Hydroxyapatite/polylactide composites for reconstructive surgery

An *in vitro* and *in vivo* biocompatibility study

Alice van Sliedregt

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cover: The Moeraki Boulders, located near Hampden, Otago, New-Zealand.

The Maori Legend

The maori name for the boulders is 'TE KAI-HINAKI'. According to Maori folklore, the KAI (food)-HINAKI (baskets) were washed ashore at Moeraki from the canoe ARAI-TE-URU, which was wrecked a few miles along the coast on a voyage South in search of the precious stone of TE WAI POUNAMU.

The scientific explanation by Assoc. Prof. CA Landis, University of Otago, New-Zealand

The moeraki Boulders are very large and remarkably spherical, they began to form about 60 million years ago, when Eastern Otago was covered by sea. Mud and organic matter deposited on the seafloor were buried and compacted to form a mudstone which is called the Moeraki Formation. Sea water was trapped in the muddy sediment during deposition. Calcium and carbonate ions moved through the trapped water towards local crystallization centres (perhaps fossil molluscs or pieces of wood or bone), forming the mineral calcite. The growth time for the largest concretions (2 metres) is estimated to be about 4 million years. About 10 million years ago, after the concretions had fully formed, the Moeraki Formation was slowly uplifted from the sea and the processes of erosion began. Steep cliffs, cut by wave erosion, became unstable and allowed the mudstone to slump seaward carrying the concretions with it. The Moeraki Boulders visible on the beach today have been exposed as the sea eroded away the slumped mudstone. Others have yet to be uncovered.

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PROEFSCHRIFT

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This tideless silence...

yet unheard, but for the screech

of a searching gull.

J.W. Hackett

CONTENTS

GENERAL INTRODUCTION

Biomaterials are often used for reconstruction of bone and/or cartilage defects in the head and neck region. The demands upon the material properties largely depends on the site of application and the function it has to restore. Many materials are available, some of which are presented in table I. The properties of these materials vary largely, in both mechanical characteristics and chemical composition. The combination of biocompatibility and appropriate mechanical characteristics into a single material seems difficult to achieve.

Bone and cartilage tissue can be used in reconstructive surgery. They possess similar mechanical characteristics compared to the tissue they replace, but are subject to possible resorption and unpredicted remodelling. Autografts have a high acceptance rate at the donor site, but allografts and xenografts can induce an immunogenic reaction through which the graft may even be rejected. Therefore, autografts are still the standard, but the disadvantages comprise exposure of the patient to a second surgical procedure, in order to harvest the donor material, and the restricted available donor area. The polymers indicated in table I often become encapsulated in fibrous tissue because they lack bone bonding properties, which might lead to unstable implants in the long term. Metals, although sometimes the only alternative, possess the advantage that they can be mechanically anchored into bone, but this will lead to the phenomenon of stress shielding which eventually, causes to resorption of the bone tissue [Sumner '91, Huiskes '91, Bobyn '91]. Corrosion of metals might affect the immune system, inducing a sensitivity reaction [Remes '92]. These shortcomings may eventually lead to a necessary removal of the implant, resulting in a second operation for the patient. Bioactive ceramics, such as hydroxyapatite, are highly biocompatible and possess bone bonding potential, but unfortunately lack appropriate mechanical characteristics. They are used to replace tissue in non-load bearing areas, such as the ear-canal wall and the ossicular chain. These ceramics are also used to fill bone defects and are coated on titanium devices to enhance bone bonding.

In short, an ideal material for reconstructive surgery should possess bone bonding

properties, be degradable and have appropriate mechanical characteristics for the site of application. The material should have bone bonding properties, because contact of the material with bone tissue would prevent fibrous tissue encapsulation. A degradable material, if bonded to bone could be replaced by new bone tissue at the implantation site. These characteristics are thought to be met in the combination of polylactide, a degradable polymer with satisfactory biocompatibility, and hydroxyapatite, a ceramic with bone bonding potential. Hydroxyapatite/polylactide composites are tested upon their ability to be used in reconstructive surgery, as is described in this thesis.

Hydroxyapatite

Hydroxyapatite is clinically used and has proven to be a successful material in reconstructive surgery (table I) as well as in other disciplines [Geesink '90, Uchida '90]. It is similar to the inorganic component of bone, and is one of a range calcium phosphates that have been studied for application as bone replacement materials [Harms '79, Denissen '80, Jarcho '81, Winter '81, Hoogendoorn '84, Block '85, Alexander '87, Ducheyne '87]. Other calcium phosphates such as tricalcium phosphate, tetracalcium phosphate, magnesium whitlockite and fluorapatite differ from hydroxyapatite in calcium phosphate ratio, crystal structure and in dissolution properties [Klein '83, Driessens '88, Patka '89]. The dissolution properties of these materials are thought to be related to their bone bonding capacities [Kotani'91, LeGeros '92, Ducheyne '93, Radin '93].

The mechanism by which bone bonding with calcium phosphates takes place has been thoroughly investigated. The process involved is thought to be as follows: a dissolution of the calcium phosphate will lead to the formation of carbonate apatite microcrystals similar to bone apatite on its surface. The simultaneous mineralization of the collagen fibril containing extracellular matrix, which is deposited by osteoblasts on the surface of the calcium phosphate, will lead to a unique strength at the interface [Daculsi '90, Legeros '92, van Blitterswijk '92]. This process of bone bonding can be studied *in vitro* by using the bone forming system of Maniatopoulos '88. For example, the effect of small variations in dissolution properties, of calcium phosphates, on bone formation rate can be studied using this system. Hydroxyapatite can vary in its dissolution rate, depending on its structure; amorphous hydroxyapatite degrades at a faster rate than 100%

MATERIALS		APPLICATION SITE	AUTHOR
Bone	autograft (tissue from the patient)	nasal dorsum, chin augmentation, mandibular and facial bone reconstruction	JS Adams '87 CS Maas '90 EH Huizing '85 H Holmström '86
	allograft (tissue from another patient, or in general, tissue from the same species)		EH Huizing '85 J Marquet '76
	xenograft (tissue from another species)		JS Adams '87
	demineralized bone	nasal dorsum	DM Terkumi '90
Cartilage	autograft (tissue from the patient)	nasal augmentation	JS Adams '87 B Petrusen '88 EH Huizing '85 J Conley '85
	allograft (tissue from another patient, or in general, tissue from the same species)		JS Adams '87 EH Huizing '85
	xenograft (tissue from another species)		JS Adams '87
Polymers	teflon (polytetrafluorethene)	injectable; facial augmentation	JS Adams '87
	silastic (organosilicone polymer)	many sites; mentoplasty, nasal dorsum, columellar implantation, malar augmentation, orbital floor	JS Adams '87 CS Maas '90 RPG Sandon '83 TM Milward '72
	proplast (porous teflon with vitreous carbon fibres)	nasal dorsum, mentoplasty, molar and maxillary augmentation, orbital floor reconstruction, mandibular onlay graft, ossicular chain reconstruction	JS Adams '87 CS Maas '90 JGN Swart '84
	supramid (organopolymer related to nylon and dacron)	nasal dorsum, chin, maxilla, auricular reconstruction	JS Adams '87 CS Maas '90
	medpor		CS Maas '90
	polyhydroxymethacrylate		JGN Swart '84 (rats)
	collagen (heterograft) Zyderm I and II, Zyplast	injectable at several sites	JS Adams '87
	polylactic acid polylactide	dental extraction wounds, absorbable bone-plates and screws for fracture fixation	JH Brekke '83 FR Rozema '88 RRM Bos '87
	polyactive (poly(butyleneterephtalate)/poly(ethyleneoxide)	tympanic membrane	JJ Grote '91
Metals	chromium-cobalt alloys	fraxture fixation, wire	E Schepers '86
	stainless steel	fracture fixation, wire	E Schepers '86
	titanium alloys	dental implants, mandibular defects	T Albrektsson '86
	gold alloys	transmandibular	H Bosker '86
Ceramics	aluminium oxide	nasal dorsum, septum, maxillo-facial, middle ear, dental	P Friedberg '84 (rabbits) WL Mang '87 GS Godbersen '85 T Trager '92
	hydroxyapatite (calcium phosphate)	facial osteotomy gaps, augmentation alveolar ridges periodontal osseous defects (dogs, human) obliteration radical cavity canal wall prosthesis, incus prosthesis (coating) dental implants	HM Rosen '90 SS Rothstein '84 M Minabe '88 (dogs) H Decher '92 CA van JJ Grote'85 '90 T Trager '92 (human) JGN Swart '84 K de Groot '88

Table I Materials used in reconstructive surgery of the head and neck region

crystalline hydroxyapatite. The effect of variations in crystallinity on bone bonding has recently been assessed [de Bruijn '92a '92b, van Blitterswijk '92] and it was found that the type of interface formed was related to the crystallinity of the hydroxyapatite. It was concluded that dissolution of hydroxyapatite, to a certain extent, seems to positively affect the bone forming process. The impact of the crystallinity of hydroxyapatite on *in vivo* bone formation is currently under study by van Blitterwijk.

Since hydroxyapatite is subject to degradation at the ultrastructural level one might expect that degradation of the bulk will eventually become apparent. The rate of degradation will depend on several factors, which can be classified into implant related factors and into those concerning the biological environment. The implant related factors comprise both physical (form, porosity, surface area and crystallinity) and chemical properties (composition, ionic substitutions, impurities). The biological factors include a pH decrease due to cell-mediated factors, infection, bone contact, bone type, animal species, age, sex, hormone levels and genetic predisposition [LeGeros '88]. It is therefore difficult to predict the lifetime of a hydroxyapatite implant.

Polylactide

Polylactide belongs to the family of poly-alpha-hydroxyacids (table II). These polymers are of interest for application as biomaterials because they will degrade in the body, due to hydrolysis, to physically familiar substances [Barrows '86]. Different combinations of poly-alpha-hydroxyacids can be obtained by varying the polymerization process and the choice of hydroxyacids (table II). Low molecular weight polymers are obtained by direct condensation of the alpha-hydroxy acids, whereas high molecular weight polymers are obtained by ring opening polymerization of cyclic lactones, such as glycolide, lactide or mixtures thereof [Miller '77, Gilding '79, Kohn '83, Rak 85', Juni '87, Nakamura '89, Gogolewski '92]. By varying the molecular weight and composition of the polymers the degradation characteristics can be varied.

The degradation rate of these polymers will depend on their molecular weight, molecular orientation, crystallinity, and chemical and physical structure, but the mechanism of degradation will be mainly via hydrolysis of the ester bonds [Anderson '74, Jamshidi '86]. A highly crystalline polymer, like poly-L-lactide, will take longer to degrade than an amorphous polymer, such as poly-DL-lactide or a copolymer of poly-DL-lactide/poly-glycolide, due to the sterical hinderance water molecules encounter by the

attacking crystalline areas.

The degradation mechanism of polylactide is much more complicated than initially assumed [Li '90a,'90b, Vert '91]. Most researchers assume that the degradation rate will remain constant until complete elimination is established. This led to an extrapolation of the rate at which the initial decrease in molecular weight took place, to an estimation of the total time necessary for degradation [Brady '73, Chawla '85/86, Bos '87, Nakamura'89]. However, these estimations have not been verified by long term follow up studies. The degradation of the polymers was expected to be via surface erosion thereby releasing the degradation products consistently, but is was demonstrated by Therin'92 that autocatalytic degradation in the centre of the material can occur. This kind of degradation can lead to a sudden collapse of an implant, with the subsequent release of large amounts of degradation products. The dimension of the bulk of the polymer is thought to be of importance whether bulk hydrolysis occurs or not. Since degradation in amorphous regions proceeds faster than in crystalline areas [Anderson '74] one might expect fragmentation with crystalline remnants which are highly resistant to hydrolysis.

The above paragraph predominantly focused on degradation related to material properties, however the influence of degradation products on living tissue should also be addressed. Assuming that polylactide degrades into a metabolic intermediate, it could be eliminated by the body through conversion into pyruvate, this would than enter the tricarboxylic acid cycle and finally be exhaled as CO_2 (fig. 1). This route was suggested by Kulkarni for degradation products of polylactide [Kulkarni '66]; however, it assumed an intracellular uptake of degradation products, since the tricarboxylic acid cycle takes place in the mitochondria. Phagocytosed polylactide particles might be eliminated in this manner, but degradation products of extracellular degraded polylactide will probably be eliminated by excretion in urine or conversion into glycogen, as indicated by studies on extracellularly administered L- and/or D-lactic acid [Cori '29, Tubbs '61, Judge '62, Alpert '65, Brin '65, Giesecke '81, Conner '83, Brandt '84].

In the past, very few *in vitro* cytotoxicity tests with polylactide have been performed, probably because Kulkarni had already successfully demonstrated its *in vivo* biocompatibility 1966. Based on these findings many *in vivo* studies with polylactide were performed [Getter '72, Visscher '80, Hollinger 86, Chavanaz '86, Leenslag '87, Schakenraad '90,'91, Bos '91, Majola '91, Matsusu '91]. This led to some clinical applications, including the use of polylactide in sutures and in bone surgery devices such

as pins and plates [Rokkanen '85, Bos '87, Eitenmuller '88].

Many studies performed with polylactides have revealed major discrepancies between data obtained under apparently similar conditions [Cutright '71a '71b, Cutright '72, Christel '84, Christel '84, Chaval '85/86, Jamshidi '86, Leenslag '87, Nakamura '89]. These differences are most likely caused by variations in the manufactoring of the polylactides used, or they may have been introduced after production, since these polymers are water, heat and irradiation sensitive [Gupta '83].

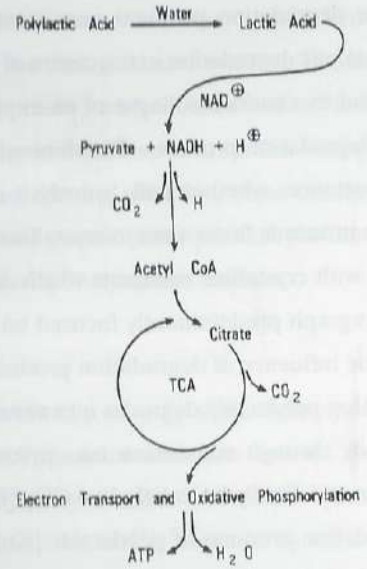


Fig. 1 The monomeric units of lactic acid become incoperated in the tricarboxylic acid cycle. (JO Hollinger, GC Battistone, Biodegradable bone repair materials, synthetic polymers and ceramics, Clin Orthop, 207:290-305, 1986)

Composites

Hydroxyapatite is a calcium phosphate with bone bonding potential but unfortunately it does not possess sufficient mechanical properties for load bearing applications. Polylactide is a degradable material which apparently has satisfactory tissue compatibility for medical application and can be produced into a material with suitable mechanical properties for load bearing applications. The combination of these two biomaterials is thought to be a bone bonding, biocompatible, degradable material that might possibly possess improved mechanical properties.

