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Bioabsorbable Tyrosine-derived Polycarbonate in Guided Bone Regeneration

An experimental study

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ACADEMIC DISSERTATION
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Abstract

The present study was designed to evaluate the suitability of a novel bioabsorbable material in guided bone regeneration. Experimental bone defects were created bilaterally in the mandibular angles of 20 New Zealand White rabbits. A poly (desamino ethyl-ester-carbonate) (PDTE carbonate) membrane was used to cover the defects. On one side the defects were additionally filled with bioactive glass 13-93 mesh. The healing of the defects was evaluated by radiological examination and histological sections.

PDTE carbonate membrane and bioactive glass mesh were implanted into dorsal subcutaneous pouches in rabbits. Soft tissue reactions caused by the biomaterials were evaluated by means of histology and immunohistochemistry. The tensile strength of the membranes was analysed after *in vitro* degradation.

The ossification of the experimental defects was initially faster without filling the defect with bioactive glass mesh. However, toward the end of the follow-up period the difference decreased. In the unfilled 52-week controls no ossification of the defects was detected. Therefore it can be concluded that PDTE carbonate membranes are sufficient to enhance ossification of critical-sized defects in rabbit mandibles.

PDTE carbonate membranes elicited a very modest foreign-body reaction in the soft tissues. This reaction was uniform throughout the study. Varying amounts of calcification were seen in the fibrous capsule surrounding the membrane. The exact nature of this phenomenon remained unsolved.

Bioactive glass 13-93 coated with chitosan implanted in soft tissues elicited a foreign-body granuloma with eosinophilic granulocytes. By 24 weeks most of the material was absorbed, but whenever there were any particles left the foreign-body reaction was notably milder; yet a mild chronic inflammatory infiltrate was still present. The presence of eosinophilic granulocytes in some of the samples suggests the possibility of an allergic reaction either to bioactive glass 13-93 or to chitosan. Matrix metalloproteinases -3 and -13 are suggested to participate in the host reaction to either bioactive glass or chitosan.

The results of the present study encourage further testing of PDTE carbonate membranes in experimental protocols where the use of conventional materials has led to compromised reconstruction.

List of original articles

The present study is based on the following articles:

I. **Asikainen AJ**, Noponen J, Mesimäki K, Laitinen O, Peltola J, Pelto M, Kellomäki M, Ashammakhi N, Lindqvist C, Suuronen R. Tyrosine derived polycarbonate membrane is useful for guided bone regeneration in rabbit mandibular defects. J Mater Sci Mater Med 16:758-758, 2005

II. **Asikainen AJ**, Noponen J, Lindqvist C, Pelto M, Kellomäki M, Pihlajamäki H, Suuronen R. Tyrosine derived polycarbonate membrane in treating rabbit mandibular defects. J. R. Soc. Interface, 3:629-635, 2006

III. **Asikainen AJ**, Pelto M, Noponen J, Kellomäki M, Pihlajamäki H, Lindqvist C, Suuronen R. *In vivo* degradation of poly (DTE carbonate) membranes. Analysis of the tissue reactions and mechanical properties. J Mater Sci Mater Med, in press

IV. **Asikainen AJ**, Hagström J, Sorsa T, Noponen J, Juuti H, Kellomäki M, Lindqvist C, Hietanen J, Suuronen R. Soft tissue reactions to bioactive glass 13-93 combined with chitosan. J Biomed Mater Res: Part A, in press

The listed articles are referred to in the text by their Roman numerals.

List of abbreviations

µm	micrometre
$CHClF_2$	difluorochloromethane
ABG	autogenous bone grafts
BAG	bioactive glass
BFGF	basic fibroblastic growth factor
BMP	bone morphogenetic protein
BP	un-demineralized bone powder
BSA	bovine serum albumin
CaO	calcium oxide
CF_2	carbon fluoride
CRP	C-reactive protein
CT	computerized tomography
d	day
D	Dalton
DA	degree of acetylation
DBP	demineralized bone powder
PDTE carbonate	poly (desamino tyrosyl-tyrosine ethyl ester carbonate)
EGF	epidermal growth factor
ePTFE	expanded polytetrafluoroethylene
g	gram
GBR	guided bone regeneration
GI	gingival index
GPa	giga Pascal (10^9 Nm^{-2})
GPC	gel permeation chromatography
GTR	guided tissue regeneration
H_2O_2	hydrogen peroxide
HA	hydroxyapatite
HDDM	homogenized demineralized dentin matrix
HPLC	high pressure liquid chromatography
IGF-1	insulin like growth factor-1
im	intramuscular
iv	intravenous
K_2O	potassium oxide
kg	kilogram
kGy	kilo Grey
kV	kilo volt
mA	milliampere

MgO	magnesium oxide
mm	millimetre
mm^2	square millimetre
MMP	matrix metalloproteinase
Mn	number average molecular weight
MPa	mega Pascal (10^6 Nm^{-2})
MR	magnetic resonance
ms	millisecond
M_w	weight average molecular weight (g/mol)
Na_2O	sodium oxide
NMP	N-methyl-2-pyrolidine
P_2O_5	phosphorus pentoxide
PBS	phosphate buffer saline
PD	polydispersity
PDGF	platelet-derived growth factor
PDS	polydioxanone
PGA	polyglycolic acid or polyglycolide
PLA	polylactic acid or polylactide
PTFE	polytetrafluoroethylene
rhBMP	human recombinant bone morphogenetic protein
sc	subcutaneous
SiO_2	siliconeoxide
SR	self-reinforced
T_g	glass transition temperature
TGF-b	transforming growth factor-b
TIMP	tissue inhibitor of matrix metalloproteinase
wk	week
wt-%	weight per cent

Introduction

Bioabsorbable devices for guided bone regeneration are developed today for various applications. Traditionally, the main goal has been to develop a material to replace metallic fracture fixation implants to overcome problems encountered with non-absorbable implants. The new and old materials invented for fracture fixation appliances have also been tested in other applications. However, during the last decade the materials science has made enormous progress, and nowadays it is aimed to find and tailor an ideal material for every treatment (Suuronen et al. 2000, Rikli et al. 2002).

Advances in materials science have enabled the manufacturing of progressively demanding implants with a complex structure. The mechanical properties and degradation time of the implant can be greatly tailored according to their application. However, all bioabsorbable materials need to meet some criteria to have clinical relevance. The material has to be non-immunogenic, non-toxic, non-allergenic, non-teratogenic, easy, and cost-effective to manufacture and is has to degrade after a reasonable healing time. In addition, the material should induce and guide regeneration of the hard tissues treated.

Autologous bone transplants have long been the only widely acceptable treatment method for extensive bone defects. However, as autologous bone needs to be harvested from another operation site, it increases the patient morbidity, treatment time, and costs of the treatment. This has aroused a need for a substitute which has all the good properties of autologous bone, but does not need a second operation. Despite extensive research in bone tissue engineering, the golden standard in reconstructing bone defects is still autologous bone (Banwart et al. 1995, Goulet et al. 1997, St John et al. 2003).

Various approaches have been taken to reduce the need for autologous bone transplants. Different bioabsorbable filling materials alone or in combination with different bioabsorbabele membranes or plates have been tested. The range of materials is broad, and with some materials, i.e. polylactides and glycolides, the number of studies is huge. None of the tested bioabsorbable materials has been a complete success; so research for new materials continues.

Tyrosine-derived polycarbonates, synthesized from natural molecules found in the body, were first introduced in 1987 (Kohn et al. 1987). The material is amorphous and can be processed with various techniques to different kinds of implants. The broad range of available processing techniques enables the manufacturing of thin implants with high tensile strength. In earlier studies tyrosine-derived polycarbonates have shown *in vitro* to be tissue compatible and they elicit a very modest foreign-body reaction and support the attachment and growth of various cell types (Ertel et al. 1994, Fiordeliso et al. 1994). The material also elicits direct bone apposition when in contact with bone .

Review of the literature

Treatment of bone defects

Cause of bone defects

Bone defects can result from several different causes. A rough division into three categories can be made: 1) congenital abnormalities, 2) trauma, and 3) medical treatment. The latter two are more common than the first.

Congenital bone defects can result from defective genes regulating, for example, osteoclasts (Takeshita et al. 2002). The underlying disease needs to be treated first, but treatment of the bone defects is often necessary to rehabilitate the patient.

It is generally known that high impact trauma on bones can result in comminuted fracture and tissues can become contamined with soil or bacteria. In such patients contamined bone fragments need first to be removed, the area debrided, and, finally, replaced to aid healing. The fragments should be fixated to enable healing.

A very common cause of a bone defect is tooth extraction. The extraction results in a defect shaped as the roots of the tooth. In molar teeth the defect can be large. Also, in some cases drilling of bone is required to remove the whole tooth to be extracted. What is generally not so well known is that persistent infection around the tooth or at the apex of the tooth leads to bone tissue resorption. The edentulous alveolar ridge may undergo severe resorption during the years following tooth extraction. The resorption needs to be treated to restore the masticatory function of the patient. Cancer can also affect the bone directly or spread from soft tissue into the bone. During cancer ablation bone often needs to be removed and later replaced to aid the final healing of the tissues.

Healing of bone tissue

The healing of a bone defect starts with a three-dimensional process where capillaries and perivascular tissues grow into a blood clot. Osteoprogenitor cells differentiate into osteoblasts and start the formation of new bone (Goldberg et al. 1987). However, a bone graft or an implant can be used to enhance the process. The material used in these cases is called osteoconductive.

Osteoinduction refers to a process where host mesenchymal cells differentiate into osteoprogenitor cells and initiate either direct ossification or endochondral ossification. This starts with local stimulating factors, called bone morphogenetic proteins (BMP) (Urist 1965). If the defect is occupied with faster growing soft tissue, the process is inhibited (Goldberg et al. 1987). Therefore bone grafts, substitutes, and membranes have been used to prevent the soft tissue invasion. Bone formation by osteoinduction is, however, a far more complicated process including the action of systemically and locally produced growth factors, hormones, and vitamins. Different factors contribute to the process at its different stages. The BMPs are the main factors initiating the morphogenetic phase which consists of cell disaggregation, migration, reaggregation and proliferation and is essential to new bone formation. Cytokines, hormones, and nutritional factors of the environment are involved in the cytodifferentiation phase causing the formation of mature functional tissue. Growth factors which contribute to the process are, in addition to BMPs, platelet-derived growth factor (PDGF), transforming growth factor-b (TGF-b), insulin like growth factor-1 (IGF-1), epidermal growth factor (EGF), and basic fibroblastic growth factor (BFGF). The exact mechanism by which the different molecules contribute to this process is highly complicated. However, if the relation of molecules to one another is not correct, the whole process is affected (Aaboe et al. 1995, Solheim 1998). The concentration of BMPs in bone transplants is not always high enough, and therefore techniques to collect and purify the proteins have been developed. The purified extract can be administered directly into a graft or incorporated into carrier materials to achieve therapeutic level concentrations (Aaboe et al. 1995). Different members of the BMP family have been studied with a special emphasis on human recombinant forms of BMP-2 and -7 (Cacciafesta et al. 2001, Jung et al. 2003, Kontaxis et al. 2004, Southwood et al. 2004).

Bone substitutes

Autogenous bone transplant is still considered the most suitable grafting material due to the natural transfer of osteoprogenitor or osteoblast cells and the osteoinductive capacity of bone in a given defect (Arrington et al. 1996). Additionally, as a scaffold, it serves as mechanical support to the forming new bone. However, the bone graft can undergo unpredictable resorption. The exact nature of this phenomenon is not yet known (Aaboe et al. 1995), but it will result in a compromised reconstruction. Allogenous and xenogeus bone grafts have been mainly studied in large defects. Their use is partly limited by the risk of the immunological response of the host. The risk of viral or other contaminations of allogenic material should not be forgotten either (Simonds et al. 1992, Aaboe et al. 1995, Conrad et al. 1995).

Especially in the cranio-maxillofacial region only a small amount of bone is often needed. Therefore a number of filtering devices have been developed to

collect bone powder aquired during drilling of the bone, for example, in dental implant treatment. This has been an efficient and convenient way to harvest small amounts (0.8 – 1.5 g) of autologous bone (Kainulainen et al. 2006).

Platelet rich plasma (PRP) has been used with autologous bone to reconstruct the mandible. The administration of PRP in the transplant was considered advantageous (Fennis et al. 2002, Fennis et al. 2004). However, controversies exist. It has been reported that adding PRP into an autologous bone transplant delayed new bone formation in an animal study (Choi et al. 2004). In a consecutive *in vitro* study it was suggested that the difference in the treatment outcomes depends on the varying PRP concentrations. It has been shown *in vitro* that PRP concentrations over 5 % affect negatively the viability and proliferation of alveolar bone cells. Furthermore, concentrations between 1 and 5 % have been shown to stimulate alveolar bone cells *in vitro* (Choi et al. 2005).

Microvascular grafts can be used to reconstruct extensive defects resulting, for example, from cancer ablation or major trauma. However, the operation requires skilled personnel as well as specialized equipment and facilities, and the risk of failure also exists in the procedure (Wei et al. 2003). The risks and morbidity of a second (donor) operation site need to be considered (Richardson et al. 1997). However, new techniques to reduce donor site morbidity are being developed (Avery et al. 2001).

With synthetic bone substitutes researchers aim to overcome the problems encountered with autologous bone transplants. The amount of suitable bone to harvest is limited, and a second operation site increases patient morbidity. The treatment time is also prolonged. Furthermore, the social and economic impact must not be forgotten (St John et al. 2003). Several synthetic bone substitutes have been studied of which hydroxyapatite (HA), collagen sponges, and bioactive glass are in routine clinical use (Bucholz et al. 1989, Delloye et al. 2003, Geiger et al. 2003, Aitasalo et al. 2004).

Another benefit with synthetic biodegradable materials is the possibility to incorporate a variety of locally active molecules into the material in the manufacturing process (Saito et al. 2005). These molecules are then released into tissues, while the implant degrades. By selecting a material with the right degradation kinetics the concentrations of the molecules can be maintained at therapeutic levels. Good results have been achieved with human recombinant bone morphogenetic protein-2 (rhBMP-2) in augmenting the alveolar ridge (Barboza et al. 2004). Anticancer drug therapy combined in bioabsorbable material is presently being developed (Cheung et al. 2005).

Favourable results have been achieved with demineralized bone powder (DBP), lyophilized bone chips, un-demineralized bone powder (BP) (Kaban et al. 1981), polyphosphazene microspheres (Veronese et al. 1999), and homogenous demineralized dentin matrix (HDDM) (Carvalho et al. 2004).

Bioactive glass (BAG) composites and ceramics in different forms and particle sizes have been studied as possible fillers in hard tissue defects since the 1970s (Greenlee et al. 1972, Hench et al. 1973, Clark et al. 1976, Hench 1990, Brink 1997, Chan et al. 2002, Kokubo et al. 2003). Until recently, there have

been two main problems associated with the BAGs. 1) The use of bioactive glasses has been restricted in non-load-bearing applications due to their brittleness. As a solution to this problem polymer-based composites with bioactive glass have been developed (Ruuttila et al. 2005). 2) The other problem has been the narrow working range which has limited the use of different forming methods and available products. Brink (1997) has presented a possible solution and a totally new range of bioactive glasses to overcome this problem.

Polylactides, widely used in bone fixation devices, have also been tested for use as bone substitute. However, the material has been found to degrade too slowly for this application (Wallkamm et al. 2003). Possibly the newest materials invented are the injectable composites which are settled into the cavity after a certain time. Good results have been achieved with a combination of bioactive glass and poly(epsilon-caprolactone-co-D,L-lactide) in rabbits. Osteoconductivity and easy handling of the material have also been confirmed (Aho et al. 2004b).

Inorganic bovine bone biomaterial has been studied in animal and human models in bone defects which were covered with bioabsorbable collagen membranes. The material was considered highly osteoconductive establishing excellent bone healing even without a membrane. The implant material could still be detected after two years of the follow-up (Artzi et al. 2003, Ersanli et al. 2004).

New bone substitutes are continuously being developed, and even Juniperus communis wood has been studied as possible bone substitute. Surprisingly, it displayed good acceptance by the tissues in rabbits up to three years, indicating bone apposition, abutment into pores, and growth into drilled cavities (Gross et al. 2003). Natural products harvested from the sea have also been studied as bone substitutes. The use of fluorohydroxyapatite obtained from sea algae, coral granules from sea corals, and skeletal elements from the seastar have been reported (Sándor et al. 2003, Martina et al. 2005, Simunek et al. 2005).

Protective membranes

Protective membranes for guided bone regeneration purposes have been developed to inhibit the fast regenerating soft tissues from invading the bone defect. It has been shown that bony healing is impaired if the defect becomes occupied by soft tissues (Goldberg et al. 1993). The membrane must be strong enough to withstand the pressure created by overlying tissues. Collapse of the membrane may lead to a diminished volume of bone regenerate jeopardizing the final clinical outcome (Schliephake et al. 2000).

The protective membranes can be either bioabsorbable or non-absorbable. However, in cases where the size of the defect exceeds the critical size the capacity of the osteoblast to form bone is also exceeded. In such cases no inert membrane is able to ensure complete bony healing (Aaboe et al. 1995).

Initially, the search for biomaterials concentrated on biologically inert materials and stable materials. In the 1980s it was concluded that "truly biologically inert" materials do not exist and certain interactions between tissues and implant material can be beneficial (Pulapura et al. 1992). Therefore, currently a broad range of synthetic bioabsorbable materials with both osteoconductive and inductive properties have been developed.

Several materials have been tested to aid bony healing including non-absorbables such as polytetrafluoroethylene (PTFE), alkali-cellulose, and bioabsorbables such as polyphosphazene, collagen, alginate, polylactic acid, polydioxanone, and polyglycolic acid (Antikainen et al. 1994, Ashammakhi et al. 1994a, Bosch et al. 1995, Vasconcelos et al. 1997, Veronese et al. 1999, Dietz et al. 2001, Salata et al. 2001, Baumann et al. 2002, Loos et al. 2002, Ueyama et al. 2002, Oh et al. 2003, Rothamel et al. 2005). However, in some cases the usefulness of the material has not been confirmed (Loos et al. 2002, Ueyama et al. 2002). A major problem in the oral cavity is also premature membrane exposure when treating periodontal bone defects. However, this phenomenon can be seen with both bioabsorbable and non-absorbable materials. Some materials may enhance the bone regeneration even if exposed to oral microbes (Moses et al. 2005).

Non-absorbable materials

The term "non-absorbable", in this context, refers to materials which are not processed or excreted by tissues. In some cases these materials need to be removed from the body after enough healing is accomplished. Non-absorbable materials include metallic and synthetic devices.

The most commonly used non-absorbable material in bone surgery is titanium. It has been considered bioinert, i.e. it does not dissolve in tissues and generate any kind of cellular response. Recently this has been proved not to be completely accurate. Release of metallic ions has been described and these particles have been detected in local lymphatic tissues. The foreign particles are suggested to increase the risk of cancer (Assad et al. 2002). Despite the risk, no routine removal is recommended unless complications are encountered, since surgery and anaesthesia also create a risk for the patient (Howell et al. 1998, Jalonen 2004).

