a biostable, non-metallic alternative material for reconstructing artificially created bone defects in frontal and calvarial areas of rabbits.

The experimental part of this work describes the different stages of the product development process from the first *in vitro* tests with resin-impregnated fibre-reinforced composites to the *in vivo* animal studies, in which this FRC was tested as an implant material for reconstructing different size bone defects in rabbit frontal and calvarial areas. In the first *in vitro* study, the FRC was polymerised in contact with bone or blood in the laboratory. The polymerised FRC samples were then incubated in water, which was analysed for residual monomer content by using high performance liquid chromatography (HPLC). It was found that this *in vitro* polymerisation in contact with bone and blood did not markedly increase the residual monomer leaching from the FRC. In the second *in vitro* study, different adhesive systems were tested in fixing the implant to bone surface. This was done to find an alternative implant fixing system to screws and pins. On the basis of this study, it was found that the surface of the calvarial bone needed both mechanical and chemical treatments before the resin-impregnated FRC could be properly fixed onto it. In three animal studies performed with rabbit frontal bone defects and critical size calvarial bone defect models, biological responses to the FRC implants were evaluated. On the basis of these evaluations, it can be concluded that the FRC, based on E-glass (electrical glass) fibres forming a porous fibre veil enables the ingrowth of connective tissues to the inner structures of the material, as well as the bone formation and mineralization inside the fibre veil. Bone formation could be enhanced by using bioactive glass granules fixed to the FRC implants. FRC-implanted bone defects healed partly; no total healing of defects was achieved. Biological responses during the follow-up time, at a maximum of 12 weeks, to resin-impregnated composite implant seemed to depend on the polymerization time of the resin matrix of the FRC. Both of the studied resin systems used in the FRC were photopolymerised and the heat-induced postpolymerisation was used additionally.

Key Words: fibre-reinforced composite, resin, residual monomers, bone defect.

TIIVISTELMÄ

Sari Tuusa: Valokovetteiseen kuitukomposiittiin perustuvan implantin kehittäminen kallon ja kasvojen alueen luupuutosten korjaamiseen. Kokeellisia *in vitro*- ja *in vivo* –tutkimuksia. Hammasprotetiikan ja biomateriaalitieteen oppiaine, Hammaslääketieteen laitos, Turun yliopisto. *Annales Universitatis Turkuensis,* Turku, Finland, 2007.

Kallon ja kasvojen alueen luupuutosten korjaus on perustunut tähän mennessä pääasiassa luusiirteiden sekä metallisten fiksaatiolevyjen ja ruuvien käyttöön. Erityisesti syöpäkasvainten, synnynnäisten epämuodostumien tai tapaturmien aiheuttamien laaja-alaisten luupuutosten korjaamisessa kallon ja/tai kasvojen alueella tarvitaan luotettavia biomateriaaleja, koska luusiirteillä tai metalleihin perustuvilla menetelmillä ei pystytä vauriokohtaa parhaalla mahdollisella tavalla korjaamaan. Tässä väitöskirjassa tutkittiin kuituvahvisteisen komposiittimateriaalin (FRC) käyttökelpoisuutta biostabiilina, metalleja korvaavana vaihtoehtoisena materiaalina luupuutosten korjaamisessa käyttäen mallina kanin otsan ja kallon luihin tehtyjä defektejä.

Väitöskirjan kokeellisessa osassa kuvataan implantin tuotekehityksen vaiheita alkaen ensimmäisistä *in vitro* -testeistä resiini-impregnoiduilla kuitukomposiiteilla, ja päätyen *in vivo* -eläinkokeisiin, joissa kuituvahvisteista komposittia testattiin implanttimateriaalina erikokoisten luupuutosten korjauksessa kanin otsan ja kallon alueella. Ensimmäisessä *in vitro*-tutkimuksessa FRC-materiaali valokovetettiin laboratorio-olosuhteissa luu- ja verikontaktissa, ja kovetetut näytekappaleet inkuboitiin vedessä, jonka jäännösmonomeeripitoisuudet analysoitiin nestekromatografia (HPLC)-menetelmällä. Tutkimuksen mukaan luu- ja verikontaktissa polymerointi ei lisännyt merkittävästi jäännösmonomeerien vapautumista FRC-materiaalista. Toisessa *in vitro*-tutkimuksessa testattiin erilaisia adheesiomenetelmiä implantin luuhun kiinnittämisessä. Tämän tutkimuksen tarkoituksena oli löytää vaihtoehtoja implantin kiinnittämiselle luuhun ilman ruuveja ja nastoja. Tutkimuksessa havaittiin, että kuitukomposiitin riittävä kiinnitys kallon luun pintaan adhesiiveja käyttäen vaati luun pinnan mekaanista ja kemiallista esikäsittelyä. Kolmessa kanin otsaluu- tai kriittisen koon kallodefektimalliin perustuvassa eläinkokeessa arvioitiin kuitukomposiitti-implantin aiheuttamia biologisia reaktioita. Näiden tutkimusten perusteella voitiin havaita, että sidekudokset kasvavat E-lasikuituun (E=electrical, sähkölasikuitu) perustuvan kuituhuopamateriaalin sisään. Myös uudisluun muodostuminen ja sen mineralisaatio on mahdollista huovan sisällä. Bioaktiiviset lasirakeet edesauttoivat luunmuodostusta kuitukomposiitin yhteydessä käytettynä. Täydellistä luudefektien paranemista ei eläinkokeissa saavutettu, vaan defektit paranivat vain osittain. Biologinen vaste resiini-impregnoidulle komposiitti-implantille maksimissaan 12 viikon implantoinnin jälkeen oli riippuvainen myös muovimatriisin polymerisaatioajasta. Molemmat kokeissa käytetyt resiiniseokset jälkilämpöpolymeroitiin valokovetuksen lisäksi.

Avainsanat: kuituvahvisteinen komposiitti, resiini, jäännösmonomeerit, luupuutos.

CONTENTS

ABBREVIATIONS

APS	aminopropyltriethoxysilane
BAG	bioactive glass
Bis-GMA	bisphenol-A- glycidyl-dimethacrylate
Bis-PEDMA	bisphenol-A-polyethoxy-dimethacrylate
BDDMA	1,4 –butanediol-dimethacrylate
BMPs	bone morphogenetic proteins
BRMs	bone replacement materials
CMF	craniomaxillofacial
CQ	camphorquinone
CSD	critical size defect
CT	computer tomography
DBM	demineralized bone matrix
DD1	hyperbranched methacrylate polyester, dendrimer
DC%	the degree of monomer conversion
DMAEMA	2-(N,N-dimethylamino) ethyl-methacrylate
DRIFT	diffuse reflectance infrared Fourier transform
3D	three-dimensional
EDS	energy dispersive spectroscopy
EGDMA	ethyleneglycol-dimethacrylate
FRC	fibre-reinforced composite
FTIR	Fourier transform infrared spectrometry
GC	gas chromatography
GMP	good manufacturing practice
HA	hydroxyapatite
HBO	hyperbaric oxygen therapy
HEMA	hydroxyethyl-methacrylate
HPLC	high performance liquid chromatography
HS	headspace
HS-GC/MS	headspace-gas chromatography/mass spectrometry
ICS	isocyanatopropyltriethoxysilane
LED	light-emitting diode
MDP	methacryloyloxy-decyldihydrogen-phosphate
4-META	4-methacryloxy-ethyltrimellitate-anhydride
MPa	megapascal
MPS	methacryloyloxy-propyl-trimethoxysilane
MMA	methyl-methacrylate
NZW	New Zealand white
PAC	plasma arc
PDTE	poly (desamino ethyl-ester) -carbonate
PGA	polyglycolic acid
PLA	polylactic acid
PMMA	polymethyl-methacrylate
QTH	quartz-tungsten-halogen

RT	room temperature
S53P4	bioactive glass with a silica content of 53 weight%
SEM	scanning electron microscopy
TEGDMA	triethyleneglycol-dimethacrylate
UDMA	urethane-dimethacrylate

DEFINITIONS

Autogenic graft:	A tissue graft taken from one part of the body and placed in another site on the same individual.
Allogenic graft:	A tissue graft transplanted from one person to another of the same species, but not to an identical twin.
Alloplastic:	A synthetic material used to replace the body´s own tissues.
Anisotropic:	Mechanical properties of a material are different depending on the direction of the load.
Biocompatiblity:	The ability of a material to perform with an appropriate host response in a specific application.
Bioactivity:	Spontaneous communication of a material in a biological environment resulting in strong adhesion between tissue and the material.
Composite:	A material that consists of two or more distinct parts.
Device:	A general term for medical implant or equipment.
Implant:	Artificial substitute for body parts; material inserted into organism.
Impregnate:	To saturate material with some active element by diffusion.
IPN	Interpenetrating polymer network, a three-dimensional chemically crosslinked polymeric material that includes two independent networks without any covalent bonds between them.
Isotropic:	Mechanical properties of a material are the same irrespective of direction of the load.
Krenchel´s factor:	The reinforcing efficiency of the fibres in FRC. Estimates the strength of FRC.
Matrix:	The continuous phase of FRC, in which the reinforcement is embedded.
Osteoconductivity:	The ability of a structural scaffold (i.e. implant) to guide bone ingrowth.

Osteoinductivity:	The ability of a structural scaffold (i.e. implant) to induce bone growth in non-osseous tissues.
Photopolymerisation:	Polymerization brought about by the action of light.
Reinforcement:	The harder and stronger phase in FRC.
Resin:	Any synthetic organic polymeric material (solid or liquid) used as a plastic.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which are referred to in the text by the Roman numerals I-V.

I **Tuusa S, Puska M, Lassila L and Vallittu P.** Residual monomers released from glass-fibre-reinforced composite photopolymerised in contact with bone and blood. Journal of Materials Science: Materials in Medicine 15 (2004) 1-6.

II **Tuusa S, Lassila L, Matinlinna J, Peltola M and Vallittu P.** Initial adhesion of glass-fibre-reinforced composite to the surface of porcine calvarial bone. Journal of Biomedical Materials Research, Part B: Applied Biomaterials 75B (2) (2005) 334-342.

III **Tuusa S, Peltola M, Tirri T, Lassila L and Vallittu P.** Frontal bone defect repair with experimental glass-fibre-reinforced composite with bioactive glass granule coating. Journal of Biomedical Materials Research, Part B: Applied Biomaterials 82 (1) (2007) 149-155.

IV **Tuusa S, Peltola M, Tirri T, Puska M, Röyttä M, Aho H, Sandholm J, Lassila L and Vallittu P**. Reconstruction of critical size calvarial bone defects in rabbits with glass-fibre-reinforced composite with bioactive glass coating. Journal of Biomedical Materials Research, Part B: Applied Biomaterials, *in press*.

V **Tuusa S, Peltola M, Tirri T, Röyttä M, Aho H, Lassila L and Vallittu P.** Biological responses to two different glass-fibre-reinforced composite implants in critical size calvarial bone defects in rabbits. Submitted.

1. INTRODUCTION

The reconstruction of defects in calvarial and maxillofacial bones caused by trauma, diseases or congenital anomalies has been done with many types of materials, as well as bone grafts. The use of autogenic and allogenic bone grafts has been the most general method (Shumrick *et al*.,1994; Aho *et al*., 1998), but the risks of infections and immunological reactions, uncontrolled bone resorption and donor site morbidity (Moreira-Gonzales *et al*., 2003; Sailer *et al*., 1998; Matsuno *et al*., 2006) have led to the search for alternative methods.

Screws are used for the fixation of plates or devices onto bone or to hold bone fragments together (Prein et al., 1998). They can be divided into different groups depending on the production material (Konttinen et al., 2001). Plates and meshes are also used in craniofacial surgery, and most often they are made of titanium (Kuttenberger *et al*., 2001; Schug *et al*., 2000; Dietz *et al*., 2001; Langford *et al*., 2002, Hughes et al., 2003). In past decades, stainless steel was the metal of choice for maxillo-facial implants, but nowadays practically all cranio-maxillo-facial implants are available in titanium. Titanium is an insoluble, inert and biocompatible metal, and has high corrosion resistance due to the spontaneously forming thin oxide layers on the surface (Prein et al.,1998). Implants made of titanium, or of metals in general, are not ideal for moulding to anatomical shapes. Mechanically they are hard, durable and stand hard strain, but the flexural strength and modulus of elasticity of metals in general differ from those of bone (Nomura et al., 2005). For example, the ultimate strength of bone is approximately one-tenth that of steel (Prein *et al.,* 1998). These differences in mechanical properties between bone and metals can cause problems during the implantation, like in the cases when the bone tissue to be repaired is very thin and metal is possibly too hard and non-bendable to the anatomical form. Metallic implants can also react to temperature changes occurring outside the body, causing the patient uncomfortable sensations. In addition, the surface of a metallic implant does not always properly attach to the surface of the bone, and thus, may need bioactive ceramic coating to improve the attachment, like in dental implants (Moritz *et al*., 2003).

Ceramics, glasses (Magee Jr. et al., 2004; Cole et al., 1996; Aitasalo et al., 1997) and *synthetic polymers* (Chiarini et al., 2004; Wolff et al., 2004) have brought new ideas and solutions to defect reconstruction. Ceramics and glasses have the advantage of chemical composition near to the mineralized component of natural bone, and they can chemically bind to bone (Hench et al., 1971). For example, hydroxyapatite coatings have been found to improve bone healing in experimental animals (Soballe et al., 1999), and thus, they are also used clinically. However, they have neither flexibility nor durability of the bone, but are very brittle.

As an example of synthetic polymers, PMMA (polymethylmethacrylate) has been one of the most widely used alloplastic materials in bone defect reconstruction in head and neck surgery during the past decades. It is well tolerated by soft tissues, it has a density similar to that of bone and acceptable strength. However, the material produces a significant exothermic reaction when liquid monomer and powder polymer are combined. This rapid temperature peak can cause necrosis of the surronding bone (DiPisa et al., 1976). The release of unreacted monomer MMA can cause the same

phenomenon (Revell et al., 1998). If PMMA is used in craniofacial reconstructions, the implants can be manufactured *ex vivo*, in the laboratory, before the operation. *Biodegradable polymers*, like polylactic acid (PLA) and polyglycolic acid (PGA) have been used as rods, screws and plates to fix fractures in CMF areas, where load-bearing properties are not needed.

Experience with *fibre-reinforced composites (FRC)* in dentistry (Vallittu et al., 1992; Goldberg et al., 1992) has suggested their potential use also in surgical applications, where biostable durable materials are needed, like in large calvarial defects, that can otherwise be difficult to obliterate. In dental applications, bundles of glass fibres have been impregnated with photopolymerisable resin systems (Peutzfeldt, 1997) in order to give the material good moulding properties. This moulding property means that dental FRC could also be used as a surgical reconstructive material. Adequate adhesion of the reinforcing glass fibres to the polymer matrix is an important factor for the strength of the FRC. All the fibres should be properly embedded in the impregnating resin (Vallittu, 1996). After polymerisation with blue light, the flexural strength of the isotropic glass FRC is at the level of bone (300 MPa) (Lassila et al., 2002). However, there is a lack of scientific literature on using FRC as a surgical material. This series of studies focuses on using glass FRC in calvarial bone repair material.

2. REVIEW OF THE LITERATURE

2.1. BONE

2.1.1. Composition of bone

Bone matrix is a composite of organic and inorganic constituents. The inorganic portion comprises approximately 65 % of the matrix and consists principally of hydroxyapatite, in addition to magnesium, potassium, chlorine, iron and carbonate. Of the organic constituents, 90 % are collagens, mainly type I, and the remaining 10 % are noncollagen proteins, including osteonectin, osteocalcin, sialoproteins, phosphoproteins, glycoproteins and albumin. Mechanically, the mineral portion of bone is responsible for compressive characteristics, while organic components, primarily the collagen structures, determine the tensile behaviour (Prein et al., 1998). The outer surface of most bone is covered by the periosteum, a sheet of fibrous connective tissue, and an inner cellular layer of undifferentiated cells. The periosteum has the potential to form bone during growth and fracture healing. Cortical bone lies under the periosteum. It is a dense, solid mass with only microscopic channels, and forms the outer wall of all bones, making it largely responsible for the supportive and protective function of the skeleton. About 20 % of the bone mass is cancellous bone, a lattice of large plates and rods known as trabecula, found in the inner parts of bones. Cortical and cancellous bone differ in their development, architecture, function, proximity to the bone marrow, blood supply, rapidity of turnover time, and magnitude of age-dependent changes and fractures (Jee, 2001).

Bone tissue in the developing embryo is of the woven type. In postnatal osteogenesis that occurs when fractures heal, new bone is deposited in woven bone. It consists of a matrix of interwoven coarse collagen fibres with osteocytes distributed in more or less sporadic order. Woven bone is provisional material that is eventually resorbed and replaced by lamellar bone. Lamellar bone is built up of unit layers (lamellae). Each lamella contains fine fibres that run in approximately the same direction, but whose axes can differ by as much as 90 ° in adjacent units. In adult cortical bone, circular rings of lamellae surround a longitudinal vascular channel forming the structure called the osteon or Haversian system. Between the adjacent Haversian systems, interstitial lamellae fill the gaps. Adult cancellous bone consists of two major types: trabecular packets of hemiosteon and interstitial lamellae. In both woven and lamellar bone, osteocytes are entrapped into small cavities (lacunae), that are connected to each other by tubular canals (canaliculi), where the long cytoplasmic processes of osteocytes are situated (Jee, 2001).

2.1.2. Special characteristics of cranio-maxillofacial bones

The mechanism of bone formation can be divided into two types: membraneous bone formation, where the ossification process takes place by direct mineral deposition into the organic matrix of mesenchymal or connective tissue, and on the other hand, endochonral bone formation, where cartilage formation is the first step. This cartilage is gradually transformed; it becomes mineralized and then replaced by bone. This mechanism is the main process of formation for long bones. In the cranio-maxillofacial

area the major mechanism observed is membraneous bone formation. The frontal, parietal and nasal bone, maxilla, zygoma and the mandible are all of membraneous origin. Only in the nasal septum and internal bony components of the nose, occipital bone and cranial base is endochondral bone formation seen.

The anatomy of the cranio-maxillofacial skeleton provides protection for the central nervous system, eyes and respiratory pathways. The shell of the cranial vault consists of an outer and inner compact layer connected by a cancellous intermediate **(Figure 1)**. The hemispherical design, together with the layered structure makes the cranium specially suited to protect against direct impact. The midface is also able to act as a shock-absorbing structure (Prein et al., 1998).

Figure 1. A view of the skull vault of a mature cat which, like that of a human, is formed by intramembranous ossification. The external surface is invested by the **periosteum (Px)**, which merges with the deep layers of the overlying skin. The internal surface is also lined by **periosteum (Pi)**; this layer also constitutes the outermost membraneous covering of the brain, known as the dura mater. The inner part of the skull bone consists of cancellous bone. (Hematoxylin-eosin staining, x 20). (Adapted from "Functional histology - a text and colour atlas", p.137, Wheater et al., 1979).

2.1.3. Fractures of cranio-maxillofacial bones

Bone fractures are the result of mechanical overload. The structural integrity and the stiffness of the bone can be interrupted within milliseconds. The shape of the fracture depends upon the type of load exerted and the energy released. Torque results in spiral fractures, avulsion in transverse fractures, bending in short oblique fractures, and compression in impaction and in higher comminution. The biomechanics of fractures can be divided into two categories according to the mechanism of forces applied either direct or indirect. Direct forces result in tapping, crush, or penetrating fractures, while indirect fractures result from traction, angulation, rotation, vertical compression, axial loading, or angulation with both torsional and axial loading (Greenberg, 1993). The degree of fragmentation depends upon the energy stored prior to the process of fracturing (Prein et al., 1998), and the mechanism for the production of fractures can be described by the forces of kinetic energy, applied to a particular surface area, and then absorption of these forces by tissues of different densities or resiliencies (Greenberg, 1993). A special phenomenon is the implosion which occurs immediately after disruption. Implosion is followed by marked soft tissue damage due to cavitation. The vascular damage is extended into the neighboring soft tissue regions. Concomitant injuries may include nerve damage (mandibular, infraorbital, optical or facial nerves).

In contrast to long bones, closed muscle compartments do not pose a problem in the skull. In the midfacial and cranial region, the bony walls are thin, and they remain attached to the surrounding soft tissues. Even if the soft tissues are stripped, the connection to circulation recovers rapidly. Thus, the susceptibility to infection is minimal, and the tendency for healing is good (Prein et al., 1998).

2.1.4. Bone fracture healing in CMF area

Healing is defined as restoration of original integrity. It can be divided into inductive, inflammatory, reparative and remodelling phases, which form a self-driven sequence that leads to a healed fracture (Martin et al., 1998). Clinically, this goal is reached when the bony structures can resume their full function, even though on a microscopic level, the structure of the bone has not yet reached the appearance of an unaltered bone. Biologically, the cells that are able to participate in the various phases of the healing process must reach the site of repair, and their activities must be supported by nutritional supply (Prein et al., 1998). The inductive phase begins shortly following bone disruption and may last until the stage of inflammation is complete. During induction, viable cells in the region of disruption are induced to form new bone. These indigenous bone-associated cells are modulated to become new osteoblasts. A second source of cells includes fibroblasts, endothelial cells, muscle cells, and various primitive mesenchymal cells, which may differentiate into bone-forming cells under the appropriate stimulus, that can be associated with the depression of pH and formation of a region of relative hypoxia around the bone "wound" (Glasgold and Silver, 1991). In the inflammatory phase, the fractured bone is immobilized. This phase is characterized by vasodilation, serum exudation, and infiltration by inflammatory cells. It lasts 3-7 days, until significant bone formation has been initiated. During this time, a hematoma is formed, attendant to local disruption of the vascular supply. Macrophages appear at this time to remove non-viable tissue, and osteoclasts and osteolysis are activated (Glasgold and Silver, 1991). In the reparative phase, which lasts about one month, mesenchymal cell proliferation and differentiation are accompanied by intense vascular proliferation. The resulting osteoblasts produce woven bone at a high rate. The reparative phase is completed, when bony union has been achieved. Now the broken bone often has a greater mass than the original bone, and is therefore less mechanically efficient. In the remodelling phase, modelling and remodelling of the fracture site gradually restore the original cantour and internal structure of the bone, although radiographically, evidence of the fracture may persist for many years. As the bone re-moldels, it is able to maintain its strength with less material than in the earlier phases of healing, thereby increasing its mechanical efficiency (Martin et al., 1998). By the end of the first week after trauma, new bone formation is observed, predominantly in the subperiosteal region. There are clearly various degrees of immobilisation of a fracture. Even in the same fracture plane a certain part may present a pattern of direct healing which is characteristic for minimal strain, or healing via a cascade of tissue differentiation which is observed under interfragmentary motion. In the case of high interfragmentary motion, the strain in the fracture cap exceeds the level tolerated by bone, and ossification is not possible **(Figure 2)**. However, some motion is beneficial; applying loads across the fracture

serves to strengthen the callus, but too much weight can lead to refracture and non-union (Martin et al., 1998).