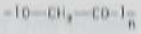
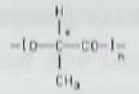
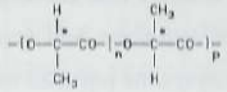
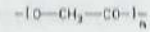
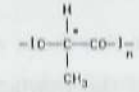
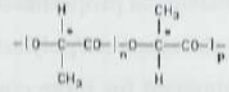
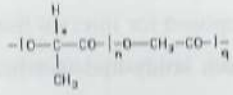
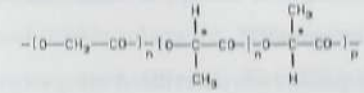
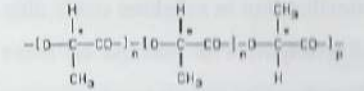
Poly-alpha-hydroxyacids	
poly(glycolic-acid)	
poly(L-lactic acid)	
poly(DL-lactic acid)	
poly(glycolide)	
poly(L-lactide)	
poly(DL-lactide) poly(meso-lactide)	
glycolide/L-lactide copolymers	
glycolide/D,L-lactide copolymers	
L-lactide/D,L-lactide copolymers	

Table II Chemical structure of different poly-alpha-hydroxyacids

Several composites of polymers with hydroxyapatite have been reported [Koeneman '86, Claes '86, Bonfield '86, Rueger '86, Alexander '87, Doyle '88a, Bonfield '88, Doyle '88b, Minabe '88, Scalzo '89], including the combination of hydroxyapatite and polylactide [Tencer '85, Ikada '85, Higashi '86]. Tencer coated porous hydroxyapatite with polylactic acid and concluded that the coating increased the mechanical properties of the material to properties equivalent to or greater than cancellous graft. Ikada mixed low molecular weight poly-DL-lactic acid with hydroxyapatite particles and an antibiotic. Samples were implanted in rat tibiae and were also investigated *in vitro* for the release of antibiotics. The results showed that the composites used had a capacity to release antibiotics, were compatible with bone, and were bioabsorbable, but evidence of a direct bone contact with the hydroxyapatite particles was not shown. However, this was reported by Higashi, who tested several polylactic acids and polylactides to which hydroxyapatite particles were added both *in vitro* and *in vivo*. Apart from the compatibility with bone, he also reported that the solubility of hydroxyapatite was markedly enhanced when mixed with polylactic acid oligomer.

The composites described in this thesis were produced by adding hydroxyapatite particles prior to polymerization of the polylactide. The hydroxyapatite particles were expected to enhance the mechanical properties of the polylactide matrix. The mechanical properties of different hydroxyapatite/polylactide composites have been tested by Verheyen '93; the values obtained for these composites are shown in table III. Daniels '90 reviewed the mechanical properties of several related biodegradable polymers and composites which were proposed for internal fixation of bone. Verheyen compared these values with those of his own study and concluded that the addition of hydroxyapatite particles did not improve the upper limits that have previously been reported for unfilled polylactide [Verheyen'92].

The composites were sterilized using ethylene oxide, because sterilization by steam or irradiation is known to affect the molecular weight of polylactides. The tests showed however, that sterilization in ethylene oxide also decreased the molecular weight, as well as the flexural strength. The storage of these composites at -20 or -80 °C did not significantly affect the flexural strength whereas storage at room temperature did. The physico-chemical properties were tested *in vitro* using different buffers to monitor the release of lactate and calcium, which are both indicators of degradation. The composites tested were composed of 30 % hydroxyapatite or 50% hydroxyapatite (w/w), and were

compared to specimens of polylactide without hydroxyapatite. The release of lactate was linear and significantly lower for the composites as compared to the unfilled polylactide. A similar pattern was obtained for the calcium release. An enhanced solubility of hydroxyapatite, due to the degradation of polylactide, was not demonstrated *in vitro*.

Table III Hydroxyapatite/polylactide composites investigated by Verheyen et al. '92

M/I	wt%HA	Mv (T=0)	Mv (ster)	Sflex (T=0)	Sflex (ster)
200/	0	130.000	110.000	83.3 ± 14.8	60.2 ± 3.5
600/	0	300.000	250.000	131.7 ± 11.0	107.7 ± 25.2
	30	150.000	125.000	93.0 ± 2.8	75.0 ± 4.5
	50	100.000	75.000	52.8 ± 12.4	50.1 ± 5.3
2000/	50	125.000	115.000	74.7 ± 8.1	69.1 ± 8.6

M/I	monomer/initiator ratio
wt % HA	% hydroxyapatite added to polylactide
Mv	molecular weight (viscosimetry)
ster.	after sterilization
Sflex	flexural strength

***in vitro* testing**

Biocompatibility testing of all materials intended for medical use should be performed prior to clinical application. These materials include any synthetic or natural polymer, metal, alloy, ceramic, or other non-viable substance, including tissue rendered non-viable, to be used as a device or any part thereof. The ISO (International organisation for standardization) has developed guidelines for the biological evaluation of medical and dental materials and devices.

Table IV gives an impression of the classification of the materials intended for medical use and the order in which the proposed tests should be performed. Significant is the fact that all materials should be tested upon their cytotoxicity prior to further evaluation. Cytotoxicity can be described as a process that injures or kills a living cell and a material can only be described as cytotoxic in relation to its effect on cells in a defined

Device categories			Biological effect												
Body contact (see 5.1)		Contact duration (see 5.2) A - Limited exposure (≤24 h) B - Prolonged exposure (24 h to 30 days) C - Permanent contact (>30 days)	Cytotoxicity	Sensitization	Irritation or intracutaneous reactivity	Systematic toxicity (acute)	Sub-chronic toxicity	Genotoxicity	Implantation	Haemocompatibility	Chronic toxicity	Carcinogenicity	Reproductive/developmental	Degradation
Surface devices	Skin	A	X	X	X									
		B	X	X	X									
		C	X	X	X									
	Mucous membrane	A	X	X	X									
		B	X	X	X									
		C	X	X	X		X	X						
	Breached surface	A	X	X	X									
		B	X	X	X									
		C	X	X	X		X	X						
External communicating devices	Blood path indirect	A	X	X	X					X				
		B	X	X	X					X				
		C	X	X		X	X	X		X	X	X		
	Tissue/bone/dentin communicating	A	X	X	X									
		B	X	X				X	X					
		C	X	X				X	X			X		
	Circulating blood	A	X	X	X	X				X				
		B	X	X	X	X		X		X				
		C	X	X	X	X	X	X		X	X	X		
Implant devices	Bone/tissue	A	X	X	X									
		B	X	X				X	X					
		C	X	X				X	X		X	X		
	Blood	A	X	X	X	X			X	X				
		B	X	X	X	X		X	X	X				
		C	X	X	X	X	X	X	X	X	X	X		

Table IV ISO guidelines for biological testing of medical materials

circumstance. In practice, it can be determined using tests which determine the lysis of cells (cell death), the inhibition of growth, and other effects on cells caused by test material(s) and/or extracts from the material(s). To identify the cytotoxicity of a material is unquestionably of major importance, but it is dependent on the assay, the cell type and the material used and is essentially a description of the material properties under defined conditions. It is dependent on several factors, therefore, many different assays are described in the literature [Guess '65, Rosenbluth '65, Sudilovsky '77, Tardiff '78, Wennberg '79, Ulreich '81, Schmalz '85, Cervinka '90, Sgouras '90, Srivastava '90, Kjellstrand '91, VanLuyn '91, Fortunati '91, Ciapetti '92]. In view of this, various cell types and preparation methods for the test materials have also been suggested [Haustveit '84, Harmand '86, Spangberg '88, McGauly '90, Rosdy '90, Saltzmann '91, Cima '91, Cornelis '91, Cenni '92, Cornelis '92].

These tests can be subdivided in to assays which indicate the viability, proliferation, activity (both cellular and metabolic), adherence, ability to synthesize, morphology, and differentiation of cells. These tests can be generally categorized into;

a) assessments of cell damage by morphological means,
b) measurements of cell damage,
c) measurements of cell growth,
d) measurements of specific aspects of cellular metabolism

Most tests indicate whether the sample is cytotoxic or not, rather than assessing the degree of toxicity. Some studies attempt to quantify the degree of toxicity, by categorizing the number of non-viable cells, but are still based on the all or nothing effect expressed in cell death. However, the cells may be affected by a material and not necessarily die; this effect is still best seen using morphology and/or by measuring metabolic activity. The proliferation of cells can be determined by counting cell numbers over a certain time period, however, it remains difficult to distinguish between cell death and inhibition of multiplication.

The reciprocal effects of the different factors involved in cytotoxicity (assay, cell and material) were investigated by Johnson et al part I '83 and II '85. He concluded that it is necessary to use several preparation/test methods and cell types to evaluate the cytotoxicity of a material, because of the discrepancies between the various test results.

The choice of which test should be used is not indicated by the ISO protocols, but should be made by the "appropriate professionals".

The intention of several authors to reduce the number of tests necessary for cytotoxicity assessment is expressed by Cingi et al '91, who found a solution in the choice and standardization of test protocols in cytoxicology by using a multicentre approach. Kjellstrand '91 compared 653 polymers intended for use in extracorporeal renal replacement therapy in a retrospective study to assess the ability of a single test or combination of tests, to predict the decisions taken on the basis of the large test battery. On the basis of this study it was concluded that only a limited number of tests have to be performed when assessing polymers intended for use in extracorporeal replacement therapy. Unfortunately these results can not be extrapolated to other materials intended for other uses.

The continuing development of new tests is related to the development of new materials, although improvements of already existing tests are also elaborated. The trend which can be extracted from the kinds of materials which are developed today is the shift from assumed non-degradable materials to biodegradable materials. The tests developed by Homsy '70 were based on the assumption that the *in vitro* degradation of polymers would be accelerated at high temperatures, thereby releasing compounds which are also expected to be released *in vivo* after long term implantation.

The proposed ISO tests today, also partially comprise an accelerated test, although the temperatures are lower and the exposure time is divided into different intervals up to 60 days as compared to the test of Homsy. If however, polymers are degraded *in vitro* in order to investigate the effect of degradation products on cells, one must consider that degradable polymers release more components into the surrounding fluid than would occur for the non-degradable materials. Apart from the type of material released (toxic adjuvants such as plasticizers and antioxidants or impurities) which can affect cells even in low concentrations, one should also consider that high quantities (high concentrations) of essentially non-toxic materials may still suggest toxicity.

OBJECTIVES OF THE STUDY

The aim of the present study was to test the suitability of hydroxyapatite/polylactide composites for application in reconstructive surgery in the maxillofacial area. The mechanical properties, as well as the tissue compatibility, are important factors for determination of a final application of such a composite. The tissue compatibility is investigated in this thesis.

The hydroxyapatite/polylactide composite is expected to be degradable and to possess bone bonding potential. The degradable properties of the material are closely related to the polylactide part. Therefore, an eventual tissue reaction will most likely be caused by the degradation products of this material. The possible short and long term effects of polylactide are investigated *in vitro* by presenting polylactides to cells in different stages of degradation. Different cell types will be in contact, directly or indirectly, with these polylactides when implanted, therefore several cell types were used in the *in vitro* experiments. To assess the *in vitro* biocompatibility of polylactide, different assays were used; these experiments are described in chapter II, III and IV. Chapter II focuses on the quantitative aspects of these experiments, whereas chapter III focuses on the qualitative facets. The results of these chapters formed the basis for the experiment described in chapter IV. The effect of large amounts of degradation products of polylactides (represented by lactic monomers) on cells was investigated.

Of course, the *in vivo* reaction is much more complex than can be investigated in an *in vitro* model, since one of the determinants for tissue compatibility is related to the inflammatory response evoked by the material. The inflammatory response of polylactide was determined by using an intraperitoneal injection model (chapter V). The main advantage of this model is that the wound reaction which normally occurs after a more invasive surgical procedure, is largely avoided.

The bone bonding potential of hydroxyapatite/polylactide composites will be attributable to hydroxyapatite. In this respect, an *in vitro* bone forming system was used to investigate whether the percentage of hydroxyapatite present on a polylactide surface indeed had an effect on the mineralization of the matrix (chapter VI).

Finally, in chapter VII, an *in vivo* experiment is described which investigated the bone bonding potential of hydroxyapatite/polylactide composites after implantation in bone tissue. The polylactide part was varied to create composites with different degradation rates.

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IN VITRO BIOCOMPATIBILITY TESTING OF POLYLACTIDES

PART I. PROLIFERATION OF DIFFERENT CELL TYPES

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ABSTRACT

Four polylactides, P-L-LAs 100 KD, 240 KD, 500 KD and a P-DL-LA 400 KD were tested *in vitro* by using five cell types. Middle ear, ear canal and nasal septum epithelial cells were used as well as fibroblasts and osteosarcoma cells. The proliferation of cells was studied by culturing on polylactide films and by culturing with media based on artificially aged PLA. The fibroblasts and the nasal septum epithelial cells were also cultured with media containing L- or D- monomers in different concentrations. Significant differences in cell numbers of polylactide cultures and/or controls were observed. These differences varied per cell type and experimental setting. In general the biocompatibility of the PLAs was satisfactory. Some inhibitory effect on the proliferation of high (10 mg ml^{-1}) monomer concentrations in culture media was seen.

INTRODUCTION

Polylactide (PLA), a degradable polymer, has found a limited clinical application [1-4]. Polylactide is degraded by a random hydrolytic chain scission process; this is described both *in vivo* and *in vitro* [5,6] and it has also been found that enzymatic degradation occurs *in vitro* [7] and *in vivo* [8]. The degradation rate of PLA polymers seems to depend on experimental setting, whereas the molecular mass and the composition of L- and/or D- enantiomers are the main determinants of the degradation rate [9-11]. Some studies revealed that after almost a year of implantation polylactide implants can still be

present, hardly changed in shape or mass [9,12-15]. Other implant studies show that a relatively high degradation could occur [16,17].

Studying the effects of the degradation products on individual tissues *in vivo* encounters difficulties because implantation is attended with a wound reaction. *In vitro* cell culture studies have the advantage of relatively well controlled variables. Most of the cell types normally present at the implantation site can be individually studied *in vitro*. It is known that *in vitro* cell testing is a very sensitive method for biocompatibility testing [18,19]. Generally, *in vitro* toxicity is in concordance with *in vivo* results [20], but discrepancies were also observed [21,22].