Nevertheless, several reasons can lead to the decision to remove metallic devices. Especially in the northern countries cold winter can cause uncomfortable feelings of cold in the craniofacial area. Metallic devices can also be palpated through thin skin or they can cause eyeglass pads to irritate the tissues overlying the implant (Iizuka et al. 1992, Schmidt et al. 1998). In the growing children non-absorbable implants may disturb the normal growth pattern and therefore need to be usually removed after the bone has healed enough to bear the mechanical stress (Peltoniemi 2000). A second operation is

always a risk to the patient and it also increases the costs and duration of the treatment (St John et al. 2003). While removing the device, the periosteum has to be stripped and this might cause resorption, especially in the easily resorbed bones of the dentofacial area (Stoffel et al. 2000).

While new imaging systems have been developed, metallic implant devices have become an increasing problem. They cause distortions and artifacts in imaging the operated area in x-ray, computer tomography (CT), and magnetic resonance (MR) imaging. With bioabsorbable materials this is not the case; on the contrary, MR imaging can be used to evaluate possible fractures in implants (Lohman et al. 1999). Even ultrasound can be used to evaluate the state of the implant (Heidemann et al. 2002).

Metallic devices have been shown to migrate into the bone and even dura mater after healing (Kosaka et al. 2003). In long bones they may weaken the bone and result in new fractures (Suuronen et al. 2000). This leads to problems especially in the growing calvarium and, if left untreated, even to deformities (Peltoniemi 2000). In several studies bioabsorbable materials are considered more suitable when treating growing bones (Eppley 2000, Peltoniemi 2000, Wiltfang et al. 2000).

Even though titanium is the most commonly used material in maxillofacial bone fixation, the most used membrane to cover bone defects in the dentofacial area is expanded polytetrafluoroethylene (ePTFE) (Buser et al. 1996, Becker et al. 1999). It is a synthetic, chemically and biologically inert polymer which does not degrade by the enzymes in the body (Becmeur et al. 1990).

Recently non-absorbable implants which do not possess the mechanical disadvantages of metallic implants have been developed. The non-absorbable fibre-reinforced composite of polymethylmethacrylate and bioactive glass has been successfully used to treat rabbit segmential bone defects (Aho et al. 2004a).

Expanded polytetrafluoroethylene (ePTFE)

Polytetrafluoroethylene was invented by Dr. Roy Plunkett in 1938. The monomer is a gas which is produced by pyrolysis of difluorochloromethane ($CHClF_2$). The polymerization is of complex nature but eventually leads to a very strong binding between carbon and fluorin atoms. The CF_2:s are equidistantly disposed in the polymer. As PTFE is chemically inert, no chemical product is able to degrade the polymer. It is also a biologically inert material and able to resist all enzymatic or microbiological attacks (Becmeur et al. 1990).

The first animal studies using PTFE were reported in 1949 and the first clinical studies in 1962. PTFE has been widely used ever since, both in clinical and animal studies. The material has been tested for diverse applications, and tissue reactions are well documented. Authors have described inflammatory granulomas as well as local and distant migration, but, surprisingly, not cytotoxity (Laustriat et al. 1990). E-PTFE membranes have been used in GBR especially in dentistry (Becker et al. 1994, Buser et al. 1996, Becker et al. 1999).

Membranes have been used with autogenous bone transplants to fixate bone and prevent soft tissue invasion. However, in the oral cavity membranes tend to expose prematurely, usually within three weeks. It was found that the longer the exposed membrane is left in place - even if the clinical signs of infection are absent - the less augmentation is gained (Haas et al. 2000). Lately it has been suggested that the e-PTFE membrane is the membrane of choice if non-absorbable membranes are used (Salata et al. 2001).

Bioabsorbable materials

"Bioabsorbable materials", in this context, refer to materials which degrade in the body to natural metabolites and are eventually excreted out. These materials have to be non-immunogenic, non-toxic, non-allergenic, non-teratogenic as well as easy and cost-effective to manufacture. Physical support for the underlying tissues should also last long enough to enable sufficient healing without collapse of the membrane (Gilding 1981).

A clear benefit compared to non-absorbable implants is that bioabsorbable material is removed by the body after the original purpose has been served. The implant will not remain in the tissues disturbing the natural environment in the future. Also, the future imaging of the body is not disturbed by the implant (Lohman et al. 1999).

Bioabsorbable fixation techniques are considered advantageous in treating the growing skeleton (Kumar et al. 1997). When treating the growing bones with bioabsorbable materials, the mechanical properties and the rate of degradation of the implant are important. If an implant that is too thick and slowly degrading is used, similar problems and tissue reactions as those with metallic implants are encountered (Bahr et al. 1999, Eppley 2000).

The first bioabsorbable implants were considered too big and their handling was not easy (Champy et al. 1992). New manufacturing processes, such as self-reinforcing, have increased the mechanical properties, and the size of implants has decreased. Self-reinforcing is a manufacturing process where the material is heated again near to its melting point and extruded through a hole with a small diameter to orientate all the polymer chains to the same direction. By this method very strong implants can be produced. Some of the materials need no heating to be contoured, and they can be bent into the correct shape in room temperature (Törmälä et al. 1987). As metallic implants also biodegradable implants can be palpable postoperatively. However, this diminishes gradually with the degrading of the material (Edwards et al. 1998).

Various studies have shown that tissue-active molecules can be incorporated into bioabsorbable polymers during the manufacturing process. They include antibiotics, anti-inflammatory drugs, hydroxyapatite (HA), bioactive glass, bone morphogenetic proteins (BMPs), calcitonin, and platelet-derived growth factor (PDGF) (Agrawal et al. 1995, Jacob et al. 1997, Park et al. 1998, Veronese et al.

1999, Park et al. 2000, Aldini et al. 2001, Heidemann et al. 2001, Tiainen et al. 2002, Pyhältö et al. 2005, Saito et al. 2005). However, controversy still exists regarding their effect on biocompatibility (Heidemann et al. 2001).

In the oral cavity a major problem has been the exposure of the membranes. Therefore bioabsorbable membranes have been studied according to their ability to create a barrier against oral bacteria. Bacterial colonization *in vitro* through the PLA/PGA membrane was proved in less than four weeks (Simion et al. 1997). However, PLA combined with N-methyl-2-pyrolidine (NMP) has been shown to inhibit bacterial colonisation on the membrane (Chogle et al. 2003).

Polyglycolide (PGA)

The first reports on polyglycolic acid are found in the literature as early as in 1893. A plastic form of this polymer was first synthetisized in the 1950s by Higgins (1954) and Schneider (1955). This form of polyglycolic acid is important for the biotechnology. In 1971 Kulkarni and his co-workers reported how to synthesize high-molecular-weight polymers from cyclic diesters, from which bioimplants can be manufactured.

PGA is a hard, crystalline polymer which has a melting point of 224–226 °C and a glass transition temperature (T_g) of 36 °C (Törmälä et al. 1998). It does not dissolve in most polymer solvents. However, it is soluble in, for example, hexafluoroisopropanol and hexafluoroacetone sesquihydrate (Schmitt et al. 1973). The molecular weight of PGA must be between 20000 and 145000 D to fabricate fibres (Frazza et al. 1971).

In vivo degradation results from simple hydrolysis and occurs in two distinct phases: 1) in approximately six weeks the implant loses its ability to endure mechanical stress, 2) between three and 12 months the implant degrades gradually. According to *in vitro* studies, enzyme activity, buffer solutions, pH of the surroundings, sterilization, and sample handling all have an effect on degradation (Vasenius et al. 1990, Böstman et al. 1992).

The first reports on the foreign-body reactions and cytotoxity of polyglycolic acid implants were published in the 1970s (Dardik et al. 1971, Böstman et al. 1990, Böstman 1991). All of these confirm the minor nature of the reactions. Most complications have associated with fast degradation of PGA, which caused an uncomfortable swelling and sterile fluid accumulation at the implant site. The complications mainly appear in adults after a few months of implantation when the degradation is at its fastest. Normal healing has been achieved after treating the sterile cysts with simple incision (Rozema 1991).

The degradation of PGA implants *in vivo* is fast, and therefore the adjacent tissues are not capable to metabolize effectively with all the free monomer being released. This might lead to local drop of pH. The local low pH values can cause tissue necrosis around the implants and eventually lead to a failure of implantation. A typical non-specific foreign-body reaction and osteolytic widening of the implant cavity described is associated with an increase in

osmotic pressure. The raising osmotic pressure causes PGA particles to spread wider in the tissues (Böstman et al. 1992). Characteristic of this reaction is giant cell proliferation, which is at highest between three and six weeks and has a peak in macrophage expression after 12 weeks. Fewer polymorphonuclear granulocytes and mononuclear round cells have been seen (Päivärinta et al. 1993). Normal clinical findings associated with the foreign-body reactions have been local oedema and pain. The bacterial cultures are negative and C-reactive protein (CRP) normal, which confirms the local nature of the reaction. The predominate cells in the infiltrate are inflammatory monocytes and lymphocytes, which verifies that the process is not an infection (Santavirta et al. 1990). This reaction is suggested to be more prevailing and common in so-called high metabolism tissues, for example, in connective tissues (Casteleyn et al. 1992).

Only a few reports exist on the use of PGA and SR-PGA in membranes. In the mid-1990s the material was tested in animal experiments for suitability in covering osteotomy sites (Ashammakhi et al. 1994a) and experimental craniosynostosis (Antikainen et al. 1994). PGA membranes were found to elicit modest foreign-body reactions and had a positive inductive effect on bone formation (Ashammakhi et al. 1994b, Ashammakhi et al. 1995a). The membranes were also found to lose their mechanical strength faster *in vitro* than *in vivo*. This was thought to be due to the formation of fibrous tissue around and inside the implant (Ashammakhi et al. 1995b). The membranes degraded completely by 30 weeks *in vivo* (Ashammakhi et al. 1995a). Despite the promising results with tissue reactions, research among the PGA homopolymer membranes has been slowing down. By combining PGA with other polymers more favourable degradation kinetics have been achieved in new copolymers.

Polydioxanone (PDS)

Polydioxanone is a colourless, crystalline polymer. It has a T_g of -16°C. In this temperature the material is brittle and solid. The melting point of the polymer is 110°C. In room temperature the material is rubber-like and resilient (Vainionpää et al. 1989). Therefore its applications are mainly in sutures, membranes, and thin plates.

The monomer p-dioxanone is analogous to glycolide, but it forms a poly (ether-ester) bond. The *in vivo* degradation of PDS occurs hydrolytically in seven to 12 months. However, the mechanical strength is gradually weakened so that 80 % remains after six weeks and 60 % after eight weeks (Hutmacher et al. 1996). There are only a few foreign-body reactions reported in the literature, but the material can cause transient osteolytic reactions (Kontio et al. 2001). Gamma sterilization has been shown to weaken the mechanical properties, as it does nearly with all polymers. Enzymes do not seem to participate in the degradation of PDS (Vainionpää et al. 1989).

The studies on PDS membranes concentrate mainly on the repair of the orbital wall and the use in periodontal surgery. Promising results have been

reported in the treatment of periodontal intrabony defects with PDS membranes (Dörfer et al. 2000, Christgau et al. 2002). Although more membrane exposures are reported with PDS than with controls, the authors have concluded that the material provides favourable outcome in the treatment of periodontal defects. A similar outcome has been reported in the treatment of implant dehiscence in dogs. The control groups have had fewer membrane exposures and better bone regeneration results (Schlegel et al. 2000).

PDS has been used in the repair of blow-out fractures and in the reconstruction of internal and medial orbital walls. Initial results in clinical studies have been promising (Iizuka et al. 1991, Dietz et al. 2001, Baumann et al. 2002). However, limitations in the treatment of large defects have been clear (Iizuka et al. 1991, Baumann et al. 2002). The amount and shape of new bone as well as the orbital volume have been considered unsatisfactory. Therefore an autologous bone graft is still considered a better alternative (Kontio et al. 2001). On the other hand, there has been success in treating extensive experimental defects of the orbital wall in guinea pigs. Irritation of the surrounding tissue and foreign-body granulomas have, however, been reported (Merten et al. 1994).

Polylactide (PLA)

Polylactide implants have been in clinical use since the 1990s. They have been used in the craniofacial area as osteofixation plates and screws in treating facial bone fractures, fixating osteotomies, protecting bone graft harvest sites, fixating craniectomy segments, guiding soft tissue growth, and in plastic surgery in treating congenital deformities (Suuronen et al. 1994, Weisberger et al. 1997, Enislidis et al. 1998, Moe et al. 2001). Bioabsorbable screws and sutures have been used in intermaxillary fixation (Eppley 2000). There are several reports on their use in orthopaedic surgery (Böstman 1991, Mosier-Laclair et al. 2001). Growing bones have also been fixated successfully (Tharanon et al. 1998).

As a pure polymer polylactic acid has a T_g of 57 °C and a melting point of 174 °C (Vert et al. 1981). A polymer used to manufacture bioimplants must have a molecular weight of 250–530 kD (Eling et al. 1982). PLA has four different forms depending on the orientation of the molecule: D- and L-antipodes, DL-isomer, and DL+LL copolymer (Holten 1971).

In the literature there are similar reports on the problems with PLA implants as with PGA (Bergsma et al. 1995a). A sterile abscess formation and loosening of the fixation screws have been reported (Mosier-Laclair et al. 2001). They have been suggested to result from the exceeded metabolism capacity of the tissues and the accumulation of the degradation products. The problems, however, are mainly local and therefore seen around the implants. Reported reactions usually relate to homopolymeric implants (Törmälä 1992). The reporting of the complications has, however, tended to be incomplete. Reports on adverse tissue reactions are rare today, because the focus has moved on to co-polymers (Hoffmann et al. 2002).

In foreign-body reactions the material particles are typically surrounded by a tight fibrous capsule where small crystalline particles are seen inside different cells (Tams et al. 1996). Polymer particles with a high grade of crystallinity have been detected as late as after five years of implantation (Bergsma et al. 1993, Bergsma et al. 1995a). During the degradation process osteolytic reactions around the implants can be present without affecting the radiological or clinical healing (Kallela et al. 1999, Mosier-Laclair et al. 2001). PLA has proved to have osteogenic potential (Nordström et al. 2001). In some cases ossification has not been complete after six months, though the healing has been uneventful (Tatum et al. 1997).

The sterilization of the implants has not been shown to increase the cytotoxity of the material (Cordewener et al. 2000).

The resorption rate of the implant can be increased by heating and chemical pre-treatment (Bergsma et al. 1995b). There is, however, evidence that a long degradation time does not necessarily interfere negatively with healing. The analysis of the results after five years of *in vitro* and *in vivo* degradation of polylactide plates showed that the foreign-body reactions were few and the material was almost completely degraded. The degradation of the implants *in vitro* was found to be faster than *in vivo* (Suuronen et al. 1998).

Poly (desaminotyrosyl-tyrosine-ethyl ester carbonate)

Tyrosine-based pseudo-peptide polymers were first introduced in 1987 by Kohn and Langer (Kohn et al. 1987). Ertel and Kohn describe polymeric amino acid systems that contain non-amide linkage pseudo-peptides. Various tyrosine polycarbonates derived from diverse alkyl esters of desaminotyrosyl-tyrosine can be prepared by condensation polymerization. All polymers manufactured in this fashion are amorphous, and no evidence of crystallinity is seen by X-ray diffraction (Ertel et al. 1994). The physical properties of PDTE carbonate and some traditional materials are presented in Table 1 and the structure of PDTE carbonate in Figure 1.

Tyrosine-derived polycarbonates incorporate two *in vivo* hydrolytically labile bonds in each repeating unit, a carbonate bond that connects the monomer units and an ester bond connecting a pendent chain. The degradation rate and products of the polymer are determined by the relative hydrolysis rate of these two labile bonds. The carbonate bond is hydrolyzed at a faster rate than the pendent chain ester bond (Tangpasuthadol et al. 2000a). According to Ertel and Kohn, the least hydrophobic polymer with a short alkyl ester pendent chain is the most stimulating substrate for cell growth *in vitro* (Ertel et al. 1994).

The shape and porosity of the tyrosine-derived polymer implants do not affect the degradation rate significantly. Since the hydrolytic cleavage of the carbonate backbone is the rate-determinating step in degradation, the surface to volume ratio has only a minimal effect (Tangpasuthadol et al. 2000b). However, the addition of bioactive glass into SR-PDTE carbonate rods has been reported to

enhance the degradation by increasing the porosity and facilitating the bone to penetrate the implant (Pyhältö et al. 2005).

Table 1. *Physical properties of PDTE carbonate (Ertel et al. 1994), PGA, and PLA. M_w in kD, T_g, and melting point (T_M) in (°C). Tensile modulus (TM) in GPa and tensile strength (TS) in MPa. Acid degradation products (ACD) in per gram of device.*

	M_w	T_g	T_M	TM	TS	ACD
PDTE	176	81	amorphous	1.2-1.6	67	2.6
PGA	20-145	36	225	6.5		15.5
PLA	250-530	50-57	170-190	0.6-4	68-75	11.4

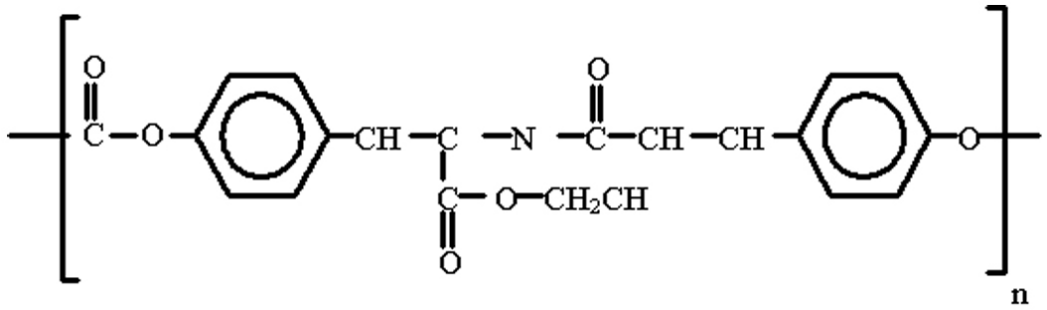

Figure 1. *Chemical structure of PDTE carbonate (Ertel et al. 1994).*

The degradation of the PDTE carbonate polymer is mainly caused by random hydrolytic cleavage of the carbonate backbone. No enzyme or cellular activity is detected to participate in the degradation process (Ertel et al. 1994, Hooper et al. 1998). At neutral or slightly acidic pH, the hydrolysis rate of the carbonate bond is faster than the rate of ester bond cleavage. As the degradation continues, the amount of acidification of the interior of the polymer matrix increases and the further degradation of the polymer can be expected to slow down. Tangpasuthadol et al. (2000a) describe this as the "lag period" of degradation. However, as more pendant chains are eventually hydrolyzed, the reaction rate increases after the pH within the polymer matrix falls below 3. The acid-catalyzed hydrolysis of the ester bonds becomes a dominant factor, and the pendant chain ester hydrolysis outpaces the rate of hydrolysis of the backbone carbonate bonds only below the pH 3. This leads eventually to the disintegration and complete degradation of the implant. However, the degradation process can take up to four years before the resorption is complete (Tangpasuthadol et al. 2000b).

While degrading *in vivo*, PDTE carbonate maintains its shape at least until 1280 days, and changes in appearance (turns opaque) are seen only inside the

material. It has been suggested that the opaque appearance of the implant is related to the small amount of water uptake into the polymer matrix (James et al. 1999). With tyrosine-derived polycarbonates no noticeable swelling is evident; they do not take up more than five per cent of water during any stage of degradation. The lack of water uptake means that tyrosine-derived implants maintain their profile for longer periods than the derivatives of lactide or glycolide and may thus be used in applications where swelling could result in a failure of implantation (Hooper et al. 1998, James et al. 1999).