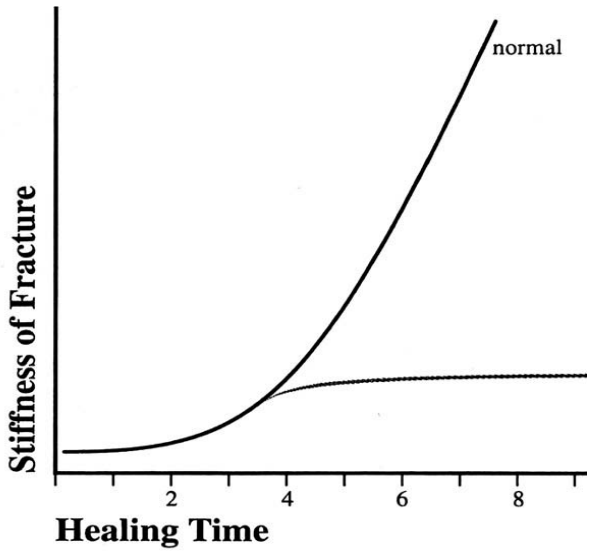


Figure 2. *Fracture healing*: recovery of mechanical function. During the fourth to sixth weeks a dramatic change in mechanical properties occurs towards normal bone. In an undisturbed situation, mineralization across the fracture plane takes place at this time. Loading of such a uniting fracture at a critical moment may disturb the mineralization process and lead to a delay in bony union, or, if compensatory healing mechanisms fail, to a non-union. *(Figure adapted from: Manual of internal fixation in the cranio-facial skeleton, p.11, Springer-Verlag, Berlin, 1998).*

Here, a tissue differentiation cascade is seen to be in progress: granulation tissue differentiates into connective tissue, fibrocartilage, mineralized cartilage, woven, and finally, into compact bone. During this differentiation cascade, strength and stiffness of these tissues gradually increase, while at the same time, the tolerance for strain is reduced. Radiologically, the progress of healing can be judged by the amount of callus formed. After internal remodelling has begun, there is a gradual reduction of radiological density in the fracture area, which is due to the internal remodelling activities. With time, the fracture site appears increasingly diffuse and gradually disappears. In midfacial and cranial regions, bony union of fragments may be observed even after one month due to the good circulatory conditions in this region and to the thin dimensions and the cancellous character of bone, allowing a more rapid recovery of the interrupted blood supply (Prein et al., 1998).

2.2. EXPERIMENTAL BONE DEFECTS AS ANIMAL MODELS FOR IMPLANTATION STUDIES

2.2.1. Calvarial bone defects

Experimental bone defects are created to assess the bone healing properties of implants and tissue-engineered constructs. The size of such inflicted defects is of particular

importance, since spontaneous healing of bone occurs in defects smaller than so-called critical size defects (CSD). A critical size defect can be defined as a defect which has less than 10 percent bony regeneration during the lifetime of the animal. Practically, if this level is not reached in one year, it is unlikely that it will ever occur. Another definition is that CSD is the smallest intraosseous wound that will not heal by bone formation during the lifetime of the animal (Hollinger et al., 1990). CSDs heal mostly by fibrous connective tissue formation, not by bone growth that creates bony union. Fragments of newly formed bone can remain isolated from each other by fibrous tissue, resulting in a physiological state known as *bony non-union* or *fibrous union* (Schmitz and Hollinger, 1986). The tissue factors are scarce in the centre of the CSD, resulting in a low incidence of differentiation. The osteoblasts fail to mineralize the matrix and are eventually replaced with fibrous connective tissue. The CSD optimizes the normal physiological response and fails to heal only because it exceeds the body´s ability to regenerate bone. Thus, CSD is filled with new bone only in the presence of bone replacement materials (BRMs) (Hollinger et al., 1990).

There are a number of variables that can influence the bony wound healing of CSDs and should be taken into account before choosing an appropriate animal model: 1) species-specific differences in bony wound healing, 2) skeletal age of the individual, 3) anatomical location of the CSD. CSD size in the rodent calvaria ranges from 5 to 8 mm in diameter, while in larger animal models the CSD size ranges from 15 mm in rabbits and non-human primates to 25 mm in cats. When an animal model for calvarial CSD study is selected, some general factors should be considered: available data concerning skeletal anatomy, bone physiology and biomechanical properties and osseous wound healing, cost and availability of the model, generalizability of the results across species and ethical and societal implications. Rodents and rabbits are the most common species used in calvarial experiments. Rabbits are more suitable than rodents for calvarial implant studies, because the anatomy of the bones and hormonal patterns in rodents differ to some degree from those of humans. In fact, non-human primates, dogs, cats, sheep, goats and swine show many physiological properties more similar to humans compared to rabbits. However, cost, availability, and genetic heterogeneity of, for example, non-human primates can be problematic. Bony defects in skeletally immature animals of most species heal at a faster rate than skeletally mature individuals, and thus, immature animals can give falsely high expectations of the osteoconductive properties of the material being studied. The craniofacial complex is much more vascular and more richly innervated than the long bones and vertebral column. Even within species, CSD size varies across these anatomical locations, and CSD sizes in the skull and mandible are relatively smaller than those produced elsewhere (Mooney and Siegel, 2000).

The method of creating an artificial, critical size calvarial defect in rodents´or rabbits´ parietal bones is usually the so-called “trephine drilling” method. In this method, a cylindrical bur is used in drilling the defect at low speed, under saline irrigation. Trephine calvaria CSDs have been shown to be both more predictable in size and more reproducible than CSDs in long bones (Schmitz et al., 1990). Recently, rabbit calvarial CSD has been used as a study model in the implantation study of Clokie et al. (2002). In that study, demineralised bone matrix putty was found to be superior compared to commercially available bone substitutes based on calcium bone cements.

The same Canadian research group has also used the rabbit CSD model in studies concerning the effect of hyperbaric oxygen therapy (HBO) on the spontaneous healing of CSD, and supra-critical-sized, 18 mm defects, and found HBO to be effective in enhancing the bony healing (Jan et al., 2006). One of their studies dealt with the comparison of demineralised bone matrix putty combined with a resorbable plating system with the calcium phosphate cement combined with native partially purified bone morphogenetic protein (BMP) (Haddad et al., 2006). Both the combinations were found to effectively enhance the CSD healing.

2.2.2. Frontal bone defects

In the development of implant materials for bone defect reconstruction, one experimental design is implantation of artificially made defects into rabbit´s frontal bone. In this model, round defects, 5 mm in diameter, are drilled into the frontal bone of an adult rabbit. Drilling this size of defect into frontal bone is not such an invasive technique as creating a CSD in the rabbit´s calvarium. Calvarial defects as a study model is a more challenging technique, not only because of the larger size of the defect created, but also because of the close proximity of the brain underlying the defect. When new, experimental implant materials are developed, it is practical to obtain information about the tissue reactions to the material using the frontal defect model, because it offers the possibility to test the first reactions of the material to bone and underlying as well as overlying soft tissues without contact to the central nervous system. After determining these reactions, the more invasive and challenging technique of calvarial implantation is reasonable in craniofacial implant development. The frontal bone defect reconstruction has been the technique of choice in the studies of Peltola (2001), in which frontal sinuses were obliterated with bioactive glass and hydroxyapatite.

2.3. AN OVERVIEW OF CLINICALLY USED MATERIALS IN CRANIOFACIAL RECONSTRUCTIONS

2.3.1. Bone grafts

The natural source of bone for reconstruction bone defects is autograft, i.e. bone taken from one place and inserted into another within the same individual. Inserting autologous bone into a defect site does not cause immunogenic problems, like rejection of the graft. Autologous bone can be used as *free bone grafts*, i.e. bone grafts that are harvested typically from iliac crest, either as particulate cancellous marrow to be used as a filler substance in small defects, or larger bone blocks which can be used as a graft material in the reconstruction of mandibular segmental defects (Glasgold and Silver, 1991). The cancellous nature of the graft allows the rapid ingrowth of blood vessels and soft tissue, ensuring adequate integration with the osseous tissue at the recipient site. Free bone grafts can be harvested also from ribs (Leake, 1976) or calvarium (Powell and Riley, 1987). The disadvantage of using free bone grafts is either partial or complete resoption of the grafts. The reason for resorption is the death of transferred bone cells prior to adequate vascular ingrowth. A viable alternative to free bone grafts is a *microvascular bone graft*. In this graft type, obtained by microvascular surgery, the graft is harvested with its arterial and venous supply, and the vessels are anastomosed

to vessels at the recipient site. Because the graft has its own blood supply, resorption is minimal, and it can be placed into a radiated field with a high degree of success (Glasgold and Silver, 1991). The anastomosis is performed with the aid of the operating microscope and very fine microinstrumentation. Proper training and constant practice are required to assure correct arterial and venous anastomosis.

The disadvantage with the use of autologous bone grafts is the donor site possibly suffering from morbidity. In addition, autograft bone is not the method of choice in the case of very large defects. Allograft bone from a tissue bank is relevant when more bone is needed than can be dissected from the patient´s own sources. Allograft bone can be found in many configurations: cortical chips, cancellous cubes, cortical struts and even powder (Stevenson, 1999). Allograft bone is principally osteoconductive, although it may have some osteoinductive capability, depending on how it is processed. In processed allograft, living cells are not present, and thus, it cannot elicit an osteogenic response (Skowronski and An, 2003). Bone processing by chemical treatment, antibiotic wash or gamma irradiation reduces the risks of microbial transmittance with the graft to the recipient, which is always a risk related to allograft bone. If massive cortical allografts are used, they can be only slowly and superficially penetrated by blood vessels in defect sites, fracturing is possible, and they can remain mechanically weaker than comparable autografts, and have more unremodelled necrotic bone (Stevenson, 1999). Immunogenicity of allograft bone can also cause undesired reactions, leading to graft rejection. This immunogenicity can be markedly reduced by thorough removal of cells and by freeze-drying the bone (Bauer et al., 2000 and Oka et al., 2001). Haimi et al. (2007) have recently studied the effect of chemical cleansing procedures combined with peracetic acid-ethanol sterilization (PES) on the biomechanical properties of cortical allograft bone. They found that the different processing methods combined with PES had only slight effects on the biomechanical properties of cortical bone samples freeze-dried to <5% of residual moisture. Their results also suggest that it is advisable not to freeze-dry processed bone grafts too dry, under 1% residual moisture. The biomechanical weakening of allograft bone may lead to unsuccessful healing in situations such as fracture repair. Administration of immunosuppressive agents to the graft recipient has been shown to improve graft performance in an animal model (Stevenson et al., 1991; Welter et al., 1990). However, immunosuppression is not indicated or required with clinical treatment. In clinical cases, patients can show chronic inflammatory cells at the implantation site (Bauer et al., 2000). Xenografts, derived from animal sources, would be available in virtually unlimited supply, but the extreme histocompatibility mismatch between human recipients and xenograft bone materials severely limits the use of this material in clinical practice (De Vito Dabbs et al., 2000).

2.3.2 Demineralized bone matrix

Demineralized bone matrix (DBM) can be produced by extracting the mineral phase of bone with 0.5 or 0.6 N HCl, leaving the organic matrix intact (Hollinger et al, 1991 and Pietrzak et al, 2003). The extraction converts mineralized allograft bone from an osteoconductive scaffold to one that exhibits significant osteoinductive behaviour. Typical DBM matrix formulations are essentially devoid of calcium (Zhang et al., 1997). The demineralizing acid treatment inactivates possible viral contaminants and

effectively removes blood elements from the matrix and thus, risks of disease transfer and immunogenic reactions can be even fewer than those of undemineralized human bone (Skowronski and An, 2003). The factors responsible for the osteoinductivity of DBM have been discovered to be bone morphogenetic proteins (BMPs), which are associated with the organic matrix of bone (Cheng et al., 2003).

2.3.3. Ceramics

Calcium phosphate and calcium sulfate ceramics represent a group of materials with different handling, morphological, mechanical and degradation properties (Le Geros, 1993; Tay et al., 1999). Biologically, these ceramic compounds are meant to substitute for the mineral phase of bone, and are purely osteoconductive by nature. They can also be combined with autogenous bone marrow to provide osteogenic potential. Clinically, use of these materials is limited to the filling of bony defects in which the surrounding host environment is conductive to bony healing (Tay et al., 1999).

Calcium phosphate is a generic term for a multitude of compositions. Of them, the calcium phosphates of greatest interest are those with a calcium-to-phosphate ratio close to 1,67, which is the stoichiometric ratio of hydroxyapatite (HA), the primary mineral component of bone. Surgical implants composed of hydroxyapatite have been available for some years. HA may be created in either ceramic or nonceramic forms. The preformed implants are typically of the ceramic variety; formed by heating a calcium phosphate preparation at a high temperature (Baker et al, 2002). Porosity of the implants is limited, and can be increased by mixing soluble particles with the calcium phosphate powders during production (Jarcho, 1981). Non-ceramic forms of HA can be molded during the operation to the needed shape. These formulations of HA differ from the ceramic type in that they undergo crystallization when mixed with a phosphate solution. Resulting HAs are not heated at high temperatures. Most of the available products are different formulations of calcium phosphate, which are mixed with either saline or dilute phosphate solution. Norian^{TM} (Synthes), however, is a carbonated calcium phosphate paste (Baker et al, 2002). HA implants have proved somewhat resistant to infection, as documented by numerous clinical studies (Baker et al., 2002; Byrd et al., 1993; Johnson et al., 2000; Rosen, 1991). One common clinical indication for calcium phosphate bone cements has been in the reconstruction of cranial defects. Another indication is to facilitate the process of guided bone regeneration in cranial reconstruction (using a membrane that prevents soft tissue prolapse into the defect site) (Eppley et al., 2002; Stal et al., 2001). HA cements have also been used for obliteration of the frontal sinus, even though there seems to be quite a high risk of contamination of the material by communication with the ethmoids and nasal cavity below (Matic et al, 2002).

2.3.4. Glasses

The history of bioactive glass, BAG, goes back to the early 1970s. Hench et al. (1971) discovered that bone can bind chemically to certain compositions of glass, which is surface-reactive in aqueous environments. The surface forms a biologically active carbonated HA layer that provides the bonding interface with tissues. Bonding to bone was first demonstrated for a compositional range of bioactive glasses that contained SiO_2 , Na_2O, CaO and P_2O_5 in specific proportions (Hench et al., 1972). There are three

key compositional features of these bioactive glasses that distinguish them from traditional soda-lime-silica glasses: 1) less than 60 mol% SiO_2 , 2) high Na_2O and CaO content, 3) high CaO/P_2O_5 ratio. These features make the surface highly reactive when it is exposed to an aqueous medium. Many bioactive silica glasses are based upon the formula called 45S5, signifying 45 wt.% SiO_2 (S=the network former) and 5 : 1 ratio of CaO to P_2O_5. Glasses with lower ratios of CaO to P_2O_5 do not bond to bone (Hench, 1996). The compositional dependence of bone and soft tissue bonding on the Na_2O-CaO-P_2O_5-SiO_2 glasses is illustrated in **Figure 3**.

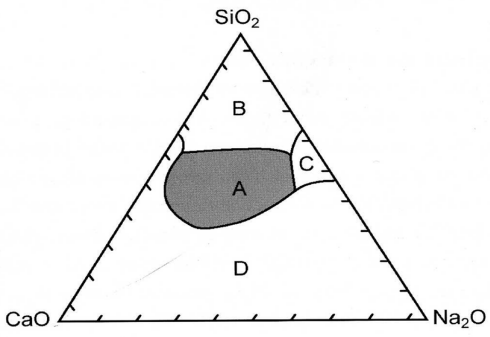


Figure 3. Compositions in the region A form a bond with bone (bioactive bone-bonding boundary). Silicate glasses within region B (window or bottle glass, microscope slides) behave as inert materials causing fibrous capsule formation at the implant-tissue interface. Glasses within region C are resorbable and disappear within 10 to 30 days of implantation. Glasses within region D are not technically practical and therefore have not been tested as implants. All compositions in this diagram have a constant 6 wt% of P_2O_5 (Hench and Wilson, 1984).

When bioactive glass bonds to bone, a series of complex reactions occurs: alkali ions (Ca^{2+}, Na^+, K^+) and PO_4^{3-} dissolve from the surface of the glass and are exchanged with hydrogen or hydronium ions (H^+, H_3O^+) from the body fluid. The silica network of the glass starts to dissolve along with the attack of hydroxyl ions. Soluble silica is lost from the glass to the solution, and Si-OH or $Si(OH)_4$ groups are formed on the surface of the glass. Later, a SiO_2 layer is formed on the surface of the glass. The Ca^{2+} and PO_4^{3-} groups migrate through the SiO_2 –rich layer and an amorphous CaO-P_2O_5 –rich film forms on top of the SiO_2 layer (calcium phosphate precipitation stage). The SiO_2 layer continues to grow. Later on, the amorphous CaO-P_2O_5 –rich film is crystallized to apatite. Organic components are incorporated, and bone minerals are able to bond to BAG (Rawlings, 1993).

Bioactive glasses have been manufactured in different shapes and forms: solid plates for reconstructing orbital floor fractures (Aitasalo et al., 2001; Kinnunen et al., 2000), microspheres for frontal sinus obliteration (Aitasalo et al., 1997, 2000, Peltola et al., 2006), maxillary sinus augmentation (Tadjoedin et al., 2002), for contour restoration of the facial skeleton (Duskova et al., 2002), or reconstruction of orthopaedical defects (Heikkilä et al., 1995). Bioactive glass has also been used as an adjunct to conventional surgery for the treatment of osseous periodontal defects (Lovelace et al., 1998). Thus, BAG is already in clinical use. In addition, glass microspheres have earlier been used as a constituent in experimental FRC implants

(Aho et al., 2004), and found to have evoked direct contact with bone in the PMMA/FRC application used. The safety and efficay of BAG for bone replacement in CMF reconstruction have been reviewed by Gosain (2004).

Glass-ionomer cements

Glass-ionomer cements (polyalkenoates) have been the materials with contradictions in clinical bone defect repair. Initially, they were developed for dental restorations, as a material having the ability to undergo specific adhesion with hydroxyapatite. The key properties, fluoride release over a prolonged period and specific adhesion to enamel and dentine, are related to their characteristics as aqueous polyelectrolyte systems (Smith, 1998). Glass-ionomer cements have been successful in repairing bony defects in the skull base and in craniofacial surgery (Cole et al., 1996; Baier et al., 1998), but have also caused serious complications, like encephalopathy with seizures caused by aluminium (Hantson et al., 1994). Because of the risks related to glass-ionomer cements, Lubben et al. (2001) performed cytotoxicity tests for glass-ionomer cements using the fibroblast cell culture system. In that study, cytotoxic effects were observed when ionomeric cement was not carefully protected from fluid contact for the first two hours after mixing. The cytotoxic effect was due to elution of large amounts of cement-constituting fluoride and aluminium ions. As a conclusion, ionomeric cement should be kept dry and protected from any fluid contact for at least 30 minutes after mixing to avoid complications in clinical use.

2.3.5. Polymers

Synthetic absorbable polymers

The implants made of biodegradable polymers provide temporary, mechanical support until the natural tissue heals and regains its strength. As the natural tissue heals, the degradable implant should gradually weaken. Biodegradable polymers, like polyglycolic acid, polylactic acid and their copolymers have been used as internal fixation devices such as pins, screws, tacks and plates for the past 20 years or so (Eppley et al., 2005, Suuronen et al., 2000, 2004a and 2004b, Cohen et al., 2001, Ashammakhi et al., 2004, Waris et al., 2004, Peltoniemi et al., 2002, Bergsma et al., 1995). They degrade by hydrolysis, and are then metabolically converted to water and carbon dioxide (Williams, 1987). These polymers alone possess limited osteoconductive potential (Cornell, 1999), but in conjunction with other technologies their potential can be improved. In tissue engineering, biodegradable polymer scaffolds have been used either seeded with cells or integrated with growth factors to generate bone, cartilage and other tissues (Freed et al., 1994; Ma et al., 1995). They can also be used as absorbable membranes in guided bone regeneration. Lendeckel et al. (2004) have used large, resorbable macroporous sheets as a soft tissue barrier in the reconstruction of widespread calvarial defects, together with autologous cancellous bone, autologous adipose-derived stem cells kept in place with autologous fibrin glue. In this case report, the result was good, seen as new bone formation and almost complete calvarial continuity three months postoperatively. This technique has been used extensively in oral surgery (Christgau et al., 1997). Asikainen et al. (2005, 2006) have studied a poly (desamino ethyl-ester-carbonate) (PDTE) carbonate membrane in

the ossification of critical-sized defects in rabbit mandibles, and found that these membranes enabled the ossification of the defects. They elicited a very modest foreign body reaction in soft tissues, and the fibrous capsule around the membrane was of satisfactory width.

Polycaprolactone is one of the biodegradable polymers that is being intensively studied in many research groups around the world. Schantz et al. (2006) performed a clinical pilot cranioplasty study using porous polycaprolactone implants in reconstructing the trephine-drilled holes made in calvaria of chronic subdural hematoma patients. According to follow-up scans up to 12 months, the implants seemed well integrated into the surronding calvarial bone with new bone filling the porous space. Different polycaprolactone applications have been developed and tested by cell culture as well as by animal implantation models. Ekholm et al. (2003, 2006) have studied the tissue reactions to a mixture of ε-caprolactone-lactide and tricalcium phosphate, and found that this mixture caused a mild inflammatory reaction, with the presence of fibroblasts, macrophages and eosinophils, when implanted in the dermis of sheep. The same material did not enhance bone formation in critical size mandibular bone defects in sheep. 3D-polycaprolactone scaffolds seeded with calvarial osteoblasts or mesenchymal progenitor cells have been tested in a rabbit´s critical size calvarial bone defect model. Osseous tissue integration was found within these tissue-engineered implants, and the restoration of the calvaria was functionally stable (Schantz et al., 2003). Jiang et al. (2005) have tested poly-ε-caprolactone-bioglass fibre composite *in vitro*, and found it not to have a negative effect on the biological responses measured by IL-1beta release on the macrophage activation test and by the craniofacial osteoblast attachment test.

Polymethylmethacrylate (PMMA)

PMMA as a prosthetic material in cranioplasty is one of the inert materials which meets some of the requirements for alloplastic material: it is biocompatible, not thermal conducting, radio-lucent, non-magnetic, light in weight, rigid, simple to prepare, easily applicable and inexpensive. In orthopaedical applications, PMMA is polymerized *in situ,* as a sterile bone cement. This polymerization is an exothermic reaction, producing heat in tissues, which can cause the necrosis of bone tissues and impair local blood circulation (Di Pisa *et al*.,1976; Mjoberg, 1986). During the curing of bone cement, the unpolymerised MMA can leach out from the polymerised cement and cause the chemical necrosis of tissues and cardiovascular side effects (Spealman *et al*., 1945; Revell *et al*., 1998).

Because of the risks related to *in situ* polymerization, cranioplastic PMMA implants have usually been preformed in the laboratory, using CT images of the patient´s cranium with a defect. CT images are used to create a 3-dimensional (3D) model of the cranium by using, for example, epoxy resin or the special technique used in the study of Chiarini et al. (2004). A laser-beam-based technique, stereolithography, is also used when cranial models are manufactured (D'Urso et al., 2000; Wurm et al., 2004). In the study of Chiarini et al (2004), PMMA implants showed good adaptation in terms of size, shape and convexity, during the operation. The follow-ups did not record any clinical signs of a foreign body reaction to the PMMA implant, nor any fracture or instability of the prosthesis, and no major complications occurred. Gosain (2005)

describes some disadvantages of PMMA as a cranioplastic material: it is an inert and fixed substance that will not adapt to the changing craniofacial skeleton of a growing child. Additionally, there is no bone incorporation or ingrowth, making it susceptible to infection throughout the reconstruction. If the patient had experienced a previous infection in the region of implantation, the risk of infection after PMMA cranioplasty increased markedly. Thus, this material is a good choice for adult patients with good soft tissue quality and no previous infections (Manson et al., 1986).