In the present study the effect of polylactides, with different degradation rates, on cell proliferation was quantitatively evaluated *in vitro*. Three poly-L-lactides were used as well as a poly-DL-lactide. Three distinct experiments were performed by using five different cell types. Three types of epithelial cells were used; middle ear-, ear canal- and nasal septum-epithelial cells. An osteosarcoma cell-line with osteoblastic properties (representing a bony implantation site) and fibroblasts were used. The osteosarcoma cells were derived from tumour cells and might therefore respond differently from non-neoplastic (bone derived) cells. These five cell types were representatives for an ear, nose and throat implantation site. To test the polylactides, various experiments were undertaken. In the first, cells were directly exposed to the polylactides by culturing on PLA films. In the second, the long term degradation of polylactide was simulated by exposure off cells to degradation products of artificially aged polylactides. The solution mimicking the long term degradation was used as a basis for culture medium. Finally, in the third experiment the cells were given different concentrations of the end-products of PLA degradation. The L- and D- monomers were added in different concentrations to the culture media to investigate whether an effect on the proliferation could be defined.

MATERIALS AND METHODS

Polylactides

The polylactides examined in this study were supplied by Purac biochem bv, Gorinchem, The Netherlands. Three poly(L-lactides) with molecular masses of 100,000, 240,000 and 500,000 Dalton and a poly(DL-lactide) with a molecular mass of 400,000 Dalton were used and two lactic acid solutions, D-lactic acid and L-lactic acid both 90 % w/v, were supplied by Purac.

Cell types and culture conditions

The cell types used in the experiments were rat epithelial cells originating from the middle ear, the ear canal and the nasal septum. Human fibroblasts and a human osteosarcoma cell line [23] were also used. All the cultures, except the osteosarcoma cells, were originally obtained from explants in our laboratory [24]. The osteosarcoma cells are a gift from S. Rodan, Merck, Sharp and Dome Laboratories, West Point, USA.

The cells were cultured with Dulbecco's Modified Eagles Medium and F12 in a 3:1 ratio to which hydrocortisone (0.4 μg/ml), isoproterenol (10^{-4} M), penicillin (100 U/ml), streptomycin (100 μg/ml) and 5% foetal calf serum had been added. Epidermal Growth Factor (10 ng/ml) was added after three days of culture. The medium was changed twice a week. The cells were cultured in 10 % CO_2 at 37 °C.

The cells were harvested from 14 cm culture dishes in their 5 th or 6 th passage by trypsinization, except for the osteosarcoma cells [23] of which the number of passages is not known. The epithelial cells were plated at a density of 1 x 10^5 cells per 35 mm dish together with lethally irradiated 3T3 feeder cells at the same density. The 3T3 cells were exposed to 3000 Rad (30 Gy) during 6.2 minutes by cobalt 60 gamma irradiation. The fibroblast and the osteosarcoma cells were plated at the same density but without the 3T3.

The test conditions in which the cells were studied were:
(a) culture of cells on polylactide films, (b) culture of cells with media based on the artificially aged PLAs, (c) culture of cells with lactic acid monomers.

Exposure of the cells directly to the polylactides

All the cell types were cultured on polylactide films for up to 2 weeks. The films were prepared by a solvent casting method using 5% PLA (P-L-LA 100, 240, 500 KD or P-DL-LA 400KD) in chloroform (w/w ratio). The initial thickness of the films was 500 μm. The films were cut into circular pieces to fit into 35 mm culture dishes (Greiner). They were then thoroughly rinsed in running tap water overnight and then washed in distilled water and air dried. The night before cell culturing the dishes with the films were sterilized by using UV light. The normal culture plastic (TCPS) served as a control.

Artificial aging media

Polymers degrade faster at elevated temperatures and thereby liberating additives

and toxic low molecular compounds, when introduced during processing and/or as a result of degradation itself. The additives and/or toxic low molecular compounds will diffuse out the material into the extraction fluid. Parts of the same films as used in the first experiment were cut into pieces and exposed to 115 °C in a pseudo-extracelluar fluid (PECF) for 60 hours.

ion concentration (meq/l) of PECF	
Na^+	154.5
K^+	5.4
Cl^-	118.5
HCO_3^-	44

The surface area of the polylactide films was 0.38 cm^2 ml^{-1} extrusion fluid. This method has been described in earlier publications [20,25]. The heat-exposed solutions were used as a basis to prepare nutrient media for cell culturing. These media will be referred to as artificial aging media. The heat-exposed pseudo-extracelluar fluid without PLA served as a basis for the control medium.

Concentrations of L- and D- monomers

In the third experiment only nasal septum epithelial cells and human fibroblasts were cultured in medium containing 0.001, 0.01, 1, or 10 mg ml^{-1} L- or D-monomers, also for two weeks. The lactic acid solutions were, after adjusting the pH with NaOH, added to routine culture medium in the different concentrations. Routine culture medium served as a control as well as routine medium to which sodium chloride ions (NaCl) were added. The amounts of sodium chloride were 2.18 mg ml^{-1}, resulting in an osmolarity of 378 mOsm l^{-1}. However, the concentrations of 10 mg ml^{-1} increased the osmolarity to about 500 mOsm l^{-1} (Fig.1), because the monomers were added to media which already had osmolarities of about 337 mOsm l^{-1}. Therefore, in a subsequent experiment the media were prepared by adding ingredient after ingredient thereby reducing the amount of sodium chloride so that the monomers could be added, resulting in a limited increase of osmolarity (Fig.2). Controls were made by adding normal amounts of sodium chloride to the media (resulting in medium similar to routine culture medium) and by adding 5 mg ml^{-1} sodium chloride resulting in an osmolarity of 500 mOsm l^{-1}.

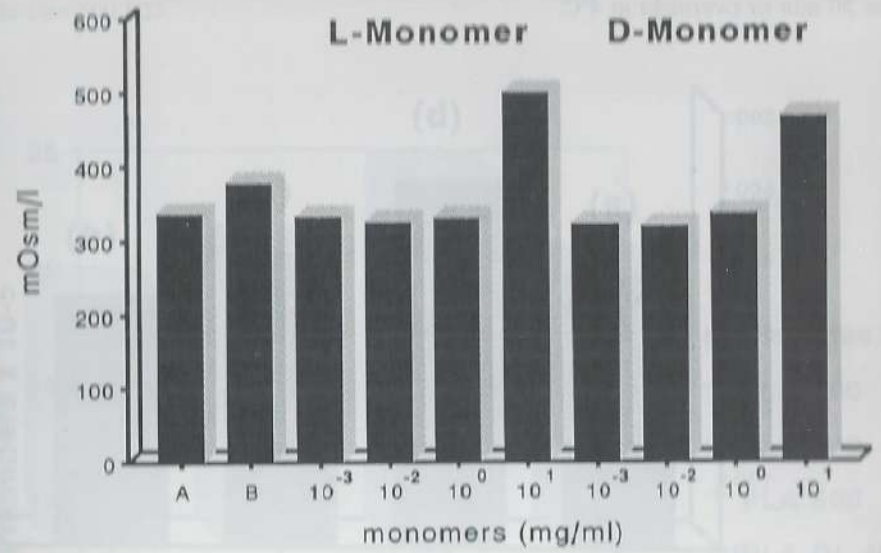

Fig.1 The osmolarities of media (mOsm l^{-1}) to which different concentrations of L- or D-monomer were added. Control A is routine culture medium, Control B is routine culture medium + 2.18 mg ml^{-1} NaCl. The L- and D-monomers are added in the concentrations 0.001, 0.01, 1 and 10 mg ml^{-1}.

Quantitative evaluation

For all three test conditions cells were counted on day 1, 4, 6, 10, and 14. For the second and third experiments, cells were also counted on day 3. Three dishes for each sample were used. Statistical analysis was performed for each cell type per experiment by using multi-analysis of variance (MANOVA) of the logarithmic transformed cell numbers at a confidence level of 95 %. On the basis of the calculations made by the MANOVAs multiple range tests using the Sheffé method were performed. Multiple range testing is useful to obtain an impression of which condition(s) was or were significantly different. The multiple range test ranks the cell numbers. Statistical calculations were performed with the software package STAT GRAPHICS.

Light microscopy

The cell cultures were observed by using a phase contrast microscope. Cell cultures were fixed in 1.5 % glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.4, 4°C) for

at least 2 hours and post fixed with 1% osmium tetroxide solution at room temperature for 30 min or overnight at 4°C.

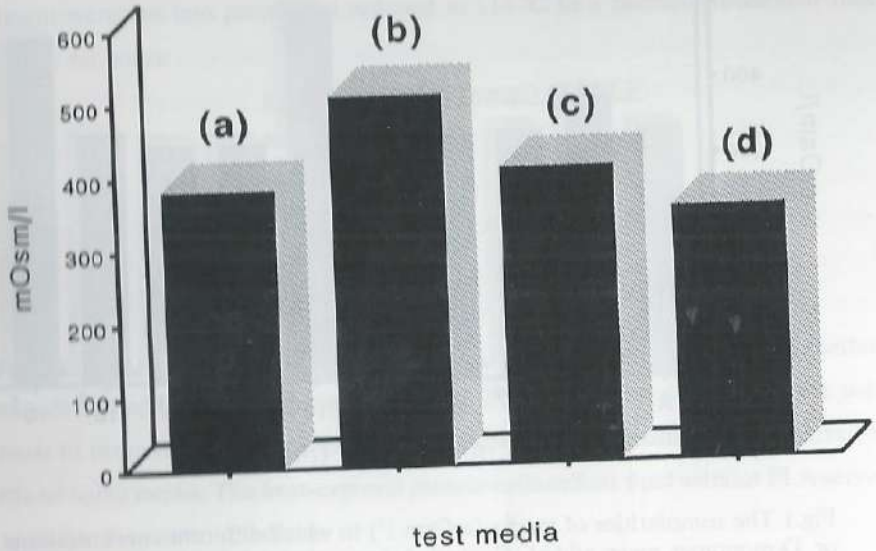

Fig.2 The osmolarities of media (mOsm l^{-1}) to which L-monomer (c) and D-monomer (d) were added (10 mg ml^{-1}) without increasing the osmolarity. Medium with extra NaCl (b) (5 mg ml^{-1}) served as a control as well as medium with normal amounts of NaCl (a).

RESULTS

Exposure of the cells directly to the polylactides

Proliferation of the cells on the polylactide films was studied by counting cell numbers after 1, 3, 6, 10 and 14 days of culture. The cell numbers were statistically evaluated and the shapes of the growth curves were comment upon; not all data are shown in figures. The cell numbers of the controls (tissue culture polystyrene) were the highest during the two weeks of culture for all cell types, except for the middle ear epithelium. The middle ear epithelium and the fibroblasts revealed no significant differences in cell numbers for the control and the polylactides. The growth curves of the human fibroblasts are shown in Fig.3. The nose epithelial cells and the osteosarcoma cells showed only significant differences between the control and polylactide 100 KD. The ear

canal epithelial cells showed only a significant difference between the control and polylactide 500 KD.

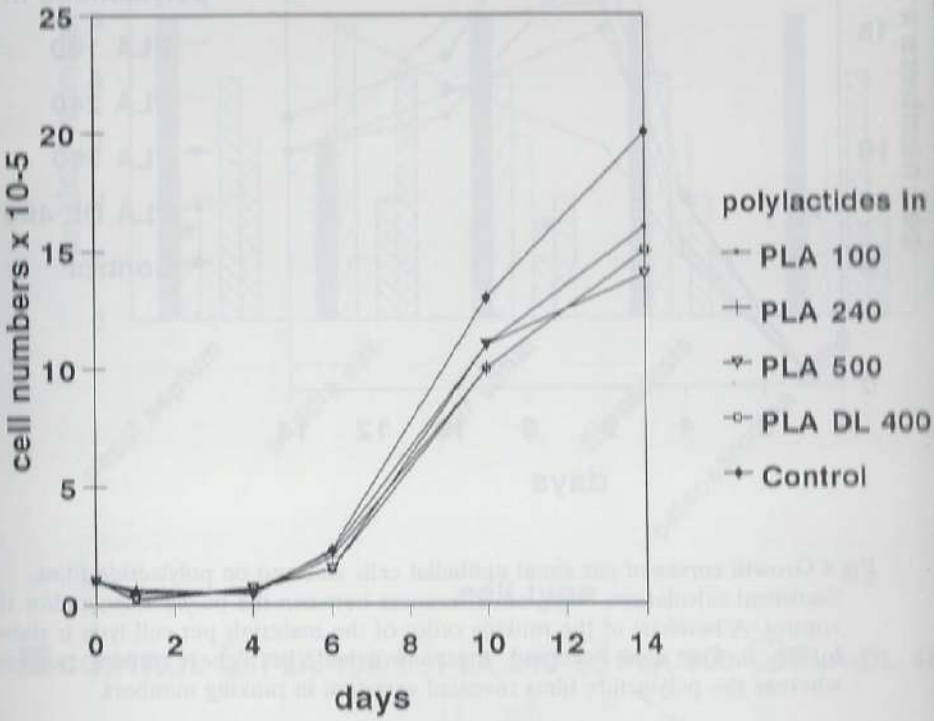

Fig.3 Growth curves of human fibroblasts cultured on polylactide films.

In general, the proliferation of the cells on the polylactide films as compared with the control revealed a similar pattern although some retardation could be observed. The ear canal epithelium revealed a differently shaped growth curve for cells cultured on the polylactide film 100 KD as compared with the others. The cell number increased throughout the two weeks whereas the others already revealed a slight decrease or a plateau after 6 days of culture (Fig.4). Proliferation of cells on the films was difficult to examine by light microscopy because the films were in general insufficiently transparent. With increasing culture time the films became more opaque.

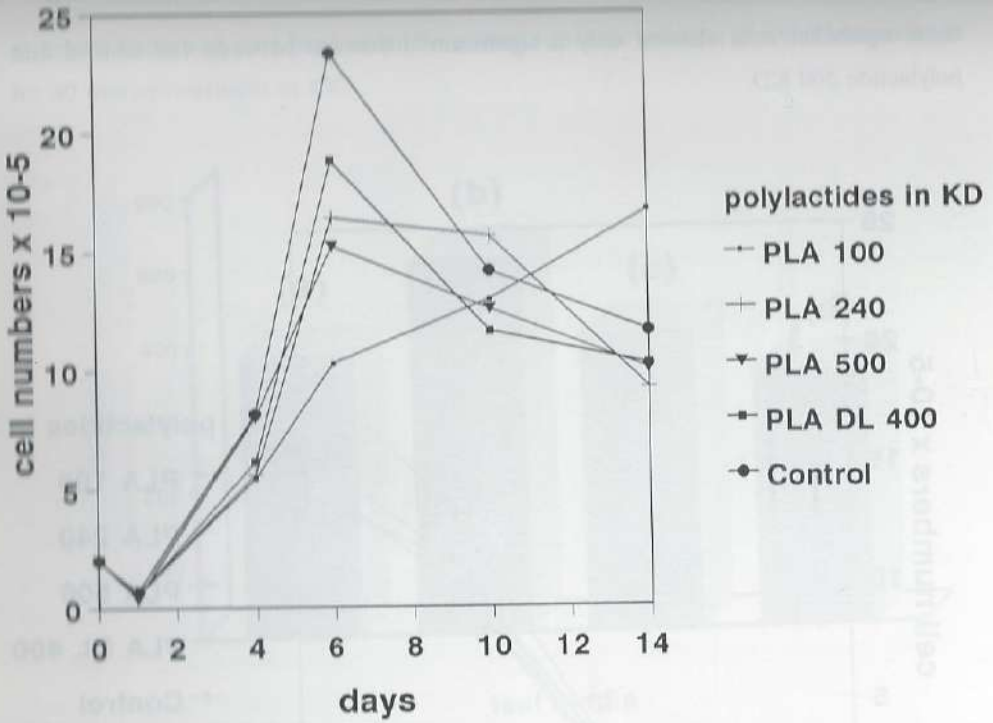

Fig.4 Growth curves of ear canal epithelial cells cultured on polylactide films. Statistical calculations revealed differences between the polylactides and/or the control. A barchart of the ranking order of the materials per cell type is shown in Fig. 5. One case excepted, the control had the highest ranking numbers whereas the polylactide films revealed variation in ranking numbers.