Besides the water uptake, another difference between tyrosine-derived polymers and PLA or PGA is related to the beginning of the mass loss. Tyrosine-derived monomers are not readily water-soluble, and the mass loss occurs very slowly at the end of the degradation process (James et al. 1999). It has been suggested that the only degradation products formed will be pendant alkyl alcohol and desaminotyrosyl-tyrosine (Tangpasuthadol et al. 2000a). Even though the material significantly loses molecular weight during degradation, the mass loss is not seen before 1090 days. Moreover, before 180 days of degradation, no significant amounts of degradation products have been detected (James et al. 1999). The degradation of PDTE carbonate to 50 % of the initial M_w takes 366 days compared to PLLA with 157days (Hooper et al. 1998).

The hydrolyzing of the PDTE carbonate pendant chains results in free carboxylate groups on the implant surface. This is a layer of free carboxylate groups that has a potential to act as a nucleation site for the formation of a hydroxyapatite surface layer. According to James et al. (1999), the layer may first lead to direct bone apposition and ultimately to bone bonding. Also, the frequency of direct bone bonding is related to the rate at which the polymer pendant chain is hydrolyzed on the polymer surface. However, a study by Pyhältö and co-workers (2002) does not support this finding. On the contrary, they suggest that bone, granulation tissue, and a connective tissue rim are bone-tissue responses to the injury and operation trauma. Also, micro-movement between the implant and bone seems to increase granulation tissue production.

The acid groups formed during the degradation of tyrosine-derived polycarbonates are polymer-bound and not able to diffuse into the surrounding media (Tangpasuthadol et al. 2000b). Free acid groups cause pH decrease and possible osteolytic reactions around implants. This, however, is not the case with PLA and PGA. The pH decrease can be demonstrated with values of acid degradation products per gram of device. The values for PGA, PLA, and PDTEC are 15.5, 11.4, and 2.6 meq of acid per gram of polymer, respectively (Hooper et al. 1998).

The PDTE carbonate has been tested in different applications in animal models. They include anterior crucial ligament reconstruction (Bourke et al. 2004), cancellous bone fixation in rabbits (Pyhältö et al. 2005), and fixation of femoral osteotomies in rats (Pyhältö et al. 2002).

The PDTE carbonate elicits only a mild inflammatory response in soft tissues. When implanted in soft tissues specimens become surrounded by a fibrous capsule (29-42 μm) containing immature fibroblasts and some

macrophages (Hooper et al. 1998). By 48 weeks of implantation *in vivo* no indicative of cell-mediated immunology has been evident (Choueka et al. 1996).

Compared to the processing temperatures of poly-L-lactic acid (>160°C), relatively low processing temperatures of PDTE carbonate (70-120°C) can facilitate the incorporation of temperature-sensitive biomolecules in implants (Ertel et al. 1994). Tyrosine-derived polycarbonates are stiffer (tensile modulus 1.2-1.6 GPa) than several other biodegradable polymers, the values being comparable to non-reinforced poly (L-lactic acid) (Ertel et al. 1994). For example, the values for other polymers are polycaprolactone (0.5 GPa), poly(ortho esters) (<1GPa), and polyglycolic acid (6.5 GPa) (Daniels et al. 1990). The gamma irradiation of PDTE carbonate with high doses has been shown to cause reduction of the molecular weight (Ertel et al. 1994).

Bioactive glass 13-93

Bioactive glass (BAG) composites as possible fillers in tissue defects have been studied since the 1970s (Greenlee et al. 1972, Hench et al. 1973, Clark et al. 1976). Several studies of different compositions of bioactive glasses have confirmed that the material is useful for enhancing bone growth either as bulk or particles (Hench et al. 1973, Brink 1997). The bone bonding capacity of bioactive glasses depends on the concentrations of ions released. These ions interact with surrounding cells/tissues. Karlsson et al. (1989) have shown that the bond is created when a layer of amorphous silica is present on the bioactive glass. Bone mineralization is proceeding along the collagen fibrils attached to this layer (Hench 1990). According to Hench and Paschall (1973), three factors control bone bonding *in vivo*: 1) Alkaline pH development at the surface, 2) time required for the surface to become alkaline, and 3) the presence of surface-active calcium phosphate sites.

Bioactive glass has been mixed with a variety of other products such as Dextran (Chan et al. 2002), a copolymer of poly (epsilon-caprolactone-co,DL-lactide) (Närhi et al. 2003), and PLA (Ruuttila et al. 2006) with success.

As described earlier, Brink (1997) introduced a new range of bioactive glasses. According to the literature, certain bioactive glasses in the system Na_2O-K_2O-MgO-CaO-P_2O_5-SiO_2 have proved to be capable of bone bonding. These bioactive glasses in this system differ from commercially available Bioglass® by their K_2O-MgO portions. However, if the MgO concentration is less than 7.8 wt-%, their bone bonding abilities are similar to those of the bioactive glasses developed so far (Brink 1997, Brink et al. 1997).

BAG 13-93 (Table 2) differs from the commercially available Bioglass® and BAG 53P4 by the protein absorption behaviour. BAG 13-93 absorbs a broad spectrum of plasma proteins, including albumin, immunoglobulins, and fibrinogen (Brink et al. 1995). The resorption starts within one week, and rods (0.8-1.2 mm) degrade to 50 % of the original mass in six months. During the degradation a large amount of phagocyting macrophages are seen on the surface (Andersson et al. 1990, Brink et al. 1996).

Table 2. *Chemical compositon of BAG 13-93 in weight percentages (Brink 1997) compared to two commercially available and widely studied BAGs.The original 45S5 Bioglass® invented by Larry Hench in the 1970s and a newer BAG 53P4 invented by Andersson et al. (1990). Although BAG 45S5 is approved for use in the whole skeleton, good results have been achieved especially with BAG 53P4 in the craniofacial area.*

	Na_2O	K_2O	MgO	CaO	P_2O_5	SiO_2
13-93	6	12	5	20	4	53
45S5	24.5	0	0	24.5	6	45
53P4	23	0	0	20	4	53

Chitosan (poly-N-acetyl glucosamine)

Chitosan is a polysaccharide which can be produced by N-deacetylation of chitin. Chitin is the naturally abundant supporting material in almost every species, except in mammals. The structure is a series of copolymers of β(1 → 4)-linked glucosamine and N-acetyl-glucosamine. The difference between chitin and chitosan lies in the degree of acetylation (DA). Solubility into organic solvents is dependent on the DA of the polymer, and with values of over 60 % chitin becomes chitosan (Domard et al. 2001).

Chitin can easily be retrieved from crab and shrimp shell waste. It is produced by two consequent chemical treatments. First the calcium carbonate is removed with mineral acid treatment. Thereafter the material is heated in alkaline media to remove proteins. Both treatments need to be completed with care if the material is designed for medical use. Chitosan is also produced from chitin by N-deacetylating chitin in a heated and highly concentrated sodium hydroxide solution. The problem with these chemical treatments is that the polymers which are produced are not always of high molecular weight. Each step, especially acidic treatment, reduces the chain length of the polymer (Domard et al. 2001). Molecular weights of the polymers vary from 50,000 to 2,000,000 D depending on the origin of the chitin (Hejazi et al. 2001).

According to current understanding, mammals do not have a specific enzyme to degrade chitosan. It is degraded *in vivo* mostly by lysozyme if DA is below 30 %; after that the enzymes involved are not known. The degradation time varies and depends mainly on the pH of the surrounding tissues, the DA and M_w, the

degree of crystallinity, the water content, and the shape of the implant (Lee et al. 1995, Domard et al. 2001, Hejazi et al. 2001).

Chitosan is biodegradable and non-toxic. It has hemostatic properties which appear to function independently of the platelets and normal clotting mechanisms (Malette et al. 1983, Klokkevold et al. 1991, Klokkevold et al. 1992, Klokkevold et al. 1999). There are claims that chitosan also induces osteoblast proliferation and mineral deposition *in vitro* (Seol et al. 2004).

Aims of the study

The aim of the present study was to evaluate the suitability of poly (desamino tyrosyl-tyrosine ethyl ester carbonate) membrane in treating artificially created mandibular defects in rabbits. Half of the defects were also filled with bioactive glass. The analysis was carried out by a radiological and histological study of the mandible. PDTE carbonate and bioactive glass (BAG) were implanted in rabbit soft tissues to evaluate the tissue reactions when bone is absent. The osteoconductivity of PDTE carbonate membranes was initially considered insufficient, and therefore BAG mesh was added to fill half of the defects. The addition of BAG was thought to enhance the healing of the defects.

The specific aims of the study were the following:

I. Does PDTE carbonate membrane have enough osteogenic potential to enable healing of experimental osseous defects of the rabbit mandible?

II. Does bioactive glass 13-93 accelerate healing of the defects when used with PDTE carbonate membranes in the rabbit mandible?

III. How is PDTE carbonate membrane interacting with soft tissues when the bone is absent?

IV. What are the tissue reactions to the PDTE carbonate membrane and how long does it retain its original shape?

V. Do the soft tissue reactions of bioglass 13-93 covered with chitosane differ from those of PDTE carbonate?

Materials and methods

This experimental study was approved by The Research Animal Commission of the University of Helsinki and by The Provincial Administrative Board, according to the Finnish law.

Manufacturing of the implants

All the specimens were manufactured in the Institute of Biomaterials (Tampere University of Technology, Tampere, Finland). All samples were sterilized with gamma irradiation, minimum dose 25 kGy (Willy Rütsch AG, Kernen-Rommelshausen, Germany).

Manufacturing of the PDTE carbonate membranes

The batches of PDTE carbonates were supplied by Integra LifeSciences Corporation (New Jersey, USA). These batches with M_w from 200 000 to 220 000 Da were prepared according to previously published procedures (Kohn 1993, Fiordeliso et al. 1994). The materials were stored as powder at -18°C temperature prior to processing in airtight containers. Three days before processing the powder was ground in liquid nitrogen to eliminate larger particles. The homogenized powder was dried in a vacuum chamber at 53°C for 48 hours. Solid plates (85 x 85 x 3.3 mm) were compression-moulded at 165°C from the raw material powder. The moulded plates were biaxially oriented (Fig. 2) in one phase to a draw ratio of 2.2 x 2.2 at 75°C with a plate-stretching machine Karo IV (Brueckner GmbH, Siegsdorf, Germany).

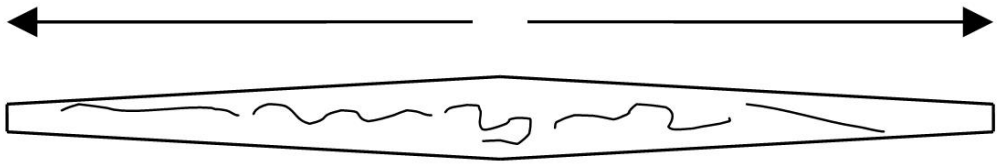

Figure 2. *Schematic drawing of the subsequent orientation step that resulted in the same-sized membranes of varying degrees of orientation and thickness. At the edges of the drawing the molecules are more oriented than in the middle (Asikainen et al. 2006b). (With the kind permission of Springer Science and Business Media)*

Manufacturing of bioactive glass meshes

Bioactive glass 13-93 (BAG) (Vivoxid Ltd., Turku, Finland) was melt spun into filaments having a diameter of 15-30 μm. The filaments were cut to stable fibres of a length of 7 mm. The non-woven (mesh) structure was made from the fibres by an air-laying process with a pilot air-laid sheet former. The non-woven (mesh) structures were bound to sheets with water dispersion of 3 wt-% medical grade chitosan Chitech™ (Medicarb, Bromma, Sweden). The samples from the non-woven were sized 12 x 6 x (2-4) mm (Fig. 3).

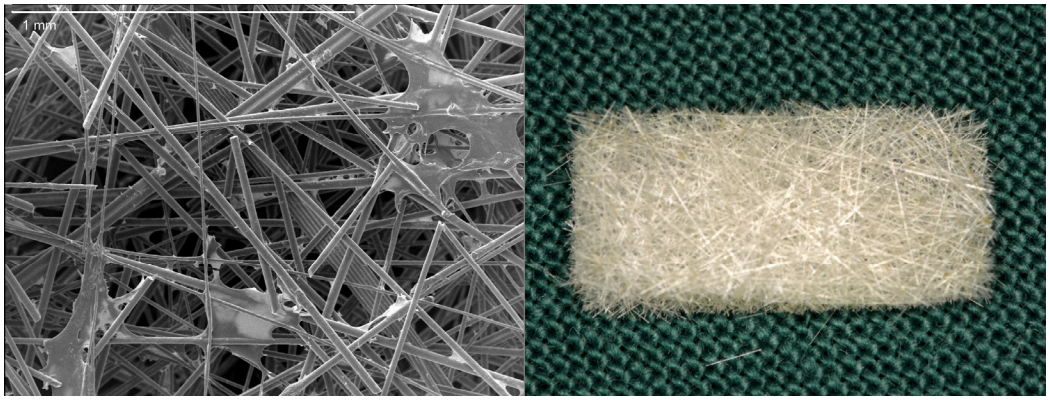

Figure 3. *Bioactive glass mesh coated with chitosan. The fine structure of bioactive glass 13-93 with chitosan can be seen on the left side. Chitosan is adhering to the glass filaments and holding the structure together. The bioactive glass mesh prior to implantation is shown on the right side.*

Experimental animals

Skeletally mature rabbits were chosen as the experimental animals, based on the form of the mandible, structure of the bone, and size of the animal. All animals were clinically healthy and conditioned at least two weeks before surgery. In the experiment, 20 female New Zeeland White Rabbits (HsdPoc strain) were operated. The animals weighed 2500-3000 g. The follow-up of the animals is summarized in Table 3.

Table 3. *Follow-up of the experimental animals and the number of animals at different healing times in the mandibular model.*

Implant type	**Follow-up time (weeks)**			
	6	12	24	52
Membrane + BAG	6[(1)]	6	6	
Membrane only	6[(1)]	6	6	
BAG only				2
Empty				2

[(1)] One animal died due to an anaesthesia complication at 3 weeks.

Pre-, per-, and postoperative procedures

Until operation, all animals were free to move around in their cages and fed ad libitum. Preoperatively, the animals received trimethoprim-sulfadiazine (Duoprim vet®, Schering-Plough, Brussels, Belgium) 0,3 mg/kg subcutaneously (sc) for infection prophylaxis. The anaesthesia was induced with medetomidine (Domitor®, Orion Pharma, Turku, Finland) 300 μg/kg and ketamine (Ketaminol vet®, Intervet International, Boxmer, The Netherlands) 25 mg/kg (sc). Methylcellulose eyedrops (Oftan-MC®, Leiras, Turku, Finland) were used to avoid ocular drying and irritation. For postoperative pain control the animals received 0.02-0.05 mg/kg (sc) buprenorphine (Temgesic®, Schering-Plough) immediately after the operation and every 12 hours for the next two days. After the operation the animals were free to move around in their cages and they were fed ad libitum.

All animals were anaesthesized after three weeks of healing to obtain native radiographs of the mandibles with same medication as described above. For euthanasia, pentobarbital (Mebunat®, Orion Pharma) 30 mg/kg was used intravenously (iv).

Surgical Procedures

Mandible (I and II)

The mandible was shaved on both sides, and the skin was rinsed and scrubbed with chlorhexidine digluconate (Klorhexol® 5 mg/ml, Leiras, Turku, Finland). A skin incision was made along the inferior border of the rabbit mandible. The periosteal flap was lifted, and standardized through-and-through defects (12x6 mm, 72 mm^2) were created with an oscillating saw bilaterally in the mandibular angle (Fig. 4). On the left side the membrane (sized 20x25 mm, thickness 160-300 µm) was mounted and wrapped around the inferior border of the mandibular angle and fixed superior to the defect with absorbable sutures (Vicryl® 3-0, Ethicon, Somerville New Jersey, USA) through holes drilled in the mandible. The periosteal flap was sutured over the implanted membrane, and the incision was closed in layers with absorbable sutures (Vicryl® 3-0). On the right side, the defect was filled with bioactive glass (BAG) mesh (Fig. 5) prior to the fixation of the membrane. In all defects blood clot formation was ensured.

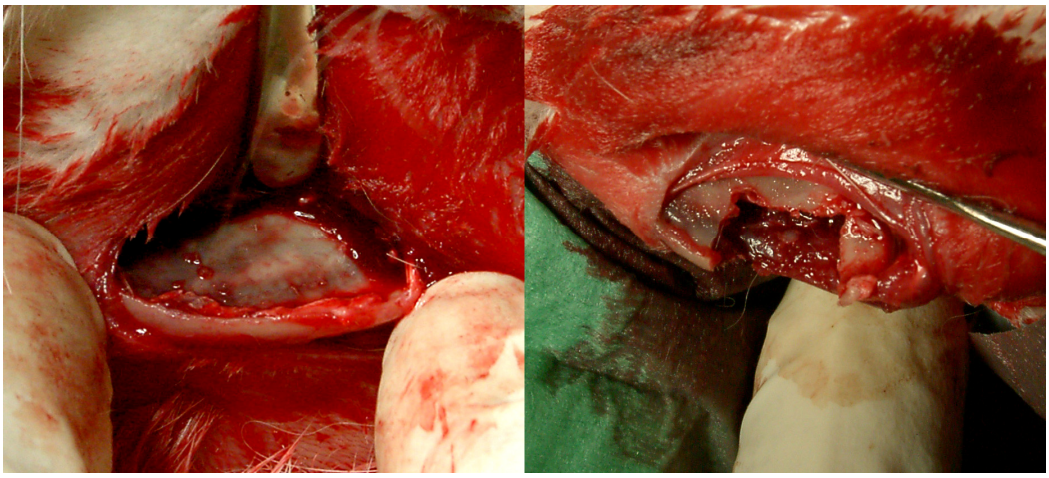

Figure 4. *Rabbit mandible with periosteum stripped is shown on the left side. The empty experimental defect can be seen on the right. The mandible in the angle is very thin.*

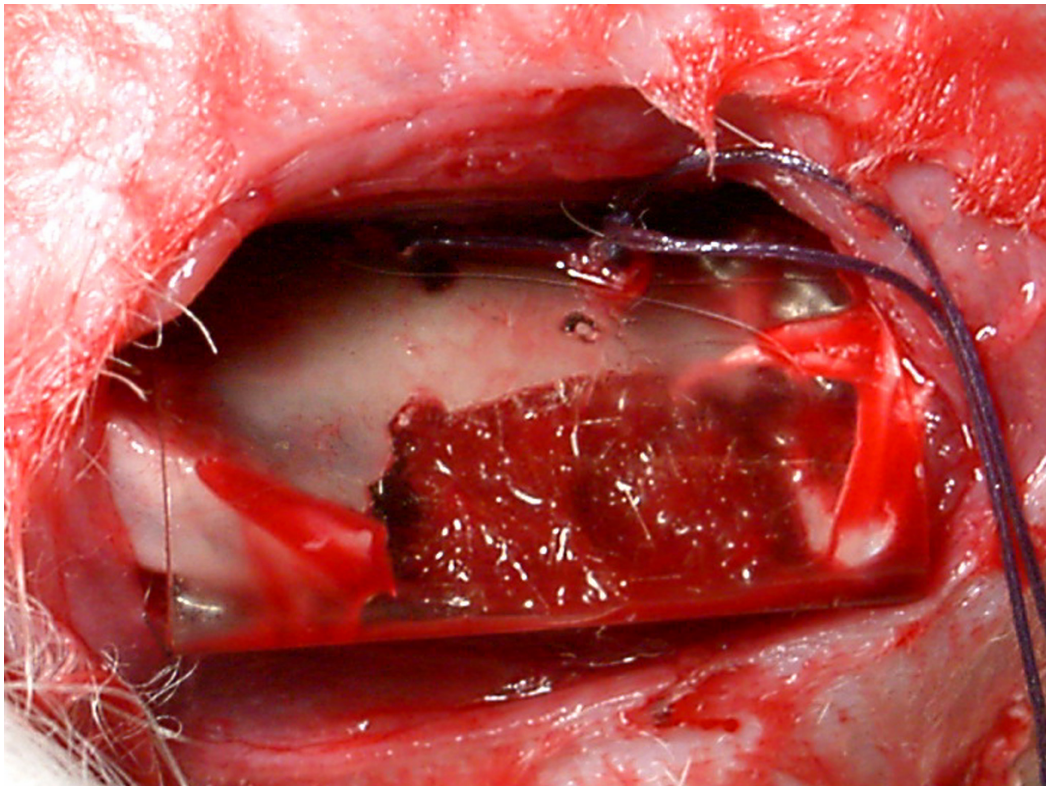

Figure 5. *Implanted PDTE carbonate membrane and bioactive glass 13-93 coated with 3 wt-% chitosan fixed with absorbable suture in the mandibular angle. Correct placement and bioactive glass are easily seen through the transparent membrane (Asikainen et al. 2005). (With the kind permission of Springer Science and Business Media)*

Subcutaneous pouches (III and IV)

Fur was shaved on the back of the rabbit, and the skin was rinsed and scrubbed with chlorhexidine digluconate (Klorhexol® 5 mg/ml). A skin incision was made at the midline, and subcutaneous pouches were created bluntly on both sides. A bioactive glass implant (12 x 6 mm) was placed on the right side and a PDTE carbonate implant (20 x 25mm) on the left side. The incision was closed in layers with absorbable sutures (Vicryl® 3-0).