Eppley et al. (1990 and 2002 (2)) have developed and studied a special kind of PMMA application as a cranioplastic material. This hard-tissue replacement polymer is a polymeric composite consisting of PMMA beads sintered with polyhydroxyethyl and a calcium hydroxide coating. In the 2002 (2) study, 14 patients with cranial or cranio-orbital defects were reconstructed with the PMMA-bead implant. The aesthetic results were very good, and no infections occurred. Fifteen months postoperatively, extensive fibrovascular ingrowth between the beads could be seen. In the study of Bruens et al. (2003), aqueous biodegradable carboxymethylcellulose gel was used to create the porosity in PMMA implants when cured. Fourteen patients with treated craniofacial defects were followed for up to 20 years postoperatively, and no side effects could be ascribed to the porous PMMA. CT scans showed bone ingrowth into the prosthesis, providing the validity behind the concept of porous PMMA.

Polyethylene (PE)

During the past ten years, polyethylene has been used in craniofacial reconstructions as a porous application (Yaremchuk, 2003, Wladis et al., 2003). This high-density polyethylene implant has been used in the treatment of facial deformities and subcutaneous defects (Frodel and Lee, 1998), frontal bone defects caused by trauma (Duman et al, 1999), and facial augmentation procedures (Gosau et al, 2006). This porous polyethylene implant can be prefabricated, based on stereolithographic reconstruction from a 3D-computed tomography scan. The porocity of this biomaterial permits bone and soft-tissue ingrowth when placed on facial skeletal defects (Gosain 2005).

2.4. AN OVERVIEW OF FIBRE-REINFORCED COMPOSITES

2.4.1. General aspects

The term “composite” means a material that consists of two or more distinct parts. In material science, composites can be considered to be materials consisting of two or more chemically distinct constituents having a distinct interface separating them. In the human body there are natural composites, e.g. tendons and bones. Fibre-reinforced composites consist of one or more discontinuous phases embedded within a continuous phase. The discontinuous phase is usually harder and stronger than the continuous phase and is called the “reinforcement”, whereas the continuous phase is termed the “matrix”. The properties of composites are strongly influenced by the properties of their constituent materials, their distribution and interaction. In describing a composite material, it is also necessary to specify the geometry of the reinforcement, its concentration, distribution and orientation (Alexander, 1996). **Figure 4** shows a commonly accepted classification of composite materials.

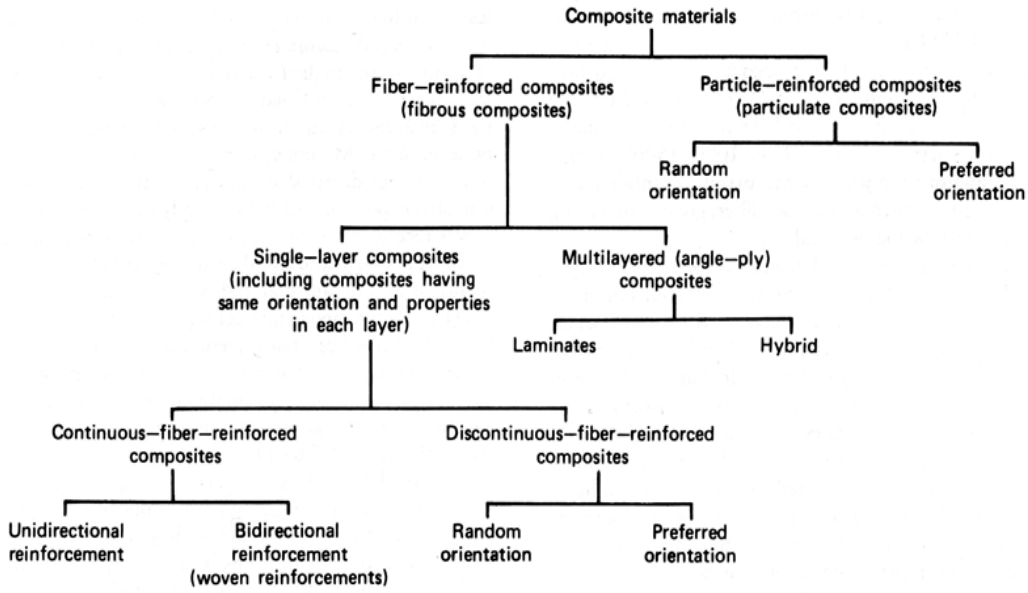


Figure 4. A commonly accepted classification of composite materials *(Figure adapted from: Alexander H (1996) Biomaterials Science, p. 95, Academic Press, San Diego).*

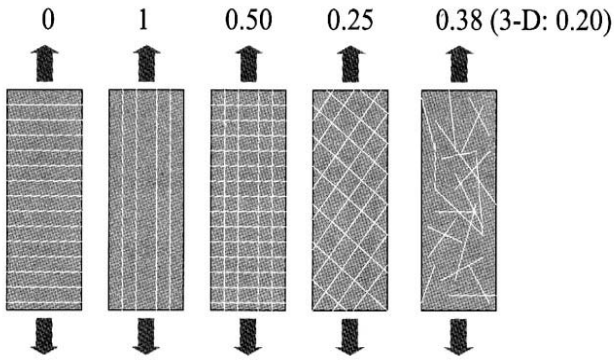


Figure 5. Reinforcing efficiency (Krenchel´s factor) of fibres with different fibre orientation in plane. *(Figure adapted from: Vallittu PK (2002) The second international symposium on fibre-reinforced plastics in dentistry, p.10, editor P. Vallittu, University of Turku)*

Mechanical properties of fibre-reinforced composites, such as strength, stiffness, toughness and fatigue resistance depend upon the geometry of the reinforcement (Alexander, 1996). The efficiency of the fibre reinforcement (Krenchel´s factor) varies in FRC laminates with different fibre orientation **(Figure 5).** The continuous unidirectional fibres give the highest strength and modulus for the FRC, e.g. the flexural strength is 700-1200 Mpa, and the modulus of elasticity 18-26 GPa with BisGMA/TEGDMA-impregnated unidirectional FRC (Lassila et al., 2002, 2004). The mechanical values for cortical bone are 80-150 MPa tension strength, and 3-30 GPa

modulus for elasticity, for comparison (Nomura et al., 2005). This property is available only in the stress direction equal to that of the direction of the fibres. Fibre weaves with fibres in two directions (bidirectional fibres) reinforce construction to some extent in all directions, and are therefore useful when it is difficult to predict the direction of highest stress (Vallittu, 1999). Fibre reinforcement with continuous fibres in random orientation is called a fibre mat, whereas reinforcement with randomly oriented short fibres is called a chopped fibre mat. These give isotropic mechanical properties for the FRC in plane. The reinforcing capacity of fibres can be divided into two or more directions, and the corresponding FRCs are called orthotropic and isotropic with regard to the mechanical properties (Vallittu, 2002). The term isotropic means that a material has similar physical properties regardless of the direction of the load, while anisotropic material has different physical properties depending on the direction of the load.

2.4.2. Properties of some resin systems used in FRCs

When fibre-reinforced composite is being considered for use as bone defect reconstruction material it should have good moulding properties for fitting to different anatomical shapes. FRC itself can not adequately fulfill this criterion. The plasticity is achieved by impregnating (=saturating) the fibre material with resins. The resin system commonly used to impregnate dental FRCs is a combination of monomers BisGMA and TEGDMA. BisGMA (bisphenol-A-glycidyl –dimethacrylate) is a hydrophobic and rigid molecule containing methacrylate groups, and the polymerized resin is highly cross-linked and rigid (Darvell, 2002). Highly viscose BisGMA monomer needs dilution to achieve handling properties suitable for composites. Typical diluents are TEGDMA (triethyleneglycol-dimethacrylate), which is a flexible and hydrophilic molecule, and EGDMA (ethyleneglycol-dimethacrylate). Based on the experience in dentistry (e.g. Vallittu, 2000) one possibility to produce shapable and partly plasticised FRC material for bone reconstructions is the impregnation of the FRC with the BisGMA/PMMA system, which forms a so-called *semi-interpenetrating polymer network (semi-IPN).* This is defined as a network composed of two chemically independent polymers. It is formed from a polyfunctional monomer that is mixed with a thermoplastic polymer, after which the monomer will be polymerized to a network polymer (Stevens, 1990). New experimental resin systems have also been developed for possible use in dentistry. One alternative is to use dendritic polymers, dendrimers, as impregnating resins for fibres and particulate fillers. Chemically, they are highly branched, three-dimensional, tree-like structures that consist of three major components: core, branches and end groups (Viljanen, 2005). A copolymer consisting of an experimental DD1-dendrimer/MMA (methylmethacrylate)/BDDMA (butanediol-dimethacrylate) system was used to impregnate the FRC implant in one of our animal studies.

The polymerization initiation for the resin systems can be based on either light activation, chemical activation or heat activation. The wavelength of the light used to activate the initiator system is 400-500 nm (Viljanen, 2005). Different types of photopolymerization units are used to produce the light needed: quartz-tungsten-halogen (QTH), light-emitting diode (LED), and plasma arc (PAC) curing units. The conventional intensity of light is 100-1000 mW/cm^2 (Uctasil et al., 2005). The photochemical basis of the visible light-curing system is almost invariably

camphorquinone (CQ), that is capable of initiating the polymerization reaction. Tertiary amines, such as 2-(N,N-dimethylamino)ethyl methacrylate (DMAEMA), are used as accelerators and form initiating radicals via electron and proton transfer (Asmussen and Peutzfeldt 2002). In room-temperature polymerization reaction of dimethacrylates, only a part of the existing double bonds are reacted. The degree of monomer conversion describes the ratio of all potentially reactive double bonds versus those reacted in the polymerization. Thus, the degree of conversion of monomers is an important factor influencing the physical properties of the polymers. In general, the higher the conversion of double bonds, the greater the mechanical strength of the resulting polymer. The unreacted double bonds may either be present in free monomer or as pendant groups on the network. The unreacted monomer may leach from the polymerized material and irritate tissues (Sideridou et al., 2002). The presence of oxygen inhibits the free-radical polymerization, because the reactivity of oxygen to a growing radical is much higher than that of a monomer (Ruyter, 1981).

The presence of residual monomers within the surgical FRC implant material with photopolymerisable resin systems is undesirable not only because of irritation reactions to tissues, but also because residual monomers can cause allergic reactions (Ruyter, 1995) or possibly have unpredictable hormonal effects on the human body, such as acting as weak estrogen (Pulgar et al., 2000; Nathanson et al., 1997). These risks make it important to determine the amount of residual monomers releasing from the composite implant material. One possibility is to analyse the amount of releasing monomers from the material by gas chromatography (GC), another by high performance liquid chromatography (HPLC). For HPLC analysis, the material is incubated e.g. in water, for different time periods, after which the residual monomers are eluted to solvent from the incubating water, the solvent is evaporated and the residual monomers are determined by the HPLC dissolved in solvents. A third system for determining the amount of residual monomers is to use headspace-gas chromatography/mass spectrometry (HS-GC/MS). In headspace (HS) sampling, the extraction of analytes from a solid or a liquid sample is achieved in a closed vial with an inert gas that carries the vaporized analytes straight into a gas chromatographic column. The sampling method is simple and convenient and does not necessitate extensive manipulation of the sample prior to analysis (Viljanen et al., 2006).

The design and choice of material for large calvarial implants poses a challenge for the researcher: the close proximity of the brain and possibly the large size of the defect make it vitally important to develop an implant which is not toxic to neural tissue and, at the same time, effectively promotes the osseous union of the bone fractures. Biostable glass-fibre-reinforced composites have not been used in fixing large cranial defects in clinical cases. Thus, it is important to find applications for experimental implants that enhance the formation of new bone in contact with the implant, and healing of the defect in the cranio-maxillofacial area. One alternative to increase the bioactivity of the FRC implant material is to add bioactive glass (BAG) to the surfaces of the implant or inside it.

2.4.3. Initial attachment of implant to bone

The traditional method of implant fixation to bone is to use screws and plates. The advantage of this method is the good immobilisation of the fractured parts of bone and

the reliability of the method, which has been in clinical use for decades. Titanium and previously also stainless steel have been the metals of choice for manufacturing these fixing systems (Heikkilä et al., 2003). In addition to metals, biodegradable screws and plates, especially made for polylactide (Bergsma et al., 1995) and polyglycolide (Ashammakhi and Rokkanen, 1997) applications, have been developed.

Direct adhesion of bone reconstruction material to bone surface is a new idea for implant fixation. The advantage of using chemical adhesive agents for fixation is to avoid the use of non-degradable and hard metals as fixing devices, that remain in tissues, and possibly have to be removed. Dental adhesives are a versatile group of chemical agents being used in dental restorations. Two main classes of material are involved, the glass-ionomer cements and the composite resins. Glass ionomers, which are naturally adhesive to both dentine and enamel, develop a zone of interaction with the tooth as they age, which produces an extremely strong bond. By contrast, bonding of composite resins is more complicated and possibly less effective, although these materials have better wear resistance. Composite resins are generally based on BisGMA, which is diluted mostly with TEGDMA. For enamel, the acid-etch technique is used for bonding. This results in bonding via micromechanical attachment, as the resin flows into the increased surface volume created by the etching process. For dentine, the bonding process is much more complicated, because the dental tubules are full of fluid. Bonding must thus develop under wet conditions (Nicholson, 2000). The same kind of wet conditons is found on cranio-maxillofacial bones *in vivo*. Thus, if dental adhesive systems are considered to be used as fixative agents with bone, they have to be able to fix the bone defects in wet and bloody conditions. Cytotoxicity of dentin adhesives (Szep et al., 2002) potentially limits their use in surgical applications. The risk of tumorgenicity of these adhesives can not be excluded, because especially TEGDMA has been shown to have mutagenic properties *in vitro* (Schweikl et al., 2006).

Another chemical group used for years as an adhesive for bone fixation is *cyanoacrylates.* They have been found to have good bonding strength and to work in a wet environment. The form most commonly used for bone fixation in clinical studies consists of butyl-2-cyanoacrylate, also known as Histoacryl®(Loctite Corp., Dublin, Ireland). It polymerizes in the presence of moisture, and sets with minimal inflammatory response, and can therefore be placed between healing bone fragments (Gosain et al., 2002). In the histotoxicity studies of Toriumi et al. (1990), Histoacryl was found to have minimal histotoxic effect and good bone graft-cartilage binding ability, whereas Krazy Glue® (Aron Alpha, Columbus, OH, USA) based on more toxic ethyl-2-cyanoacrylate, demonstrated severe histotoxicity. Generally, shorter-chain-derivatives (methyl- and ethyl-cyanoacrylate) have proved to be histotoxic, while longer-chain derivatives (butyl- and isobutyl-cyanoacrylates) are much less histotoxic (Toriumi et al., 1990). When the bond strength of Histoacryl® to cortical bone of pig mandible *in vitro* was compared to dental adhesive Clearfil® New Bond (Kuraray Co.Ltd., Kurashiki, Japan), the dental adhesive produced a higher bond strength to bone than Histoacryl® (Maurer et al., 2004).

Another possibility for fixing the fractures is to use a so-called “surgical glue” based on fibrinogen and other coagulation factors. Thus, the adhesion is based on clot formation. Many hospitals use “house-made” fibrin glues that can be manufactured

from patients´ own blood. There are also a few commercially available products, e.g. Tisseel® (Baxter, Beltsville MD, USA), Beriplast® (ZLB Behring, Hattersheim am Main, Germany), Hemaseel® (Haemacure Corp, Montreal, Quebec, Canada), and Biocol® (Alcabideche Portugal), which differ from each other in the concentrations of different coagulation factors. Primarily, they have been used as hemostatic agents and to assist tissue sealing and wound healing (Albala, 2003). When different adhesive systems were compared to each other as regards tensile strength of the bone surface (gluing dry bone or gluing bone in moist conditions simulating *in vivo)* the adhesive capacity of fibrin glue in moist and bloody conditions was poor compared to chemical-based adhesives (Rimpler, 1996). A gelatin-resorcinol-aldehyde system was developed and tested also *in vivo* with rats and rabbits, and was found to be useful in bone fixation. Most of the adhesive was already resorbed after four weeks, and only some necrotic reactions were found.

3. AIMS OF THE STUDY

This study was an attempt to develop an implant based on fibre-reinforced composite material for reconstruction of bone defectsin the cranio-maxillofacial area. *In vitro* evaluations consisted of determinations of residual monomer release and the adhesion properties of this FRC material. *In vivo* studies with rabbits were performed with frontal and calvarial defect reconstruction models using the FRC as an implant material. The hypothesis of this study was that this FRC material has potential to be developed into a biostable material alternative for reconstructing bone defects in the head and neck area.

The specific aims were:

1. To determine whether bone or blood disturb the monomer conversion, when the further-impregnated FRC with BisGMA and TEGDMA is photopolymerised in contact with them *in vitro*, leading to increased leaching of the residual monomers from the FRC (study **I**).

2. To test *in vitro* whether mechanical and chemical treatments of the surface of the porcine calvarial bone affect the bone-adhesion properties of the FRC impregnated with different adhesive systems (study **II**).

3. To study *in vivo* whether BisGMA/MMA/PMMA-impregnated FRC implants coated with BAG granules promote the bony healing of the rabbit experimental frontal bone defects compared to empty controls, and to specify the inflammatory reaction caused by the implants (study **III**).

4. To study *in vivo* whether DD1/MMA/BDDMA–impregnated FRC implants coated with BAG granules promote the bony healing of the rabbit experimental critical size calvarial bone defects *in vivo,* compared to empty controls, and to specify the biological reactions caused by the implants (study **IV**).

5. To study *in vivo* whether the biological reactions to two structurally different FRC implant types with BisGMA/MMA/PMMA impregnation differ from each other using rabbit critical size calvarial bone defects as a test model (study **V**).

4. MATERIALS AND METHODS

4.1. MATERIALS

4.1.1. Glass-fibre-reinforced composites and bioactive glass

The E-glass-fibre-reinforced composite material (FRC, experimental material, StickTech Ltd, Turku, Finland) used in all of the studies was manufactured from silanized electrical glass (diameter ca.16 μm, chemical composition: SiO_2 55 %, Al_2O_3 + Fe_2O_3 14.5 %, CaO 21.5 %, MgO 0.5 %, Na_2O and K_2O < 1.0 %, B_2O_3 7.5 %). FRC materials were used as combinations of veils of random-oriented fibres and laminates of woven fibres. In studies **I, II** and **III,** the FRC material consisted of one random oriented veil and one laminate of woven fibres fixed together with the impregnating resin **(Figure 1a)**. The thickness of the veil was 1.5 mm, and the laminate of woven fibres 0.06 mm. The outer appearance of the test samples in study **III** is seen in **Figure 1a**: round discs, diameter 10 mm, were coated with particulate bioactive glass granules (BAG S53P4, granule size 315-500 μm, Vivoxid Ltd, Turku, Finland). In studies **IV** and **V,** the FRC formed a double-veil structure that consisted of two veils of random-oriented fibres and one laminate of woven fibres lying between these veils **(Figure 1b),** all fixed together with impregnating resins**.** In studies **III, IV** and **V,** the FRC was coated with particulate bioactive glass, BAG S53P4 **(Figure 1c).** In study **V,** one new glass-fibre material consisting of two laminates of woven fibres with highly porous inner structures was also tested as an implant material (Parabeam®, thickness 3 mm, 3D Glass Fabrics, Helmond, The Netherlands) **(Figure 1d)**. It was filled with S53P4-type particulate bioactive glass and pure fused quartz fibres (Quartzel® wool, Saint-Gobain Quartz, Nemours Cedex, France). These fibres were used for filling the empty spaces of this implant, and for preventing the movements of the BAG granules. Thus, instead of adding impregnating resin, fibres were used, because the resin content was intentionally retained at a low level. **Table 1** summarizes the constituents of the test specimen and implants used in studies **I-V.**

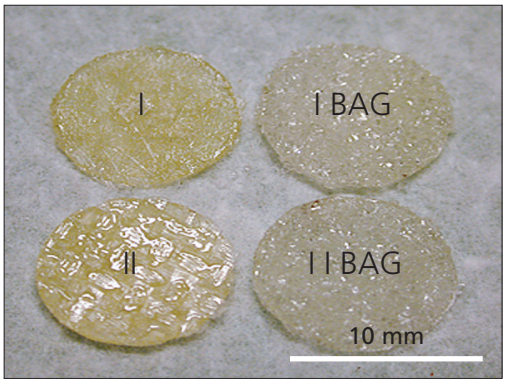


Figure 1a)

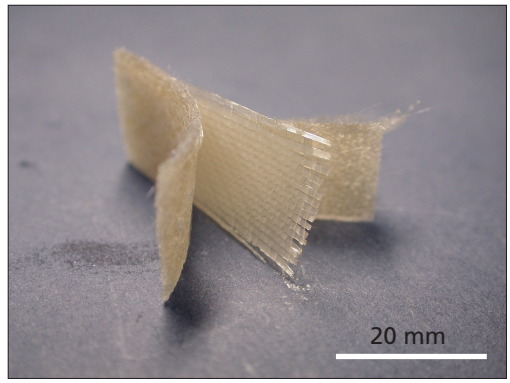


Figure 1b)

Figure 1c)

Figure 1d)

Figure 1a) Top left: The veil side of the material (**I**) showing random-oriented fibres. Bottom left: The laminate side of the material (**II**) showing the net of woven fibres. Both pieces were impregnated with BisGMA/MMA/PMMA resin. On right: respective materials (**I BAG** and **II BAG**) coated with bioactive glass granules. Diameter of one piece is 10 mm. **Figure 1b)** Fibre-reinforced composite (FRC) implant material made of two layers of E-glass fibre veil and a laminate of woven fibres lying between these two veils, impregnated with DD1/MMA/BDDMA resin system (or BisGMA/MMA/PMMA resin system). Here fibre-reinforced composite (FRC) is cut into 20 x 30 mm sample piece. **Figure 1c)** Particulate bioactive glass, BAG S53P4, manufactured to the size 315-500 µm. **Figure 1d)** The surface view of the double-laminate FRC. The fibre bundles are more irregularly oriented than in the laminates used to reinforce FRC material in all the other studies (magnification 25 x).

Table 1. The structural properties of FRC, BAG and resin systems used in the test specimen and implants in the studies **I-V**.