Artificial aging media

Cell proliferation was studied by counting cell numbers after 1, 3, 4, 6, 10 and 14 days of culture. The cell numbers were statistically evaluated and the shapes of the growth curves were comment; not all data are shown in figures. Statistical calculations showed that the osteosarcoma cells proliferated less in the artificial aging media based on the polylactides 100 and 240 KD than in the other artificial aging media (Fig.6). The other cell types did not show this phenomenon.

In general, the proliferation pattern of the cells cultured with the PLA artificial aging media were similar to the control artificial aging medium. The artificial aging medium based on polylactide 240 KD displayed a slightly different growth curve for the middle

ear epithelium. A plateau was not reached but an increase in cell number up to day 14 was seen (Fig.7).

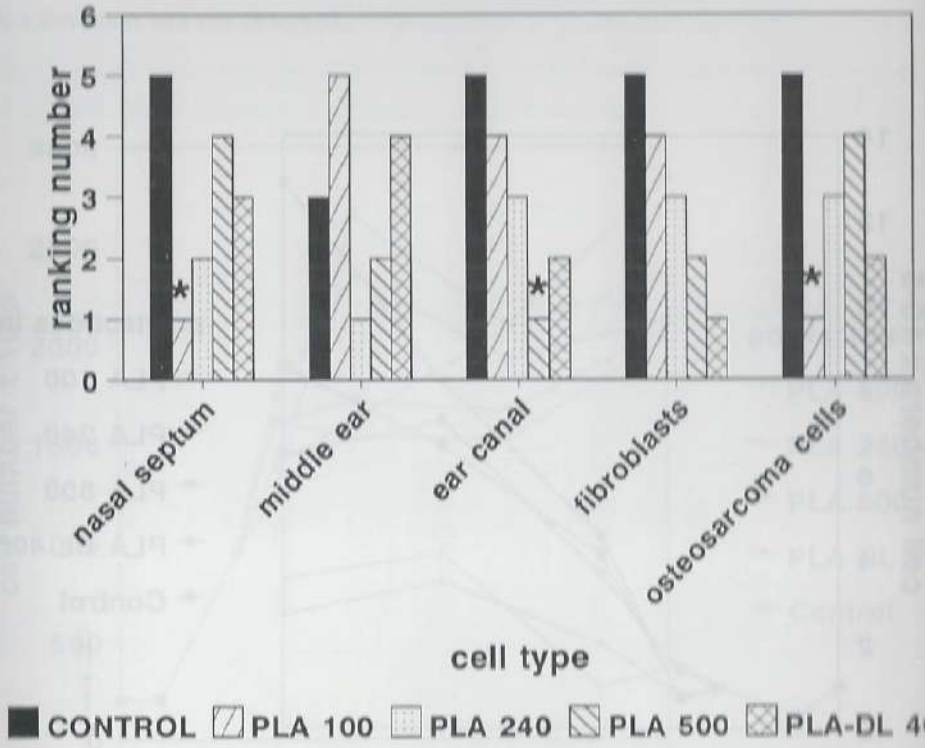

Fig.5 The polylactides and the control with the corresponding ranking numbers per cell type are shown. Ranking number 1 indicates lowest cell number. * Significantly different from the control ($p<0.05$).

The cultures could be well observed by using light microscopy, in contrast with the first experiments where the films hindered observation. The proliferation of cells cultured with the PLA based artificial aging media was similar to those cultured with the control artificial aging medium. The increase of cell numbers was generated from colonies eventually resulting in confluent cultures. Confluent cultures were already present after six days of culture, except for the ear canal epithelial cells which were confluent after 10 days of culture. The fibroblasts and the osteosarcoma cells developed a stratification of cells combined with a parallel orientation of cells into tracks.

Statistical calculations revealed differences between the polylactides and/or the control. A barchart of the ranking order of the materials per cell type is shown in Fig 8. A variation in ranking numbers is seen for all conditions.

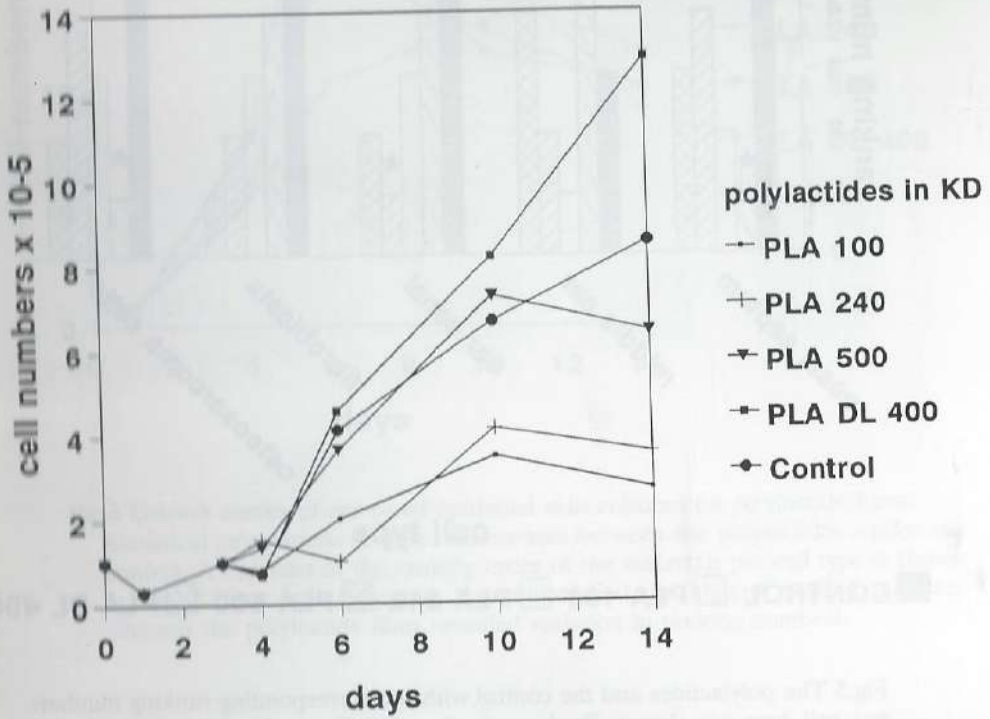

Fig.6 Growth curves of osteosarcoma cells cultured with the artificial ageing media.

Monomer concentrations

In this experiment we investigated the effect of different monomer concentrations on cell proliferation. The cells were counted after 1, 3, 4, 6, 10 and 14 days of culture. Of the used concentrations only the highest concentrations, both L- and D-monomers, revealed an apparent retardation in proliferation. Fig.9 shows the ranking numbers of both cell types for each culture condition. Some discrimination in the ranking order is present for the human fibroblast. All the D-monomer concentrations have lower cell

numbers as compared with the corresponding L-monomer concentrations. Statistically however, besides the highest concentrations, only the 1 mg ml^{-1} D-monomer was significantly lower from the other L-monomer concentrations. For the nose epithelial cells such a deviation was not observed.

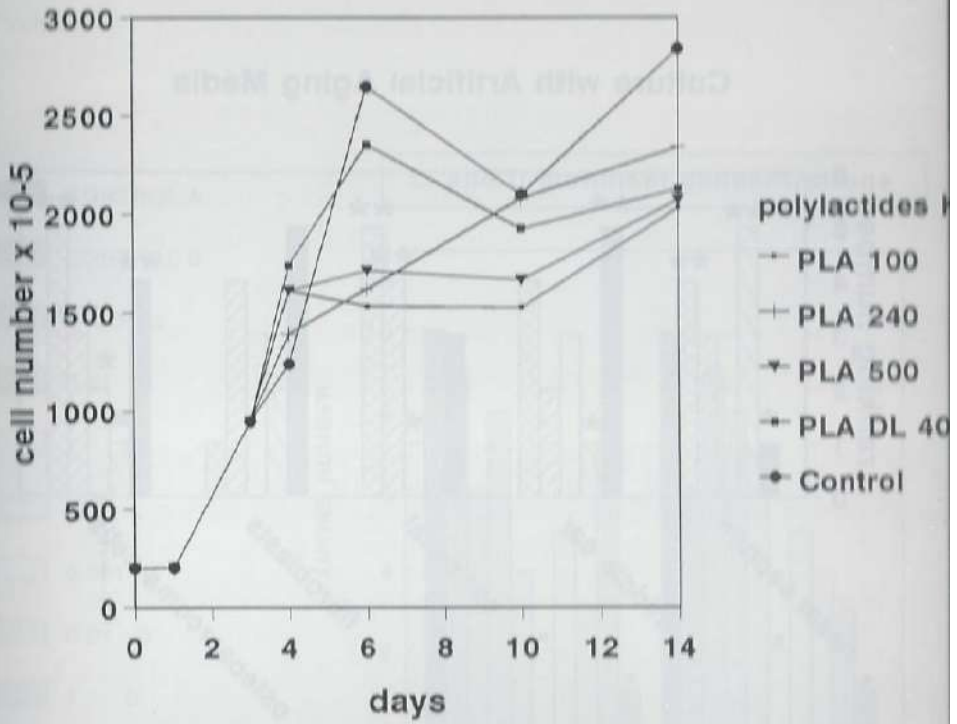

Fig. 7 Growth curves of middle ear epithelial cells cultured with artificial ageing media.

As mentioned before, of the concentrations used only the highest concentrations, both L- and D-monomers, revealed an apparent retardation in proliferation. However, the osmolarities were increased to a level of approximately 500 mOsm l^{-1}. To investigate whether this was caused by the high osmolarities or the high monomer concentrations, the cells were also cultured with routine culture medium to which sodium chloride was

added to the same level of 500 mOsm l^{-1} and compared to cells cultured with media to which 10mg ml^{-1} monomer was added without increasing the osmolarity considerably (by lowering the amount of sodium chloride and replacing it by high concentrations of monomer). The osmolarities were 406 mOsm l^{-1} for the L-monomer and 347 mOsm l^{-1} for the D-monomer. The cell numbers of the cultures with high monomer concentrations

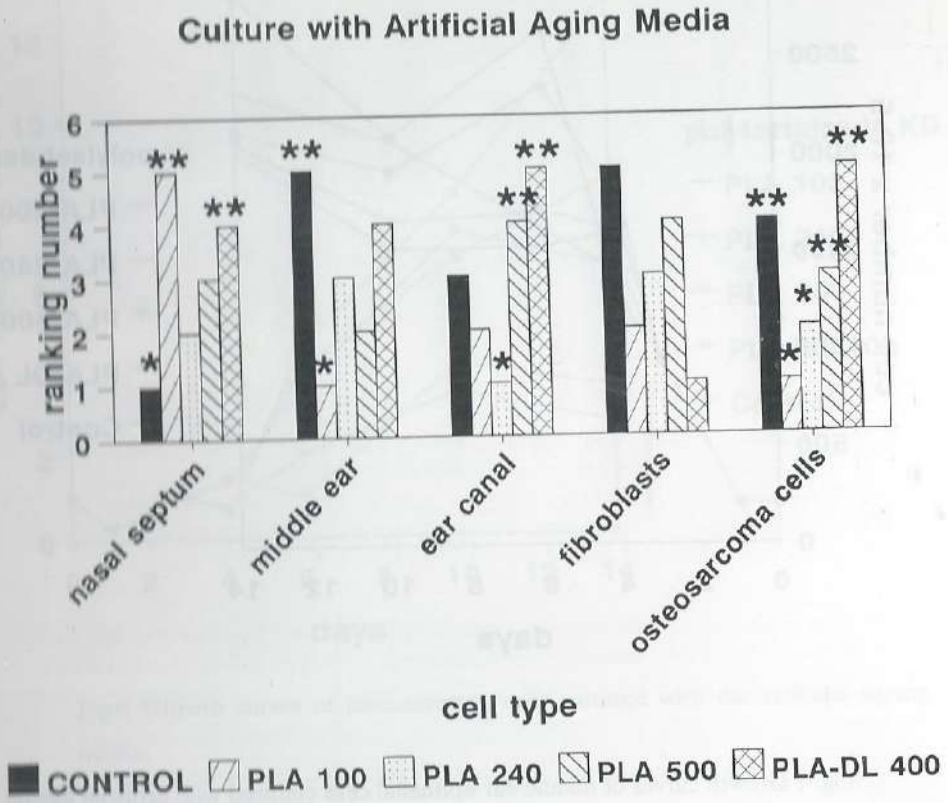

Fig.8 The polylactides and the control with the corresponding ranking numbers per cell type are shown. Ranking number 1 indicates lowest cell number. * and ** are significantly different ($p<0.05$) from each other.

again revealed retardations which were not significantly different from the medium of which the osmolarity was increased to a level of 500 mOsm l^{-1} (Fig.10). This was observed for both cell types. The cells cultured with the routine culture medium had significantly higher cell numbers than cells given media with the monomers or high sodium chloride. Both cell types used, nose epithelium and fibroblasts, showed a normal proliferation pattern using light microscopy. The nose epithelium was confluent already after 4 days of culture.