Immunohistochemical analysis (IV)

Immunohistochemistry was performed with monoclonal MMP-2 antibody (Chemicon International Inc, Temecula, CA, USA, clone 42-5D11) (1 :400 in 2 % bovine serum albumin (BSA) in phosphate buffer saline (PBS)), monoclonal MMP-3 antibody (Chemicon International Inc, clone 148-1A3) (1 :300 in 2 % BSA/PBS), and monoclonal MMP-13 antibody (NeoMarkers, Fremont, CA,

USA, clone VIIIA2) (1:1000 in 0.2 % BSA/PBS), using the VECTASTAIN 1 Elite ABC Kit PK 6101 (Vector Laboratories, Burlingame, CA, USA). The monoclonal antibodies for TIMP-1 (Chemicon International Inc, clone 147-6D11) (1:250 in 2 % BSA/PBS) and for TIMP-2 (Chemicon International Inc, clone 67-4H11) (1:100 in 2 % BSA/PBS) were used as described previously by Choi et al. (2002). A summary of the antibodies and dilutions used is presented in Table 4.

Table 4. *Specification of the antibodies used in the immunohistochemical part of this study.*

Antibody	Clone	Dilution	Manufacturer
MMP-2	42-5D11	1:400	Chemicon
MMP-3	148-1A3	1:300	Chemicon
MMP-13	VIIIA2	1:1000	NeoMarkers
TIMP-1	147-6D11	1:250	Chemicon
TIMP-2	67-4H11	1:100	Chemicon

After normal deparaffinization the tissue sections were treated with pepsin for 45 minutes. Endogenous peroxidase activity was blocked with 0.6 % H_2O_2 in methanol for 30 minutes. The sections were incubated and blocked with 2 % BSA in 10 mM PBS (pH 7.4) and normal horse serum (1:50 VECTASTAIN ABC kit). The sections were incubated with primary antibodies overnight at +4°C in a humid chamber, after which the immunohistochemical assays were performed following the manufacturer's instructions of the VECTASTAIN ABC kit. Positive immunoreactivity was revealed with 3-amino-9-ethylcarbazole (Sigma, St Louis, MO, USA), diluted in N,N-dimethylformamide (Merck, Darmstadt, Germany). Thereafter the tissue sections were counterstained with Mayer's hematoxylin and mounted in DAKO's glycergel (DAKO Corporation, Carpinteria, CA, USA). All incubations were performed at room temperature, and the sections were washed three times in PBS between each step. Replacement of the primary antibody with the immunoglobulin fraction of sera from healthy mice was used as staining control and replacement of the primary antibody with buffer as control the for procedure.

Histological analysis (II, III, and IV)

Subcutaneously implanted BAG samples were freshly dissected and fixed in formalin and embedded in paraffin. Five-µm-thick tissue sections were deparaffinized according to standard laboratory practice and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS).

The rabbit mandibles and subcutaneously implanted poly (DTE carbonate) membranes were freshly dissected, fixed in 70 % alcohol and embedded in methylmetacrylate blocks (Schenk 1965). Five-µm-thick sections were prepared with a Leica SM2500 heavy-duty sliding microtome and stained both with Masson-Goldner thrichrome and hematoxylin and eosin. All sections were analysed with a light microscope to record cellular reactions, membrane placement, new bone formation, and localization.

Radiographic analysis (I and III)

Radiographs were taken of all animals three, six, 12, and 24 weeks postoperatively. The samples from 12, 24, and 52 weeks were also radiographed with a "standard pearl ∅ 5 mm" after dissection, using a Heliodent DS® (Siemens Co, Munich, Germany) unit at 60 kV, 7 mA and 5 ms on a Digora® digital imaging plate (Soredex Co, Helsinki, Finland). The imaging plate was read by a Digora FMX® (Soredex Co) laser scanner. On the digital radiographs a specialist in oral radiology marked areas of visually complete or partial ossification using Adobe Photoshop® 7 (Adobe System Inc, San Jose, USA) software. The marked areas were then calculated with Matlab® (MathWorks Inc, Natic, USA) and divided into two groups: 1) radiographically completely non-ossified areas and 2) completely non-ossified areas together with partly ossified areas (where bone mineralization was not yet radiologically complete).

Material properties (III)

Mechanical testing and molecular weight determination were done in the Institute of Biomaterials, Tampere University of Technology, Tampere, Finland.

The weight average molecular weight (M_w), number average molecular weight (M_n) and polydispersity (PD) of the poly (DTE carbonate) membranes were studied using conventional gel permeation chromatography (GPC) with narrow polystyrene standards using chloroform as solvent and eluent. The equipment consisted of a differential refractometer detector (Waters 410 RI) and

and an HPLC pump (Waters 515). The injection volume of the samples was 150 µl and the flow rate in the columns 1 ml min^{-1}. Each data point presents the mean of four measurements taken from two samples at the same time point.

The biaxially oriented membranes were die-cut to achieve samples for tensile testing (Fig. 6). The tensile strengths of the membranes were measured following the standard ISO 527. The samples were in the form of a standard tensile specimen, except the size 5 x 50 mm. The membranes were tested for their tensile strength with an Instron 4411 (Instron Ltd, High Wycombe, England) materials testing instrument using a crosshead speed of 10 mm/min.

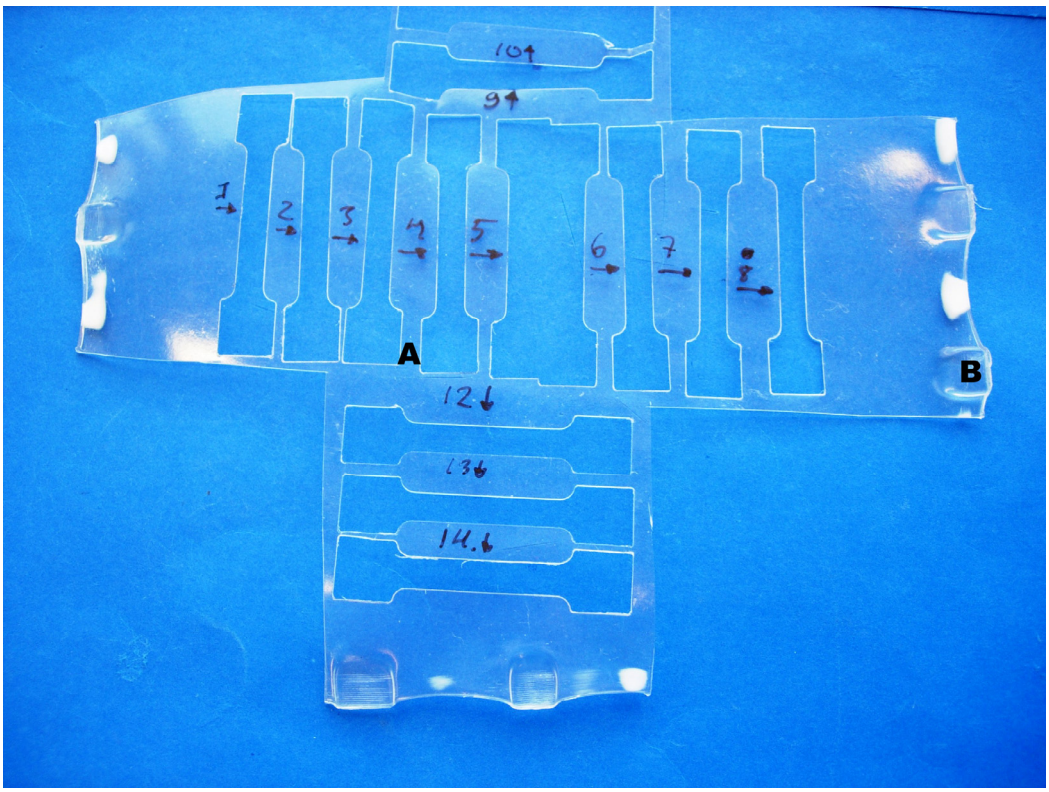

Figure 6. *Biaxially oriented plate, samples for mechanical testing removed. By decreasing the thickness of the implant results in an increased orientation inside the structure of the implant (A). Grip positions of the biaxial stretching machine are shown (B) (Asikainen et al. 2006b). (With the kind permission of Springer Science and Business Media)*

Statistical analysis (I)

The paired samples T-test was used for statistical evaluation to test the hypothesis. The working hypothesis was that the use of bioactive glass enhances the ossification in defects. Ossification on the left and right side of the mandible was compared to test the statistical significance.

The summary of the procedures and methods of analysis in the original articles is presented in Table 5.

Table 5. *The summary of procedures and methods of analysis used in the original articles of the study.*

	I	II	III	IV
Mandibular model	X	X		
Subcutaneous model			X	X
Immunohistology				X
Histology		X	X	X
Radiographical analysis	X	X		
Material testing		X		
Statistical analysis	X			

Results

Radiographical analysis (I)

The plain radiographs could reveal only a part of the actual ossification at the mandibular angle of the rabbits. This was due to the difficulties in positioning the animal. Therefore a different method was chosen to analyse the ossification. The dissected mandibles were radiographed perpendicular to the long axis of the mandible.

Three weeks

At three weeks all the wounds had healed properly, and clinically no signs of infection were seen. All animals were eating normally. Plain radiographs were taken. However, no reliable conclusions regarding the ossification could be made based on the radiographs.

Six weeks

Clinically no signs of infection were seen. After dissection a small clear fluid-filled cyst was seen in the left mandible of one rabbit (membrane only). This, however, did not seem to have any significant effect on the ossification process evaluated from the plain radiographs. No reliable conclusions of the size of the ossified defect area could be made from the plain radiographs.

12 weeks

Clinical inspection revealed no signs of infection. The radiographic analysis of the specimens (n=6) revealed that on the right side (membrane + BAG) 13.6 % (range 0.0-26.9 %) of the defect area remained non-ossified and 44.6 % (range 12.3-84.7 %) was fully ossified. On the left side (membrane only) 4.5 % (range 0.0-16.6 %) was non-ossified and 58.7 % (range 30.7-100 %) was fully ossified. The mean difference between the non-ossified areas calculated was 6.6 mm^2 (standard deviation 4.0 mm^2), which was statistically significant ($p<0.05$).

24 weeks

No signs of infection were clinically evident in any animal. The radiographic analysis of the specimens (n=6) revealed that on the right side (membrane + BAG) 10.7 % (range 0.0-31.4 %) of the defect area was non-ossified and 47.1 % (range 27.7-100 %) was fully ossified. On the left side (membrane only) 0.8 % (range 0.0-5.1 %) was non-ossified and 58.5 % (range 44.3-100 %) was fully ossified. The mean difference of the non-ossified areas was 7.1 mm^2 (standard deviation 4.1 mm^2), which was statistically insignificant ($p>0.05$). The summary of the data is presented in Figure 6.

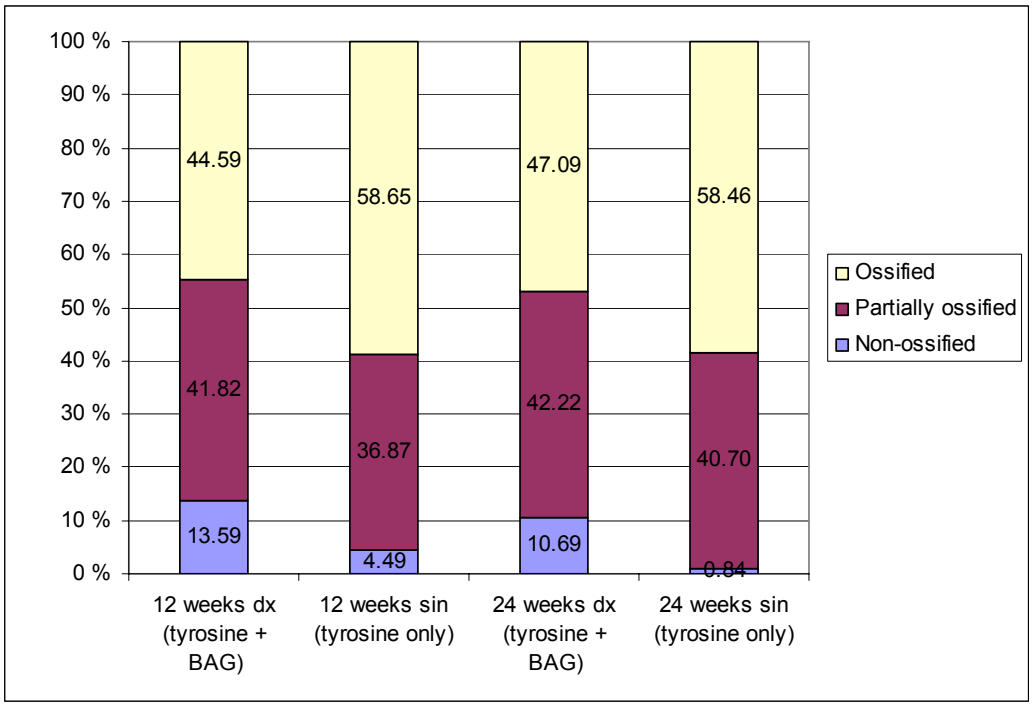

Figure 7. *Summary of the radiographical analysis. On the PDTE carbonate-treated side (tyrosine only) ossified areas calculated from the radiographs remained almost at the same level after 24 weeks as after 12 weeks. However, the partially ossified area diminished to almost zero in 24 weeks (Asikainen et al. 2005). (With the kind permission of Springer Science and Business Media)*

Histological analysis of the mandibular samples (II)

The fixation of the membranes was considered good intraoperatively in all cases. However, the histological sections revealed that in only five out of 18 cases the membrane was situated where it was originally placed.

Six weeks

One animal in this group was lost due to anaesthesia complications at three weeks when radiographs of the defects were carried out. Clinically no signs of infection were seen in any animal. After dissection a small clear fluid-filled cyst was detected in the left mandible of one rabbit (membrane only). However, the histological inspection revealed an inflammatory cell infiltrate with lymphocytes and plasma cells in the membrane surrounding the capsule in three out of five cases in the membrane-only group and in two out of five cases in the BAG + membrane group. No foreign-body reaction with giant cells was present in any sample.

In all five cases, new bone formation was detected in the membrane-only group inside the membrane at the site of the defect. New bone formation was seen at the site opposite to the defect only in one case. In the membrane + BAG group new bone formation was seen in three out of five cases. In one case the inflammatory cell infiltrate with lymphocytes and plasma cells was notably greater than in the others. In this case no new bone formation was detected on either site of the membrane. In one case new bone formation was seen only on the outside of the membrane.

12 weeks

Clinical inspection revealed no signs of infection in any animal. An inflammatory cell infiltrate with lymphocytes and plasma cells was present in three out of six cases, in the membrane-only group, and in two out of six in the BAG + membrane group. In one animal, at both sites, there was notably more inflammatory infiltrate with lymphocytes, plasma cells, and occasional macrophages than in the others. No foreign-body reaction with giant cells was present in any sample.

New bone formation in the membrane-only group was detected on the outside of the membrane in all six cases, and in the defect site in three out of six cases. In the membrane + BAG mesh group new bone formation was seen in three out of five cases on the outside and in one inside the membrane. One sample was unfortunately lost from the histological studies due to the problems in the polymerization of the methylmetacrylate.

24 weeks

No signs of infection were clinically evident in any animal. An inflammatory cell infiltrate with lymphocytes and plasma cells was seen in the membrane-only group in one out of six cases and in the BAG mesh + membrane group in two out of six cases. No foreign-body reaction with giant cells was present in any sample.

New bone formation was seen in the membrane-only group inside the membrane in three out of six cases and on the outside in all but one case. In the membrane + BAG mesh group new bone was detected inside the membrane in five out of six cases and on the outside in four out of six cases.

52 weeks

No signs of infection were clinically evident in any animal. Histologically no inflammatory infiltrate was seen. No foreign-body reaction with giant cells was present in any sample. No new bone formation was detected in any sample, either. Radiologically the defects were not ossified, and the edges of the defects were rounded (Fig. 8). This confirms the critical size of the defect.