Study	Structure of FRC	Resin system	Type of BAG
I	One veil + one woven laminate	BisGMA / MMA PMMA / TEGDMA	-
II	One veil + one woven laminate	Dental adhesives and primers, silane mixtures	45 µm granule in coating in one study group
III	One veil + one woven laminate	BisGMA / MMA / PMMA	315-500 µm granule in coating
IV	Two veils and one woven laminate between the veils	DD1 / MMA / BDDMA	315-500 µm granule in coating
V	Two veils and one woven laminate between the veils	BisGMA / MMA / PMMA	315-500 µm granule in coating
	and		
	Two woven laminates with highly porous inner structures between the laminates	BisGMA / MMA / PMMA	315-500 µm and 90-300 µm granule + pure fused quartz fibres in filling

4.1.2. Resin systems and bone surface treatment chemicals

The resin system used to impregnate the FRC material in studies **I , II, III** and **V** consisted of BisGMA/MMA/PMMA-mixture (bisphenol-A-glycidyl-dimethacrylate/ methylmethacrylate/polymethylmethacrylate; EverStick® resin, StickTech Ltd, Turku, Finland), which forms a partly plasticised composition called the semi-IPN structure (Stevens, 1990). The resin matrix contained 1 wt % of camphorquinone and DMAEMA (N,N-dimethyl aminoethyl methacrylate) as the photo-initiator. In study **I,** the slightly porous fibre veil was further-impregnated by light-polymerizable BisGMA/TEGDMA (triethyleneglycol-dimethacrylate) resin prior to use (Stick® Resin, lot 304683, Stick Tech Ltd, Turku, Finland) to make the veil adapt better to the surface of the test substrates. Information about the chemicals used in impregnation resins in study **I** is presented in **Table 2.** Further-impregnation resin was dropped onto the veil pieces, spread with forceps, and left untouched for about 2 minutes, after which the extra resin was absorbed into blotting paper. The amount of further-impregnation resin left in the test specimen was 5% of the final weight.

Table 2. Information about the impregnating chemicals in study **I.**

Resin	Lot.no	Manufacturer
BisGMA	Lot T 0109/1	Röhm GmbH & Co. KG, Degussa-Hüls Group, Chemische Fabrik, Darmstadt, Germany
PMMA	N.A.	Palapress®, powder, Heraeus Kulzer GmbH and Co KG, Hanau, Germany.
Camphorquinone (CQ)	N.A.	Fluka Chemika GmbH, Buchs, Steinheim, Germany
DMAEMA	N.A.	Sigma-Aldrich
Stick® Resin (BisGMA/TEGDMA)	N.A.	Sticktech Ltd., Turku, Finland

BisGMA= bisphenol-A-glycidyl- dimethacrylate
DMAEMA**=** N,N-dimethyl aminoethyl methacrylate
PMMA= polymethylmethacrylate
N.A:= not available

Different dental adhesives used for further-impregnation of the FRC in study **II** are presented in **Table 3.** Basically, they can be divided into the following groups: BisGMA-HEMA-based, 4-META/UDMA/bisGMA-based, UDMA/BisGMA/PMMA-based, and silane-based (experimental) adhesives.

Table 3. The adhesive resins used to further impregnate the FRC veil in study **II**.

Resin	Lot.no	Manufacturer	Chemical Composition
3 M® Scotchbond	2001-	Ultradent Products Inc	BisGMA, HEMA.
Multi-Purpose Adhesive	0911	South Jordan, Utah, USA	
UDMA/BisGMA resin with IPN-structure (10 WT-% PMMA)		Experimental resin made by Stick Tech Ltd., Turku Finland	BisGMA, UDMA, PMMA
Unifil® Bond Bonding agent	0306041	GC Corporation, Tokyo, Japan	UDMA, HEMA, 4-META
Clearfil® Se Bond adhesive, Okayama	00416A	Kuraray Medical Inc., Japan	BisGMA, HEMA, MDP

HEMA= hydroxyethyl methacrylate
BisGMA= bisphenol-A-glycidyl di methacrylate
UDMA= urethane dimethacrylate
PMMA= polymethylmethacrylate
4-META= 4-methacryloxyethyltrimellitate anhydride
MDP= methacryloyloxydecyl dihydrogen phosphate

The chemicals used to modify the bone surface are presented in **Table 4.**

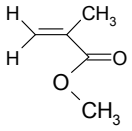
Table 4. Dental primers and experimental silane mixture used to modify the bone surface in study **II**.

Name	Lot.no.	Manufacturer	Chemical composition
Clearfil® Se Bond Primer	00325A	Kuraray Medical Inc. Okayama, Japan	MDP, HEMA
Clearfil® Porcelain Bond Activator	00133B	Kuraray Medical Inc. Okayama, Japan	Bis-PEDMA, MPS
Unifil® Bond Self-etching primer	0306041	GC Corporation, Tokyo, Japan	4-META, HEMA, ethanol
Experimental silane, (APS-ICS-MPS –mixture)		Produced by the FRC Research Group at the University of Turku	APS, ICS, MPS-silanes

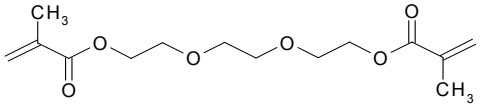
MDP= methacryloyloxydecyl dihydrogen phosphate
HEMA= hydroxyethyl methacrylate
Bis-PEDMA= bisphenol-A-polyethoxy dimethacrylate
MPS=methacryloyloxy propyl trimethoxysilane
BisGMA= bisphenol-A-glycidyl dimethacrylate
APS= aminopropyltriethoxysilane
ICS= isocyanatopropyltriethoxysilane
META= 4-methacryloxyethyltrimellitate anhydride

No further-impregnation (in addition to the BisGMA/MMA/PMMA preimpregnation) of the FRC material was used in studies **III** and **V**. In study **IV,** the resin system used was DD1-dendrimer/MMA/BDDMA (butanediol-dimethacrylate)–

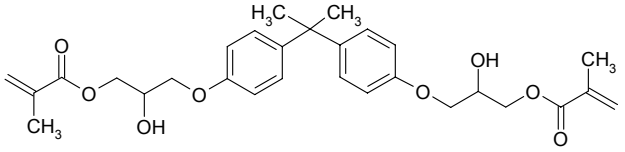
mixture. The DD1-monomer contained 20 % of MMA (methylmethacrylate) as a diluent, because of the high viscosity of DD1 (hyperbranched methacrylate polyester). In the final monomer mixture, BDDMA (1,4-butanediol-dimethacrylate) was added to the DD1/MMA in the proportions: 32 % BDDMA and 63 % DD1/MMA. 5 % of the weight was PMMA. PMMA powder was added to produce the partly plasticised consistence for this impregnation resin. The chemical structures of the monomers used in impregnation are presented in **Figure 2**.



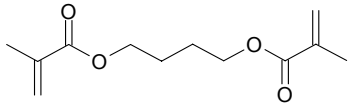
Methyl methacrylate (MMA) (repeating unit of PMMA)



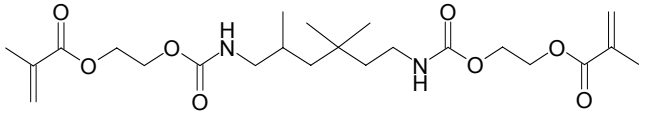
Tri(ethyleneglycol) dimethacrylate (TEGDMA)



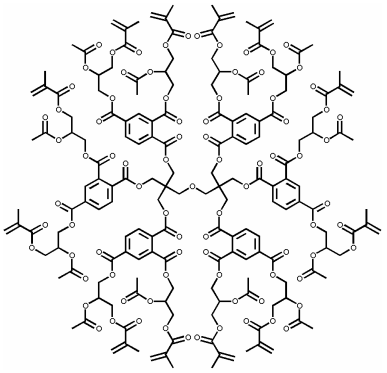
Bisfenol-A-glycidyldimethacrylate (BisGMA)



1,4-Butanediol dimethacrylate (BDDMA)



Urethane dimethacrylate (UDMA)



Hyperbranched methacrylate polyester (DD1-dendrimer)

Figure 2. The chemical structures of the monomers used in impregnation of reinforcing fibres.

4.2. METHODS

4.2.1. Test specimen and implant fabrication

In study **I,** the preimpregnated FRC veil was cut into square-form pieces (surface area 1 cm^2), which were further-impregnated with Stick® Resin. Excess of resin was absorbed into blotting paper. The test specimens were then photopolymerized according to the test procedure; in contact with bone or blood, between glass slides (negative control) and freely in the air (positive control), for 40 s with a handcuring unit Optilux 501 (SDS, Kerr/Demetron, Danbury, CT, USA). Irradiation intensity was 800 mW/cm^2, and wavelength 400-500 nm.

In study **II,** the FRC was cut into *round discs* with a cutting chisel, diameter 6 mm. These FRC discs were further-impregnated by light-polymerisable adhesive resins, and photopolymerized for 40 s in contact with the cadaver porcine calvarial bone according to the test procedure **(Figure 3).**

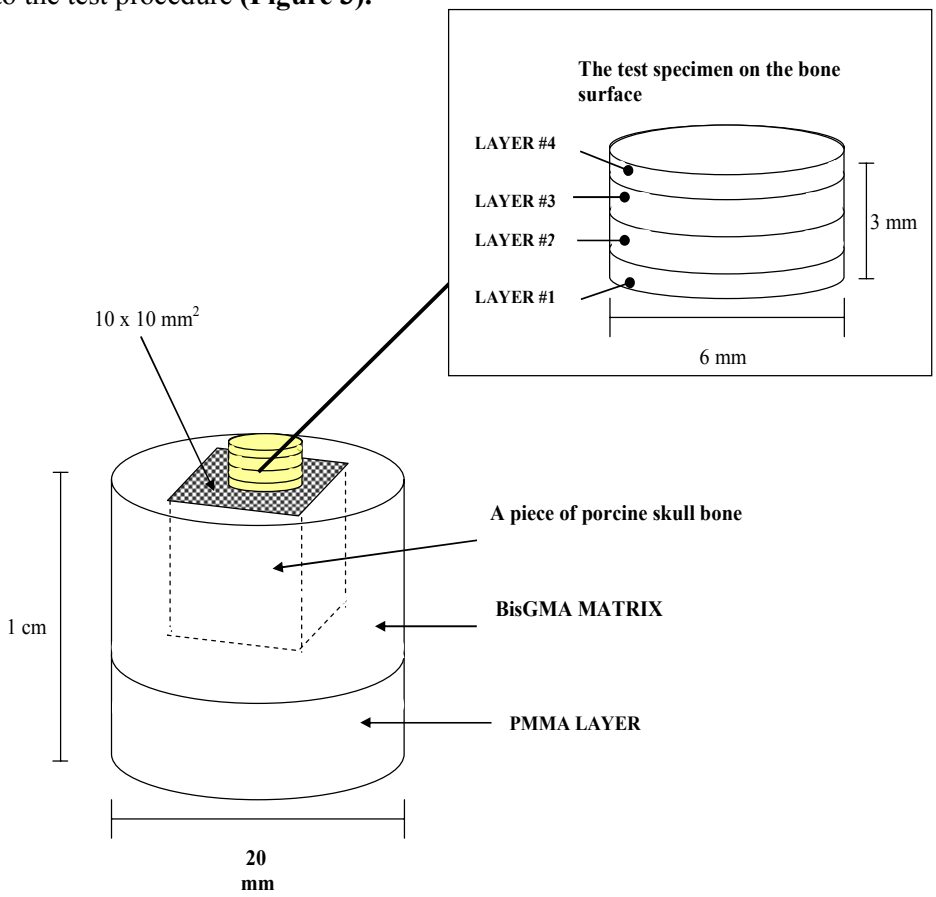


Figure 3. A diagrammatic presentation of the shear bond strength testing device. Layer 1 represents the veil disc to be tested. Layers 2 to 4 are the same veil material as layer 1; they were photopolymerised first to each other and then in contact with the first layer to be tested. Layers 2-4 were added to make the test device high enough for the shearing instrument. The shaded surface on the bone block represents the compact cortical layer of the calvarial bone. In one test group, BAG granules were added between the surface of the bone and layer 1 (Figure by M. Puska), (study **II**).

In study **III,** the preimpregnated FRC had originally been cut into round discs with a cutting chisel, diameter 10 mm, and coated with bioactive glass granules (315-500 µm), photopolymerized for 40 s on both sides with a handcuring unit (Optilux 501), and autoclaved at 121°C to sterilize and post-polymerize the material with heat. During the surgical operation, these coated discs were cut into rectangular pieces (6 x 9 x 1.5 mm) to fit better onto rabbit frontal defects **(Figure 4)**.

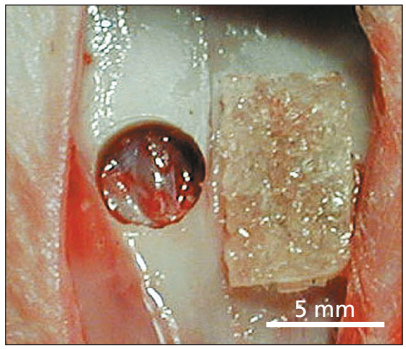


Figure 4. The FRC was cut into rectangular pieces (6 x 9 x 1.5 mm3) during operation, and applied over the frontal bone defect, 5 mm in diameter. (Study **III**).

For the studies **IV** and **V,** the rabbit skull model made of epoxy **(Figure 5a)** was manufactured according to the CT (computerized tomography) images using rapid prototyping technology. The impregnated FRC was cut into 25 x 20 mm oval pieces, coated with bioactive glass granules (315-500 µm), and photopolymerised (Visio Alfa-curing unit, ESPE, Seefeld, Germany) on the skull model to copy the anatomical form of the calvarium to the implant **(Figure 5b)**.

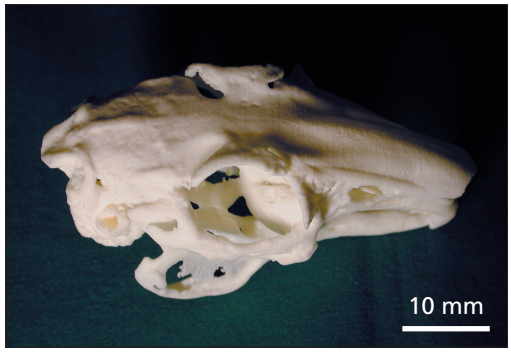


Figure 5a)

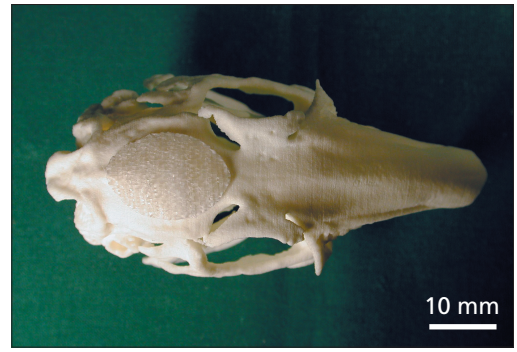


Figure 5b)

Figure 5a) The epoxy model of a rabbit skull made by rapid prototyping technique; length 100mm, width 45 mm (study **IV** and **V**). Scalebar represents 10 mm. **Figure 5b)** The impregnated FRC implant (20 x 25 mm) on the skull model before the photopolymerization. (Study **V**).

In addition, the oval-shaped implants **(Figure 6a-c)** were photopolymerized in a vacuum for 15 min (Visio Beta Vario vacuum curing unit, ESPE, Seefeld, Germany) and in the lighcuring unit for 15 min (Liculite curing unit, Dentsply, Dreieich, Germany).

In study **IV**, FRC implants were autoclaved (121°C, 20 min in 0.1 MPa pressure) after photopolymerization, to sterilize the material for an animal study, and for heat-induced post-polymerization of the resin matrix. In study **V**, the material was post-polymerized and sterilized in a dry hot air incubator for 24 hours at 121° C, after the photopolymerization period. In that study, the first test group consisted of double-veil structure (veil-woven laminate-veil) FRC coated with BAG granules (size 315-500 µm). The second test group was the double-laminate FRC implant filled with BAG granules and pure fused quartz fibres **(6c).**

Figure 6a)

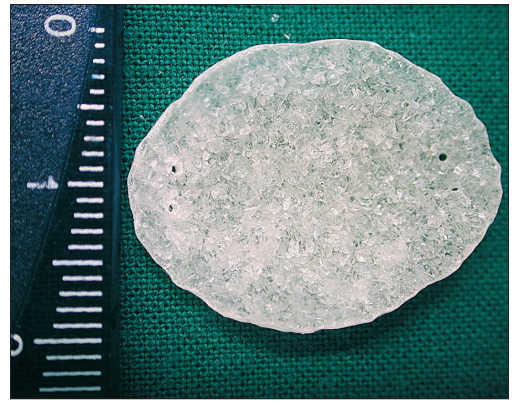


Figure 6b)

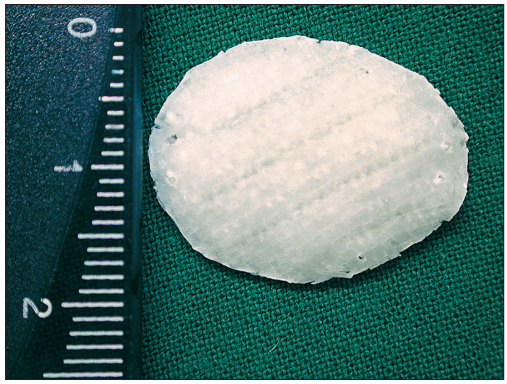


Figure 6c)

Figure 6. The BAG-coated FRC-implant **6a)** with DD1/MMA/BDDMA-impregnation (study **IV**), **6b)** with BisGMA/MMA/PMMA-impregnation (study **V**), both cut to oval shape, size 20 x 25 mm. **6c)** The double-laminate FRC implant with BisGMA/MMA/PMMA –impregnation cut to oval shape and filled with quartz fibres and BAG-granules; implant size 20 x 25 mm. (Study **V**).

FTIR-spectroscopy (I)

In study **I**, the degree of monomer conversion (DC%) of the resin matrix of the veil prepreg of negative and positive controls (material photopolymerized between glass slides and in air, respectively) was measeured by Fourier transform infrared spectroscopy (FTIR) (Spectrum One, Perkin Elmer, Beaconsfield Bucks, UK) using the DRIFT sampling accessory (diffuse reflectance infrared Fourier transform). The DC% was calculated from aliphatic C=C of the reactive methacrylate group peak at 1638 cm^{-1}, and normalized against the aromatic C=C peak at 1608 cm^{-1} according to the following equation:

$$DC\% = \left[1 - \frac{C_{aliphatic} / C_{aromatic}}{U_{aliphatic} / U_{aromatic}} \right] \bullet 100\%$$

where: $C_{aliphatic}$= absorption peak at 1638 cm^{-1} of the cured specimen.
$C_{aromatic}$= absorption peak at 1608 cm^{-1} of the cured specimen.
$U_{aliphatic}$= absorption peak at 1638 cm^{-1} of the uncured specimen.
$U_{aromatic}$= absorption peak at 1608 cm^{-1} cm of the uncured specimen.

Each spectrum was recorded with 16 scans using a resolution of 4 cm^{-1}. In addition, three parallel spectra were recorded per test group.

4.2.3. Determination of residual monomer content (I, IV)

In study **I**, the amounts of residual monomers of BisGMA and TEGDMA released from the FRC samples in different study groups (polymerisation in air = *positive control,* between glass slides = *negative control*, in contact with *bone,* and in contact with *blood*) were analyzed with high performance liquid chromatograph (HPLC) using the following components (connected to a computer): a system controller (SCL-10Avp), a liquid chromatograph pump (LC-10Advp), a UV-VIS detector (SPD-10Avp), an on-line degasser (DGU-14A), and an auto injector (SIL-10Advp). The incorporated columns used in the system were Phenomenex`s C18 precolumn (Phenomenex, Torrance, CA, USA) and Phenomenex's C18 analysis column (type: RP18, length: 150 mm, internal Ø: 2 mm, and particle size: 5 μm). Finally, the collected data were processed using Shimadzu´s CLASS VPsoftware.

A sample of each extraction solution (5 μl) was injected into the chromatograph and three parallel determinations were done *per* test group. The used mobile phase contained methanol, HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland, UK) and Milli-Q water. The analysis was carried out using a gradient run, where the concentration of methanol was changed from 40 to 90 vol%, while, at the same time, the concentration of Milli-Q water (18 MΩ cm) was changed from 60 to 10 vol% within the run time. The used flow rate was 0.3 ml/min, the run time was 25 min, and the used wavelength (λ) of UV-light was 227 nm.

The amounts of residual monomers of BisGMA and TEGDMA were calculated from the areas under the curve at peaks produced by the monomers. In the sample solutions, the concentration of residual monomers ($\mathbf{c}_{monomer}$ (mg/ml), $\mathbf{c}_{bis\text{-}GMA}$ or $\mathbf{c}_{TEGDMA}$) was determinated using linear regression equations obtained from calibration graphs.

The following equation was used to calculate the total amount of bis-GMA or TEGDMA monomers in the sample solutions, $\mathbf{m}_{monomer}$ (mg) = ($\mathbf{m}_{bis\text{-}GMA}$ or $\mathbf{m}_{TEGDMA}$).

$$\mathbf{m}_{monomer}\,(mg) = [\mathbf{c}_{monomer}\,(mg/ml) \times 3\ ml]$$

This value was used to calculate the weight percentage of the residual monomer (bis-GMA or TEGDMA) using the following equation:

$$\text{Residual monomer (wt\%)} = \mathbf{m}_{monomer}\,(mg) \times 100 / \text{mass of specimen (mg)}$$

In study **IV,** the amounts of residual monomers of MMA and BDDMA released from the same FRC material that was also used in the animal experiment, were analyzed by HPLC. After each incubation period (3,7 or 28 days in water, being shaken), the specimens were removed and 1 ml of HPLC-grade methanol (Rathburn Chemicals Ltd., Walkerburn, Scotland, UK) was added to the incubation water. After mixing the solvent with water, the HPLC samples were filtered using a 0.45 mm GHP membrane syringe filter (Pall Gelman Laboratory, Ann Arbor, MI, USA), and the HPLC analyses were performed with Shimadzu's (LC-2010) modular high performance liquid chromatograph (HPLC) system (Shimadzu Corporation, Kyoto, Japan). The collected data were processed using Shimadzu´s CLASS VP software.

In detail, a sample of each extraction solution (5 µl) was injected into the chromatograph, and six parallel determinations were done *per* test group. The used mobile phase contained methanol, HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland, UK) and Milli-Q water. The analysis was carried out using a gradient run, where the concentration of methanol was changed from 66 to 85 vol%, while, at the same time, the concentration of Milli-Q water (18 MΩ cm) was stepwise changed from 34 to 15 vol% within the run time. The used flow rate was 0.3 ml/min, the run time was 40 min, and the used wavelength (λ) of UV-light was 205 nm.

The amounts of residual monomers of MMA and BDDMA were calculated from the areas under the curve at peaks produced by the monomers. In the sample solutions, the concentrations of residual monomers ($\mathbf{c}_{monomer}$ (mg/ml), $\mathbf{c}_{MMA}$ or $\mathbf{c}_{BDDMA}$) were determined using linear regression equations obtained from calibration graphs.

The following equation was used to calculate the total amount of MMA or BDDMA monomers in the sample solutions, $\mathbf{m}_{monomer}$ (mg) = ($\mathbf{m}_{MMA}$ or $\mathbf{m}_{BDDMA}$).

$$\mathbf{m}_{monomer}\ (\text{mg}) = [\mathbf{c}_{monomer}\ (\text{mg/ml}) \times 4\ \text{ml}]$$

This value was used to calculate the weight percentage of the residual monomer (MMA or BDDMA) using the following equation:

$$\text{Residual monomer (ppm)} = \mathbf{m}_{monomer}\ (\text{mg}) \times 10^6 / \text{mass of specimen (mg)}$$

The methodology for these HPLC analyses were developed by the FRC research group (M. Puska, PhD).