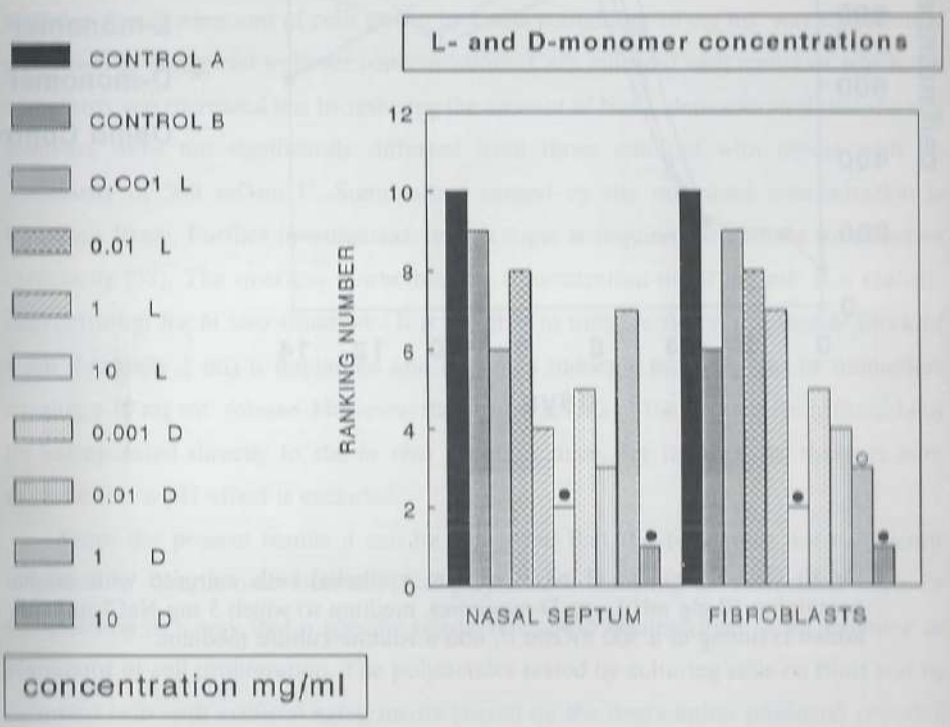

Fig.9 Ranking numbers of both cell types for each culture condition. Ranking number 1 indicates lowest cell number. (0)Significantly ($p<0.05$) lower cell numbers than the other concentrations and the control. (0) Significantly different from the L-monomer concentrations and the controls but not from the other D-monomer concentrations.

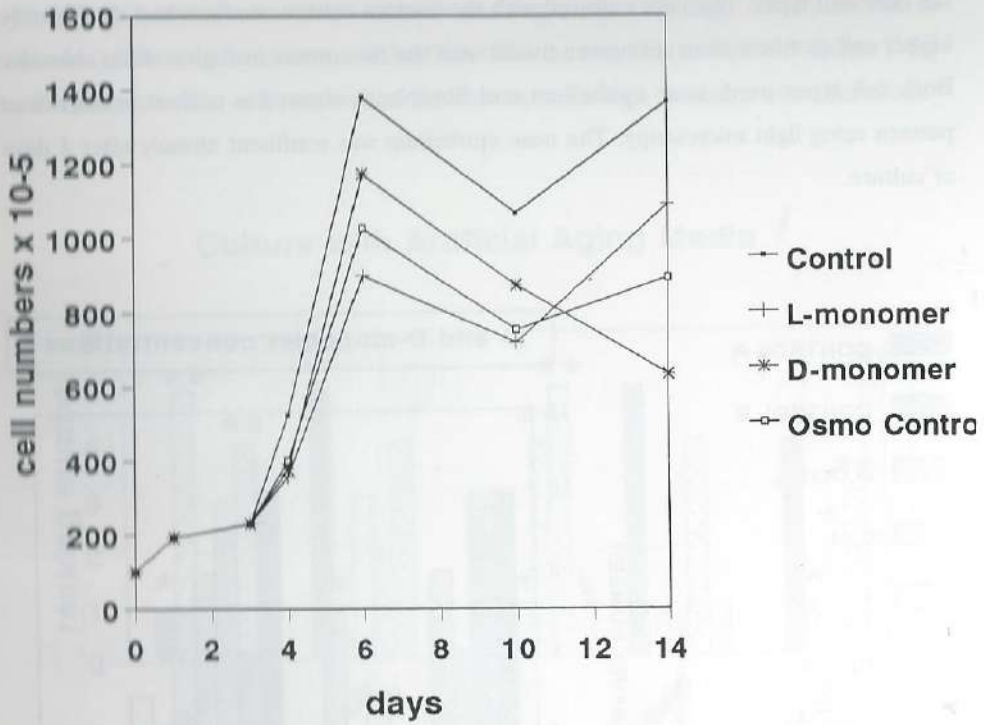

Fig.10 Growth curves of nasal septum epithelial cells cultured with media containing 10 mg ml^{-1} L- or D-monomer, medium to which 5 mg NaCl ml^{-1} was added resulting in a 500 mOsm l^{-1}, and a routine culture medium.

DISCUSSION AND CONCLUSIONS

The polylactides tested in the present study showed differences in cell proliferation. The differences between the polylactides and/or the control were dependent on cell type and experimental condition. A prominent retardation or stimulation caused by the polylactides was not observed. This was seen for the exposure of cells directly to the films as well as for the exposure to the artificial aging media. Although *in vitro* cell testing is

a very sensitive method for biocompatibility testing, to discriminate toxic from non-toxic materials seems to be easier than to determine the degree of toxicity [20,25-28]. Since dissimilarity in reactions of distinct cell types towards the same material occurs *in vitro* [29,30] as well as *in vivo* [31], it is necessary to test with the cell types present at the implantation site. Epithelial cells will normally not be in contact with an implant. However, diffusion of toxic compounds out of the implant could eventually reach epithelial cells. Therefore epithelial cells should be included in testing materials which will not be in contact with epithelium *in vivo*.

The experiment in which the cells were cultured with the monomers of polylactide revealed that the amount of cells grown in media containing 10 mg ml^{-1} was significantly decreased as compared to lower concentrations. Cells cultured with media of which the osmolarity was increased less by reducing the amount of NaCl, demonstrated that the cell numbers were not significantly different from those cultured with media with an osmolarity of 500 mOsm l^{-1}. Some effect caused by the monomer concentration is therefore likely. Further investigation on this topic is required to exclude an effect of osmolarity [32]. The question is whether the concentration of 10 mg ml^{-1} is a realistic concentration for *in vivo* situations. It is possible to imagine that a polylactide block of 1 cm^3 (virtually 1 ml) is implanted and degrades suddenly for 1 % into its monomers causing a 10 mg ml^{-1} release. However, the *in vitro* results of the present study should not be extrapolated directly to the *in vivo* situation since, for instance, in these *in vitro* experiments a pH effect is excluded.

From the present results it can be concluded that the tests were not sufficiently sensitive to discriminate between the polylactides differing in molecular mass or composition in a way that a specific polylactide could be described as an inhibitor or stimulator of cell proliferation. The polylactides tested by culturing cells on films and by culturing cells with artificial aging media (based on the degradation products) revealed a satisfactory biocompatibility. Significant differences were observed, but these varied per cell type and/or experimental setting. The monomer experiments need further investigation.

Quantitative testing, although very useful in evaluating large numbers of cell types under various conditions, is a restricted approach to biocompatibility testing. Qualitative aspects also need to be considered. In chapter III the morphology of the cells using light microscopy, transmission and scanning electron microscopy is described.

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IN VITRO BIOCOMPATIBILITY TESTING OF POLYLACTIDES

PART II. MORPHOLOGIC ASPECTS OF DIFFERENT CELL TYPES

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ABSTRACT

Four high molecular weight polylactides, three poly-L-lactides of 100, 250, 500 kDa and a poly-DL-lactide 400 kDa were tested qualitatively *in vitro*. Cells were cultured on polylactide films and with media based on the artificially aged polylactides, as well as with different concentrations of the final degradation products (monomers). Implant site related cell types were selected. Three kinds of epithelial cells (middle ear, ear canal and nasal septum) as well as fibroblasts and osteosarcoma cells were used. Characteristic of all four polylactides was the normal morphology of cells when cultured on polylactide films and with the artificially ageing media. Although the polylactide films revealed still uncovered spots after 14 days of culture, the control cultures were already confluent. The different concentrations of monomers revealed normal cell morphologies except for the 10 mg ml^{-1} concentration, which showed larger fibroblasts and the nasal septum epithelium showed more signs of terminal differentiation for the 10 mg ml^{-1} D-monomer than was seen for the L-monomer even if the osmolarity was adjusted. The degradation products of polylactide are not expected to cause adverse reactions when implanted, since cell cultures with monomer concentrations up to 1 mg ml^{-1} resulted in normal morphologies. In the present study were the cells not able to cover the polylactide films completely.

INTRODUCTION

Polylactides are already used clinically in different disciplines such as maxillofacial surgery, dentistry and orthopaedics [1-3]. Research is still extending to improve the characteristics of the polymer. As a first approach of testing newly obtained polymers *in*

vitro studies should be used [4-6]. However, biocompatibility studies of polylactides are regularly performed *in vivo* but seldom *in vitro* [7,8].

In vitro biocompatibility studies have the advantage that cell-material interactions can be studied without the predominant wound reaction that normally occurs after implantation. Such a wound reaction interferes with the events caused by the material itself. Besides the lack of an interfering wound and inflammatory reaction, the sensitivity of *in vitro* biocompatibility tests is known to be equal or greater than that of *in vivo* studies [5,9,10]. In general, *in vitro* studies use quantitative measurements to determine an effect of the material on the cells, as was described in [11]. Qualitative evaluations such as the morphology of cells are supplementary to quantitative measurements [12]. Normal phenotypic expression of cells is a major indication of its well being. The cell surface appearance and intracellular accumulation of degradation products, for instance in lysosomes, are areas of interest that can be studied. Culture dependent occurrences such as confluency, stratification and differentiation can also be observed.

In this study high molecular weight polylactides, three poly-L-lactides of 100, 250 and 500 kDa and a poly-DL-lactide 400 kDa were tested qualitatively *in vitro*. Cells were cultured on polylactide films, as an approach to the *in vivo* situation shortly after implantation (degradation was hardly expected in the culture period of 14 days). Two other experimental settings were chosen in such a way that different stages of degrading implants were imitated *in vitro*. Therefore, the cells were either cultured with media based on the artificially aged polylactides, mimicking long-term implantation periods, or in different concentrations of the monomers resembling the release of final degradation products within a particular time period. Implant site-related cell types were selected to evaluate the polylactides, because the sensitivity can depend on the cell type. The *in vivo* phenomenon that different cell types can respond differently towards the same material [13] was also demonstrated *in vitro* [14]. In the present experiments three kinds of epithelial cells, a stratifying squamous-, a pseudostratified-, and a simple squamous epithelium were used. Fibroblasts and osteosarcoma cells were also used. The osteosarcoma cells are derived from tumour cells and might, therefore, respond differently from non-neoplastic (bone derived) cells. The cells were evaluated with light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Polylactides

The polylactides used in this study were supplied by Purac biochem BV, Gorinchem, The Netherlands. Three poly-L-lactides with molecular weights of 100,000, 240,000 and 500,000 were used and a poly-DL-lactide with a molecular weight of 400,000. Two lactic acid solutions were used as representatives of the final degradation products of polylactides. D-lactic acid and L-lactic acid [both 90 % w/v] were supplied by Purac biochem bv, Gorinchem, The Netherlands.

Cell types and culture conditions

The cell types used in the experiments were rat epithelial cells originating from the middle ear [15], the ear canal [16] and the nasal septum. Human fibroblasts and a human osteosarcoma cell line [17] were also used. The cells were originally obtained from explants in our laboratory [15], except the osteosarcoma cells, which were a gift from S. Rodan, Merck, Sharp and Dome Laboratories, West Point, New York, USA. Culture conditions were described in [11]. In short, cells were cultured with Dulbecco's modified Eagles medium and F12 in a 3:1 ratio to which 5% foetal calf serum had been added. Epidermal growth factor (10 ng ml^{-1}) was added after three days of culture. The medium was changed twice a week. The cells were cultured in 10 % CO_2 at 37 °C. The epithelial cells were plated at a density of 1 x 10^5 cells per 35 mm dish together with lethally irradiated 3T3 feeder cells at the same density. The 3T3 cells were exposed to 3000 rad for 6.2 minutes by Co^{60} gamma irradiation.

Experiments

Experimental conditions are described in detail in part I [11]. In short, three types of experiments were used. In one experiment the cells were directly exposed to the polylactides by culturing on films. In another experiment the cells were cultured with artificial ageing media. These media were obtained by exposing polylactide films to elevated temperatures in a pseudo-extracelluar fluid. The extraction fluids were used to prepare culture media. The third experiment investigated the effect of different concentrations (0.001, 0.01, 1, 10 mg ml^{-1}) of D- and L- monomers. The monomers (the pH was adjusted with NaOH) were added to normal culture media as well as to media

of which the osmolarity initially was lowered, after which the monomers were added. Routine culture medium served as a control as well as medium to which extra NaCl was added. The osmolarities of the media obtained were determined (table I).

All experiments lasted up to 14 days.

Normal culture medium	337	
Control culture medium	378/508	
Normal culture medium + monomer mg ml^{-1}	L-monomer	D-monomer
0.001	333	323
0.01	326	320
1	331	337
10	500/406	468/347

Table I. Osmolarities of the media with the different monomer concentrations. The osmolarities are given in mOSm/L. Up to 1 mg ml^{-1} the osmolarities are hardly changed. The second value given for the 10 mg ml^{-1} concentrations are of media of which the osmolarities were initially lowered.

Preparation for morphological study

Cell cultures were fixed by immersion in 1.5 % glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.4, 4 C) for at least 2 h and post fixed with 1% osmium tetroxide solution at room temperature for 30 min or over night at 4 C. The cell cultures which were first used for light microscopy, were either dehydrated, critical-point dried and gold sputter-coated for SEM or dehydrated, embedded in epon and cut into ultra-thin sections for TEM.

The morphology of the cells was studied using phase contrast light microscopy, SEM and TEM (SEM, Cambridge 180 Stereoscan or a Philips SE 525; TEM, Philips EM 201).

Light microscopy was not possible for the cells cultured on the films as the films were generally too opaque. In general, the cultures from day 14 were used for extensive evaluation, the shorter culture periods being checked with light microscopy when possible, but not always with SEM and/or TEM.

Normal morphology

By SEM, flat cells with various amounts of microvilli were seen for all three types of epithelia. The cells also varied in size and form. Cell-cell contact was 100 % for the middle-ear and nasal-septum epithelia, but less for the ear-canal cells (fig.1).

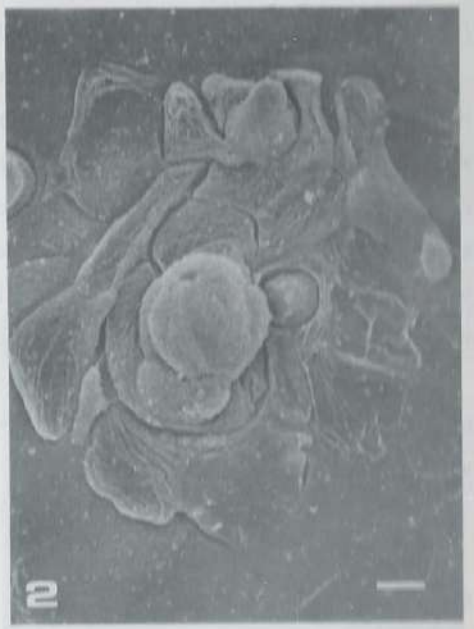


Fig.1 Normal ear canal epithelium after 6 days of culture, note the irregular cell shape and the partially cellular contact. SEM-micrograph, bar is 5 μm.