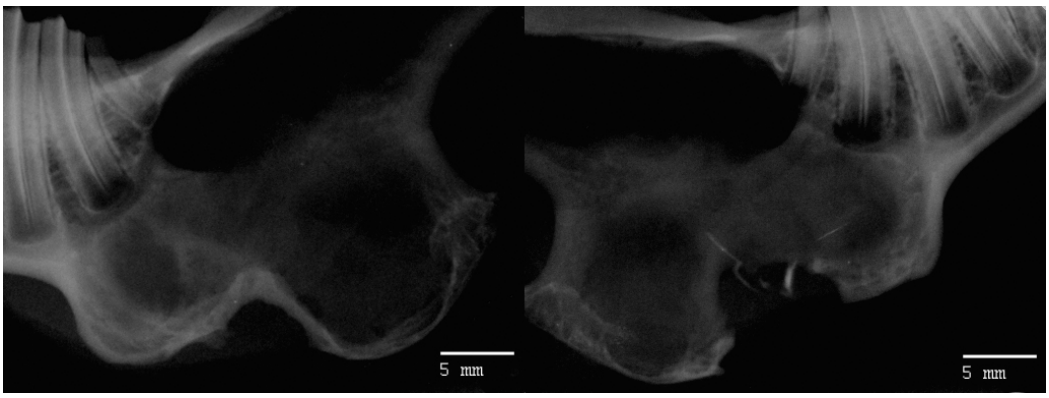

Figure 8. *Radiographs of the controls after 52 weeks of healing. The mandible on the left side was neither covered with membrane nor filled with BAG. On the right side a BAG-treated mandible is shown. The edges of the defects are rounded in both cases, and the ossification is not complete (Asikainen et al. 2006a). (With the kind permission of J Royal Society Interface)*

Table 6. *Summary of the radiological and histological analysis of the mandibular samples after six, 12, and 24 weeks. (+) moderate amount detected, (++) slightly more detected, and (-) not detected. If no value, the sample has been lost from the study. Inflammation (IF), membrane attached (MA), radiologically non-ossified area in per cent (NO), and radiologically partially ossified area in per cent (PO)*

		PDTE carbonate membrane						**membrane + BAG 13-93 mesh**					
					new bone formation						new bone formation		
		IF	MA	inside	outside	NO	PO	IF	MA	inside	outside	NO	PO
6	1	+	-	+	-			+	-	+	-		
weeks	2	-	-	+	-			++	-	-	-		
	3	-	-	+	-			-	-	-	+		
	4	+	-	+	-			-	-	+	-		
	5	+	-	+	+			-	-	+	-		
12	1	++	-	-	+	0	69.4	++	-	+	++	12.2	53.6
weeks	2	+	+	+	+	0	53.5		-	-	++	11.5	41.6
	3	-	-	-	++	0	54.4	-	-	-	-	0	15.3
	4	+	-	+	++	10.3	44.3	+	+	-	+	25.7	71.1
	5	-	-	+	+	16.6	26.6			-		26.9	63.2
	6	-	+	-	++	0	0	-	-	-	-	5.2	87.7
24	1	-	-	-	++	0	28.6	++	-	+	++	31.4	72.3
weeks	2	+	+	+	++	5.1	0	-	-	-	+	25.3	67.5
	3	-	-	+	++	0	0	-	-	+	++	0	0
	4	-	-	-	+	0	52.9	-	-	+	-	0	66.1
	5	-	+	+	+	0	40.9	+	-	+	-	0	42.3
	6	-	-	-	-	0	55.7	-	-	+	+	7.4	69.3

Histological analysis of the soft tissue reactions to membranes (III)

The implanted membranes elicited a fairly uniform tissue reaction throughout the study. The membrane can be seen as an opaque structure in all histological sections. After the first six weeks of implantation, the membranes became surrounded by a 200-300-µm-thick fibrous capsule containing mature fibroblasts. The fibrous capsule was thicker at the edges of the membrane. The presence of a few macrophages was confirmed in only one 24-week sample.

After six weeks of implantation, varying amounts of calcified deposits were seen uniformly distributed at the membrane site in the surrounding fibrous capsule (Fig. 9). The number of calcified bodies was not found to correlate with the implantation time. There was no concomitant bone formation or inflammation in the surrounding tissue.

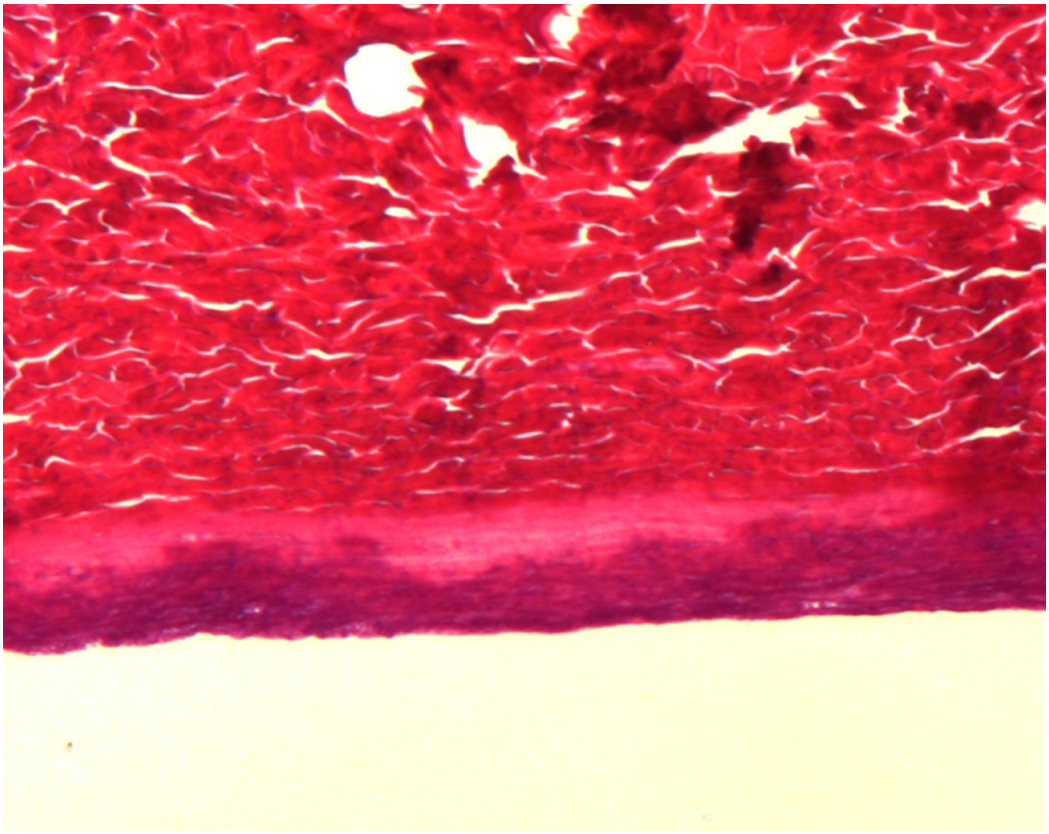

Figure 9. *Opaque PDTE carbonate membrane at the bottom of the picture. Calcified deposits (dakly staining) next to the membrane. (Original magnification x 50)*

Testing of material properties (III)

No changes in appearance or shape of the membranes were recorded. All samples remained transparent and were not bent or otherwise distorted.

The tensile strength of the membranes varied from 84 to 142 MPa (average 114 MPa, standard deviation 26 MPa). The determined strength values were adequate even for demanding load-bearing applications but had no significant contribution to the animal model used. Due to the limited number of samples, plates were not selected according to mechanical properties. The strength values varied according to different degrees of molecular orientation, caused by variation of the preform dimensions. The retrieved membranes from weeks 12, 24, and 52 were tested to evaluate molecular weight reduction. After 52 weeks, the viscosity average molecular weight (M_w) was reduced to 38 % of the initial value (Table 7 and Fig. 10).

Table 7. *Measured reduction in molecular weight (M_w) in per cent. For better accuracy, the standard deviation (SD) is multiplied by three (Asikainen et al. 2006b). (With the kind permission of Springer Science and Business Media)*

Weeks	M_w %	SD *3
12	60.60	1.35
24	56.10	1.35
52	37.90	0.64

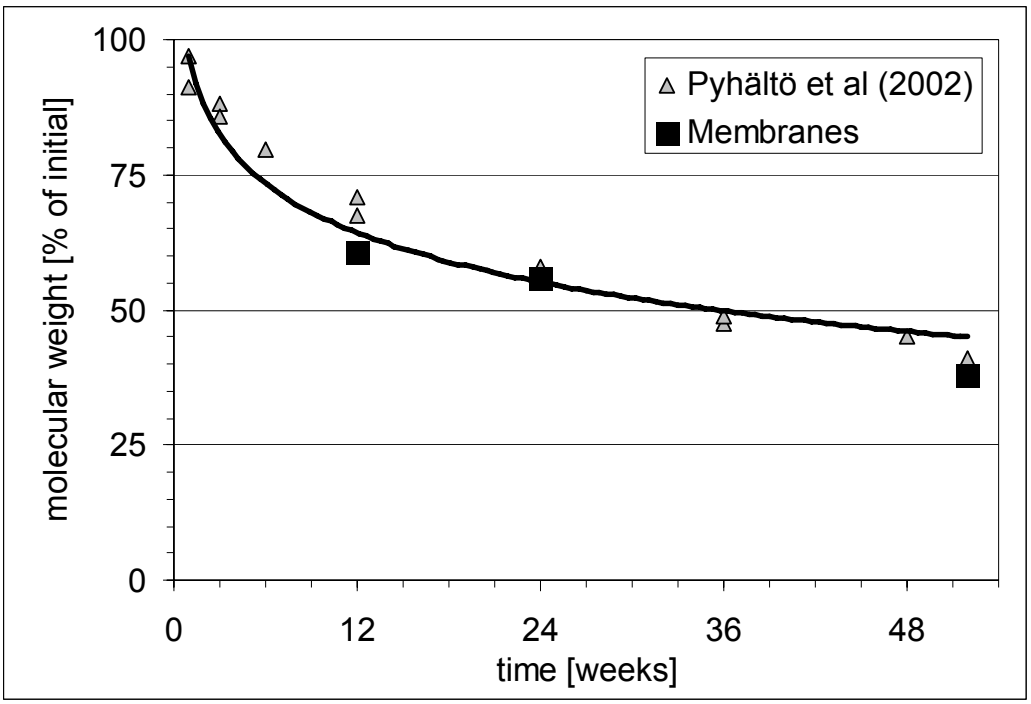

Figure 10. *Molecular weight retention of membranes retrieved in vivo in comparison to rods used by Pyhältö et al. (2002). The trend line is fitted to data using logarithm equation as suggested by Hooper et al. (1998) (Asikainen et al. 2006b). (With the kind permission of Springer Science and Business Media)*

Histological and immunohistological analysis of the soft tissue reactions to bioactive glass (IV)

At six weeks in the tissue slides the bioactive glass material (BM) remnants (mainly non-polarizing crystalline pieces, most likely chitosan) were surrounded by foreign-body reaction including multinucleated giant cells with chronic

inflammatory infiltrate. In some cases clusters of eosinophilic granulocytes were present.

At 12 weeks the foreign-body tissue reaction was still seen in the slides, but BM was mainly resolved. Occasionally BM debris was seen inside multinucleated giant cells.

At 24 weeks in most of the slides no more BM could be seen, though chronic inflammatory infiltrate was still present. Wherever BM was seen, it was surrounded by foreign-body tissue reaction.

Positivity for PAS staining was not detected in any sample.

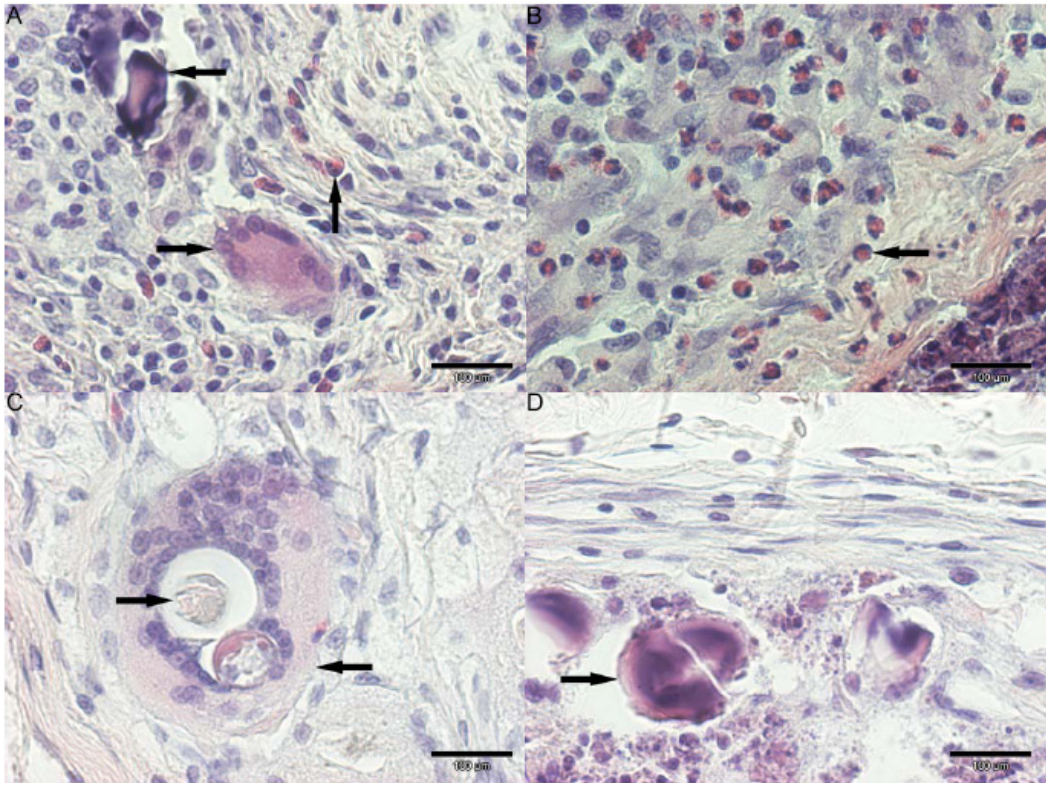

Figure 11. *A) After six weeks of healing the bioactive glass fibres (arrow left) were surrounded by multinucleated giant cells (arrow right) and, in some cases, by eosinophilic granulocytes (arrow up). B) After 12 weeks of healing the bioactive glass was mainly absorbed, but chronic inflammatory infiltrate was still present with eosinophilic infiltration (arrow left). C) After 12 weeks bioactive glass debris (arrow right) is still detectable inside the multinucleated giant cells (arrow left). D) After 24 weeks most of the bioactive glass particles (arrow right) were absorbed. If bioactive glass was still seen, it was surrounded by milder foreign-body reaction than in the six- or 12-week samples.* ***IV*** *(Copyright ®2006 Wiley Periodicals, Inc.)*

Immunohistochemistry

Immunohistochemistry revealed positivity for MMP-13 in the endothelial cells after six weeks; otherwise only occasional positivity in the fibroblasts at six, 12, and 24 weeks could be observed. MMP-2 positivity was not seen at six and 12 weeks, but occasionally in the slides at 24 weeks and mainly in the endothelial cells. MMP-3 (stromelysin-1) immunopositivity was detected in the multinucleated giant cells and associated with collagen fibres. Occasionally MMP-3 expression was detected also in the macrophage-like cells and fibroblasts. With time the MMP-3 expression became milder but was still present in the multinucleated giant cells and again associated with collagen and elastin fibres.

TIMP-1 and -2 (tissue inhibitory matrix proteinase-1 and -2) positivity was detected occasionally in the multinucleated giant cells up to 12 weeks. However, positivity was also detected throughout the follow-up period in the luminal endothelial cells and in the endothelial basement membrane.

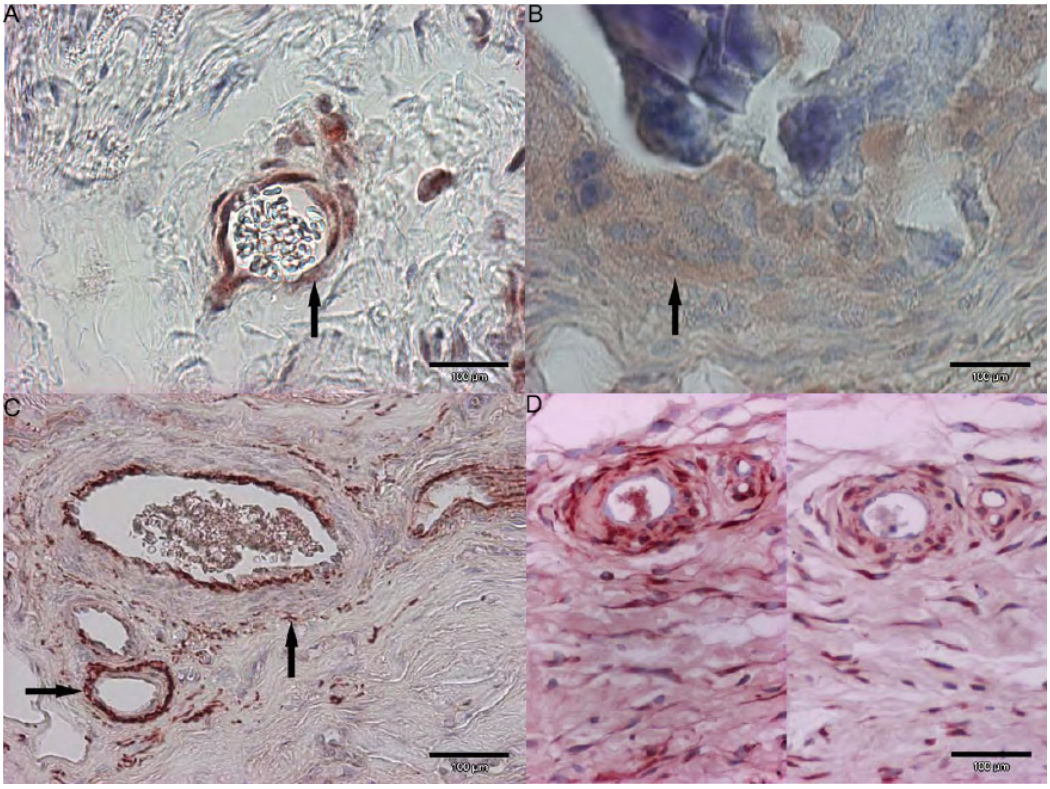

Figure 12. *A) MMP-13 immunopositivity is seen in endothelial cells (arrow up) after six weeks. B) TIMP-1 is occasionally seen in multinucleated giant cells (arrow up). C) TIMP-1 immunoexpression after 24 weeks is detected in luminal endothelial cells (arrow right) and in endothelial basement membrane (arrow up). D) TIMP-1 (on the left side) and -2 (on the right side) expression after 12 weeks.* ***IV*** *(Copyright ®2006 Wiley Periodicals, Inc.)*

Discussion

Several bioabsorbable materials are already in clinical use, including polylactides, polyglycolides, and polydioxanone. These materials have been approved for clinical use, though none of them is considered an optimal material for guided bone regeneration. All materials currently available for clinical use are associated with a foreign-body reaction in the host tissue. However, some create more adverse reactions than others.

Osseus tissue engineering, especially in the dentofacial area, is challenging due to the many thin bones, thin periosteum, and constant masticatory load. Moreover, the facial soft tissues are well vascularized and innervated. Nerves and vessels enter the calvarium through narrow canals in which even slight oedema of the surrounding tissues can cause problems, such as discomfort, paresthesis, pain, impaired function, and, in severe cases, even necrosis (Cornah et al. 1981, Renzi et al. 2004).

The environment of the dentofacial area sets strict criteria for the implant materials. The material should not create an unnecessarily strong foreign-body reaction, the peri-implant fibrous capsule should be as thin as possible if not absent, and the material should not induce late tissue reactions after the original defect has healed. The material should also be strong enough to withstand pressure caused by the soft tissues. All the above have been challenges in the development of an ideal material, for example, to repair blow out fractures (Kontio 2005).

Good vascularization and, subsequently, rapid growth and healing of the soft tissues of the head and neck need a firm barrier to enable ossification of the defect. The bones of the skull are generally only a few millimetres thick. The implant cannot be notably thicker than the bones, as it should serve as support without interfering intact tissues around the bony defect (Goldberg et al. 1987, Goldberg et al. 1993).

A common problem with all guided bone regeneration materials is the handling and correct placement of the membrane. The membrane used in this study showed excellent bending and handling properties during surgery. The edges of the membrane could be rounded with scissors. The needle, used to introduce the fixating suture, perforated the membrane easily. Blood clot formation and correct positioning of the membrane could be ensured, because the membrane was transparent.