4.2.4. Determination of shear bond strength (II)

In study **II,** the shear bond strength of the further-impregnated FRC samples fixed to the moist surface of porcine calvarial bone blocks *in vitro* after 24 h water incubation in 37°C were measured by adapted standards (ISO/TR 11405) with a material-testing machine (model LRX, Lloyd Instruments, Fareham, England) at room temperature, and recorded using PC software (Nexygen, Lloyd Instruments Ltd., Fareham, England). The specimens were secured in a mounting jig (Bencor Multi-T shear assembly, Danville Engineering Inc., San Ramon, CA) with the shearing rod against and parallel to the bonding site **(Figure 7).** The bonded adherend was loaded at 1.0 mm/min crosshead speed until fracture, and shear bond strengths were calculated and recorded in megapascals (MPa).

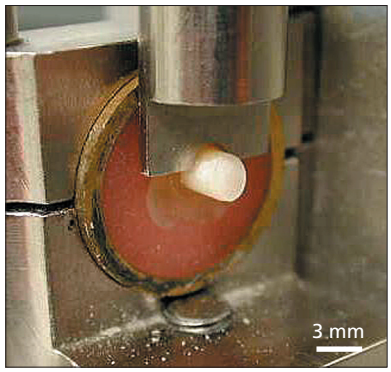


Figure 7. A schematic representation of the material-testing machine showing the position of the shearing rod against and parallel to the bonding site. Here the sample is only 3 mm in diameter (courtesy of Arzu Tezvergil-Mutluay, DDS).

4.2.5. Scanning electron microscopy, SEM (II,III)

After shearing off the veil discs from the bone surface (**II**), both the surfaces of the bone and the veil discs were coated with a gold layer using a sputter coater (Model BAL-TEC SCD 050 Sputter Coater, Balzers, Liechtenstein), and then studied using scanning electron microscopy, SEM (Model JSM 5500, JEOL Ltd, Tokyo, Japan), to detect possible differences between the shearing surfaces of the variously prepared samples. Bone surface was also studied as such and after different treatments, without being in contact with the FRC veil. Bone was studied with SEM before mechanical treatments**,** after grinding and roughening**,** after Clearfil Se Bond Primer treatment**,** and after acetone-drying, and treatment with the experimental silane mixture (APS, ICS, MPS) prepared by the FRC research group (J. Matinlinna, PhD).

In study **III**, SEM/EDS analysis was performed for one good sample in order to demonstrate the reactive layers of bioactive glass granules after implantation, as well as to find differences between the chemical compositions in different parts of the surface between the implant and surrounding tissues. Scanning electron microscope (SEM) (Model JSM 5500, JEOL Ltd, Tokyo, Japan) and energy dispersive X-ray spectroscopy (EDS) analyses (Spirit, Princeton Gamma-Tech Inc., Princeton, NJ, USA) were carried out on the ground surfaces of the test specimens after they were dried in a desiccator and coated with a carbon layer using a sputter coater (Model BAL-TEC SCD 050 Sputter Coater, Liechtenstein). SEM micrographs and EDS analysis were carried out using a standardless method with an accelerating voltage of 20 kV, at a working distance of 20 mm. A liquid-nitrogen-cooled Lithium-drifted Silicon (Si(Li)) X-ray detector with a 30 mm^2 active area (PRISM 2000, Princeton Gamma-Tech Inc., Princeton, NJ, USA) was used to collect X-ray spectra. The topographical and linescan analyses of EDS were evaluated with SEM.

4.2.6. Animal experiments (III, IV, V)

All the animal experiments in this study were performed according to the Licence for Animal Experimentation set by the Lab-animal care and use committee, University of Turku, Central Animal Laboratory (Licence number 1485/05). In study **III**, twelve adult female Hsdlf:NZW (New Zealand White) rabbits, mean weight 4000 g ±100 g (Harlan, France) were operated under standard aseptic conditions. In study **IV**, ten adult male Hsdlf:NZW rabbits were used, and in study **V,** twelve adult male NZW rabbits (Linköping, Sweden) were used in the implantation studies. Study **III** dealt

with frontal defect reconstruction with FRC, while studies **IV** and **V** concentrated on calvarial defect reconstruction with FRC.

Anesthesia and surgical procedure in animals (III, IV, V)

Rabbits in all the implantation studies were premedicated with midazolam 1 mg/kg, by intramuscular injection (Dormicum® 5 mg/ml, Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany). General anesthesia was given by intramuscular injections of medetomidin hydrochloride 0.25 mg/kg (Domitor® 1mg/ml, Orion Pharma, Espoo, Finland) and ketamin hydrochloride 10 mg/kg (Ketalar® 50 mg/ml, Pfizer Oy, Espoo, Finland). Lidocain hydrochloride, 0.8 ml subcutaneously, was used for local anesthesia (Lidocain® 5 mg/ml, Orion Pharma, Espoo, Finland). Buprenorphine, 0.9 ml subcutaneously, was used for post-operative analgesia (Temgesic® 0.3 mg/ml, Schering-Plough Oy, Espoo, Finland). One drop of Oftagel® (2.5 mg/g, Santen OY, Tampere, Finland) into each eye of the animal was used to prevent drying of the cornea during the operation.

In study **III**, after shaving the skin of the frontal bone area, the surgical field was cleaned with antiseptic chlorhexidine gluconate (Klorhexol® 5 mg/ml, OY Leiras Finland AB, Tammisaari, Finland). A U-shaped incision was made, and the skin-periosteum flap was stripped off over the frontal bone and retracted. Two round trepan bur (Gebr. Brasseler, Lemgo, Germany)-drilled holes, 5 mm in diameter, were prepared in frontal bone. Physiological saline solution was used as irrigation during the drilling, which was done at low speed (<700 rpm). The BAG-coated FRC plate was applied randomly over one defect with the veil facing the defect; the other hole was left without implant, as a control defect (**Figure 4**). Implants were fixed to bone with titanium pins (Stabilok® medium diameter dentine pins, Fairfax Dental, Ireland). The musculoperiosteal flap was closed with resorbable polyglycolic acid sutures (Safil® 4/0, Braun, Aesculap, Germany) and the skin with non-resorbable polyamid monofilament sutures (Dafilon® 4/0, Braun, Aesculap, Germany).

In study **IV,** after shaving and desinfecting the calvarial skin as described above, the cranium of the rabbit was operated by making a vertical incision over the cranial bone, and the skin and musculoperiosteal tissues were retracted. A 15 mm-diameter round defect was drilled in the midline of fronto-parietal bones using a round stainless steel template fixed to the cranial bone by titanium screws **(Figure 8)**. Thereafter, a round standard-size bone resection according to the template outer margin was performed with a dental drill. BAG-coated implants were fixed to the calvarial bone with two titanium pins (Stabilok®, medium diameter dentine pins, Fairfax Dental, Ireland), one pin at each end of the implant **(Figure 9).** The closure of the wound was performed as described above. In study **V,** all the other methods were the same as in study **IV,** except for the calvarial bone drilling procedure. A tailor-made "critical size calvarial bur" **(Figure 10)** was used for drilling the round defect of 15 mm in diameter, in the midline of fronto-parietal bones.

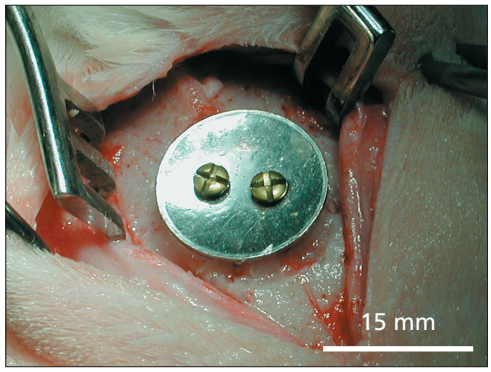


Figure 8. The stainless steel plate used as a template in calvarial defect drilling in study **IV**.

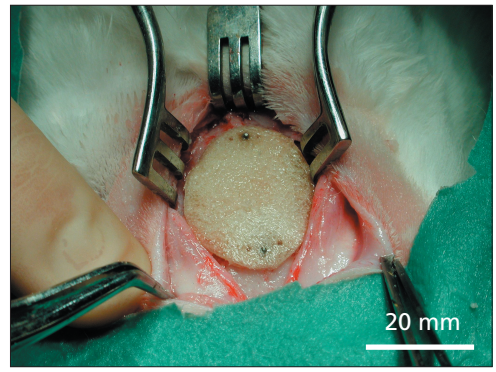


Figure 9. Implant fixed to calvarial bone with titanium pins in the studies **IV** and **V**.

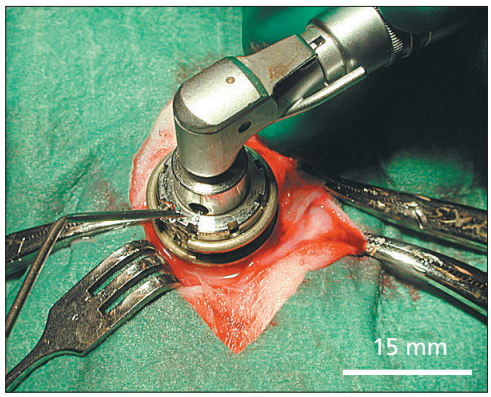


Figure 10. Calvarial bone drilling with tailor-made "critical size calvarial bur" in the study **V**.

Dosage of fluorescing dyes to rabbits (IV)

In study **IV,** fluorescing dyes precipitating calcium were injected subcutaneously (4 ml) 1, 2 and 3 weeks postoperatively (4-week group), and 8, 9, 10 and 11 weeks postoperatively (12- week group) to label sites of active bone formation. The dyes were injected as follows: *oxytetracycline* (**Terramycin**®, oxytetracycline 200 mg/ml, Pfizer OY, Helsinki, Finland) 25 mg/kg, at 1 or 8 weeks postoperatively depending on the study group, *calcein* (**Calcein**, Sigma c-0875, Sigma-Aldrich Finland Oy, Helsinki, Finland) 7.5 mg/kg, at 2 or 9 weeks, *alizarine complexone* (**Alizarin-3-methyliminodiacetic acid**, Sigma A-3882, Sigma-Aldrich Finland Oy, Helsinki, Finland) 25 mg/kg, at 3 or 10 weeks, and *calcein* 7.5 mg/kg, at 11 weeks postoperatively. These dyes have spectrally separable emission wavelengths.

4.2.7. Histological processing of bone (III, IV, V)

In all animal experiments, the rabbits were sacrificed by administration of Mebunat® 80mg/kg (Orion-Farmos, Turku, Finland) intravenously. In the laboratory, the

implanted frontal or calvarial bone was resected en bloc, and sawn with an Exakt Apparatebau saw (Exakt, Kulzer, Norderstedt, Germany) to a suitable size. The samples were fixed first in 70 % ethanol at room temperature for two weeks, being slowly shaken, then in an increasing concentration of ethanol (96 %, 99 %, 2-3 days in each), and finally in an increasing concentration of photopolymerisable resin based on isobornyl-methacrylate (Technovit® 7200, Exakt, Kulzer, Norderstedt, Germany) 30:70, 50:50, 70:30 Technovit : ethanol, 2-3 days each, then 100 % Technovit for 1-2 weeks. Then the fixed samples were moulded to the cast by filling the cast with Technovit and were photopolymerised with an Exakt Kulzer Histolux instrument (Norderstedt, Germany); 2 hours in visible light and 2 hours in blue light, at 430 nm. Three 20 μm-thick histological sections from the middle of the defects in an anterior-posterior direction were prepared (Exakt Apparatebau, Hamburg, Germany) by the cutting and grinding technique (Donath, 1982). In study **V,** the implanted calvarial bones were embedded in autopolymerisable MMA in the hard tissue laboratory of Histola Ltd., Tampere, Finland. The calvarial bones were passed through the ethanol series at increasing concentrations: 70 % → 80 % → 96 % → 100 %, with 2-3 days in each concentration, and then immersed in xylene. After this, the bone tissue was mounted on two separate aliquots of methylmethacrylate, MMA, in RT. Finally, the bone tissue was mounted on a third MMA aliquot, and left to polymerize for 2 weeks with the temperature being gradually raised to 37 ° C, at a rate of 2-3 degrees celcius per day. The polymerised block was then ready for the cutting process. Histological sections 5 μm thick were then prepared.

In studies **III** and **IV,** the sections were stained with a modified Masson-Goldner-Trichrome method (developed by Pierre Masson in Montreal, Canada, 1951, established by F.B. Mallory). In study **V**, the Van Gieson staining method for connective tissues was also used. Stained sections were examined with a light microscope to define the state of the bone healing process, as well as the magnitude of the inflammatory and foreign body reaction. The staining results of the different tissue types produced by the Technovit and PMMA histological preparation methods used in processing the calvarial bones were finally compared and evaluated.

4.2.8. Imaging procedures of bone

Faxitron-X-ray photography (V)

Fixed calvarial blocs with implants in study **V** were radiographed using a Faxitron® MX-20 DC-2 X-ray cabinet (35 kVp, 10s, Faxitron X-ray Corp., Wheeling Il, USA).

Wide-field imaging (IV,V)

Wide-field images were acquired with a Zeiss® Axiovert 200M microscope (Carl Zeiss MicroImaging Inc., Oberkochen, Germany) equipped with an AxioCam MRc5 colour CCD camera. Data were recorded with AxioVision 4.3 software using the MosaiX option.

Confocal imaging (IV)

Confocal images were collected with a Zeiss® LSM510 META confocal microscope (Carl Zeiss MicroImaging Inc., Oberkochen, Germany). Oxytetracyclin, calcein and alizarin red were excited with 405nm, 488nm and 543 nm lasers, respectively, and the

emission was collected with 500-530nm, 500-550nm and 650-710nm lasers, respectively.

4.2.9. Histomorphometry of bone (III, IV)

Images were captured using a stereo light microscope (magnification 6.5 x) with a digital camera (Leica® DC300, Leica Microsystems GmbH, Wetzlar, Germany) connected to a personal computer (PC). Computer- assisted histoplanimetric analyses were performed with PC image analysis software (Leica® Qwin v3.00, Leica Microsystems GmbH, Wetzlar, Germany). The amount of new bone formation within the bone defect was measured as an area% of the defect cross-section. Only sections with a defect length greater than 3.5 mm on cross-section qualified for the quantitative analysis in study **III,** while in study **IV** only sections cut in the middle of the defect with conserved structures of the implant material facing the defect qualified for the quantitative analysis.

New bone formation based on histoplanimetric results was statistically analysed with two-way ANOVA (SPSS v12, SPSS Inc., Chicago, USA) to evaluate the effect of time and type of implant material. In study **V,** the amount of bone formed in defects and inside the implants in two study groups were histomorphometrically evaluated.

4.2.10. Neuropathology of the brain (IV, V)

The brains of one implanted and one control defect rabbit, as well as the brain of one rabbit with no surgical operations in the head area in the 12-week implantation group (study IV) were carefully removed and fixed in 4 % phosphate-buffered formaline solution for one week. In study **V**, the brain of one rabbit in each study group was removed and fixed as described above. The transverse sections were taken from different levels of the brain hemispheres, as well as from the cerebellum and brain stem. The sections were embedded in paraffin and 4 µm sections were cut for histochemical stainings. The following stainings were used: Hematoxylin-eosin (HE, Davenport, 1960) for routine histological changes and cells, Luxol fast blue (LFB, Klüver and Barrera, 1953) for myelin changes, and Bielschowsky silver stain (Bielschowsky, 1902) for axonal changes. The histological samples were scanned with an Olympus® BX51 microscope (Olympus® Corporation, Tokyo, Japan).

4.2.11. Statistics

In study **I**, statistical analysis was performed using SPSS Systems for Windows. One-way analysis of variance (ANOVA) was used to compare test groups using Tukey`s Post Hoc test. P-values less than 0.05 were considered to be statistically significant in all test. In study **II**, the statistical analyses were conducted with the nonparametric Kruskal–Wallis test, followed by Man-Whitney U pair-wise comparison. In study **III,** new bone formation based on histoplanimetric results was statistically analysed with two-way ANOVA (SPSS v12 , SPSS Inc., Chicago, USA) to evaluate effect of time and type of implant material. In study **IV**, the differences between the release of two monomers from the polymerised FRC at different incubation time points were analysed with Tukey´s Post Hoc test (Oneway ANOVA, SPSS 11.0 for windows).

5. RESULTS

5.1. FTIR-SPECTROSCOPY (I)

When the degree of conversion-% between the negative and positive controls in study **I** was compared by the FTIR analysis, the results showed that the DC % clearly increased when the formation of an oxygen-inhibition layer was prevented by polymerising the veil prepreg between objective glasses (negative control, DC: 62.8 % versus positive control, DC: 34.0 %)($p<0.001$).

5.2. DETERMINATION OF RESIDUAL MONOMERS (I, IV)

In study **I,** the total quantity of residual monomers released in the bone contact group (0.55 wt%) was clearly lower than in the positive control group (0.97 wt%)($p=0.021$), and only slightly exceeded that of the negative control group (0.42 wt%) ($p=0.717$)). The groups´ negative control, bone and blood did not differ statistically from each other ($p>0.05$). Released monomers in the blood contact group were on the level of negative control. In all groups, the main residual monomer released was TEGDMA, the amount of which clearly exceeded that of released BisGMA (**Figure 11**).

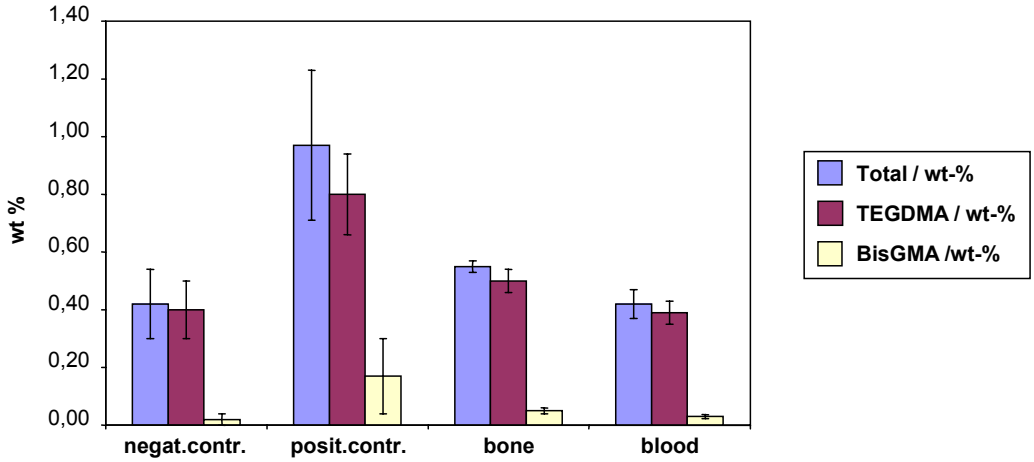


Figure 11. The quantity of released residual monomers as a percentage of the weight of photopolymerised veil. Bone and blood refer to polymerisation in contact with them (Study

In study **IV,** the length of incubation time (3, 7 or 28 days) in water had no significant effect ($p>0.05$) on the release of BDDMA from the polymerised FRC. In the case of MMA, there were no statistical differences in the release of this monomer from FRC between the incubation times 3 and 7 days ($p=0.896$), but there was a tendency toward increased release of MMA from the FRC when the incubation times of 3 and 28 days and, on the other hand, 7 and 28 days were statistically compared ($p=0.012$ and $p=0.029$, respectively). Overall, the amounts of total residual monomers released from the polymerised FRC were low; the highest value did not reach 900 ppm (0.09 wt%) **(Figure 12)**.

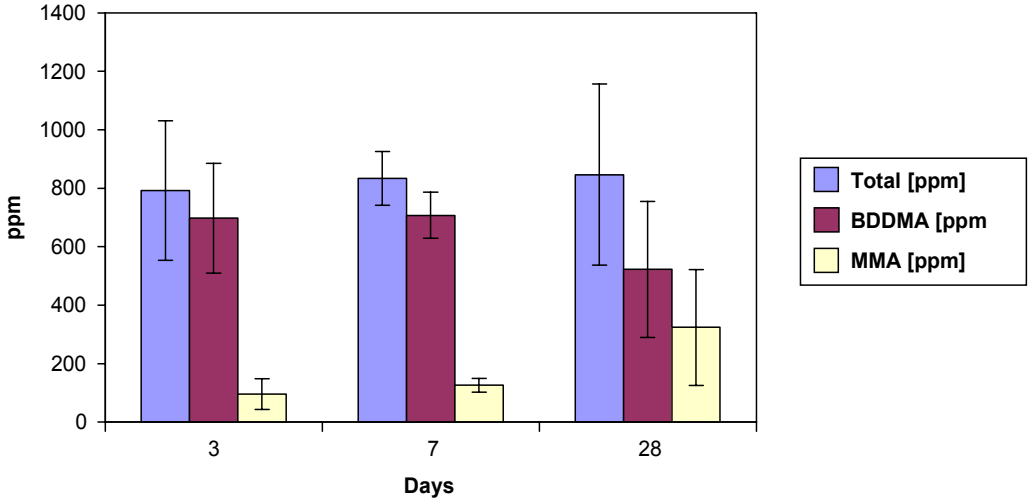


Figure 12. Monomers MMA and BDDMA released from the polymerised FRC after 3, 7 an 28 days incubation in water (study **IV**).

5.3. DETERMINATION OF SHEAR BOND STRENGTH (II)

The impregnation of the FRC veil with a hydrophilic adhesive, 3M® Scotchbond Multi-Purpose Adhesive (group 1), or with UDMA/BisGMA/PMMA experimental adhesive (group 2), did not produce good adhesion between the veil and the surface of the bone. The shear bond strengths were 0.58 and 0.40 MPa, respectively (**Figure 13 and Table 4**). With the impregnation of the FRC veil with Unifil® Bond Bonding Agent (group 8) and the chemical treatment of the surface of bone with Unifil® Bond Self-etching primer, the shear bond strength was higher: 3.40 MPa ($p<0.05$, compared to groups 1 and 2). The treatment of the FRC veil with Clearfil® Se Bond adhesive containing phosphate groups, and the treatment of the surface of bone with Clearfil® Se Bond Primer (group 3), raised the shear bond strength to 6.19 MPa ($p<0.05$, compared to groups 1 and 2). When Clearfil® Porcelain Bond Activator was mixed with Clearfil® Se Bond primer (group 4), and the mixture was used in the chemical treatment of the surface of bone, the shear bond strength was 9.50 MPa ($p<0.05$, compared to groups 1 and 2, but $p>0.05$ compared to groups 3,5,6 and 7). The addition of the bioactive glass granules to the surface of the FRC veil (group 5) (with the same impregnation and bone surface treatments as described above) lowered the shear bond strength to 6.72 MPa ($p>0.05$ compared to groups 3,4,6 and 7) (**Figure 13** and **Table 4**). When Clearfil® Se Bond Primer was mixed with the experimental silane mixture (group 6), and this combination was used in the chemical treatment of the bone surface, the shear bond strength was 6.14 MPa ($p>0.05$ compared to groups 3,4,5 and 7). When the bone surface was first acetone-dried and then treated with the experimental silane mixture, and then with Clearfil® Se Bond Primer (group 7), the shear bond strength was 7.40 MPa ($p>0.05$ compared to groups 3,4,5 and 6). All the shear bond strength test results are presented in **Figure 13**.