Fig.2 Normal ear canal epithelium after 6 days of culture already showing differentiation and cornification signs. Ruffled cell surfaces and a clod of cells. SEM-micrograph, bar is 10 μm.

The three kinds of epithelial cells possess some differences in culture dependent occurrences such as confluency, stratification and differentiation. The cultures of the epithelia started by seeding epithelial cells with lethally irradiated feeder cells. Cultures of the three epithelial cell types revealed colonies of cells intermingled with lethally

irradiated 3T3 feeder cells on day 1. The colonies grew until confluent cultures were present. The 3T3 feeder cells were pushed out of the cultures when the epithelial cells occupied more of the surface.

The middle-ear epithelium (a simple squamous epithelium) developed into a monolayer. Confluency was reached in 6 days. The nasal-septum epithelium (a pseudo-stratified epithelium) developed into a pseudo-multilayer. Confluency was reached within 6 days. The ear-canal epithelium showed clear signs of differentiation and cornification (fig.2). Confluency was reached within 10 days.

In short, the osteosarcoma cells as well as the human fibroblasts developed into tracks of parallel-orientated cells while multilayering. The osteosarcoma cells had a spindle-like appearance but, flatter, rounder cells were also seen (fig.3). These flat, round cells were often seen at crossings of tracks. Some cells had microvilli and bleb-like structures on the cell surface. TEM revealed that these bleb-like structures were filled with the same cytoplasm as was seen for the rest of the cell, and no secretory vesicles were observed as might have been expected. The packing of the cells was loose.

The human fibroblasts were spindle-like in appearance and had hardly any microvilli on their surface. TEM often showed invaginations of the cell membrane, and no blebs were seen.

Culture on polylactide films

The first observation that was characteristic to all four polylactides was the normal morphology of cells cultured on polylactide films.

However, the three kinds of epithelial cells showed however some differences in culture dependent occurrences such as confluency, stratification and differentiation when cultured on films, compared with their control cultures. The epithelial cells cultured on the films never reached complete covering of the films during the culture period of 14 days. Cells surrounding uncovered parts of films were clustered and seemed unable to spread on the film (fig.4). Cornification of the ear-canal cells occurs normally after confluency is reached, but also in cultures grown on films that were not confluent (parts of the films were uncovered). TEM revealed basal cells on which a few cell layers were present, and cornification (fig.5).

Generally the cells cultured on the films were similar to those seen in the control

cultures.

The fibroblasts detached easily from the films when being prepared for SEM or TEM. Whether full covering of the films had occurred could therefore not be concluded. The cells that could be evaluated were similar to those of control cultures. The osteosarcoma cells revealed uncovered spots of the films, but the morphology was normal (fig.6).

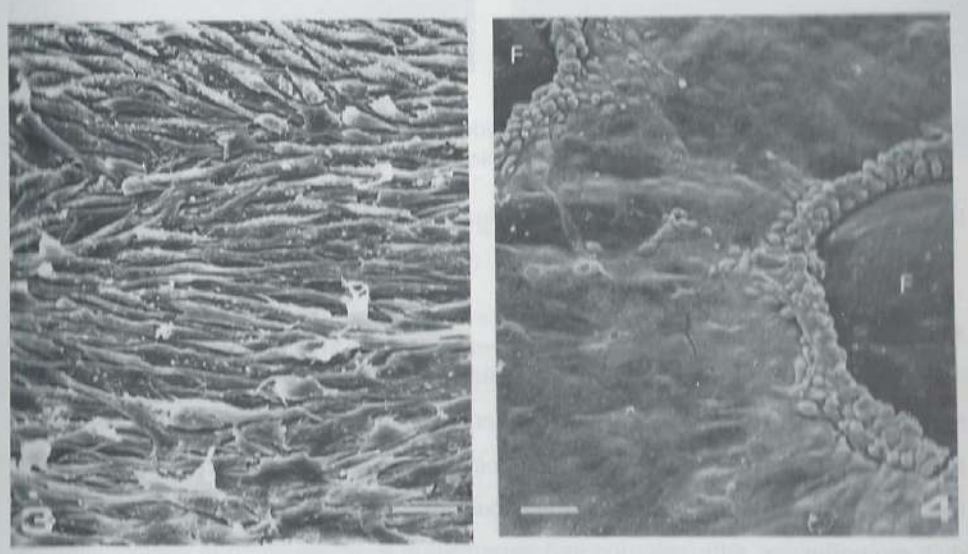


Fig.3 Control culture of osteosarcoma cells after 14 days. SEM-micrograph, bar is 30 μm.

Fig.4 Middle ear epithelium cultured on the DL-polylactide film (F) for two weeks. Note the clustered cells around the bare spots. SEM-micrograph, 30 μm.

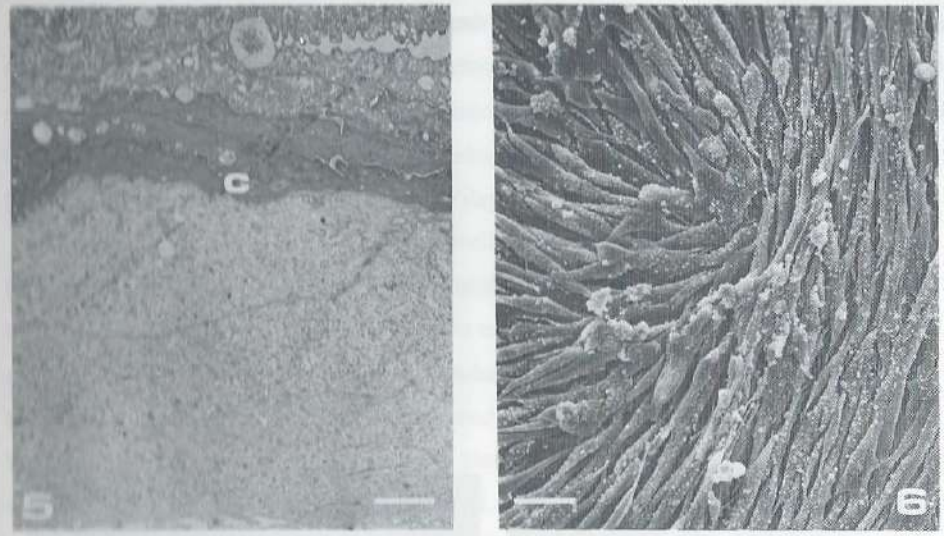


Fig.5 Ear canal cells cultured on Polylactide film (Mw 250.000). Cross-section of the multi-layer, cornification (C) is visible. TEM-micrograph, bar is 0.14μm.

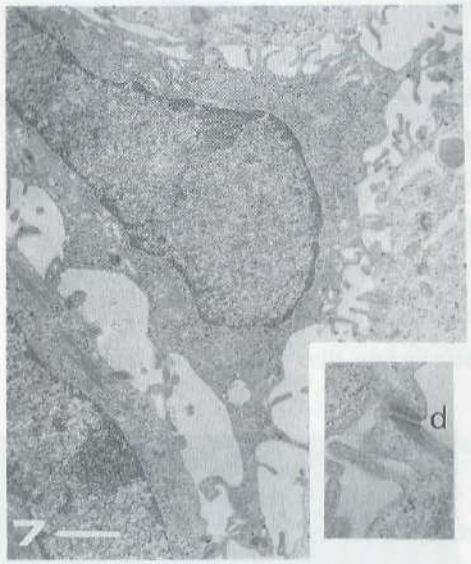
Fig.6 Osteosarcoma cells cultured on polylactide film (Mw 250.000). SEM-micrograph, bar is 30 μm.

Artificial ageing media

The same cell types were also cultured with artificial ageing media. These media mimicked the release of materials of long term implanted polylactide. The same characteristic was found as was seen for the culture on films; all cell types cultured with the artificial ageing media revealed normal morphologies. Culture dependent occurrences such as confluency, differentiation and cornification were similar to in control cultures (fig.7).

Culture with different L- and D-monomer concentrations

The nasal septum epithelium and the fibroblasts were cultured with different concentrations of monomers. The cells cultured with these media proliferated well compared to the control cells, except for the highest concentrations (10 mg ml^{-1}).



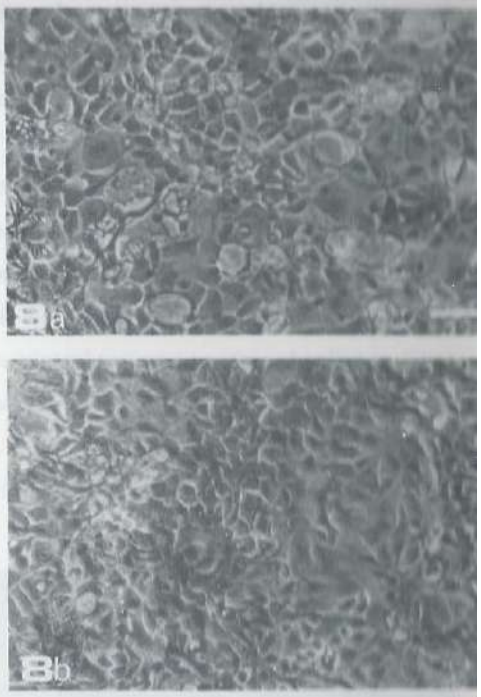


Fig.7 Nasal septum epithelium cultured with control artificial ageing medium for 14 days. Inset shows a desmosome (d). TEM-micrograph, bar is 0.2 μm.

Fig.8 Nasal septum epithelium cultured with 10 mg D-monomer/ ml medium (a) for 14 days. Note the more cornefied envelopes as is seen for the 10 mg L-monomer/ ml medium (b). Phase-contrast light-micrograph, bar is 32 μm.

The fibroblasts had a normal appearance but developed into less-dense tracks for the 10 mg ml^{-1} concentrations. Cross-sections (light microscopy and TEM) revealed that cell development into multi-layers occurred, as was seen for the other concentrations and the controls. The TEM morphology of the cells had a similar appearance to that seen for control cells. The nasal-septum epithelial cells revealed more variation in cell size for the 10 mg ml^{-1} concentration, and sometimes more vacuoles were seen in the cytoplasm. The cells were already confluent at the beginning of the experiment (at day 4) when they were cultured with routine culture medium and showed no decrease in confluency after adding the experimental media.

When both cell types were cultured again with 10 mg ml^{-1} monomer concentration but with a osmolarity correction, the morphologies were more like those of control cultures. An exception was the nasal septum epithelium, which revealed more manifest cornefied envelopes with 10 mg D-monomer ml^{-1}. This was best seen with phase contrast light microscopy (fig.8).

When the cells were cultured with media to which 500 mMol/l extra NaCl was added the fibroblasts were big and flat, and the nasal septum epithelium showed the same deviations as was seen earlier with the 10 mg ml^{-1} monomer without osmolarity corrections (fig.9 & 10).

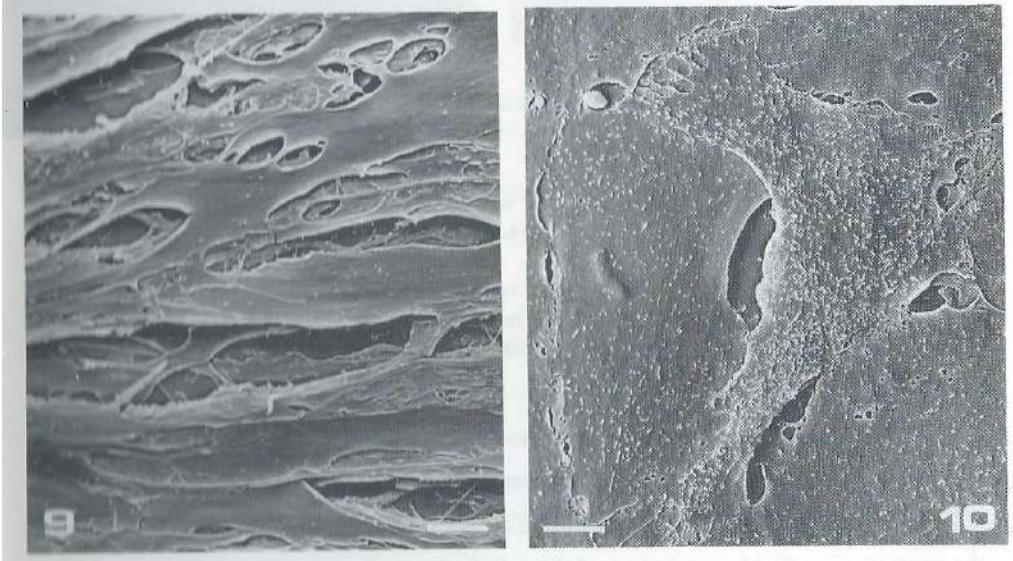


Fig.9 Human fibroblasts cultured for 14 days (control culture). SEM-micrograph, bar is 10 μm.

Fig.10 Human fibroblasts cultured with medium with an osmolarity of 500 mOsm/L. The shape of the cells is different from that seen in the control cultures. SEM micrograph bar is 10 μm.

DISCUSSION AND CONCLUSIONS

The four high molecular weight polylactides were tested *in vitro* using cell cultures, and the morphology was evaluated. The experimental setting in which cells were cultured on polylactide films can be regarded to as a short term real time experiment. The results of culturing on films indicated that polylactide has no negative effect on individual cells, but some unfavourable potentials are encountered when used as a substrate for cell culture. The inability of cells to cover the whole films might be caused by local impurities. However, in a study of Cima et al [18] a similar inability of covering was seen. Hepatocytes were cultured on films of poly-DL-lactic acid-co-glycolic acid (PGLA) and on a blend of PGLA and poly-L-lactic acid, as a control served a coating of collagen. The hepatocytes cultured on the polymers were globular at the edge of cell colonies whereas they were fully spread on the control cultures. In the present study all four polylactides showed parts uncovered by cells, and no clear difference in covering related to molecular weight or type (L versus DL) was seen.

In a previous article [11] the proliferation was described for the same cells cultured on polylactide films. Of these five cell types used, four types revealed lower cell numbers for cultures on films than for the control, but the differences between the controls and the cultures on films were minor. Apparently the proliferation was not inhibited, but were adherence and spreading inhibited on some parts of the films. This is in concordance with the study of Cima et al [18] who found a similar inability of covering and an unaffected proliferation as in dicated by the DNA synthesis levels which were the same for cultures on the polymers as for the collagen coated controls.

The artificial ageing experiment can be regarded as an accelerated time experiment. The degradation of the polylactides was expected to be accelerated in the pseudo-extracelluar fluid when heated [19,20]. The products released are supposed to be similar to the released products when polylactide is implanted for longer periods. Cells cultured with the artificial ageing media were similar to control cultures. This means that degradation products of polylactide are expected to be biocompatible at longer intervals.