Economical aspects

Non-absorbable materials for guided bone regeneration often need to be removed after the tissues have healed (Iizuka et al. 1992). This subjects the patient to another operation which may require additional hospitalization time and cause postoperative pain and discomfort by the patient. During the recovery period the patient is not able to work and perform daily tasks normally. This leads to an economically unfavourable situation, though the non-absorbable implants are normally cheaper than the bioabsorbable (Bouwman et al. 1999).

With bioabsorbable materials the treatment time can be reduced, but the operation times might be slightly longer than those with non-absorbable implants. This is partially due to the limited experience of the operating staff with new techniques. With time and practice the operation times are likely to be reduced close to those with traditional methods. However, the patient benefits the most from shorter overall treatment times. By using bioabsorbable materials the future surgical treatment of the patient will not differ from the treatment given to a previously unoperated patient, and medical imaging will not be disturbed as it is with metallic implants (Juutilainen et al. 1997, Suuronen et al. 2000).

The optimal degradation time for biodegradable implants depends on the nature of the tissues being treated. A larger bone defect needs more time to heal than a smaller one. The load-bearing requirements of the treated area set criteria for the mechanical properties of the implant. A site where the load is only caused by the pressure of the soft tissues does not need a membrane with similar properties to resist deformation as does the membrane used for augmenting the alveolar ridge. In the latter case the treatment site is under constant loading and needs to be a mechanically stable structure to resist collapse (Buser et al. 1996, Busenlechner et al. 2005).

Problems and limitations in the present study

The membranes were fixated with Vicryl® 3-0 sutures, as the rabbit mandible was too thin for commonly used tack or pin fixation. The fixation was considered good in all cases intraoperatively but was later found inadequate, since at the end of the follow-up time in only five out of 18 cases the membrane was situated where it was originally placed. Possible reasons for the loosening of the fixation could be the holes through which the sutures were inserted. The mechanical stress between the bone and membrane may have caused the shearing of the sutures, which has led to the loosening of the fixation. In the cases where the fixation was adequate a notably thinner fibrous capsule surrounded the membrane. This supports the theory that micromovement of any implanted device increases the granulation tissue around the device (Pyhältö et al. 2002).

None of the membranes used was found to have collapsed during the follow-up. The mechanical stability of the membranes used was therefore considered adequate. However, a thinner membrane would probably have been more advantageous, since it would not have interfered with the neighbouring tissues. Except for the loosening of the fixation, no significant interference with tissues around the membranes was detected. Even if the membranes had been thinner, the present follow-up would have been too short to detect complete degradation of the membranes.

The PDTE carbonate has not been studied extensively so far. In the literature there is no previous similar experimental protocol with bioabsorbable membranes. To evaluate the efficacy of a new material compared to older materials, a study with a traditional, well-known material such as PLA or PGA could have been used as a control. However, the results in this study with PDTE carbonate are encouraging, and the comparison to conventional materials could also be used in a protocol where treatment using traditional materials has led to compromised results.

In the literature the degradation of the PDTE carbonate has been reported to take more than 1280 days (James et al. 1999). The relatively short follow-up time in the present study (52 weeks) is not long enough compared to the complete degradation of the material. Therefore, in the future, experiments with long term controls close to the complete degradation of the material should be designed.

The use of visual inspection in marking ossification on the radiographs can be considered a weakness in this study. Visual inspection is always an objective analysis. The possible error caused by the method used was, to some extent, compensated by using a single radiologist and pathologist to analyse the entire series. Also the lack of a quantitative analysis can be considered a disadvantage.

Advantages of PDTE carbonate

Decrease in tissue pH activates osteoclasts and stimulates bone resorption (Meghji et al. 2001). PDTE carbonate has not been shown to cause accumulation of acidic degradation products into the tissues surrounding the implant. The acid groups formed during the degradation of tyrosine-derived polycarbonates are polymer-bound and not able to diffuse into the surrounding media (Tangpasuthadol et al. 2000b) to cause pH decrease and possible osteolytic reactions around implants. This, however, is not the case with PLA and PGA (Böstman 1991). With PLA and PGA the degradation products are acidic and not polymer-bound. They have the potential to decrease pH in the tissues surrounding the implant, though the buffer systems of the body can rapidly compensate for local decreases of pH if the implant degradation rate is not too fast.

The lack of water uptake of the tyrosine-derived implants explains why they maintain their profile for longer periods than the derivatives of lactic or glycolic

acid and can thus be used in applications where swelling may result in a failure of implantation or cause esthetic problems (Hooper et al. 1998, James et al. 1999). PDTE carbonate is also more hydrophobic and would therefore be a better alternative than PLA in coating or drug-release applications (Hooper et al. 1998).

The degradation of PLA and PDTE carbonate occurs through random hydrolysis of the carbonate backbone (Ertel et al. 1994). While PLA degrades, the tissue response and implant behaviour vary depending on the capability of tissue to clear metabolites. First, PLA elicits a foreign-body reaction containing large amounts of macrophage-like cells and almost twice as thick a fibrous capsule as the PDTE carbonate (Hooper et al. 1998). Then the number of cells in the capsule decreases until the beginning of mass loss and the eventual disintegration of the implant material. This latent inflammatory response is assumed to be associated with the release of lactic acid (Gogolewski et al. 1993, Hooper et al. 1998). With PDTE carbonate the mass loss occurs very slowly, and tissue reactions remain constant during the degradation (Hooper et al. 1998). This is consistent with the findings of the present study and makes a clear difference compared to PLA.

In the present study, PDTE carbonate implanted in soft tissues induced calcifications in the surrounding fibrous capsule. A possible explanation of this might be the formation of carboxylate groups formed on the implant surface during the degradation (James et al. 1999). They can act as as nucleation sites for the HA surface layer. However, the calcifications were not attached to the implant material. It is also possible that they have been loosened from the implant surface due to the chemical treatments needed for preparation of histological sections. This phenomenon was seen only in the soft tissue samples and was absent in the bony mandibular model, though new bone formation was detected already after six weeks along the membranes.

The mechanical data observed in the present study (III) is consistent with the previous publications, in which identical polymers have been used for orthopaedic applications (Pyhältö et al. 2002). The molecular weight levels reached in the previous studies as well as in the present one did not provide information of any mechanisms responsible for the final absorption. However, the rod-formed implants used by Pyhältö et al. (2002) had comparable degradation rates with the membranes in the present study. Therefore it can be concluded that the surface area of the implant appears to have a negligible effect on the degradation rate. The present results are consistent with those of earlier publications (Tangpasuthadol et al. 2000b).

According to the literature, PDTE carbonate elicits a very modest foreign-body reaction and becomes surrounded by a thin (29-42 μm) fibrous capsule (Hooper et al. 1998). No cell-mediated immunology has been shown earlier (Choueka et al. 1996). In the present study (II, III) PDTE carbonate caused a very similar reaction throughout the follow-up period as reported earlier. However, it was found that the fibrous capsule was notably wider in the present experiment than in previous publications. This might have been caused by the shape of the implant, because in previous studies mainly rods were used. The

thicker fibrous capsule may also have been the result of micromovement under the masseter muscle which was not prevented by the bioabsorbable suture fixation of the membrane. The present results support the theory that micromovement of the implant increases the thickness of the fibrous capsule (Pyhältö et al. 2002).

MMP contribution

Matrix metalloproteinases (MMPs) are a group of more than 20 genetically distinct, but structurally related, proteases capable of degrading almost all extracellular matrix proteins and also able to modulate many other substrates that are involved in cellular signalling and immune responses (Kähäri et al. 1999, Uitto et al. 2003). They contribute to pro-inflammatory tissue destruction and tumour metastasis as well as to many other physiological actions, such as wound healing and bone remodelling. MMPs have been shown to be expressed by both osteoclasts and osteoblasts and to contribute to bone remodelling, both in pathological and physiological states (Rifas et al. 1989, Meikle et al. 1992, Reponen et al. 1994).

TIMP-1 and -2 expressions were detected occasionally in the samples up to 12 weeks. In previous studies it has been shown that TIMPs can directly stimulate the bone resorbing activity in mature rabbit osteoclasts (Sobue et al. 2001) and that the osteoclast stimulating effect is independent of the inhibitory effect on MMPs (Shibutani et al. 1999). The previously suggested stimulating effect of TIMP-1 on bone resorption might explain the delaying of the ossification in mandibular defects. Therefore it would have been interesting to study TIMP-1 expression in the mandibular samples where ossification was compromised. This was, however, not possible due to the denaturation of the proteins in the polymerizing process of methylmetacrylate.

MMP-3 positivity was found to decrease with time. Furthermore, MMP-3 positivity associated with collagen fibres and multinucleated giant cells. The expression in the multinucleated giant cells suggests that MMP-3 may be participating in the degradation of either bioactive glass or chitosan. The gradual reduction of MMP-3 expression during the follow-up period supports this hypothesis. By 24 weeks only very few implant particles could be seen on the slides. Moreover, it seems that MMP-3 and MMP-13 form an inductive MMP-cascade as part of the host response against implanted material.

Unsolved issues

In the recent review by Di Martino et al. (2005) chitosan was considered a very promising material for osseous tissue engineering in orthopaedic applications. The authors, however, suggested further studies to be carried out to overcome the problem of poor mechanical properties (Di Martino et al. 2005). In the

present study (I, III), bioactive glass 13-93 containing chitosan did not enhance healing in experimental bone defects. Healing was statistically slower in the group treated with BAG 13-93 and chitosan. In the literature both materials, separately (Brink et al. 1997, Seol et al. 2004) and combined with other materials, have been reported to enhance ossification (Wang et al. 2002, Ruuttila et al. 2006).

The reason for the presence of eosinophilic granulocytes in the initial phase of degradation at six weeks in the present study (IV) remained unclear. Both chitosan and bioactive glass 13-93 were of medical grade, free of impurities, and capable of creating an inflammatory reaction to be related to chronic and allergic reactions. The presence of eosinophils suggests the possibility that these materials are at least in part capable of inducing an allergic reaction. However, chitosan has been reported to cause macrophage activation (Mori et al. 2005) and eosinophilia in tissues surrounding the implant (Peluso et al. 1994). These results suggest that the accumulation of eosinophils might be mainly caused by chitosan. However, in the initial phase of BAG degradation large amounts of macrophages have been reported. Furthermore, the possibility of a parasite infection cannot be definitively ruled out. According to the manufacturer of chitosan (Medicarb, Bromma, Sweden), high endotoxin levels and adverse reactions caused by impurities have been an earlier problem. Although chitin is still obtained from crustaceans, the current manufacturing process should have eliminated such problems.

In the present study it was found that subcutaneously implanted PDTE carbonate membranes elicited formation of small calcified bodies in the connective tissue capsule. This phenomenon was seen as early as six weeks after implantation. The amount of calcified bodies was not found to correlate with the healing time. To the knowledge of the present author, no such reaction has been described with any *in vivo* implanted bioabsorbable polymer so far. The origin of the calcified bodies remained unsolved in the present study. It might be partly due to the relatively short follow-up time.

In *in vitro* studies it has been shown that the deposition of the calcium salts can result either from osteoblast-derived ossification or from dystrophic or non-metabolic calcification. The presence of osteoblasts and the formation of a type I collagen-containing extracellular matrix marker have been considered to be the marker of osteoinductive capacity of the material (Declercq et al. 2005). In this study, it was failed to demonstrate the presence of osteoblasts in the fibrous capsule and, hence, the effect of calcification in relation to possible bone formation remained unsolved. Further studies are needed to confirm the nature of calcification.

In the future

Bioabsorbable plates and screws are in routine clinical use, and bioabsorbable membranes have been used successfully in treating periodontal defects. In the craniofacial area the repair of orbital wall fractures has been a challenge with bioabsorbable membranes. The limited space and pressure of the eye have been problematic. Complications, from moderate to severe, have been described with all materials studied, both non-absorbable and bioabsorbable. Kontio (2005) discussed this problem in his recent thesis. Deformation and fracturing of the implants as well as passive molding of the material by the pressure exerted by the orbital content were described when PDS implants were used. Clinical symptoms were the result from the problems above. In the present study no deformation, coiling or swelling of the membranes were recorded during the follow-up period in the soft tissue or mandibular models. The material was tolerated well, the tissue reactions were very mild, and the soft tissue capsule around the membrane was thin. However, PDTE carbonate degraded slowly, and slow degradation has been considered a disadvantage in some cases. This can, however, be challenged, because PDTE carbonate does not degrade as the conventional bioabsorbable materials do. Moreover, PDTE carbonate was osteoconductive, since the critical-sized defects were ossified after the follow-up period. The results of the present study are encouraging for evaluating the suitability of this material in orbital wall fracture treatment as well as in treating calvarial bone defects in the animal model.

Conclusions

I. The experimental defects created in the present study were of critical size. PDTE carbonate membranes enabled ossification of the defects within the follow-up time.

II. In the radiological evaluation, the healing of the defects treated with BAG 13-93 mesh was statistically slower by 12 weeks. After that the difference was not statistically significant. Histologically new bone formation was notably more extensive in the “membrane-only” group. Therefore it can be concluded that no additional benefit is gained by using BAG 13-93 mesh in combination with PDTE carbonate membranes.

III. PDTE carbonate membranes elicited a very modest foreign-body reaction in the soft tissues. The fibrous capsule around the membrane was of satisfactory width. The material elicited formation of small calcified bodies in the surrounding fibrous capsule. The origin and possible contribution to ossification remained unsolved.

IV. PDTE carbonate elicited a notably mild foreign-body reaction throughout the follow-up period. In soft tissues, it elicited formation of calcified bodies in the capsule. In the mandibular model these were not detected. No bending or coiling was seen in the membranes. Therefore it can be concluded that PDTE carbonate membranes are most likely to retain their original shape for at least 52 weeks.

V. BAG 13-93 with chitosan induced a modest foreign-body reaction, though, compared to PDTE carbonate, in the initial phase of degradation eosinophils and macrophage infiltration were seen. The results suggest that the delayed ossification in the mandibular model was possibly caused by the elevated levels of MMPs and TIMPs.

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Tyrosine derived polycarbonate membrane is useful for guided bone regeneration in rabbit mandibular defects

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Abstract

Standardized bilateral through-and-through defects (12x6 mm) were created extraorally in the mandibular angle of 18 New Zealand White rabbits. Animals were divided in to three groups (n=6) according to the intended healing time. On the left side, defects were covered with a poly(desaminotyrosyl-tyrosine-ethyl ester carbonate) (PDTE carbonate) membrane wrapped around the inferior border of the mandible and fixed with bioabsorbable sutures. On the right side, the defects were filled with a mesh made of bioactive glass 13-93 and 3 wt-% chitosan. The defects were covered with the same membranes. Periosteal flap was sutured over the membrane.

Radiographically, bone ingrowth was seen in all specimens at 12 weeks postoperatively. At 24 weeks, completely ossified area remained approximately at the same level as at 12 weeks, but the non-ossified area decreased to almost zero. However, the bioactive glass mesh did not improve the results. Nevertheless, enveloping the defect with PDTE carbonate membrane seemed to play a crucial role in new bone formation. Based on these results, we conclude that tyrosine polycarbonate is a promising new material for guided bone regeneration.

Keywords: PDTE carbonate, bioabsorbable membrane, rabbit, mandible, reconstruction, guided bone regeneration

Introduction

Bioabsorbable materials suitable for clinical use in oral and maxillofacial surgery have been under extensive research for over three decades [1-4]. One significant task has been the reconstruction of masticatory system when there is not enough bone available, for example, due to trauma or tumor ablation. Bone defects in both, the mandible and maxilla, create a problem in the reconstruction of fully functioning masticatory system and occlusion. Today, defects are mainly filled with autogenous bone, with either free or microvascular bone grafts, which subjects the patient to another operation site, longer operation and more morbidity [5, 6].

Various studies have shown that missing bone can be aided to regenerate by using different kinds of membranes, both bioabsorbable and non-absorbable [7-10]. They inhibit soft tissue invasion to bony defects, which is considered the most important factor contributing to bone formation [10-12]. At present, expanded polytetrafuoroethylene (e-PTFE) is a clinically widely used membrane. Because it is non-absorbable it needs to be removed from the patient in a second operation. The removal procedure subjects the patient to an unnecessary risk of infection and increases the costs of the treatment. Stripping the periosteum, to remove the membrane, might also enhance bone resorption [13]. The time needed for the whole treatment protocol is therefore often prolonged.

By using a bioabsorbable membrane instead of bone grafts, several benefits can be achieved: less morbidity and risk of infection and shorter treatment periods, not to forget the economical aspects, either [6, 14, 15]. Bioabsorbable materials differ from each other in handling properties, malleability, elasticity and absorption rates. However, no material supercedes the other one so far according to the literature[16].

An ideal bioabsorbable material in guided bone regeneration should be able to enhance bone growth in bone defects, while at the same time establish a firm mechanical barrier against soft tissue invasion into the defect area. The material should also be easy to handle and manufacture and should degrade after tissues have healed. Additionally it should not elicit too strong, clinically significant foreign body reaction [17, 18].

Currently, commercially available bioabsorbable polymers approved for clinical use, are polylactides (PLAs), polyglycolides (PGAs), polydioxanone (PDS) and their copolymers [16]. PLAs and their copolymers are today perhaps the most commonly used and studied bioabsorbable polymers in oro-maxillofacial and orthopedic surgery [3, 19]. By changing the composition of the polymer and the manufacturing procedure, the resorption time, handling properties, and mechanical durability can be adjusted to suit the needs of the patient [20, 21]. In the literature several reports have been published on sterile fluid accumulation, cyst formation and foreign body reaction in association with PLA and PGA homopolymers [22-24]. It is assumed that this may be caused by too fast degradation of impure homopolymeric implants, which exceeds the metabolizing capacity of the surrounding tissues [21].

Tyrosine derived polycarbonates are new bioabsorbable polymers suggested for use in medical applications. Tyrosine-based pseudo-peptide polymers were first introduced in 1987 by Kohn and Langer [25]. They have proven to be biocompatible, biodegradable, non-toxic, and non-immunogenic with good processing properties including solubility, thermal stability, and moldability [26-28]. Various tyrosine polycarbonates derived from the ethyl, butyl, hexyl, or octyl esters of desaminotyrosyl-tyrosine, can be prepared by condensation polymerization [29]. A polymer carrying an ethyl ester pendent chain, PDTE carbonate, has been established as a promising orthopedic implant material, exhibiting bone apposition when in contact with hard tissue [30].

Tyrosine-derived polycarbonates incorporate two in vivo hydrolytically labile bonds in each repeat unit, a carbonate bond that connects the monomer units and an ester bond connecting a

pendent chain. Degradation rate and products of the polymer are determined by the relative hydrolysis rate of these two labile bonds. Carbonate bond is hydrolyzed at a faster rate than the pendent chain ester bond [31]. They, however, are relatively stable and degrade only very slowly in vitro. No mass loss could be detected after three years in vitro degradation [32]. Tyrosine-derived polycarbonates were not found to be associated in "acid dumping" or the release of acidic residues found during the degradation of poly(D,L-lactic acid) [32].

The degree of surface hydrophobicity is related to the length of the alkyl ester pendent chain, with the polymer carrying longer alkyl ester pendent chains being more hydrophobic [29]. The least hydrophobic polycarbonate (having a short ethyl ester pendent chain) was a more stimulating substrate for cell growth than the more hydrophobic polymers (carrying longer alkyl ester pendent chains) [29]. Surface hydrophobicity has proven to contribute to reduced swelling during the degradation process compared to poly(alpha-hydroxy acids) [32].