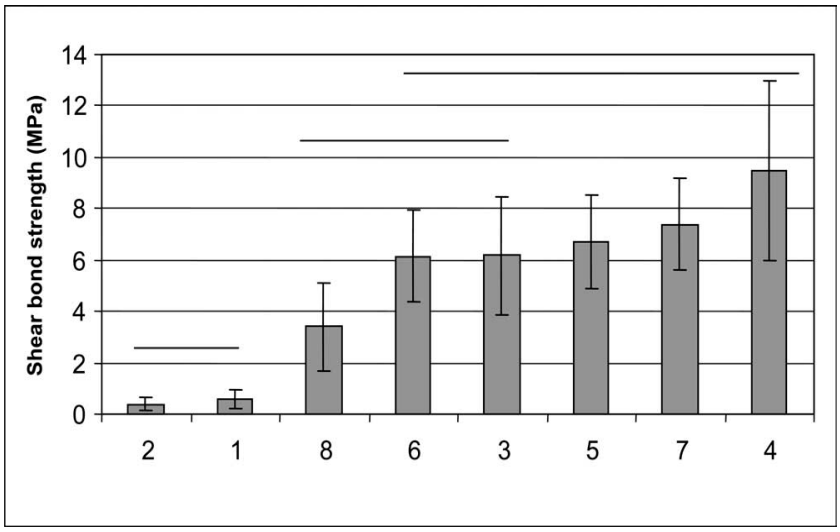


Figure 13. The results of the shear bond strength test. The horizontal lines above the sets of bars on the graph indicate the groups that have been compared to each other in statistical analyses. There were no statistical differences. ($p > 0.05$) (cf. Table 4.) (Study **II**)

Table 4. The Test Setup Groups 1–8 in Detail (cf. Figure 11). (Study **II**)

Groups	Primer agent used to treat the bone surface	Adhesive resin added to FRC veil discs
1	The surface of the bone was chemically untreated. Only mechanical roughening used.	3M® Scotchbond Multi-purpose Adhesive
2	The surface of the bone was chemically untreated. Only mechanical roughening used.	UDMA/BisGMA/PMMA -experimental adhesive
3	Clearfil® Se Bond Primer	Clearfil® Se Bond Adhesive
4	A mixture of Clearfil® Se Bond Primer and Clearfil® Porcelain Bond Activator	Clearfil® Se Bond Adhesive
5	Same as group 4	Clearfil® Se Bond Adhesive + BAG-granules
6	A mixture of Clearfil® Se Bond Primer and experimental silane mixture	Clearfil® Se Bond Adhesive
7	Acetone drying, experimental silane mixture, Clearfil® Se Bond Primer	Clearfil® Se Bond Adhesive
8	Unifil® Bond Self-etching Primer	Unifil® Bond bonding agent

5.4. SCANNING ELECTRON MICROSCOPY (II,III)

In study **II,** in addition to the SEM imaging of the shearing surfaces of the variously prepared samples, bone was studied with SEM before mechanical treatments **(Figure 14a),** after grinding and roughening **(Figure 14b),** after Clearfil® Se Bond Primer treatment **(Figure 14c),** and after acetone-drying, and treatment with the experimental silane mixture (APS, ICS, MPS) **(Figure 14d)**.

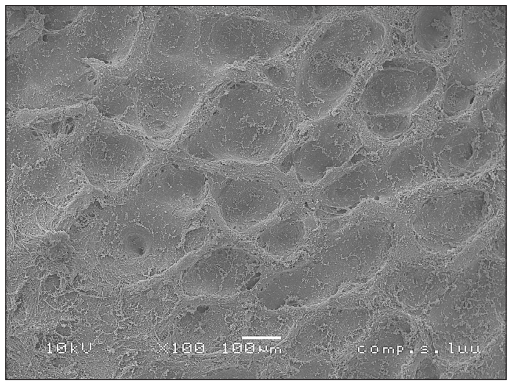


Figure 14a)

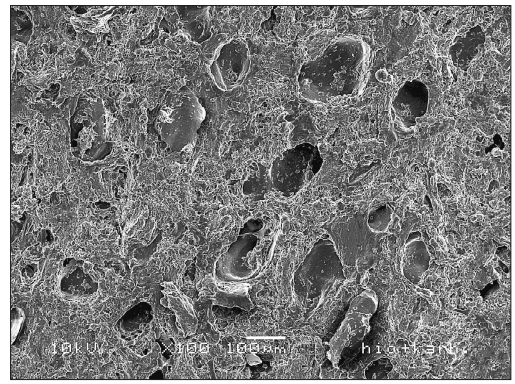


Figure 14b)

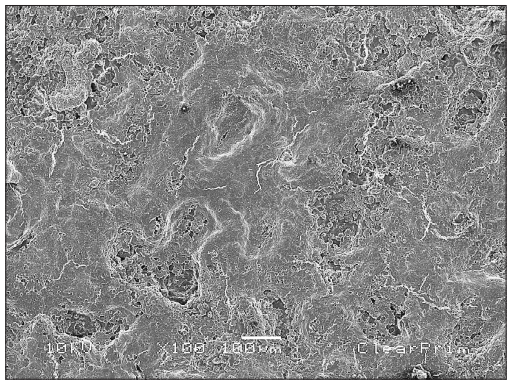


Figure 14c)

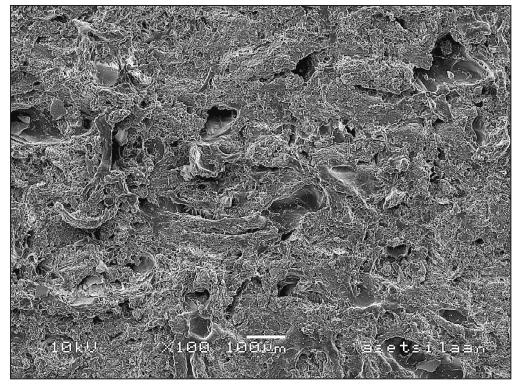


Figure 14d)

Figures 14 a-d. SEM photomicrographs of **14 a)** the surface of bone before mechanical treatments. The pores are not visible. **14 b)** the ground and roughened surface of the bone. Mechanical treatment has revealed the porocity. The ground and roughened surface of the bone **14 c)** with Clearfil® Se Bond Primer treatment, that partly covers the pores. **14 d)** after acetone-drying and treatment with the experimental silane mixture. The effect of this treatment on the bone surface topography was not clearly seen. In these figures, the surface of the bone is presented without being in contact with the FRC veil. Original magnification 100 x. Bar=100 µm. (Study **II**).

In study **III**, elementary analysis (SEM/EDS) was performed to demonstrate the reactive layers of bioactive glass granules after implantation, as well as to find differences between the chemical compositions in different parts of the surface between the implant and surrounding tissues. SEM-EDS analysis of one sample showed that the bioactive glass granules on the surface of the implant had formed typical reactive layers: *inner layer* (glass not reacted), *medium layer* (Si- rich layer), and *outer layer* (CaP-rich layer) **(Figures 15 and 16)**. The new bone formation was occasionally seen as small patches in close contact with the polymer surface and the BAG granule. However, the results of SEM-EDS analysis across these bone patches were to some extent conflicting: they showed this new bone to contain more Ca and P than the underlying calvarial bone.

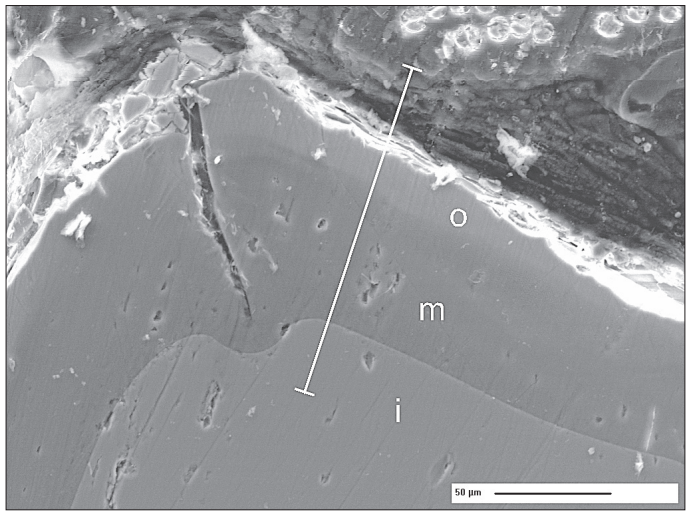


Figure 15. SEM-micrograph of reactive layers of bioactive glass granule lying on surface of implant: inner part (i) (glass not reacted), medium layer (m) (Si-rich layer), outer layer (o) CaP-rich layer). The line represents where elementary analysis (EDX) data were collected (magnification 400 x). (Study **III**).

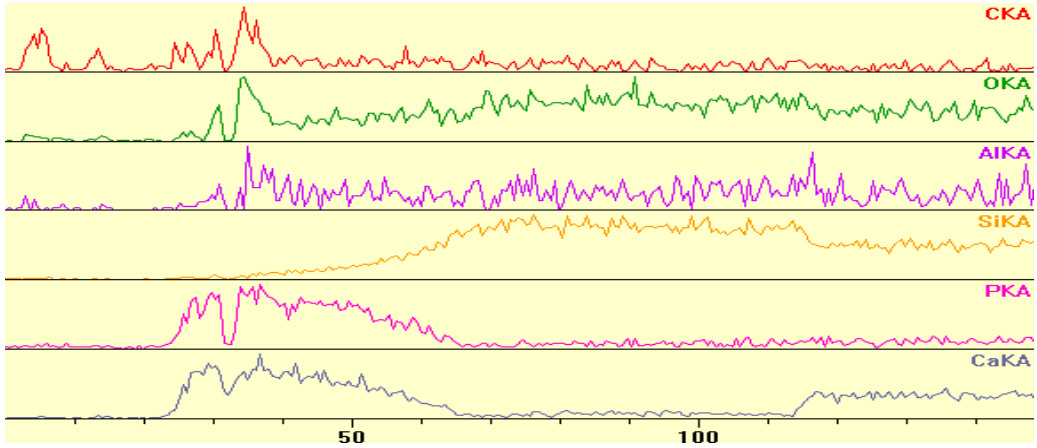
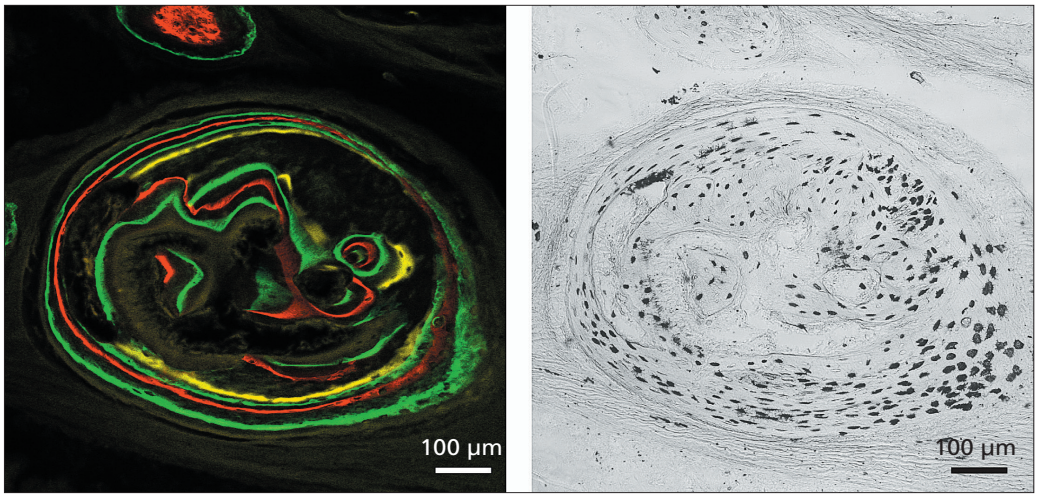


Figure 16. The results from elementary analysis (EDS) along the line in *Figure 15.* (Study **III**).

5.5. ANIMAL EXPERIMENTS

5.5.1. Anesthesia and surgical procedure in animals (III, IV, V)

In study **III,** no complications occurred during the anesthesia or surgical operation. In study **IV,** ten animals were operated and two of them died of anesthetical complications during the operation. In study **V,** one animal out of twelve died of anesthetical complications, and one 10 weeks after implantation of epileptic shock.



Figures 17 a-b) New bone formation 12 weeks postoperatively in the middle of the defect, under the implant **a)** A confocal image of four different fluorescing bands formed after serial subcutaneous administration of *oxytetracyclin* (yellow, 8 weeks), *calcein* (green, 9 weeks), *alizarin red* (red, 10 weeks) and again *calcein* (green, 11 weeks) precipitating calcium in mineralizing bone, **b)** unstained light microscopic image from the same bone islet. (Study **IV**).

In general, the combination of midazolam, medetomidin and ketamin as anesthetics, and buprenorphine as post-operative analgetic functioned as expected. Oedema of the brain tissue through the artificial calvarial defect could be seen on the histological slides of both the calvarial implant studies (**IV** and **V**). In addition to ketamin anesthesia, the burring procedure itself could induce the swelling of the brain tissue through the defect.

5.5.2. Histological processing of bone (III, IV, V)

Two different types of bone tissue processing methods were used in studies **IV** and **V,** Technovit moulding (**III**) based on photopolymerisation, and autopolymerisable PMMA moulding. Both methods yielded good results, but the staining patterns of connective tissues and newly formed bone were clearer in samples processed by the PMMA method. On the other hand, the staining of the inflammatory cells was more intensive in the Technovit-moulded samples than in the PMMA-moulded, in which the staining of the cells was faint.

5.5.3. Fluorescence imaging of bone histology (IV)

In study **IV,** fluorescing dyes precipitating calcium had been injected subcutaneously into rabbits under the implantation for labelling the sites of active bone formation and mineralization process. The light emitted from oxytetracycline (brownish-yellow) was very faint, whereas calcein (green) and alizarine complexone (red) gave strong colour emissions. Clear fluorescing bands could not be distinguished inside the implant´s inner structures. Instead, the dyes dispersed into the polymer matrix of the implant making it impossible to see the moment of dosage and mineralization. The small islets of new bone seen at the defect area were the site of clear fluorescing band formation. Dispersion of different colours did not disturb the construction, and thus, it was possible to follow the progress of the mineralization of the new bone **(Figure 17a and 17b).**

5.5.4. Histomorphometry of bone (III, IV)

In study **III**, no statistically significant difference was observed between treatment and control group ($p>0.05$). However, a statistically significant increase in new bone formation ($p<0.001$) was observed between 3 and 6 weeks. Bone formation did not increase significantly ($p=0.965$) between 6 and 8 weeks **(Figure 18).** In study **IV**, only sections cut in the middle of the defect with conserved structures of the implant material facing the defect qualified for the quantitative analysis. In the 4 week implantation group, only one animal met this criterion. The average healing measured from parallel sections by new bone in the defect area was 25.4 % in this animal. The result from sections cut nearer the edges of the defect from two other animals in the 4 week group rose as high as 40 %. In the control animal, the average healing was 3.1 %. In the 12 week implantation group, the average healing % by new bone in two animals was 37.9 %**,** while in the control animal it was 9.8 %. One animal in the 12 week group was excluded because of persistent infection.

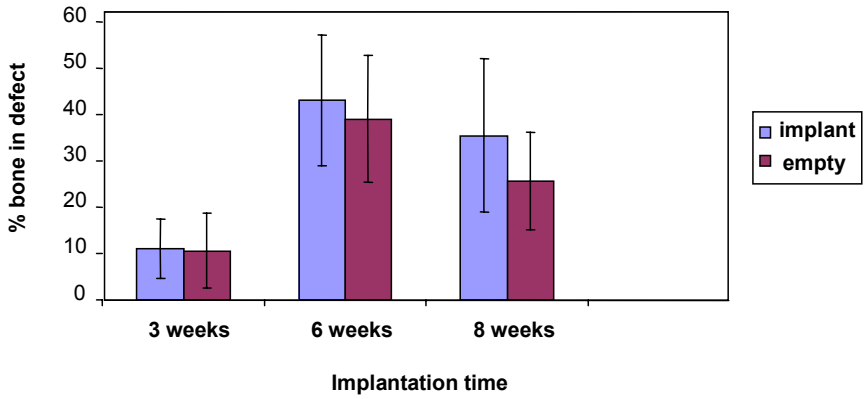


Figure 18. Amount of bone as percentage of defect area 3, 6 and 8 weeks postoperatively, (Study **III**).

5.5.5. Histological responses to implants (III, IV, V)

Frontal defect implantation (III)

Three weeks after implantation in frontal bone (study **III**), both empty and treated defects had healed by fibroconnective tissue and some trabecular bone repair from the defect margins. However, a relatively wide variation was seen between animals at each observation point. Newly formed bone was woven bone, and thin trabeculae were linked to each other with osteoid. The observed new bone formation was closer to osteoid and woven bone than mature lamellar bone at 3 weeks. Bone remodelling was observed at the defect margins. Only few inlammatory cells were observed within the empty defects, whereas in some areas, the polymer surface was associated with slight inflammation.

Six and eight weeks after implantation, some osteoid and woven bone, but also lamellar bone structures were observed, and bone bridging occurred over the defect in most cases **(Figure 19)**.

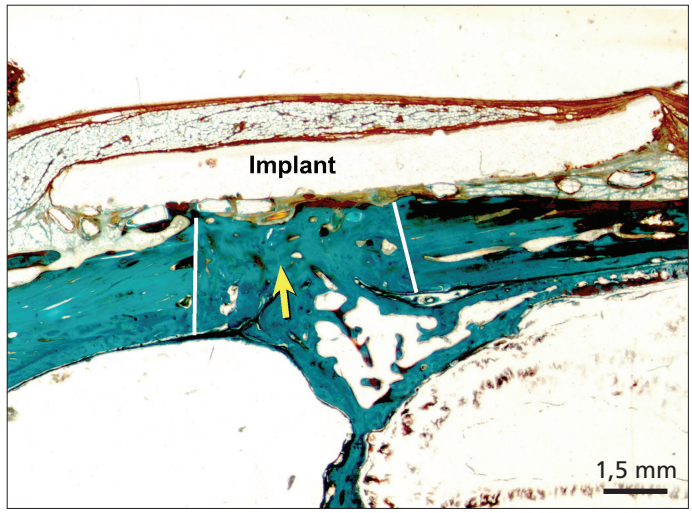


Figure 19. Implanted defect healing 8 weeks postoperatively (arrow). White lines represent original edges of defect. Masson-Goldner-trichrome staining. (Study **III**).

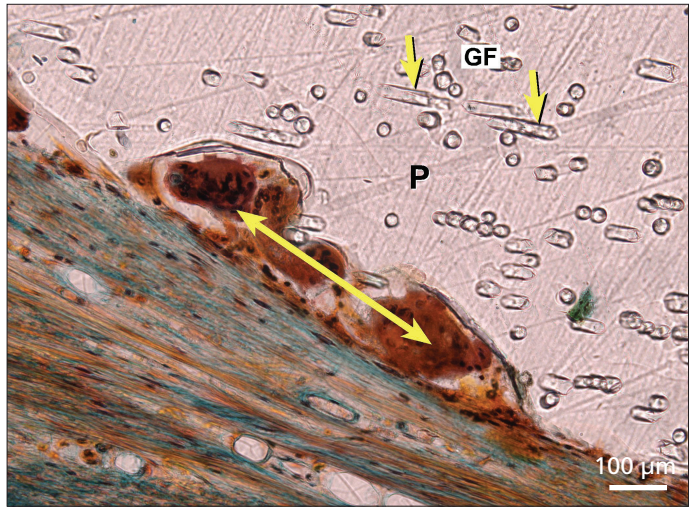


Figure 20. Inflammation cells (left-right arrow) in contact with polymer (P) surface 8 weeks postoperatively. (GF=glass fibres). Masson-Goldner- trichrome staining. (Study **III**).

No statistically significant difference was observed between treatment and control group ($p>0.05$). However, a statistically significant increase in new bone formation ($p<0.001$) was observed between 3 and 6 weeks. Bone formation did not increase

significantly (p=0.965) between 6 and 8 weeks. The polymer surface of the FRC was associated with a slight and focally moderate inflammation reaction at 6 and 8 weeks **(Figure 20)**, but part of the inflammatory cells were dying. Direct bone formation was generally observed on the bioactive glass granules facing the bone defect, while glass on the periosteal side was, in the majority of cases, surrounded by dense connective tissue **(Figure 21)**.

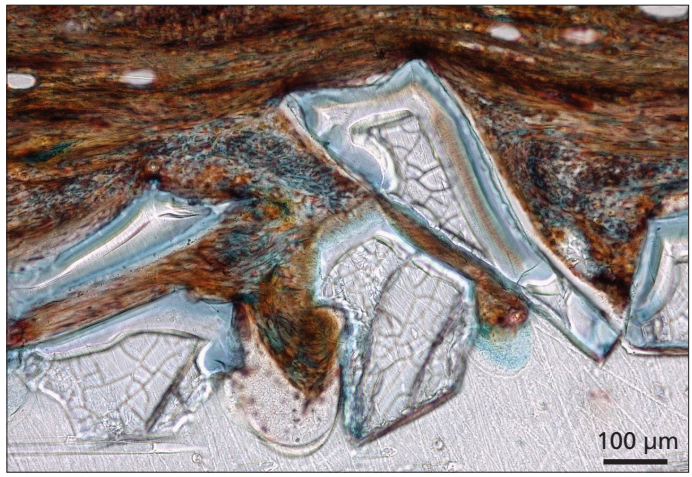


Figure 21. BAG-granules on the periosteal side of the implant surrounded by dense connective tissue; Masson-Goldner-Trichrome staining. (Study **III**).

Calvarial defect implantations (IV, V)

Bone histology at 4 weeks (IV)

At 4 weeks postoperatively, the healing process of the defects had started in the form of new bone growth from the defect margins, as well as small islands of woven bone in the middle of the defect **(Figure 22)**. These bony islands were surrounded by dense connective tissue just beneath the BAG-coated surface of the implant. The mineralization process of the newly formed bone had also started. Ingrowth of dense connective tissue into the pores of the implant was widely seen. Occasionally, mineralizing bone could be seen inside the implant structures **(Figure 23).** The bone formation inside the implant seemed to progress gradually from dense fibroconnective tissue to newly formed bone through mineralization of the tissue. A slight inflammation reaction with some lymphocytes, foreign body giant cells and macrophages was occasionally observed. However, the inflammatory reaction was minimal, and mainly fibrous connective tissue was seen inside the implant. The inflammatory response was stronger on the side of the implant facing the periosteum and skin**.** Control defects without the implant healed with a thin fibroconnective tissue layer; only a small amount of newly formed bone had started to grow from the defect margins **(Figure 24).**

Bone histology at 12 weeks (IV)

At 12 weeks postoperatively, more bony islands were seen as compared to the animals studied at 4 weeks. Part of the newly formed bone had the appearance of a lamellar structure. The porous structures of the implant were deeply filled with fibroconnective tissue **(Figure 25)**. Ingrowth of maturing bone to the implant structures could occasionally be seen, starting from the surface of the calvarial bone where the implant was fixed **(Figure 26).** The inflammatory reaction of foreign-body type, composed of macrophages, giant cells, lymphocytes and some granulocytes, as well as plasma cells, was moderate, and was mostly found inside the upper part of the implant, and between the BAG granules on the side of the pericranium. Many macrophages were poorly preserved without distinct plasma membrane among cellular debris. Fibrous connective tissue and bone formation were present despite the inflammatory reaction **(Figure 27).** Control defects without the implant had still healed mainly with a thin fibroconnective tissue layer **(Figure 28).**

Neuropathological findings (IV)

When the brains from one implanted and one non-implanted control animal (empty defect), as well as from one animal with no surgical operation were examined, *no obvious* general morphological differences could be observed. Only a focal lesion in the subarachnoid space was noted in one of the implanted animal. A foreign-body particle was seen, as well as infiltration of macrophages, and some lymphocytes surrounding the particle. A mild cortical gliosis could be seen below the foreign body reaction site **(Figure 29)**. Additionally, the cortical samples from the implanted as well as from the non-implanted animal with empty defect, showed some oedema. No inflammation reaction could be observed in the brain tissue. Neurofilament and myelin stains revealed no obvious morphological changes.