Cells cultured with the different monomer concentrations showed that up to 1 mg monomer ml^{-1} medium did not cause any alterations in morphology or culture characteristics. Although this experiment needs further research to exclude effects of osmolarity- and/or ion- changes [21] it has shown that the toxicity levels are probably not reached even at 10 mg ml^{-1} concentration. However, the proliferation was slightly

inhibited [11].

The *in vitro* biocompatibility of the polylactides seems to be good. In these experiments the pH was adjusted and maintained within the physiological range. An extrapolation to the *in vivo* situation is difficult since the exact pH deviations in the environment of a degrading polylactide implant are not known.

In this study different cell types were used to extend the information obtained from just one or two cell types. From the results it is concluded that the use of five different cell types did not add supplementary information as compared to the use of one or two cell types.

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EVALUATION OF POLYLACTIDE MONOMERS IN AN *IN VITRO* BIOCOMPATIBILITY ASSAY

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ABSTRACT

The effect of polylactide monomers on cultured fibroblasts was determined by studying their proliferation (cell numbers), morphology (light microscopy) and cellular activity (acid phosphatase assay). Both L- and D-lactic monomer were added to culture medium in concentrations of 0.01 and 0.5 %. Since the osmolarity increases if soluble particles are added to culture medium, the 0.5% monomers were also added to a modified medium with an initially lower osmolarity. Evaluation showed that fibroblasts were not affected by 0.01 % lactic monomers, but the addition of 0.5 % monomer reduced the metabolic activity of the cells by about 40%, as compared to the control, regardless of the osmolarity of the medium. Cell proliferation and morphology were only negatively affected when 0.5% monomer was added to standard medium. The addition of sucrose, however, resulted in an increased proliferation and acid phosphatase activity, as well as an altered morphology. Therefore, it can be concluded from this study that the osmolarity, as well as the nature and concentration of the added material, can exert an influence on proliferation, morphology and/or cellular activity. Thus the osmolarity of test media should be defined for *in vitro* biocompatibility assays. In this study, both the L- and D- lactic monomer caused similar effects.

INTRODUCTION

Polylactide, a degradable polymer, is applied clinically as a component of commercially available suture material (Vicryl) as well as in bone surgery devices [1-4], although to a lesser extent. The good biocompatibility characteristics of polylactides are indicated by *in vitro* studies [4-7] and by many *in vivo* studies [8-12]. Previously,

degradation of polylactides was thought to be a gradual process, due to hydrolysis, thereby gradually releasing the degradation products [13]. The degradation of high molecular weight polylactides however, seems to be more complex than initially assumed [14]. Instead of surface degradation or erosion, bulk degradation appears to take place [15-17]. This might result in a sudden collapse of the implant, and the release of large amounts of degradation products which might have a negative effect on the surrounding tissue.

As part of a previous *in vitro* study on polylactides [Chapter II and III], the effect of large amounts of ultimate degradation products on cells has been assessed. Both L- and D- lactic monomers, were added to culture medium at various concentrations to mimic the release of degradation products within a specific time period. Cells cultured in medium containing 10 mg ml^{-1} lactic-monomer showed a decrease in proliferation as compared to the control. A more detailed study was needed as the cause of this effect was unclear; it was doubted that the lactic-monomer itself was responsible. The reduced proliferation could have been due to alterations in, for example, pH and/or osmolarity. In *in vitro* biocompatibility studies, materials of interest are often added to culture medium to investigate their effect on cells. However, changes in the culture conditions, due to variations in pH and/or osmolarity are, mostly disregarded [18-21], although some investigators, such as, Wilsnack [22] and Sgouras [23] have addressed this issue.

Recently, van Blitterswijk et al. [24] assessed the effect of pH on middle ear epithelium by culturing with media varying in pH (6-9). They observed that cell proliferation ceased when cell were cultured in medium at pH 6, whereas it was optimum at pH 6.5. Therefore, even small differences may have a dramatic effect on cellular behaviour.

The effects of osmolarity changes on cells is much more complicated due to the many factors involved, such as direct interference with the ion balance or indirectly by binding of ions. A tolerable osmolarity range seems to exist for cells in culture, as illustrated by the osmolarities of commercially available media ranging from 230-340 mOsm/kg [25]. Optimum osmolarity values for cells in culture may even vary between cell types. The impact of different osmolarities on a particular cell type can manifest itself in differences in mobility, metabolic activities and proliferation [25].

In the present study, an attempt was made to control both the pH and osmolarity of the media used, and still determine the effect of high lactic-monomer concentrations.

Human fibroblasts were used as this cell type is present at almost any implantation site.

MATERIALS AND METHODS

Test media

D-lactic acid and L-lactic acid solutions, both 90 % w/v, (obtained from Purac Biochem bv, The Netherlands) served as ultimate degradation products of polylactide. The pH of both acids was adjusted to pH 7 with NaOH before they were added to culture medium (see culture conditions). Culture media with 1 mg L-monomer ml^{-1}, 1 mg D-monomer ml^{-1}, 5 mg L-monomer ml^{-1} and 5 mg D-monomer ml^{-1} were prepared. The 5 mg monomer ml^{-1} were also added to modified medium which contained 50 mM less NaCl than in standard culture medium (see under culture conditions). Control media were; standard medium, standard medium with 3.6 % sucrose, modified medium with 3.6 % sucrose and modified medium which contained 87.6 mM NaCl less than standard medium to which 6.1 % sucrose was added. The cells were not expected to metabolise the sucrose, mainly because sufficient glucose (4500 mg/L) was present in the medium.

The osmolarities were measured by freezing point depression (Roebling Osmometer, Berlin, Germany) (Table I).

MEDIUM	OSMOLARITY (mOsm/L)
standard medium	329
1 mg ml^{-1} L-monomer	343
1 mg ml^{-1} D-monomer	343
5 mg ml^{-1} L-monomer -NaCl	401
5 mg ml^{-1} D-monomer -NaCl	335 (lower than calculated)
5 mg ml^{-1} L-monomer	444
5 mg ml^{-1} D-monomer	488 (higher than calculated)
3.6% sucrose	537 (higher than calculated)
3.6% sucrose -NaCl	401
6.1% sucrose --NaCl	346

Culture conditions

Human mammary fibroblasts were obtained from explants in our laboratory, according to the method earlier described by van Blitterswijk [26] for middle ear epithelium. The eighth cell passage was used and the cells were plated at a density of 2 x 10^4 cells per 1.5 cm well and at 1 x10^3 cells per 6 mm well. The cells were cultured in

Dulbecco's Modified Eagles Medium containing 5% foetal calf serum (FCS), penicillin (100 U/ ml) and streptomycin (100 µg/ ml) (standard medium). Modified media were obtained by adding ingredient after ingredient and finally adding less NaCl than in standard medium. The fibroblasts were cultured at 37°C and in a humidified atmosphere of 90 % air/10% CO_2. On day 3 the standard medium was replaced by the test media. Three 1.5 cm wells per test medium were evaluated on day 4, 7, 10, 14 and 21 for cell proliferation, three 1.5 cm wells for the viability, and three 6 mm wells for the acid phosphatase assay.

Proliferation

Three 1.5 cm wells with cells were available for each test medium at the different evaluation days. The cells were harvested by trypsinization and counted using a Coulter Counter. The cell numbers were plotted against the culture time and the mean area under the curve ± SD was calculated and used for statistical analysis. An ANOVA at a confidence level of 95 % was performed followed by a multiple range test based on the Scheffe method. The calculations were made using the software package SPSS.

Cell viability

Three 1.5 cm wells with cells were available for each test medium at the different evaluation days. The cells were washed with phosphate-buffered saline (PBS) and incubated with 0.001% neutral red (Sigma) in PBS for 30 minutes. The vital stain was then removed and the samples were compared at the light microscopical level. Viable cells take up the stain, whereas non-viable or damaged cells are unable to retain the stain intracellularly.

Acid phosphatase assay

The acid phosphatase assay was performed in 96-wells-plates according to the method described by Martin and Clynes [27]. A pilot experiment indicated a linear relationship between the amount of human fibroblasts and the acid phosphatase activity, ranging from 50 to 25,000 cells/well (fig.1). Cell densities higher than 25,000 cells/well showed a dramatic decrease in acid phosphatase activity. Fibroblasts can show a 20 fold increase in cell numbers during 2 weeks of culture. To omit the upper threshold value of the acid phosphatase activity at the end of the experiment, human fibroblasts were

plated at a density of 1,000 cells/well at day 0. After 1, 4, 7, 10, 14 and 21 days the acid phosphatase assay was performed. The medium was removed and the wells were rinsed with PBS followed by incubation in 0.1 ml of 10mM p-nitrophenyl phosphate (Sigma) in 0.1 M sodium acetate/ 0.1% Triton X100 (pH 5.5). A chemical control was included in the procedure by performing all treatments on wells without cells. After 2 hours incubation at 37°C in a humidified atmosphere of 90% air/ 10% CO_2, 100 µl 1N NaOH was added to each well to stop the reaction. The extinction values were read on an ELISA plate reader at 405 nm and were statistically analyzed as described previously for the cell proliferation.

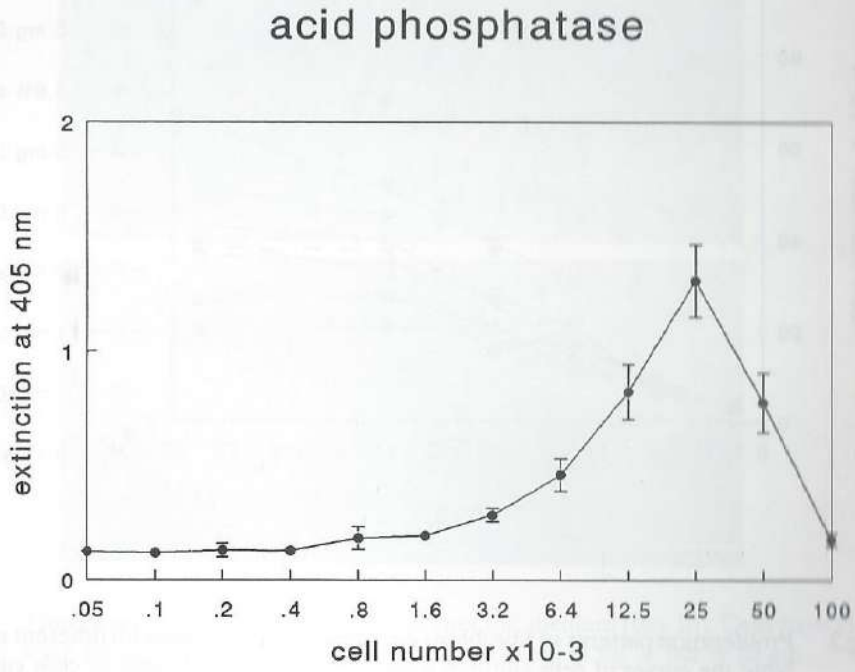

Fig.1 Acid phosphatase assay for a range of 50 to 100,000 fibroblasts/well. Note the sudden decrease in enzyme activity for cell numbers higher than 25,000 cells/well.

Monomer media and standard medium

Cells cultured with standard medium showed an increase in cell number during the 3 week culture period. During the third week, the increase in cell number was less evident resulting in a plateau phase. A similar proliferation pattern was found for cells cultured with the monomer containing media, except for two media containing 5 mg monomer ml^{-1}, which resulted in downward shifted cell proliferation patterns (fig.2).

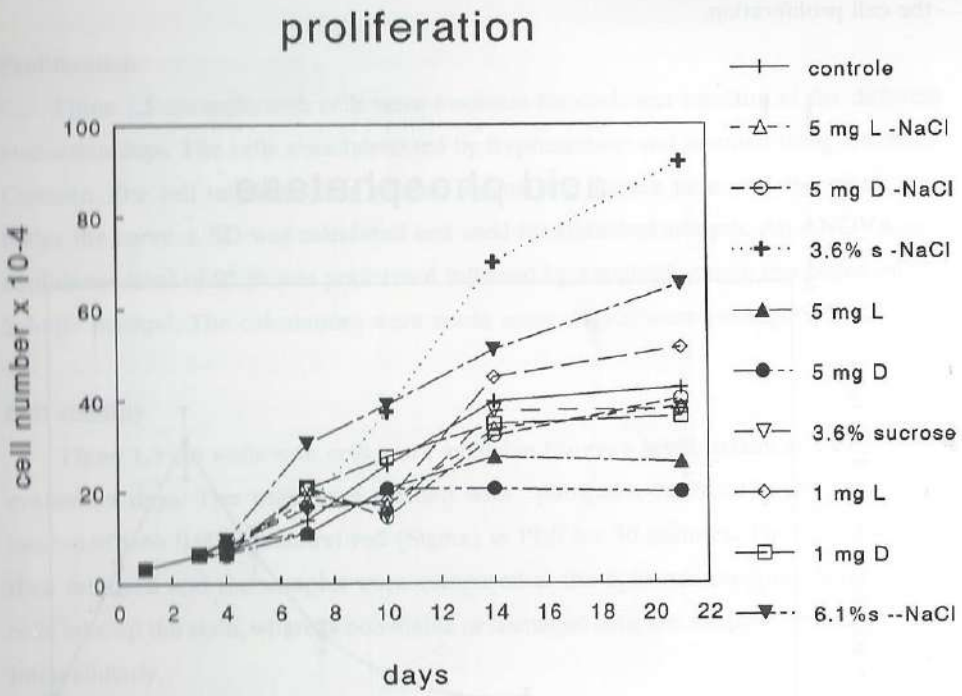

Fig.2 Proliferation patterns of fibroblasts cultured for three weeks with different media. Note the curves of cells cultured with sucrose media and those of cells cultured with medium to which 5 mg monomer ml^{-1} was added.

The osmolarity values of these media were; 444 for the L-monomer and 488 for the D-monomer. The two other media containing 5 mg monomer ml^{-1}, which had normal proliferation patterns, had lower osmolarity values (401 L-monomer, 335 D-monomer)

due to a modification of the medium (see M & M).