Bioactive glass (BAG) composites as possible fillers in tissue defects have been studied since 1970s [33]. It has been proven that bioactive glass either as bulk or as particles can be used as substrates for bone growth [34]. The bone bonding capability of bioactive glasses depends on the concentrations of ions released interacting with surrounding cells/tissues. Certain bioactive glass glasses in the system Na_2O-K_2O-MgO-CaO-P_2O_5-SiO_2 have proven to be capable to bone bonding. Bioactive glasses in this system differ from commercially available Bioglass® by their K_2O-MgO portions, however, if the MgO concentration is less than 7.8 % their bone bonding abilities are similar to the bioactive glasses developed so far [35].

The aim of the present study was to evaluate the use of tyrosine derivative polymer membrane and bioactive glass mesh in the treatment of artificially created defects in rabbit mandible. Poly(desaminotyrosyl-tyrosine-ethyl ester carbonate) (PDTEcarbonate) was selected to the study to test its suitability to guided bone regeneration (GBR). Filling the defects prior to covering them with membrane to improve the bony growth was considered as an advantageous procedure and thus bioactive glass 13-93 was selected to increase the bioactivity in the covered defect.

Materials and methods

This study was approved by The Research Animal Committee of University of Helsinki and the Provincial Administrative Board, according to Finnish law.

Materials

Batches of PDTE carbonates were supplied by Integra LifeSciences Corporation (New Jersey, USA). These polycarbonates having M_w from 200 000 to 220 000 (weight average molecular weight) were prepared according to previously published procedures [36, 37]. Materials were stored in the form of powder at -18°C temperature prior to processing in airtight containers. Three days before processing, powder was ground in liquid nitrogen to eliminate larger particles. The homogenized powder was dried in vacuum chamber at 53°C for 48 hours. Solid plates (85 x 85 x 3.3 mm) were compression moulded at 165°C from the raw material powder. Moulded plates were biaxially oriented in one phase to a draw ratio of 2.2 x 2.2 at 75°C with a plate-stretching machine Karo IV (Brueckner GmbH, Siegsdorf, Germany).

Bioactive glass 13-93 (BAG) (Vivoxid Ltd., Turku, Finland) was spun to fibers, which were prepared to mesh fixed with 3 wt-% chitosan (Chitech®, Medicarb, Sweden). Samples were sized 12 x 6 x (2-4) mm (Fig 1) and used as filling material in the defects on the right side. BAG composition in weight percentages was Na_2O 6%, K_2O 12%, MgO 5%, CaO 20%, P_2O_5 4% and SiO_2 53 %.

Manufacturing of all the specimens was done at the Institute of Biomaterials (Tampere University of Technology, Tampere, Finland). All samples were sterilized with gamma irradiation, minimum

dose 2.5 Mrad. (Willy Rütsch AG, Kernen-Rommelshausen, Germany).

Animals

Eighteen adult female New Zealand White rabbits (HsdPoc strain) weighing 2500-3000 g were used as experimental animals. No preoperative fasting was required. The animals were divided in to three groups (n=6) according to the intended healing time (6, 12 and 24 weeks).

Surgical procedure

Preoperatively, the animals received trimethoprim-sulfadiazine (Duoprim vet®, Schering-Plough, Brussels, Belgium) 0,3 mg/kg subcutaneously (s.c.) for infection phrophylaxis. Anesthesia was induced with medetomidine (Domitor®, Orion Pharma, Turku, Finland) 300 μg/kg and ketamine (Ketaminol vet®, Intervet International, Boxmer, The Netherlands) 25 mg/kg (s.c.).

Mandible was shaved on both sides and skin was rinsed and scrubbed with chlorhexidine digluconate (Klorhexol® 5 mg/ml, Leiras, Turku, Finland). A skin incision was made along the inferior border of the rabbit's mandible. The periosteal flap was lifted and standardized through-and-through defects (12x6 mm, 72mm^2) were created with oscillating saw bilaterally in the mandibular angle (Fig 2). On the left side the membrane (Fig 3) (sized 20x25 mm) was mounted and wrapped around inferior border of the mandibular angle and fixed superior to the defect with absorbable sutures through holes drilled in the mandible. Periosteal flap was sutured over the implanted membrane and the incision was closed in layers with absorbable sutures (Vicryl® 3-0, Ethicon, Somerville New Jersey, USA).

On the right side , the defect was filled with Bioactive glass (BAG) mesh prior to fixation of the membrane (Fig 4). In all defects blood clot formation was ensured. For postoperative pain control the animals received 0.02-0.05 mg/kg (s.c.) buprenorphinum (Temgesic®, Schering-Plough, Brussels, Belgium) immediately after the operation and every 12 hours for the next two days. For euthanasia, pentobarbital (Mebunat®, Orion Pharma, Turku, Finland) 30 mg/kg was used intravenously (i.v.).

Radiographical analysis

Radiographs were taken of all animals 3, 6, 12 and 24 weeks postoperatively. The samples from 12 and 24 weeks were also radiographed with a "standard pearl ∅ 5 mm" after dissection, using Heliodent DS® (Siemens Co, Munich, Germany) unit at 60 kV, 7 mA and 0,005 s on Digora® digital imaging plate (Soredex Co, Helsinki, Finland). The imaging plate was read by Digora FMX® (Soredex Co, Helsinki, Finland) laser scanner. On the digital radiographs a specialist in oral radiology[2,5] marked areas of complete or partial ossification using Adobe Photoshop® 7 (Adobe System Inc, San Jose, USA) software. The marked areas were then calculated with Matlab® (MathWorks Inc, Natic, USA) and divided into two groups: radiographically completely non-ossified area and completely non-ossified area together with partly ossified area (where bone mineralization is not yet radiologically complete).

Statistical evaluation

All 12 and 24 week specimens were included in statistical analysis. Differences between non-ossified areas on the left and right side were evaluated using paired samples T-test.

Results

3 weeks

At three weeks all the wounds had healed properly and clinically no signs of infection were seen (tai etected). All animals were eating normally. Plain radiographs were taken. However, no reliable conclusions regarding the ossification could be made based on these radiographs.

6 weeks

Clinically no signs of infection were seen. After dissection a small clear fluid filled cyst was seen in the left mandible of one rabbit (membrane only). This, however, did not seem to have any significant effect on the ossification process evaluated from the plain radiographs. No reliable conclusions about the size of ossificated defect area could be made from the plain radiographs.

12 weeks

Clinical inspection revealed no signs of infection. Radiographical analysis of the specimens (n=6) revealed that on the right side (membrane + BAG) 13.6 % (range 0.0-26.9%) of the defect area remained non-ossified and 44.6% (range 12.3-84.7) was fully ossified. On the left side (membrane only) 4.5 % (range 0.0-16.6%) was non-ossified and 58.7 % (range 30.7-100 %) was fully ossified. The mean difference between non-ossified areas calculated was 6.6 mm^2 (standard deviation 4.0), which was statistically significant ($p<0.05$).

24 weeks

No signs of infection were clinically evident in any animal. Radiographic analysis of the specimens (n=6) revealed on the right side (membrane + BAG)(Fig 5.) 10.7 % (range 0.0-31.4%) of the defect area was non-ossified and 47.1 % (range 27.7-100%) was fully ossified. On the left side (membrane only)(Fig 6.) 0.8 % (range 0.0-5.1%) was non-ossified and 58.5 % (range 44.3-100%) was fully ossified. The mean difference of non-ossified areas calculated was 7.1 mm^2 (standard deviation 4.1), which was statistically insignificant ($p>0.05$). Summary of the radiographical analysis is presented in Figure 7.

Discussion

In the current study, poly(desaminotyrosyl–tyrosine -ethyl ester carbonate) (PDTEcarbonate) was selected due to previously experimented mechanical properties and processability (unpublished data). The membrane showed excellent bending and handling properties at the operation. Edges of the membrane could be rounded with scissors and needle perforated it easily. Blood clot formation and correct positioning of the membrane could be ensured because the membrane was transparent.

Most of the bioactive glass formulations are not processable and thus need to be used either as larger blocks or as crushed particles [34]. From certain glass recipies, including the bioactive glass 13-93, it is possible to manufacture for example fibres [38]. The mesh manufactured for the current study tended to disintegrate when in contact with blood. Thus it was more difficult to handle and place, compared to the membranes.

Clinically all animals went through an uneventful healing process except one, which due to wound dehiscence, needed two additional sutures in the mandible on the first postoperative day. All animals started eating normally within 24 h after the operation. This suggests that no excessive functional damage was caused by the operation.

Plain radiographs revealed little of the actual ossification due to the difficulties in positioning rabbit's mandible at the right angle. This was why another technique was chosen to further study the animals. One animal died due to sedation complication at three weeks in radiographic examination.

Postmortem digital radiographical analysis of the non-ossified area suggested, surprisingly, that no additional benefit is gained at 12 weeks when using bioactive glass with PDTE carbonate membrane. On the contrary, it seems that BAG slowed down mineralization when it was used. Partially mineralized or non-ossified area remained almost at the same level whether BAG was used or not. According to the statistical evaluation the difference between the non-ossified areas was statistically significant ($p<0.05$) at 12 weeks.

At 24 weeks completely ossified area remained approximately at the same level as at 12 weeks. However, the non-ossified area on the left side (membrane only) diminished to almost zero (on the average 0.84 %). On the right side (membrane + BAG), non-ossified areas remained approximately at the same level as at 12 weeks. The difference between non-ossified areas on the right and left sides was statistically insignificant ($p>0.05$).

Ossification of defect areas was calculated from digital radiographs taken perpendicular to the buccal side of the mandibular angle. The tube was applied as close to the mandible as possible. This should give an optimal result especially when the dissected areas were radiographed. The final results were calculated from radiographs of dissected mandibles. The fact that marking of the partly and non-ossified areas was done manually on the screen can, however, cause some inaccuracy.

As shown above, the results from this study indicates that the ossification of the defect area proceeded well in both groups, but BAG seemed to slow it down. The reason(s) for this unexpected phenomenon remain unsolved in this. One reason for this is that thin bioactive glass fibres may resorb too fast to obtain an optimal healing response in bone. This can be confirmed by further studies comparing differently sized bioactive glass particles and fibres. It may also be that BAG resorption products while trapped in the defect under the slowly absorbable covering membrane, may exceed the capacity of the tissues to effectively clear metabolites and thus slow down the mineralization. Third theory is that otherwise beneficial and bone growth enhancing ion release from BAG [39] may influence the osteoblasts' production of regulating factors as well as covering the defect may concentrate the regulating factors turning the effect to a negative one. Confirming of these assumptions need to carried out in further experiments where more set-ups

(for example, comparison of an empty defect to BAG filled and membrane covered BAG defects) need to be cross-checked.

Studies of tyrosine derivatives for medical purposes have been in progress since 1987 [25]. However, to our knowledge, this is the first time a PDTE carbonate membrane was used to cover segmental bone defects. Our study suggests that the use of this material could benefit the ossification in bone defects without additional augmentation materials. Further studies are naturally needed to confirm its behavior in larger animals. Also, long-term behavior in healed tissues needs to be studied because tyrosine derivatives degrade very slowly [32].

Acknowledgments

Support received from the Finnish Dental Society Apollonia and the European Commission (European Union R&D Project QLRT-2000-00487) are greatly appreciated. The authors thank Mr. Juha-Matti Perkkiö for his assistance with Matlab program and Mr. Pasi Ohtonen, MSc, for statistical analysis

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Figure 1. Bioactive glass 13-93 mesh with 3 wt-% chitosan

Figure 2. Rabbit mandible and the template used to create standardized defects

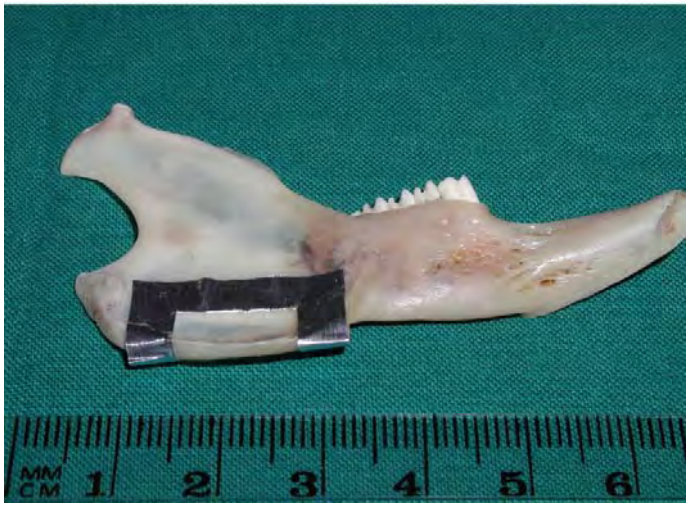
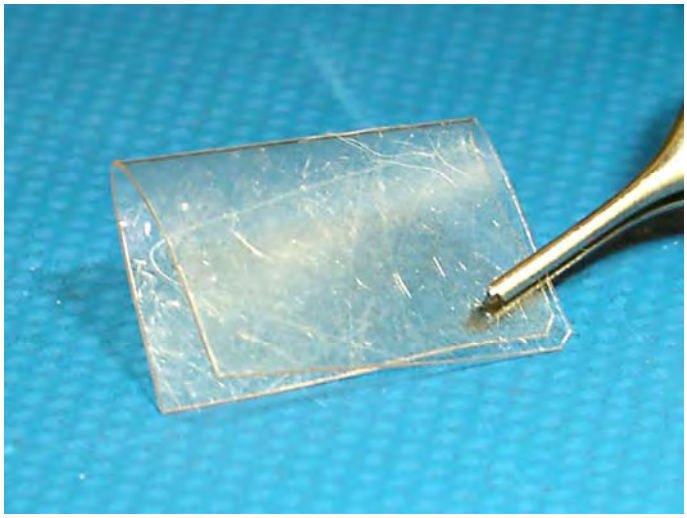


Figure 3. Folded PDTE carbonate membrane



Asikainen AJ, Noponen J, Mesimäki K, Laitinen O, Peltola J, Pelto M, Kellomäki M, Ashammakhi N, Lindqvist C, Suuronen R. J Mater Sci Mater Med 16:758-758, 2005
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Figure 4. Implanted PDTE carbonate membrane and bioactive glass 13-93 mesh with 3 wt-% chitosan fixed with absorbable suture. Correct placement and bioactive glass is easily seen through the transparent membrane.

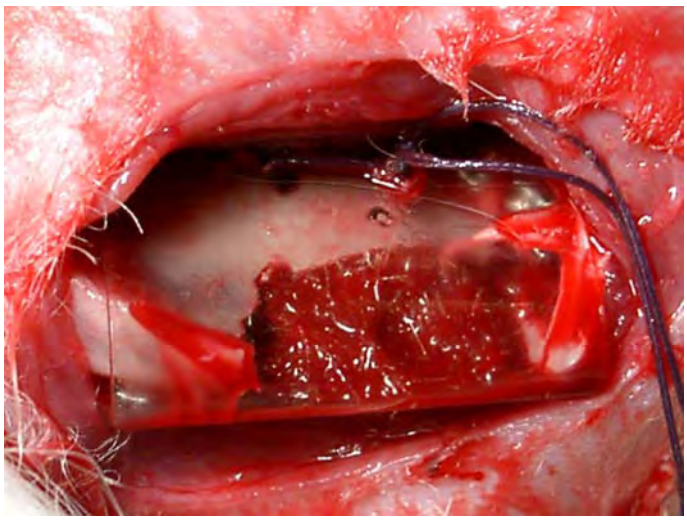


Figure 5. Digital radiograph from the right side (membrane + BAG) at 24 weeks. Mineralization of the defect is not yet complete.

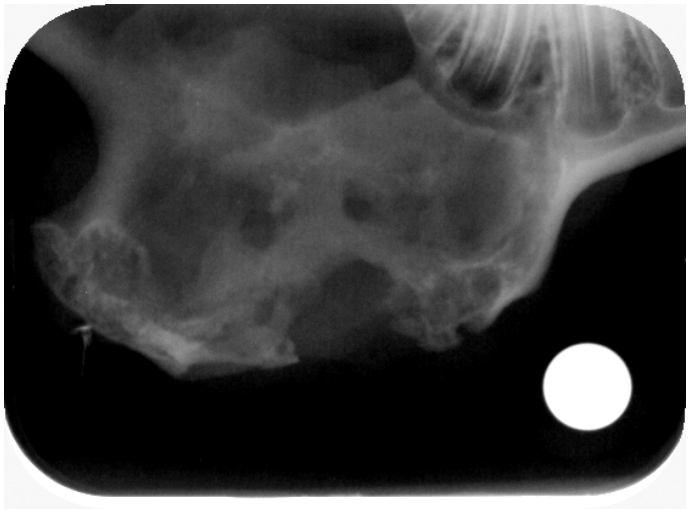
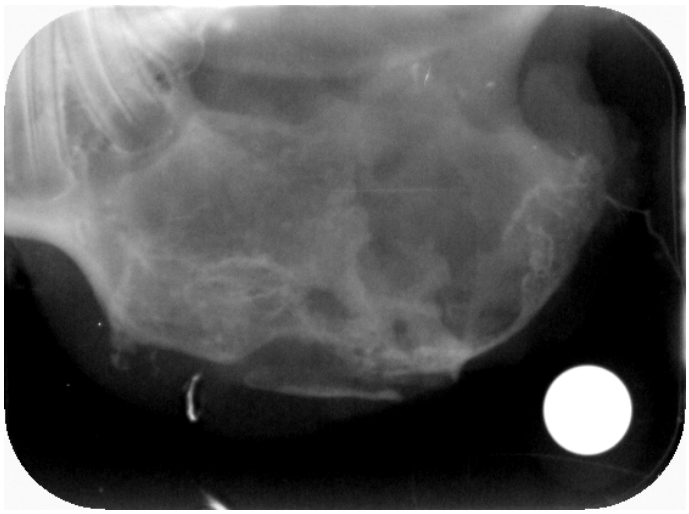


Figure 6. Digital radiograph from the left side (membrane only) at 24 weeks. The defect is almost completely ossified and it seems as if bone is growing along the membrane.



Asikainen AJ, Noponen J, Mesimäki K, Laitinen O, Peltola J, Pelto M, Kellomäki M, Ashammakhi N, Lindqvist C, Suuronen R. J Mater Sci Mater Med 16:758-758, 2005
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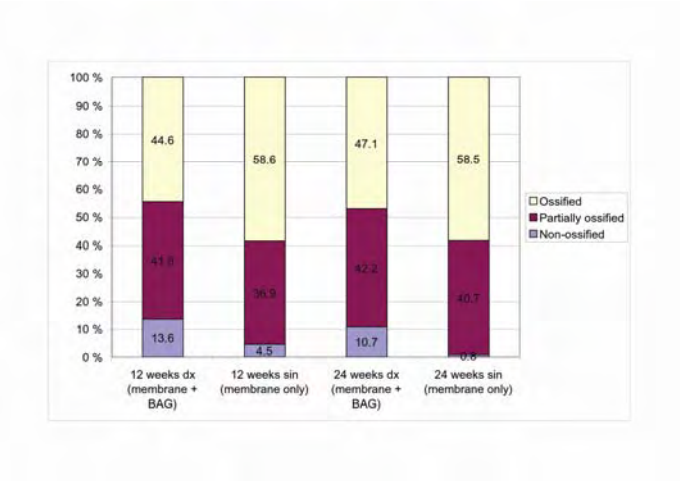
Figure 7. Summary of the radiographical analysis after 12 and 24 weeks of healing.