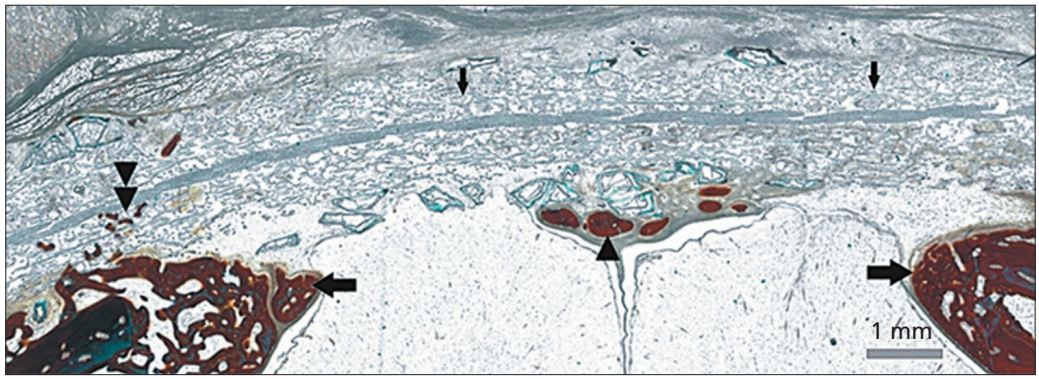


Figure 22. A wide-field histological image of the implanted calvarial defect 4 weeks postoperatively. The defect has started to heal from its margins (big arrows), as well as from the center of the defect (arrowhead). Maturing bone is seen also inside the implant (double arrowheads). The ingrowth of connective tissue to the implant´s porous structures is seen (small arrows). Masson-Goldner-Trichrome –stain. (Study **IV**).

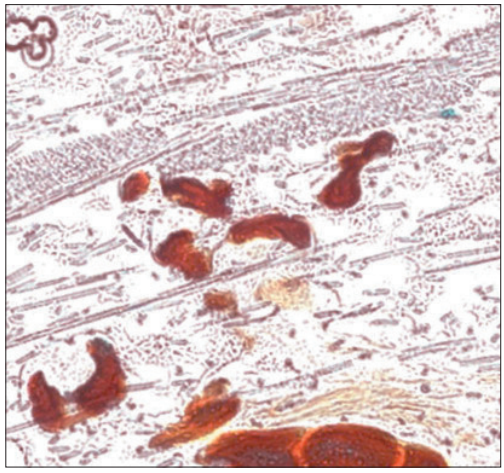


Figure 23. A detail from figure 22. Four weeks postoperatively, mineralizing bone could occasionally be seen inside the implant structures. Masson-Goldner-Trichrome stain. (Study **IV**).

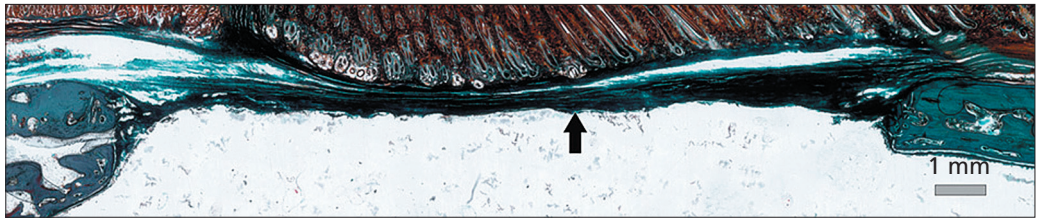


Figure 24. A widefield histological image of the control defect without the implant 4 weeks postoperatively. Control defect healed mainly with a thin fibroconnective tissue layer (arrow). Masson-Goldner-Trichrome stain. (Study **IV**).

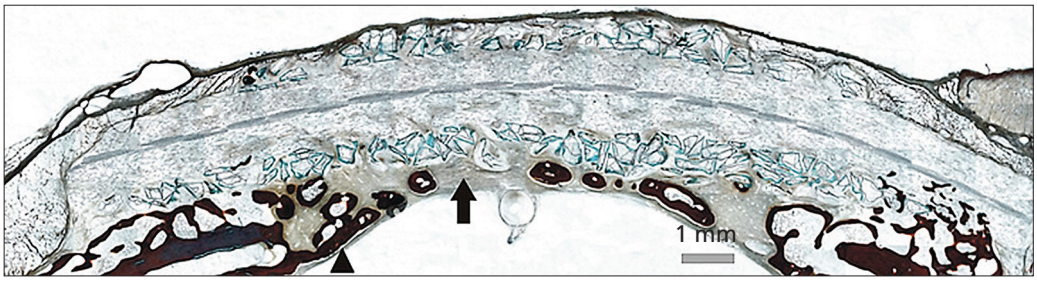


Figure 25. A wide-field histological image of the implanted calvarial defect 12 weeks postoperatively in study **IV**. More bony islands are seen in the defect area (arrowhead). These islands are growing within the dense connective tissue layer (arrow). Masson-Goldner-trichrome staining. (Study **IV**).

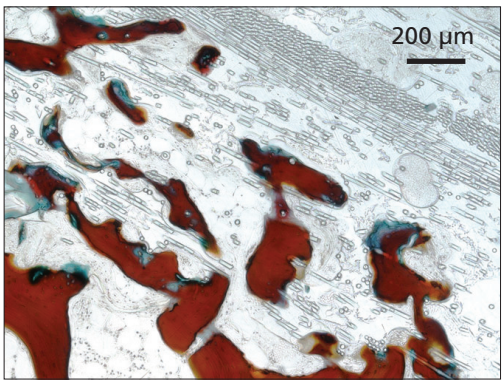


Figure 26. A detail from figure 25. Twelve weeks postoperatively, maturing bone inside the implant structures could be seen. Masson - Goldner-trichrome staining. (Study **IV**).

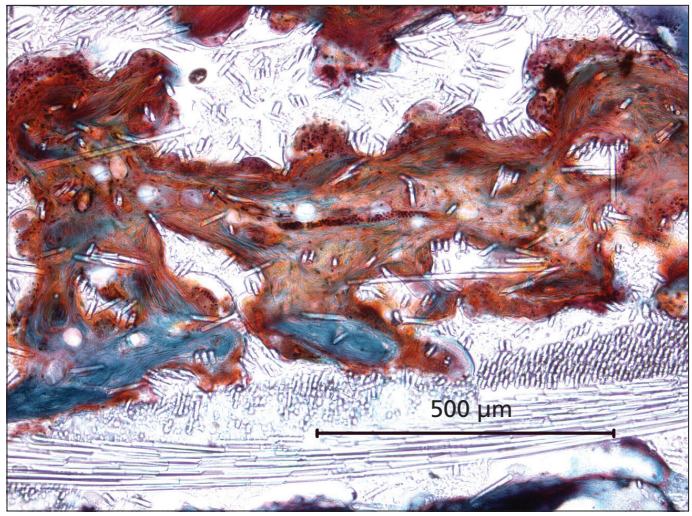


Figure 27. Twelve weeks postoperatively, fibrous connective tissue and bone formation were present inside the implant´s inner structures despite the inflammatory reaction Masson-Goldner-trichrome staining. (Study **IV**).

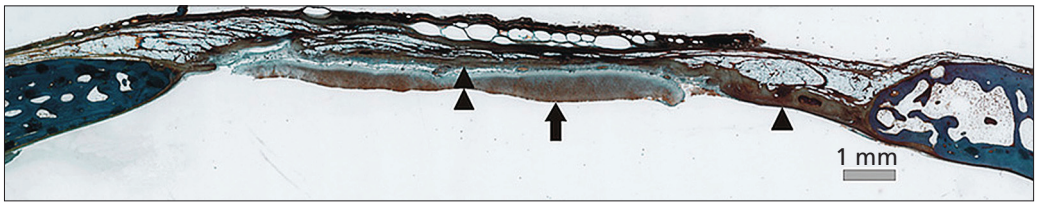


Figure 28. A wide-field histological image of the control defect without the implant 12 weeks postoperatively. Still, the control defect had healed with a fibroconnective tissue layer (double arrowhead). A small amount of newly formed bone had started to grow from the defect margin (arrowhead). A layer of brain tissue is also seen in the defect (arrow). Masson-Goldner-trichrome staining. (Study **IV**).

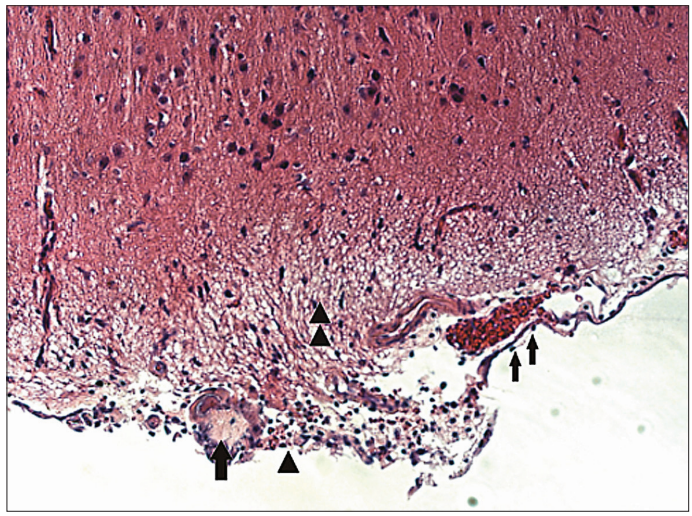


Figure 29. A foreign-body reaction in the subarachnoid space of the brain in an implanted animal 12 weeks postoperatively. A foreign body particle detached from the implant (arrow) surrounded by macrophages and some lymphocytes (arrowhead). There is a mild cortical gliosis (double arrowhead) below the foreign body. Arachnoidea is marked with small double arrows (HE stain, 20 x objective). (Study **IV**).

Bone histology at 12 weeks (**V**)

Twelve animals were used in this study. One rabbit died of anesthetical complications during the operation, and another one died of an epileptic shock at 10 weeks postoperatively. Thus, at the end of the study, there were four animals in the first study group, five animals in the second study group, and one control animal with an empty defect. These animals progressed well after implantation.

According to the Faxitron-X-ray radiographs from the fixed calvarial blocks in study **V,** it could be seen that the glass-fibre structures and the filling materials overshadowed the defects complicating the differentiation of small bony structures from glassy structures of the same size **(Figures 30a and b).** The Faxitron image of the control defect without the implant clearly shows the islets of new bone at the defect margins **(Figure 30c)**.

Based on the wide-field and other histological light microscopy images, it was found that the overall healing of the defects in this study was defective. With both the implant types, defects had started to heal at the margins as new bone formation, after which the healing process had slowed down and had not proceeded normally. Inflammation reaction caused by these two implant types was minimal.

The double-laminate FRC as a new type experimental implant material showed the ingrowth of maturing new bone into the implant´s inner structures, where the forming bone was surrounded by BAG granules and SiO_2 fibres **(Figures 31-33)**. This bone formation was seen among SiO_2 fibres despite the chronic-type inflammatory reaction there, caused by these fibres seen as an accumulation of macrophages between the fibres. This inflammatory response was not seen in the areas filled with the BAG granules.

The inner structures of the double-veil implant did not favour the ingrowth of tissues or new bone formation inside it **(Figure 34)**. Instead, new bone formation was seen on the outer surface of the implant, in close contact with the BAG granules, at some sites **(Figure 35).** Defect healing was seen at the defect margins as a small amount of new bone, and thus, healing had not proceeded normally, but was clearly delayed. The porous structures of the veils in this implant type seemed to be filled with the impregnating resin. The signs of chronic inflammation were seen on the polymer surface of minor local spots on the pericranial side of the implant, as an accumulation of macrophages in these areas, and as some giant cells attached to the polymer surface. Slight fibroconnective tissue capsule formation around the implant and its BAG granule coating was seen. The oedema of the brain tissue through the defect was clearly seen **(Figure 36)**. One animal in the double-veil group died of *status epilepticus* 10 weeks postoperatively (2 weeks before sacrifice). Histologically, the brain was highly oedemic, and a bony projection was seen to have grown towards the centre of the defect, between the implant and the oedemic brain tissue. Areas of new bone were also found to have grown in contact with the BAG granules on the outer surface of the implant. However, there was no marked inflammation reaction in the tissues in contact with the implant. The control defect had healed with a thin fibroconnective tissue layer, where islets of new bone could also be seen, mostly at the defect margins **(Figure 37)**.

Neuropathological findings (V)

The double-laminate type FRC implant did not cause any obvious pathological alterations. Neither abnormal inflammatory reaction in the meninges nor in deeper structures of the brain could be observed. Reactive gliosis or neuronal necrosis were not found. White matter seemed structurally normal and evidence of demyelinisation or any other light microscope alterations could not be seen. The brain ventricles, hippocampus, basal ganglia, brain stem and cerebellum were morphologically normal.

On the slides cut from the whole calvarial blocks with cranial bones, implant and the brain (fixed and embedded en bloc), the underlying brain tissue appeared oedemic in the defect area, and was in close contact with the fibrous structures of the implant.

The morpholgy of the brain tissue dissected from the animal with the double-veil implant showed some pathological alterations *in a focal area* of the meninges and cortex: in a cortical area of the brain, a focal thickening of the meninges could be found. Some mononuclear inflammatory cells, as well as macrophages with phagocytezed cytoplasmic material, were seen in this focal area of the cortex. In this area, white matter appeared slightly hypomyelinisated. No apparent gliosis was seen, nor any signs of inflammatory reaction. No other areas of the cortex, as well as ventricles, hippocampus, basal ganglia, brain stem and cerebellum showed any light microscope changes. Also with this type of implant, brain tissue showed oedema in the defect area. The most serious oedema was seen in the animal that died of epileptic shock.

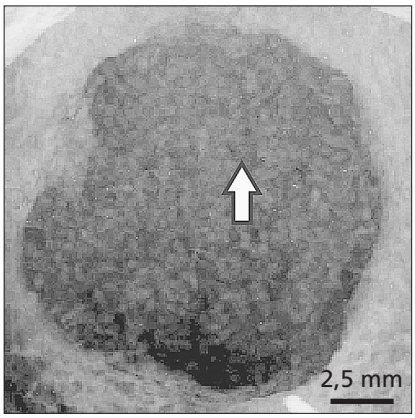


Figure 30a)

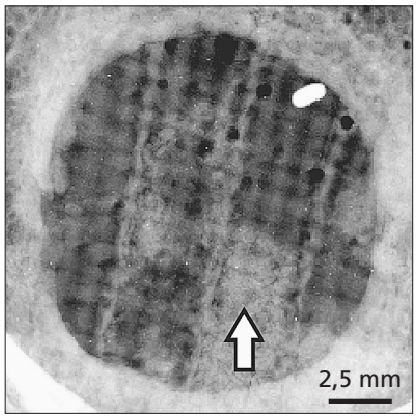


Figure 30b)

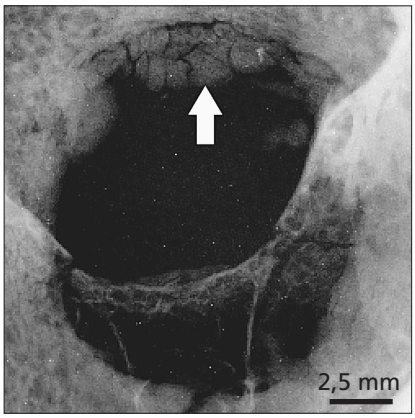


Figure 30c)

Figure 30. Faxitron radiographs of fixed calvarial blocs with two different types of FRC implant were radiographed using a Faxitron X-ray cabinet. In Figure **30a)** the double-veil implant with BAG-granule coating overshadows the defect, and in Figure **30b)** the double-laminate implant with BAG-granule filling does the same, even more so than the implant in Figure 30a. (Study **V**). **30c)** A Faxitron radiograph of the control defect without the implant. The islets of new bone can be seen at the defect margins (arrow). (Study **V**).

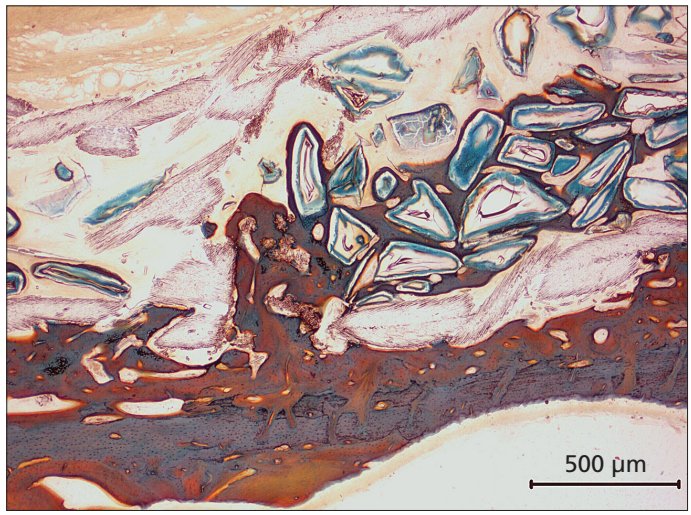


Figure 31. New bone ingrowth into inner structures of the double-laminate implant, around the BAG granules (Masson-Goldner staining). (Study **V**).

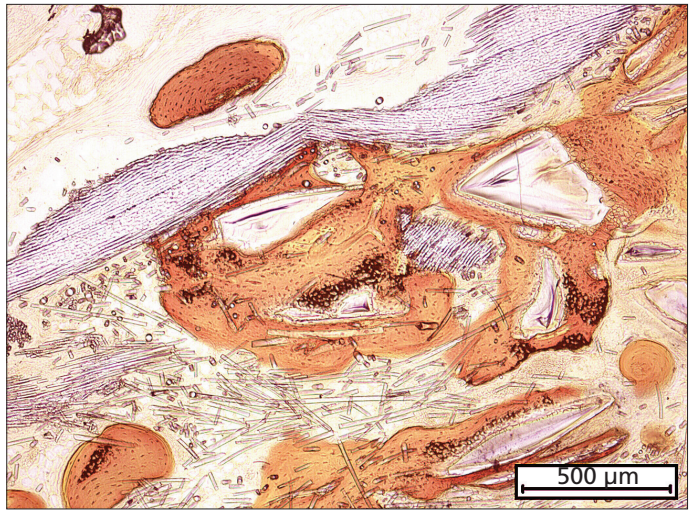


Figure 32. New bone formation inside the inner structures of the double-laminate implant; in contact with BAG granules and SiO_2 fibres (Van Gieson staining). (Study **V**).

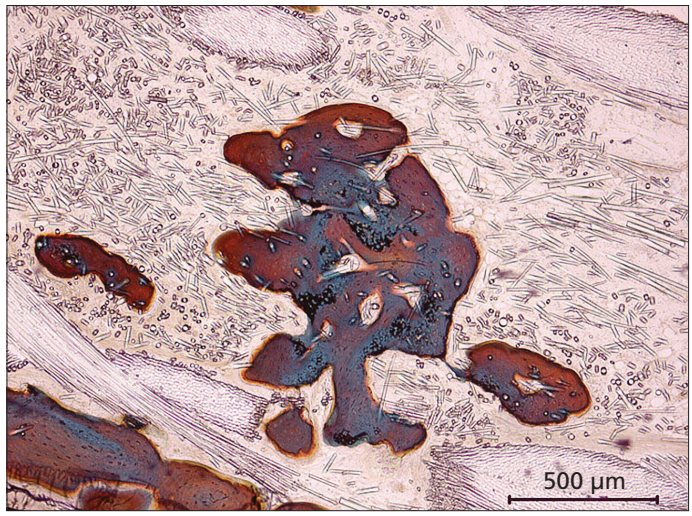


Figure 33. New bone formation inside the inner structures of the double-laminate implant; in contact with the SiO_2 fibres (Masson-Goldner staining). (Study **V**).

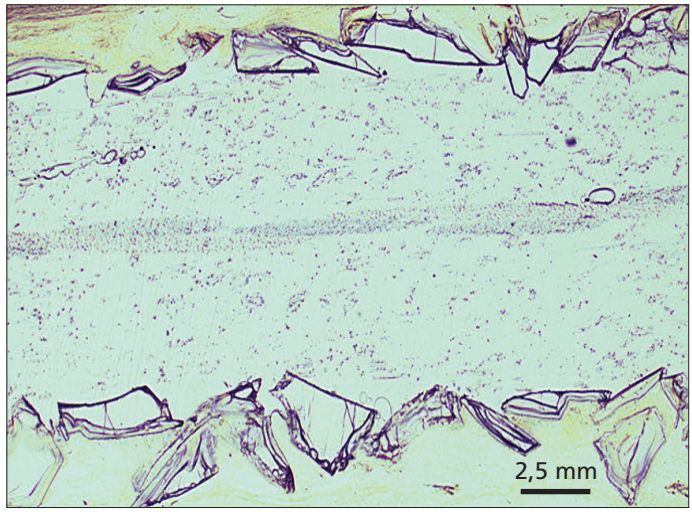


Figure 34. No tissue ingrowth into the inner structures of the double-veil implant (Van Gieson staining). (Study **V**).

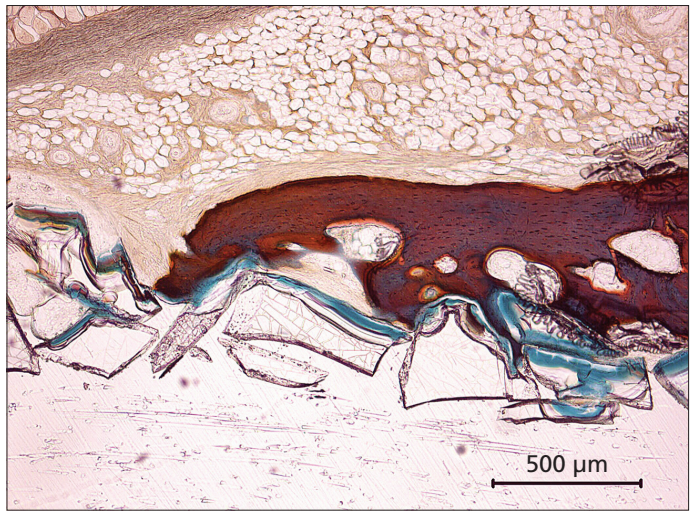


Figure 35. An islet of new bone formation in contact with the BAG granules covering the outer surface of the double-veil implant (Masson-Goldner staining). (Study **V**).

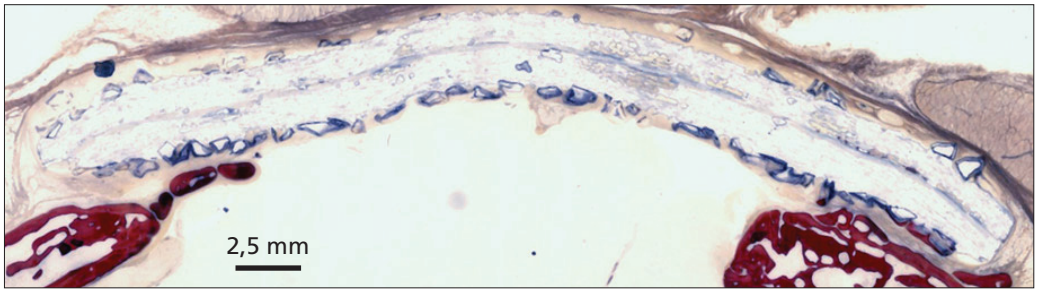


Figure 36. Oedema of the brain tissue disturbs the defect healing process. Double-veil implant, Masson-Goldner staining. (Study **V**).

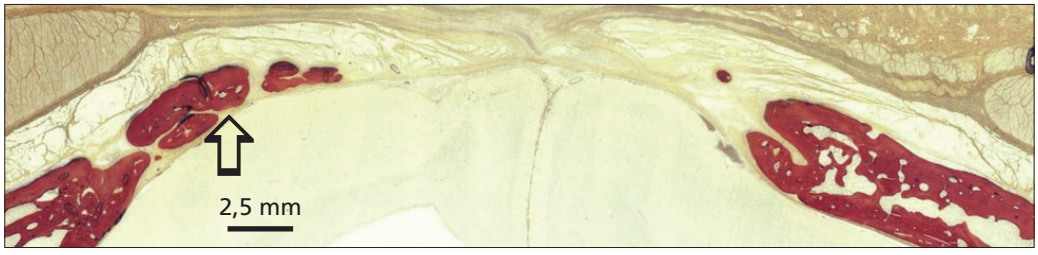


Figure 37. A wide-field image of the control defect without the implant. Oedema of the brain tissue also disturbs the defect healing process here. Islets of new bone can be seen in the defect (arrow); Van Gieson staining. (Study **V**).

6. DISCUSSION

6.1. GENERAL DISCUSSION

In the studies presented above, different aspects of biostable, shapable, glass-fibre-reinforced composites with photopolymerisable resin systems were evaluated. The ideas for the first two *in vitro* tests were based on the presumption that these FRC materials could be photopolymerised *in situ,* during the surgical operation. Thus, it was reasonable to study whether the contact with bone or blood has any effects on the polymerisation of the resin matrix of the composite. The adhesive study was based on the idea of finding a mixture of adhesives and pretreatment methods for the bone surface, that could be used during the surgical operation when the FRC implant is being photopolymerised, and hypothetically also fixed, to the bone suface. Even though these *in vitro* tests supported the idea and success of the in situ polymerisation system, they were only *in vitro* results. In fact, they did not really predict the events in a living body after implantation. No *in vitro* test can simulate all the complicated metabolic reactions occurring in a living body. The most crucial facts are whether the body accepts or rejects the material after implantation, and whether chronic inflammatory reactions are seen in the implantation site indicating that the material irritates tissues. The implantability properties of our resin-impregnated FRC implants could be evaluated only on the basis of the last three *in vivo* studies. The frontal defect study showed that short photopolymerisation is not adequate to produce an implant without tissue irritation reactions. In general, the resin systems used in our *in vivo* studies, BisGMA/MMA/PMMA and DD1/MMA/BDDMA, seem to need photopolymerisation in a vacuum *and* heat-induced postpolymerisation for creating a polymer matrix that does not cause irritation to tissues, especially soft tissues. In addition, they still need improvements. In practice, this means that the FRC implants impregnated with the resin systems studied have to be manufactured *ex vivo*, in the laboratory. By doing this, it seems to be possible to produce an implant which does not cause any remarkable inflammation reactions at the implantation site. Regardless of the implant, some inflammatory reactions can be seen inside the tissues of the implantation site, if the musculoperiosteal flap is closed with resorbable polyglycolic acid sutures. The E-glass fibre-based trunk of the composite implant seems not to be irritative on its surface contacting tissues, but on the basis of the calvarial studies, the inflammatory responses are seen mainly *inside* the implant. There, the connective tissue growing into the implant reaches the areas of the polymer matrix that are not necessarily as well polymerised as on the surface of the implant. This can be the case especially with the DD1-impregnated implants, where the inflammatory cells were seen in pouches inside the material. On the other hand, SiO_2 fibres as a filling material inside the double-laminate implants seem to cause similar inflammatory reactions. These fibres break easily when handled during the manufacture of the implant. Thus, it is possible that a mass of needle-like short fibres has caused irritation and attracted macrophages to this site. Bioactive glass granules are known to stimulate bone formation. If they are placed on the surfaces of the FRC implant, they can stimulate bone formation in contact with them, but they can also cause slight capsule formation around them, especially if they are not properly attached to the surface. The reason for capsule formation around BAG

granules can also be the slight movements of the soft periosteal tissues against the realtively sharp corners of BAG granules. Thus, these granules seem to work better when placed inside the FRC implant, where they stimulate bone formation without causing any marked undesirable reactions. In addition, placing BAG granules onto the surface of the FRC implant facing the defect and the brain tissue should be avoided, because detaching glass granules can cause mechanical and possibly also chemical irritation to the brain tissue.

Structural differences in the FRC implants also seem to be important for defect healing. The implant should sit properly in the defect site, because improper fixing can cause micromovements of the implant, that can, for one thing, interfere with the defect healing. Besides, as was seen in the calvarial studies presented above, an empty space between the implant and the brain can cause oedemic patterns in the brain tissue, and this also disturbs the defect healing process. Calvarial defect drilling procedures should be developed, standardized, and also fine-tuned, to avoid all the possible mechanical effects of the drilling procedures on the brain tissue and thus ensure the better healing of the bony defect. Furthermore, when bone reconstructive materials are developed, it is worth remembering that other tissues can also react to the materials, and that implant-derived substances can travel far from the implantation site carried by the blood circulation, lymphatic and tissue fluid. That is why the materials, investigated in this study, glass-fibres and photopolymerisable resin systems, need to be tested by toxicity tests following the rules of good manufacturing practice (GMP).

6.2. DEGREE OF CONVERSION AND RESIDUAL MONOMERS RELEASE (I, IV)

The degree of conversion in study **I** was measured only from positive and negative controls, because, in the study groups, "the polymerisation in contact with *bone* or *blood"* FTIR process using the DRIFT sampling accessory was unsuccessful as bone and blood tended to stick to the surface of the FRC during the polymerisation. Positive and negative controls showed conversion rates (34.0 % and 62.8 %, respectively), comparable to those of BisGMA-based dental resins in the literature. The residual monomer release from the FRC samples impregnated with BisGMA and TEGDMA and polymerised in contact with bone or blood were analysed by the HPLC method from the incubation water of the polymerised FRC samples. This revealed the polymerisation state of the FRC surface only indirectly: the leaching monomers came from the whole sample, and the differences between the samples were found on the surface that had been in contact with the tissues.

In study **I,** the main finding was that if the FRC is polymerised in contact with bone or blood, this contact with biological surfaces did not markedly inhibit the polymerisation of the BisGMA-based resin matrix of the FRC. In this study, the idea of *in situ* polymerisation, i.e. -polymerising the FRC implant during the surgical operation in contact with tissues; was tested *in vitro*. *If* biological conditions (contact with bone, blood or tissue fluid) would not inflict the formation of an oxygen-inhibition layer on the polymer surface, the resin-impregnated FRC could be moulded into the anatomical shape of the bony defect and photopolymerised there during the surgical operation. This *in vitro* test showed it to be possible. However, *in vitro* –tests seldom really

simulate *in vivo* conditions, where multiple factors of living body interact the foreign material implanted into tissues. The results of the animal study (**III)** did not support the applicability of briefly photopolymerised, resin- impregnated FRC as an implant.

In study **IV,** the residual BDDMA and MMA release from impregnated FRC was analysed from water after a maximum of 28 days' incubation. Even though the released amounts were low, and there is a strong possibility that they do not pose any remarkable biological hazard, there was a tendency towards an increased release of MMA from the FRC over time, which is not a desirable characteristic for a calvarial implant material. The amount of possibly released residual dendrimer could not be analysed by HPLC, but the size of the dendrimer molecule is so large and branched, that MMA as a small molecule is more likely to be the main leaching residual monomer, and not dendrimer. MMA as a constituent in the DD1 mixture could be totally replaced by BDDMA, which does not leach out so easily.

6.3. THE SHEAR BOND STRENGTH OF FRC FROM PORCINE CALVARIAL BONE *IN VITRO* (II)

In study **II,** the adhesive properties of impregnated FRC to the wet surface of porcine calvarial bone were tested *in vitro* using different kinds of adhesives in further-impregnation of the FRC, and mechanical and chemical bone surface treatments to improve the adhesion. The methods tested were adapted from dental biomaterial study protocols. This study was performed to find an adhesion method for fixing the FRC implant to bone without using metallic fixing systems. In the ideal case, the etching and adhesion chemicals could be used by an "all in one" system: the sterile surgical FRC pack could be opened during the operation, and the bone defect could be fixed with a ready-made material with all the necessary chemicals.

The untreated bone surface did not seem to favour the adhesion of impregnated FRC. The surface etching and adhesion chemicals progressed only when the surface of the bone was mechanically ground and roughened. Many of the chemical treatments tested work in dentistry, but during cranio-maxillofacial operations they could possibly irritate tissues and are toxic, leading to the unbiocompatibility of the material as a whole. The good adhesive property of Clearfil products was an interesting finding, however, because these products contain phosphate groups (MDP), known to have good binding property to apatite. The polymeric form of MDP-containing resin also forms apatite on the surface, when immersed in simulated body fluid (Hayakawa et al., 2004). This phenomenon might play an important role, when long-term adhesion to bone *in vivo* is considered. Biological adhesion systems, like *fibrin sealants* (Donkerwolcke et al., 1998), are more biocompatible than dental adhesive systems, but the bond strength they produce is not comparable, for example, to acrylates (Rimpler, 1996). Thus, the search for good and biocompatible adhesion systems for fixing non-metallic implants to bone surface without screws and pins remains an interesting challenge for the future.

6.4. FRONTAL BONE DEFECT RECONSTRUCTION WITH FRC

In study **III,** BisGMA-based resin-impregnated FRC was tested for the first time as a surgical implant. The test model used was a rabbit frontal bone defect reconstruction

model, in which trephine-drilled 5 mm round holes in frontal bones were covered with FRC implants and fixed with titanium pins. In this model, the artificial defects were quite small, and they can heal also spontaneously without implants. On the other hand, the size of frontal bones is so small that wider defects could not be drilled in them without causing extra damage to the architecture of the bone. Moreover, regardless of the small size of the defects, it is possible to see the reactions between the implant materials and the surrounding tissues.

E-glass fibres forming the mechanical trunk of the implant seemed not to elicit any immediate irritation reactions when implanted to tissues. In this frontal study, the resin matrix was the reacting part of the implant, from the first controlled timepoint to the end of the study. The surface of the polymer matrix was not stable, but started to dissolve and attract inflammatory cells onto it, the amount of which increased with the time from implantation, eliciting a moderate inflammatory reaction. On the other hand, bioactive glass granules embedded in the resin-impregnated FRC surface formed such a milieu together with the polymer matrix, that enhanced new bone formation in close proximity to them, seen as *local patches* of maturing bone. Thus, new bone formation was possible despite the tissue irritation.

In this study, short-time photopolymerisation with a handcuring unit only was combined with postpolymerisation by heat by autoclaving the material; this combination was expected to elicit acceptable biological responses *in vivo*. However, the irritative nature of the dissolving polymer surface that caused the signs of long-time inflammatory reactions forced us to improve the chemical character of the polymer matrix of the FRC implant, as well as the polymerisation processes, in the following studies with the resin-impregnated FRC implantations.

6.5. CALVARIAL BONE DEFECT RECONSTRUCTIONS WITH FRC (IV, V)

In study **IV,** the BisGMA-based resin in impregnation of the FRC implant was replaced with the experimental DD1/MMA/BDDMA mixture, which has been investigated in our laboratory as a possible new resin in restorative dentistry. In preliminary cell culture tests with primary rat osteoblasts and human gingival fibroblasts, this experimental resin was found to be promising, also because of its good biocompatibility and cell proliferation-enhancing properties. The polymerisation process was modified to prevent the leakage of residual monomers from the polymer surface to tissues. Photopolymerisation in a vacuum curing unit was presumed to be the critical modification in the process because the lack of oxygen in the polymerising chamber prevents the formation of an oxygen-inhibition layer onto the polymer surface.

At four weeks, and later at 12 weeks postoperatively, the ingrowth of fibroconnective tissue into the porous structures of the FRC implant could be seen, as well as bone formation inside the implant. The defect started to heal both from the margins and in the middle of the defect as woven bone which began to form lamellar structures. This part of the biological response was as expected. However, when tissues had reached the innermost structures of the implant, especially in the upper part of the implant, signs of chronic inflammation could be seen: macrophages, giant cells,

lymphocytes and some granulocytes, as well as plasma cells, had infiltrated into sac-like structures inside the implant. Many macrophages were poorly preserved without a distinct plasma membrane among the cellular debris. Fibrous connective tissue and bone formation were present despite the inflammatory reaction. The reason for the accumulation of inflammatory cells in these structures could be the presence of air bubbles inside the resin matrix. Thus, oxygen was possibly present to such amounts that it was able to promote the formation of an oxygen-inhibition layer on the surface of the polymer, inside the implant. Again, there was the dissolving layer which seems to be responsible for the irritation reactions in the tissues.

In neuropathological studies, no signs of chemical or mechanical irritation inside the brain tissue were found. The only lesion site was seen on the surface of the brain 12 weeks postoperatively: the dura mater seemed to be damaged and thus, the detached foreign body particle (possibly BAG granule) had the possibility to move into the subarachnoidal space and cause the formation of gliosis in the brain tissue just beneath the arachnoidea. Therefore, it is vitally important in the future to fix these granules of bioactive glass properly to the material surface to prevent the possbly formation of neural lesions. The granules could also be replaced by some other kinds of coating methods that enhance the bone formation ability of the implant. The DD1/MMA/BDDMA –polymer matrix of the implant was found not to damage brain tissue or meninges of the brain. Thus, neuropathologically, the combination of photopolymerisation and heat-induced postpolymerisation by autoclave was adequate to prevent the leak-out of residual monomers from the implant to the brain tissue. Moreover, the bone formation inside the implant and the defect was rather good. However, the persistent inflammatory response seen in the inner pouches of the upper part of the implant indicates that the polymerisation process was probably inadequate. Thus, the resin mixture should be developed in this respect. In addition, the reactions of pericranial soft tissues to the silanized E-glass-fibre material, in a form of a random-oriented fibre veil and, on the other hand, as a laminate of woven fibres *without any polymers* should be investigated to distinguish the reactions caused by the glass material from that of the polymers used.

In study **V**, the pros and cons of the BisGMA/MMA/PMMA –resin system as an impregnating agent of the calvarial reconstructive FRC was tested with the same rabbit critical size defect model that was used in study **IV**. In the frontal defect model, the combination of short-time photopolymerisation with a hand-curing unit and heat-induced postpolymerisation by autoclaving the material did **not** produce a desirable result regarding the inflammatory response. Therefore, the polymerisation process was modified on the basis of the system used in study **IV**: photopolymerisation was performed in a vacuum curing unit to prevent the formation of an oxygen-inhibition layer onto the polymer surface. A curing oven was also used. The difference in the polymerisation process compared to study **IV** was that the final heat-induced postpolymerisation (and sterilization at the same time) was performed with a dry hot-air blowing instrument for 24 h, instead of autoclaving the material in the chamber with pressure and high humidity. This long-lasting heat treatment was performed with the purpose of further improving the conversion rate of the monomers within the resin system of the FRC. With this polymerisation system, it seemed to be possible to reduce the inflammation reaction caused by the implant to a minimum, in all the tissue types

in contact with the implant. No such layer of inflammatory cells was found that was clearly seen on the polymer surface of the implants in the frontal defect study **(III)**. New bone was able to grow in contact with the BAG granules covering the outer surface of the double-veil-type implant, but not into inner structures of the implant, as was seen to happen in implants in study **IV**. The reason for this difference possibly lies in the different porosity of the implants in these two studies, and is not solely related to the chemical character of the resin matrices. The implants in study **IV** were very dry and porous, whereas the implants in study **V** contained more resin. Differences in porosity are based on the impregnation process: if the resin mixture used in impregnation is easily absorbed into the glass-fibre trunk, the result is "well and evenly impregnated FRC" that seems mechanically strong after curing, but the porosity can be inadequate for tissue ingrowth. The viscosity of the experimental DD1/MMA/BDDMA resin system was different from that of the BisGMA/MMA/PMMA system and, thus, the impregnation result of the glass-fibre material was also different. The veil-type implants in both the calvarial studies were structurally similar: one double-veil and a laminate of woven fibres between these veils. In both the studies, implants were coated with BAG granules of the same size: 315-500 µm granule size. This coating has been proved to enhance the new bone formation process onto the surface of the FRC implants in all the studies presented in this thesis. However, the size of the granule is possibly too large, and the shape is too sharp to be used in contact with the smooth and slightly moving tissues of the pericranium: the granules protruding out of the implant´s surface start to cause slight connective tissue capsule formation around them. In addition, it is possible that they mechanically and/or chemically irritate the surface of the brain tissue. If the *dura mater* is damaged for some reasons this may cause reactive gliosis in the nervous tissue.

Bearing in mind the risks mentioned above, the BAG granules were placed *inside* the porous inner structures of the experimental double-laminate implants also tested in this study. Both the surfaces of this implant type facing the tissues were relatively even: they consisted of slightly irregularly woven glass-fibre bundles. The pinholes of 1 mm size were drilled into the surface facing the defect to facilitate the outflow of the ions when the BAG dissolves inside the implant. The surfaces of the implant did not seem to cause irritation reactions in contact with the tissues. However, the pinholes, although only allow the ions to flow out, had markedly weakened the structure of the implant: it had cracked in places. In addition, drilling had caused single fibres to stick out making the hole opening irregular and rough. Quartzel wool, pure silica glass-fibre material had been packed into the implant to prevent the slight movements of BAG granules in the empty spaces of the implant. New mineralizing bone seemed to be able to grow into this fibrous material, and aroung the BAG granules, both through the open ends of the implant and through the drilled pinholes; not thoroughout the implant, but as islands seen in places in the inner compartments, indicating that both the glass types enhanced bone formation and mineralization. Mechanically, the quatrzel wool was not ideal, because the fibres tended to break easily, forming small spiky structures that are not biologically good, either: inflammatory cells were seen to have accumulated among the fibres. Thus, if glass fibres are going to be used as a filling material inside the implant, they have to be bendable without cracking.

The defect drilling method in study **V** was changed from the fixed-steel-plate model used in study IV, to the use of a custom-made, hollow trephine bur of 15 mm diameter. This change was made in order to simplify the surgical process, and to shorten the time needed for this operation and the length of anesthesis of the animal. Both these intentions were fullfilled. However, this drilling procedure slightly increased bleeding in some animals, indicating that the method is possibly too invasive at this particular site. In both the calvarial defect studies, regardless of drilling method used, oedema of the brain tissue was seen, but the BisGMA-implant study with the trephine-drilled defect, the oedemic effect was more obvious. The brain swelling observed through the defect opening towards the implant seen in this calvarial study obviously impedes the healing process of the defect. One reason for the oedema of the brain can simply be the improper fitting of the FRC implant in the defect site. Implants were slightly curved giving the skull the desired outer shape, but the inner surface was concave, possibly leaving too much space between the implant and the brain, thus complicating the defect healing. The *dura mater,* essential for normal bony healing, is always at risk of being damaged during the calvarial drilling procedure. Slight damage to the dura was seen in this study, at least in some of the animals, so there were many points complicating the defect healing process. However, even though oedema of the brain was also seen in the DD1 implant study, the defect healing was markedly better: there were many bony islands within the dense fibroconnective tissue layer, and the defects were clearly in the process of bony healing, contrary to the finding in study **V**. Thus, it can be concluded that the defective healing seen in study **V** is also related to the chemical character of the impregnating resin system used. This could also explain the observed neuropathological findings: the highly impregnated veil-type implant had caused some focal alterations in the brain morphology, while the slightly impregnated double-laminate implant had not. Thus, in conclusion, the ideal system for impregnating the FRC remains to be further investigated in the future.

7. CONCLUSIONS

1. Photopolymerisation of the further-impregnated FRC in contact with bone or blood *in vitro* did not markedly increase the leaching of residual monomers of BisGMA and TEGDMA from the FRC when compared to controls (study **I**).

2. Mechanical and chemical treatments of the surface of porcine calvarial bone enhanced the adhesive properties of the FRC impregnated with different adhesive systems, to the calvarial bone surface *in vitro* (study **II**). Biocompatibility of these treatments *in vivo* remains to be studied further.

3. When rabbit frontal bone defects were reconstructed with BisGMA/MMA/ PMMA-impregnated FRC implants with a BAG granule coating, the difference in new bone formation in the defect areas between the implanted defects and empty controls was not statistically significant. The polymer matrix of the implant had caused a moderate inflammatory reaction at the implantation site (study **III**).

4. DD1/MMA/BDDMA -impregnated FRC implants coated with BAG granules were found to have promoted the bony healing of the rabbit critical size calvarial bone defects. The islands of mineralizing bone were growing within the dense fibroconnective tissue, which filled those parts of the defects where bone formation was not seen. In addition, connective tissues ingrowth and bone formation were found inside the implant structures. A persistent inflammation reaction was found only in the inner pouches of the upper part of the implant, whereas this reaction was not observed in the lower part. Bone formation proceeded despite the inflammatory reaction. The brain morphology was mainly normal. Control defects healed with fibroconnective tissue (study **IV**).

5. When rabbit critical size calvarial bone defects were reconstructed with two structurally different BisGMA/MMA/PMMA –impregnated FRC implants, better biological responses were seen with the double-laminate implants filled with BAG granules and SiO_2 fibres: maturing bone had grown into the implants. This was not seen with the double-veil implants. Both the implant types had caused delayed defect healing, and the brain tissue showed oedemic patterns. The inflammatory reaction caused by both the implant types was only slight (study **V**).

The hypothesis of this study was to show that this FRC material has potential to be developed to a non-metallic, biostable material alternative for reconstructing bone defects at the head and neck area. Based on the results presented, it can be concluded that this potential has been reached. However, futher product development is needed for modifying the material properties so as to be used in real clinical applications.

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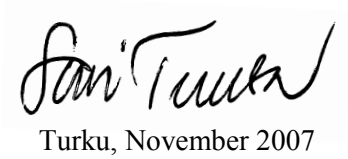

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ORIGINAL PUBLICATIONS (I-V)