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In vivo degradation of poly (DTE carbonate) membranes. Analysis of the tissue reactions and mechanical properties.

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Asikainen AJ, Pelto M, Noponen J, Kellomäki M, Pihlajamäki H, Lindqvist C, Suuronen R. J Mater Sci Mater Med, in press
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Abstract

Different bioabsorbable polymers and their co-polymers have been used to construct an optimal material for guided bone regeneration applications. Our aim was to evaluate a novel bioabsorbable material in a soft tissue environment. In this study, a poly (DTE carbonate) membrane (0.2-0.3 mm) was implanted into 20 NZW rabbits' subcutaneous pouches for 6, 12, 24 and 52 weeks. The material was evaluated by means of histological reactions to the material and mechanical properties of the membrane.

Based on this study, it can be concluded that poly(DTE carbonate) elicited a very modest foreign body reaction in the soft tissues. This reaction was uniform throughout the study. Varying amounts of calcification was seen in the fibrous capsule surrounding the implant. The number of calcified bodies did not correlate to healing time.

Introduction

Most of the early studies about bioabsorbable implant materials have been on the use of these materials in fracture fixation and surgical sutures [1-4]. Applications for thin plates, foils and membranes were also introduced in the early 1970's [5]. Clinical studies have concentrated mainly on applications of bioabsorbable membranes in dentistry or cranio-maxillofacial surgery. Thin implants have been especially required in places where there is limited space for the implant, which is often the case in the dento-facial area. Therefore, the material should not expand or create an unnecessary space-consuming connective tissue capsule. So far, thin plates have been successfully used to reconstruct orbital floor fractures, to support bone augmentation in periodontal surgery and to cover defects of the calvarium [6-8].

Different bioabsorbable membranes have been tested for guided bone regeneration purposes. These include polylactides, polyglycolides, polydioxanone, e-caprolactone , trimethylene carbonate, collagen and their co-polymers [9-12].

Bioabsorbable materials are developed for different purposes. However, the main goal is to avoid the problems encountered with non absorbable materials, and the second goal is to avoid material removal operations. By avoiding the removal of the implant, risk of infection, cost and time of the treatment, and patient morbidity have been reduced [13].

Tyrosine-based pseudo-peptide polymers were first introduced by Kohn and Langer in 1987 [14]. Ertel and Kohn have described polymeric amino acid systems that contain non-amide linkages pseudo-peptides. Various tyrosine polycarbonates derived from diverse alkyl esters of desaminotyrosyl-tyrosine, can be prepared by condensation polymerization. All polymers manufactured in this fashion are amorphous and no evidence of crystallinity is seen by X-ray diffraction [15]. Studies of various tyrosine polycarbonates, derived from diverse alkyl esters of desaminotyrosyl-tyrosine have indicated that poly (DTE carbonate) is the most suitable polymer to construct scaffolds for guided bone regeneration [16]. In the literature, the use of poly(DTE carbonate) in ACL(anterior crucial ligament) reconstruction [17] and cancellous bone fracture fixation [18] has been previously reported in rabbits.

Tyrosine-derived polycarbonates incorporate two in vivo hydrolytically labile bonds in each repeat unit, a carbonate bond that connects the monomer units and an ester bond connecting a pendent chain. Degradation rate and products of the polymer are determined by the relative hydrolysis rate of these two labile bonds. Of these two bonds, the carbonate bond is hydrolyzed at a faster rate than the pendent chain ester bond [19]. No enzyme or cellular activity has been shown to participate to the degradation process [15, 20]. Shape and porosity of the implant has not been shown to affect degradation significantly either. Because the hydrolytic cleavage of the carbonate backbone is the rate determinating step in degradation, the surface to volume ratio has only a minimal effect [21]. At neutral or slightly acidic pH, the hydrolysis rate of the carbonate bond is faster than the rate of ester bond cleavage. As the degradation continues, the amount of acidification of the interior of the polymer matrix increases and the further degradation of the polymer can be expected to slow down. Tangpasuthadol et al. describe this as the "lag period" of degradation. However, as more pendent chains are eventually hydrolyzed, the reaction rate increases after the pH within the polymer matrix sinks below 3. Only under pH 3, the acid catalyzed hydrolysis of the ester bond becomes a dominant factor and pendent chain ester hydrolysis outpaces the rate of hydrolysis of the backbone carbonate bonds [19]. This leads eventually to disintegration and complete degradation of the implant. However, degradation process can take up to four years before resorption is complete [21].

While degrading in vivo, poly (DTE carbonate) rods have been shown to maintain their shape at least until 1280 d and changes in appearance (turning opaque) has been seen only inside the material. James et al. have suggested that the opaque appearance of the implant is related to the small amount of water uptake into the polymer matrix [16]. With tyrosine-derived polycarbonates, no noticeable swelling has been evident; they do not take up more than 5% of water during any stage of degradation [16, 20].

Besides the water uptake, another difference between tyrosine-derived polymers and poly(alpha hydroxyacid) is related to the beginning of mass loss. Tyrosine-derived monomers are not readily water soluble and the mass loss occurs very slowly at the end of the degradation process [16]. Tangpasuthadol et al. have suggested that the only degradation products formed will be pendent alkyl alcohol and desaminotyrosyl-tyrosine [19]. Even though the material looses molecular weight significantly during degradation, the implants' mass loss has not been evident before 1090 days of implantation. Further, before 180 days of degradation, no significant amounts of degradation products have been detected [16]. For example, poly lactic acid starts to loose mass when molecular weight decreases to about 20000 D [20].

Based on the properties presented above and the results published by Ertel and Kohn [15], we decided to investigate the behaviour of poly(DTE carbonate) membrane in a rabbit subcutaneous model. In this study, biaxially oriented membranes 0.2-0.3 mm thick were fabricated for use in guided bone regeneration applications. These membranes were implanted in rabbit soft tissue to evaluate the soft tissue reactions to thin implants. The material was also studied for mechanical properties and shape maintenance when no bone tissue fixation was available.

Materials and methods

Batches of poly(PDTE)carbonates were supplied by Integra LifeSciences Corporation (New Jersey, USA). These polycarbonates having molecular weights from 200 000 to 220 000 D (weight averages) were prepared according to previously published procedures [22, 23].

Materials were stored in the form of powder at -18°C temperature prior to processing in airtight containers. Three days before processing, this powder was ground in liquid nitrogen to eliminate visible clots. The homogenated powder was dried in vacuum at 53°C for 48 hours.

Solid plates (85 x 85 x 3.3 mm) were compression moulded from the raw material powder at 165°C. Moulded plates were biaxially oriented in one phase to a size of 150 x 150 mm at 75°C with a plate-stretching machine Karo IV (Brueckner GmbH, Germany).

Animals

This experimental study was approved by The Research Animal Commission of the University of Helsinki and by The Provincial Administrative Board, according to Finnish law.

Twenty adult female New Zealand White rabbits (HsdPoc strain) weighing 2500-3000 g were used as experimental animals. No preoperative fasting was required. The animals were divided in three groups (n=6) according to the intended healing time (6, 12 and 24 weeks) and two animals for 52 weeks. National guidelines for care of laboratory animals were followed.

Surgical procedure

Preoperatively, the animals received trimethoprim-sulfadiazine (Duoprim vet®, Schering-Plough, Brussels, Belgium) 0,3 mg/kg subcutaneously (s.c.) for infection prophylaxis. Anesthesia was induced with medetomidine (Domitor®, Orion Pharma, Turku, Finland) 300 μg/kg and ketamine (Ketaminol vet®, Intervet International, Boxmer, The Netherlands) 25 mg/kg (s.c.).

The back of the rabbit was shaved and the skin was rinsed and scrubbed with chlorhexidine digluconate (Klorhexol® 5 mg/ml, Leiras, Turku, Finland). A skin incision was made at midline on the back and subcutaneous pouch was created for poly (DTE carbonate) membrane (10x20mm). The membrane was inserted in the pouch but was not fixed by any other means. The incision was closed in layers with absorbable sutures (Vicryl® 3-0, Ethicon, Somerville New Jersey, USA).

For postoperative pain control, the animals received 0.02-0.05 mg/kg (s.c.) buprenorphinum (Temgesic®, Schering-Plough, Brussels, Belgium) immediately after the operation and every 12 hours

for the next two days. For euthanasia, pentobarbital (Mebunat®, Orion Pharma, Turku, Finland) 30 mg/kg was used intravenously (i.v.).

Samples were carefully dissected, fixed in 70% ethanol and embedded in plastic.

Histological analysis

Five μm thick sections were prepared and stained with hematoxylin and eosin. Masson-Goldner staining was carried out as well

Mechanical testing and molecular weight determination

Mechanical testing and molecular weight determination was done at the Institute of Biomaterials, Tampere University of Technology, Tampere, Finland. Weight average molecular weight (M_w), number average molecular weight (M_n) and polydispersity (PD) of the poly (DTEcarbonate) membranes were studied using conventional gel permeation chromatography (GPC) with narrow polystyrene standards using chloroform as solvent and eluent. The equipment consisted of differential refractometer detector (Waters 410 RI) and HPLC-pump (Waters 515). The injection volume of the samples was 150 μl, and the flow rate in the columns was 1 ml min^{-1}. Each data point presents the mean of four measurements taken from two samples at the same time point.

The biaxially oriented membranes were die cut to achieve samples for tensile testing (figure 1). Tensile strengths of the membranes were measured following the standard ISO 527. The samples were in form of standard tensile specimen except the size 5 x 50 mm. The membranes were tensile tested using an Instron 4411 (Instron Ltd, High Wycombe, England) materials testing instrument using a crosshead speed of 10mm/min.

Results

Tissue reactions

The implanted membranes elicited a fairly uniform tissue reaction throughout the study. The membrane can be seen as an opaque structure in all histological sections. After the first six weeks of implantation, the membranes became surrounded by a 200-300 μm thick fibrous capsule containing mature fibroblasts. The fibrous capsule was thicker at the edges of the membrane. Presence of a few macrophages was confirmed in only one 24 week sample.

After six weeks of implantation, varying amounts of calcified deposits (figure 2) were seen uniformly distributed at the membrane site in the surrounding fibrous capsule. The number of calcified bodies was not found to correlate with implantation time. There was no concomitant bone formation or inflammation in the surrounding tissue.

Mechanical testing

No changes in appearance or shape of the membranes were recorded. All samples remained transparent and were not bent or otherwise distorted.

The tensile strength of the membranes varied from 84 to 142 MPa (average 114, standard deviation 26). The strength values varied due to the fact that the original plates had been compression molded to different thicknesses. The subsequent orientation step resulted in membranes with the same size but varying degree of orientation and thickness (figure 3). Consequently the thinnest membranes exhibited the highest mechanical properties. Determined strength levels were in the same range as high performance engineering polymers but had no significant contribution in the animal subcutaneous model used.

Retrieved membranes from weeks 12, 24 and 52 were tested to evaluate molecular weight reduction. After 52 weeks, the viscosity average molecular weight (Mw) was reduced to 38 % of the initial value (figure 4 and table 1).

Discussion

Tyrosine derived polycarbonates are a relatively new category of bioabsorbable polymers. Therefore, there are only a few reports about tissue reactions in different applications. We have been testing the material in guided bone regeneration application in rabbit mandibles [24]. These membranes enhanced the bone regeneration in the experimentally created defects, and it seemed that the bone was growing along the membrane. Small radiologically detectable calcifications were also seen at the outside of the membrane. Therefore, we decided to evaluate poly (DTE carbonates) reactions in a soft tissue model to find out whether the material has osteoinductive properties in vivo. The lack of water uptake of the tyrosine-derived implants explain the fact that they maintain their profile for longer periods than derivatives of lactic or glycolic acid, and thus may be used in applications where swelling could result in failure of implantation or cause esthetic problems [16, 20]. As a result, poly (DTE carbonate) was considered a suitable material for membrane application.

The mechanical data observed is consistent with the previous publications, in which identical polymers have been used [25]. The trend line in figure 4 was fitted to the data using logarithm equation, suggested by Hooper et al. [20]. The molecular weight levels that have been reached in previous studies and in this study did not provide information about any mechanisms responsible for final absorption. However, the rod-formed implants used by Pyhältö et al. [25] did have comparable degradation rates with the membranes in this study. Therefore, it can be concluded that the surface area of the implant appears to have negligible effect on the degradation rate. Our results are consistent with earlier publications [21].

Previous results from studies concerning soft tissues indicate that this material elicits a very modest foreign body reaction and becomes surrounded with a thin (29-42 μm) fibrous capsule [20], and by 48 weeks of implantation in vivo, no indication of cell mediated immunology has been evident [26]. We found that this material caused a very similar reaction throughout the follow-up period. This supported the earlier findings, except the thickness of the fibrous capsule was notably wider in this study. This might be explained by the shape of the implant. Hooper et al. used a small diameter rod, but in this study, we used a membrane with rather sharp edges. So, the thicker fibrous capsule noticed adjacent to the edges of the implants could be the result of the tissues' reaction to the mechanical stress caused by these edges.

However, we found that poly (DTE carbonate) membranes elicited a formation of small calcified bodies in the connective tissue capsule around the implant material. This phenomenon was seen as early as after six weeks of implantation. The amount of calcified bodies was not found to correlate to healing time. To our knowledge, no such reaction has been described with any in vivo implanted bioabsorbable polymer so far. The origin of the calcified bodies remains unsolved in this study. This might be due to the relatively short follow-up time.

In in vitro studies, it has been shown that the deposition of the calcium salts can result from either osteoblast derived ossification, dystrophic or non-metabolic calcification. The presence of osteoblasts and the formation of type I collagen containing extracellular matrix marker has been considered to be the marker of osteoinductive capacity of the material [27]. In this study, we failed to demonstrate the presence of osteoblasts in the fibrous capsule and, hence, the effect of calcification in relation to possible bone formation remains unsolved. Further studies are needed to confirm the nature of calcification.

Conclusions

Poly (DTE carbonate) membranes elicit a very modest foreign body reaction. After six weeks of implantation, small calcifications were seen in the membrane surrounding fibrous capsule. The implantation time did not correlate with the amount of calcified bodies. Based on the results retrieved

from mechanical testing and earlier publications, it can be concluded that the surface area of the implant appears to have negligible effect on degradation rate

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Figure 1. Bi-axially oriented plate, samples for mechanical testing removed. Decreasing the implants thickness results in an increased orientation inside the implant's structure (A). Grip positions of the biaxial stretching machine are shown (B).

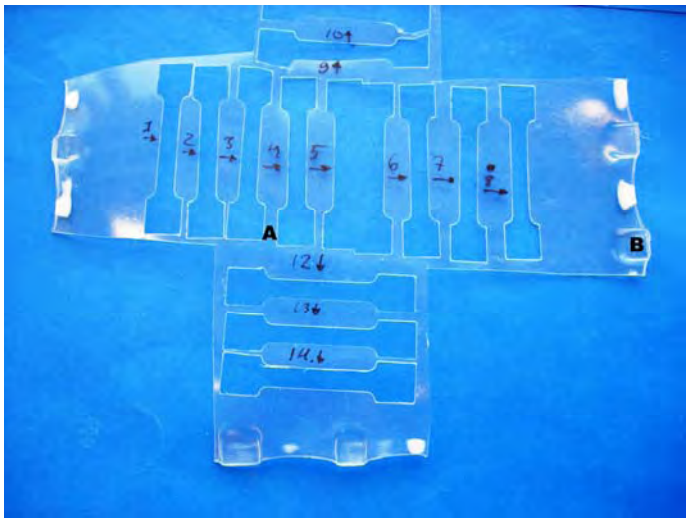

Figure 2. The opaque poly (DTE carbonate) membrane is shown at the bottom of the picture. Large amounts of calcified tissue (dark staining) is evident next to the membrane. (Original magnification x 200)

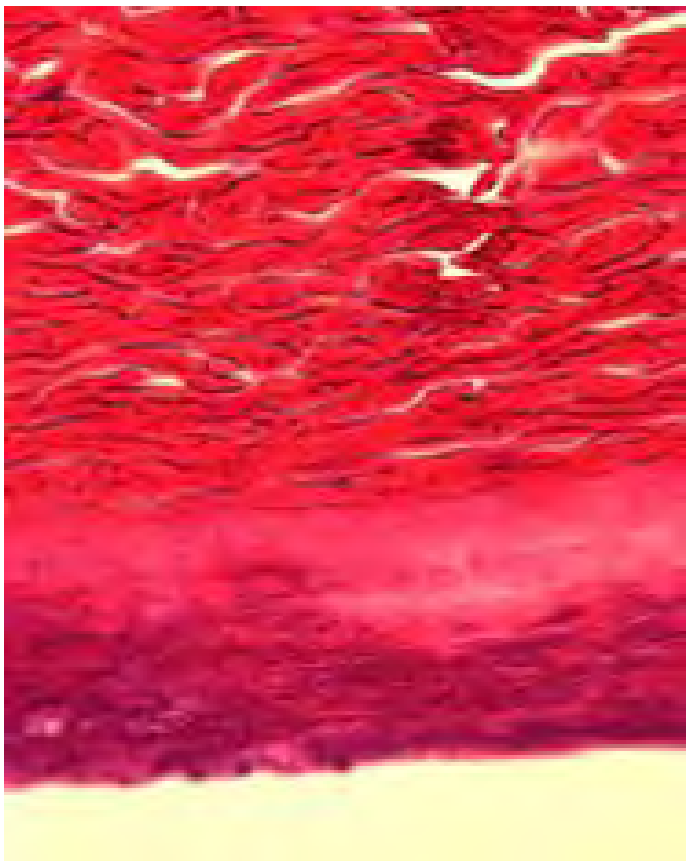

Figure 3. Schematic drawing of the subsequent orientation step that resulted in same sized membranes of varying degree of orientation and thickness. At the edges of the drawing the molecules are more oriented than in the middle.

Figure 4. The molecular weight retention of membranes retrieved in vivo in comparison to the rods used by Pyhältö et al [25]. The trend line is fitted to data using logarithm equation as suggested by Hooper et. al. [20]

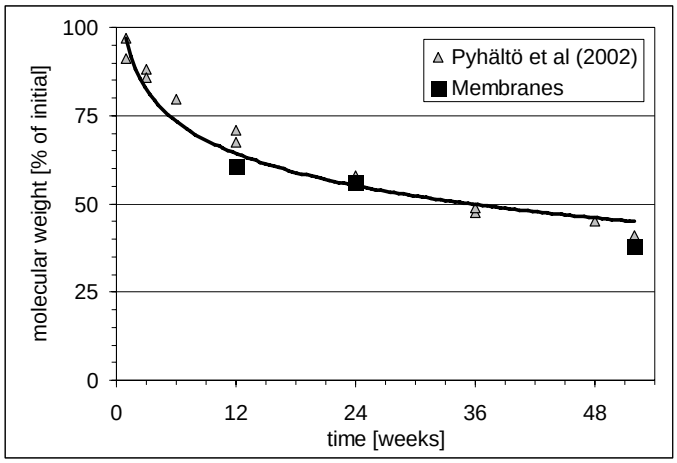

Table 1 Measured reduction in molecular weight in percentages. For better accuracy, the standard deviation is multiplied by three.

Weeks	MW %	Standard deviation *3
12	60.60	1.35
24	56.10	1.35
52	37.90	0.64