The downward shifted proliferation patterns were correlated with larger cells which were

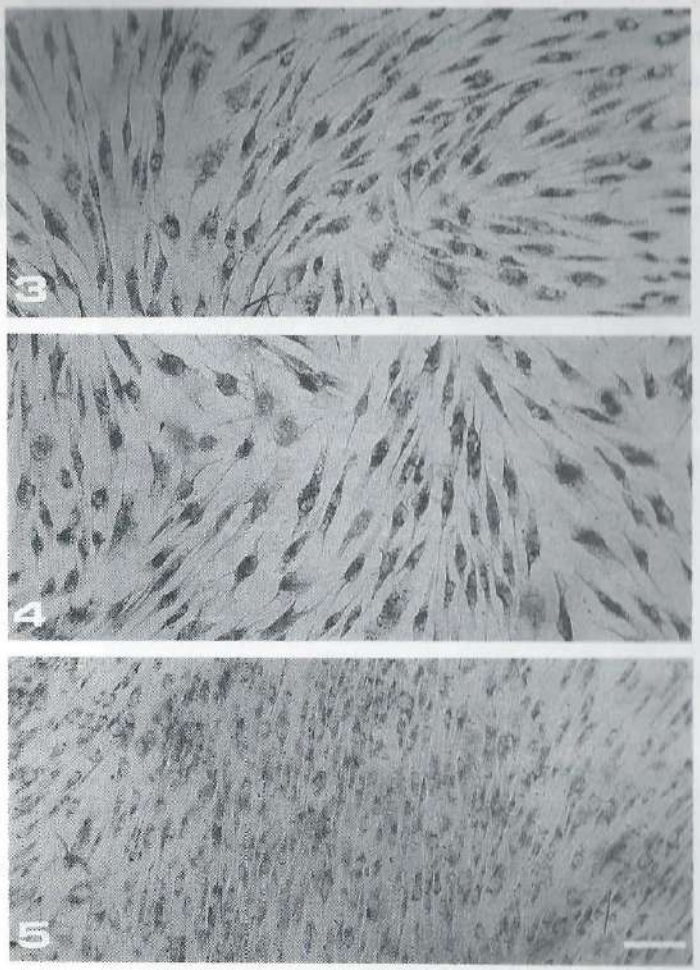

Fig.3 Fibroblasts cultured with 5 mg L-monomer ml^{-1} medium (day 10). Cells have vital stain intracellularly. The cells are similar to those in fig. 4. bar is 20 μm

Fig.4 Fibroblasts cultured with 5 mg D-monomer ml^{-1} medium (day 10). Cells have vital stain intracellularly. The cells are similar to those in fig.3. bar is 20 μm

Fig.5 Fibroblasts cultured with standard medium (day 10). Cells have less vital stain intracellularly than in figs. 3 and 4, and they are smaller and more densely packed. bar is 20 μm

less densely packed than those cultured with standard medium or with the other monomer containing media (figs. 3-5). The cells also contained the vital stain intracellularly in vesicles, mainly located in the centre of the cell, leaving the cellular extensions and pseudopodia unstained. This was in contrast to the cells cultured with standard medium or with the other monomer containing media, which all revealed less intensely stained cells.

The enzyme activities in the cells (indicated by the acid phosphatase assay) for all four media containing 5 mg monomer ml^{-1} were lower than those cultured with standard medium (fig. 6).

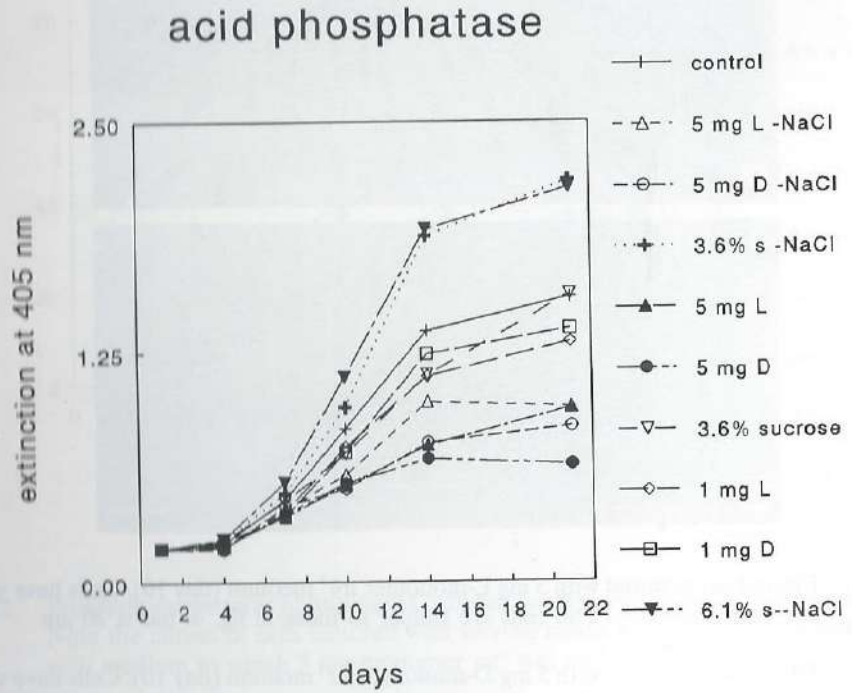

Fig.6 Acid phosphatase curves of fibroblasts cultured for three weeks with different media. Compare with the proliferation patterns (fig.2). Note the three groups which can be easily indicated.

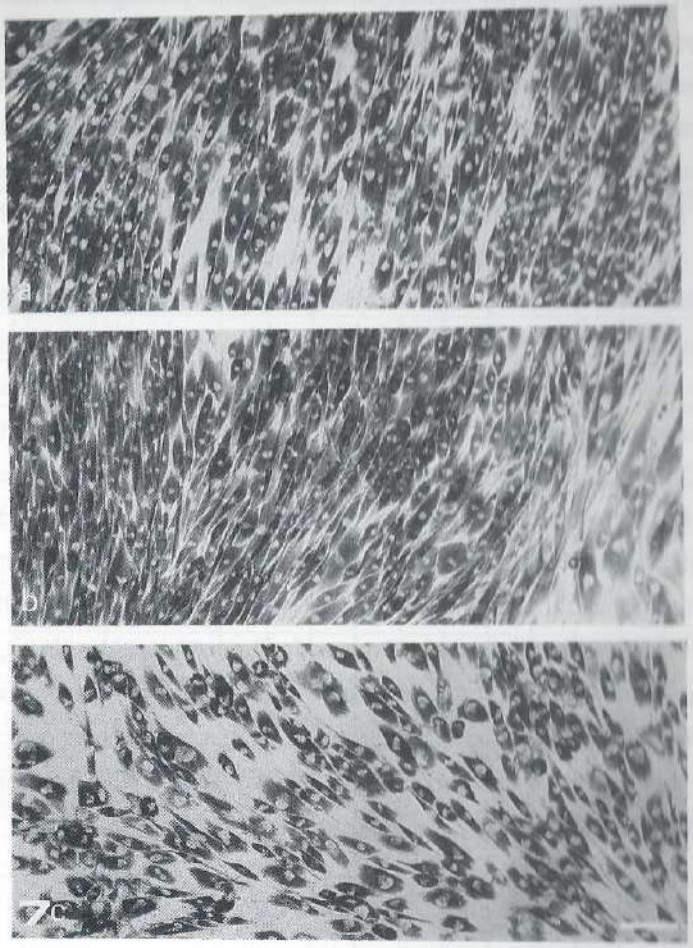

Fig.7 a-c Fibroblasts cultured with sucrose media (day 10), 3.6 % sucrose to medium with 50 mM less NaCl (a), 6.1% sucrose added to medium with 87.6 mM less NaCl (b) and 3.6 % sucrose added to standard medium (c). All three have a similar morphological appearance; the cells are larger than the control (fig.5) and contain much more vital stain. bar is 20 μm

Sucrose media

Cells cultured with the sucrose containing media showed a very different morphology than those cultured with standard medium. They were larger and contained considerable amounts of vital stain intracellularly. The stain was present in small vesicles, which were evenly distributed in the cytoplasm of the cells, leaving the nucleus visible as an unstained area (fig. 7). Besides the deviating morphology, the proliferation was highly (about 100% compared to the control) enhanced for cells cultured with 3.6% sucrose medium of 401 mOsm/L and to a somewhat lesser extent (about 50% compared to the control) for cells cultured with 6.1% sucrose medium of 346 mOsm/L. Cells cultured with 3.6% sucrose medium of 537 mOsm/L revealed a proliferation similar to that of the standard medium.

The acid phosphatase assay showed similarly high activities (both about 50% higher than the control) for cells cultured with sucrose media of 346 and 401 mOsm/L. Cells cultured with sucrose medium of 537 mOsm/L had an activity similar to the standard medium.

Statistical analysis

Statistical analysis of the cell numbers indicated three major groups. Cells cultured with the sucrose containing media, with osmolarity values of 346 and 401 mOsm/L, had the highest cell numbers, and the media containing 5 mg monomer ml^{-1} with an increased osmolarity, had the lowest cell numbers. Consequently, the other media had cell numbers in between (Table I). Analysis of the acid phosphatase values also revealed three major groups. Again, the highest activity group consisted of the sucrose media 346 and 401 mosm/L, but the lowest group consisted of **all** four media containing the 5 mg monomer ml^{-1}, irrespective of the osmolarities. The remaining media had intermediate enzyme activity levels.

DISCUSSION

The results of this study have shown that all media allowed proliferation of the fibroblasts. The main differences were found with the 5 mg monomer ml^{-1} media which had higher osmolarities, and the sucrose media.

The differences in cell proliferation and morphology that were observed with the 5 mg monomer ml^{-1} were dependent on whether standard medium or modified medium was used. The addition of 5 mg monomer ml^{-1} to standard medium affected the cells although

this was not supported by the acid phosphatase assay. The results of this test revealed no statistical difference between cultures of 5 mg ml^{-1} monomer, irrespective of the osmolarities. All four media containing 5 mg monomer ml^{-1} showed cells with lower enzyme activities as compared to the control. Apparently, acid phosphatase is a sensitive assay to detect early stage effects of monomer solutions.

A correlation between high osmolarity values and affected cells, is supported by two of the three tests. The results obtained with the sucrose media do not support this phenomenon as proliferation and acid phosphatase activity were enhanced instead of depressed. An exception was the sucrose medium with an osmolarity of 537 mOsm/L,

AREA UNDER THE PROLIFERATION CURVES													
	MEAN ± SD	mOsm/L		1	2	3	4	5	6	7	8	9	10
5 mg D	19.65 ± 1.42	488	1										
5 mg L	25.40 ± 1.32	444	2										
5 mg D -NaCl	33.32 ± 1.33	335	3	*									
5 mg L -NaCl	34.00 ± 2.61	401	4	*									
3.6 % sucrose	35.89 ± 2.84	537	5	*	*								
1 mg D	38.35 ± 0.89	343	6	*	*								
control	40.37 ± 3.26	329	7	*	*								
1 mg L	44.72 ± 4.32	343	8	*	*	*	*						
6.1 % sucrose --NaCl	64.63 ± 2.60	346	9	*	*	*	*	*	*	*	*		
3.6 % sucrose -NaCl	79.66 ± 3.08	401	10	*	*	*	*	*	*	*	*	*	

Table I The areas under the proliferation curves ± SD as well as the osmolarities of the media are expressed. The ranking order of the media is based on the areas under the curves. The asterisks indicate the significant differences ($p<0.05$). An ANOVA was performed followed by a multiple range test based on the Scheffe method.

AREA UNDER THE ACID PHOSPHATASE CURVES													
	MEAN ± SD	mOsm/L		1	2	3	4	5	6	7	8	9	10
5 mg D	9.33 ± 0.92	488	1										
5 mgD -NaCl	10.54 ± 1.35	335	2										
5 mg L	10.79 ± 0.55	444	3										
5 mg L -NaCl	13.14 ± 1.18	401	4										
1 mg L	14.83 ± 1.17	343	5	*									
1 mg D	15.60 ± 1.22	343	6	*	*	*							
3.6 % sucrose	15.89 ± 1.30	537	7	*	*	*							
control	17.60 ± 1.54	329	8	*	*	*	*						
3.6 % sucrose -NaCl	23.23 ± 1.17	401	9	*	*	*	*	*	*	*	*		
6.1 % sucrose --NaCl	24.06 ± 1.13	346	10	*	*	*	*	*	*	*	*		

Table II The areas under the acid phosphatase curves ± SD as well as the osmolarities of the media are expressed. The ranking order of the media is based on the areas under the acid phosphatase curves. The asterisks indicate the significant differences ($p<0.05$). An ANOVA was performed followed by a multiple range test based on the Scheffe method.

which resulted in values similar to the control. An explanation might be that, although the high osmolarity resulted in a depression of proliferation and acid phosphatase activity, the sucrose itself had a stimulating effect. These two effects were probably complementary, through which a normal result was obtained.

It can be concluded that the increased osmolarity in itself is not responsible for the deviating results in proliferation, acid phosphatase activity or cell morphology but that it depends on the nature and concentration of the material added to media. In the case of lactic monomers, a negative effect is caused by the monomer itself, as indicated by the

acid phosphatase assay. In the case of sucrose, stimulating effects as well as a deviating morphology were found.

The three tests; proliferation based on cell numbers, morphology in combination with the neutral red stain, and the acid phosphatase activity, all gave additional information.

If these concentrations of lactic-monomers would be released from a degrading implant *in vivo*, it is not likely that they affect the surrounding tissues. Although threshold values for lactic-monomer concentrations may be obtained *in vitro*, it remains difficult to extrapolate them to the *in vivo* situation. *In vivo* effects may be influenced by the draining capacity of the surrounding tissue and possible local changes in pH. The present *in vitro* results endorse the satisfactory biocompatibility characteristics of polylactide.

The *in vivo* elimination rate of the lactic monomers might vary for the L- or the D-monomer. Experiments indicated that L-lactic acid is utilized four times as rapidly as the D-lactic acid [28] in the rat. From other studies it could be calculated that the plasma level of the D-lactic acid is about 25 times smaller than the L-lactic acid [29,30]. The relative oxidation rates of L- and D-lactic acids were found to vary between different tissue types [30]. The consequence of these differences for the degradation of polylactide implants made of both L- and D-monomers is not known. The effects caused by the D-monomer might last longer. The present *in vitro* results however did show similar findings for the L-lactic monomer and the D-lactic monomer.

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CELLULAR REACTION ON THE INTRAPERITONEAL INJECTION OF FOUR TYPES OF POLYLACTIDE PARTICULATES

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ABSTRACT

Four types of polylactide particulates, P-L-LA 100, 250, 550 KD and a P-DL-LA 400 KD were injected into the peritoneal cavity of mice. The inflammatory reaction showed an increase in cell number (mainly neutrophilic granulocytes) up to 48 hours after which the cell numbers decreased below the control (phosphate buffered saline).
All four polylactide particulates aggregated and intermingled with inflammatory cells. The aggregates remained throughout the investigation period of 6 months. Quantitative measurements showed that standardisation of the particle form and size is essential. From this study and other experiments in which calcium phosphates and asbestos were injected intraperitoneally, it is concluded that the inflammatory response observed in the peritoneal cavity is related to the type of material injected and probably to form and size of the individual particles, but not to molecular weight.

INTRODUCTION

The peritoneal cavity of mice has been extensively used for research on material/tissue interactions [1]. It has been demonstrated that introduction of foreign materials into the peritoneal cavity evokes an inflammatory response. Research has indicated that the elicited response depends on the chemical and physical material characteristics such as size, shape and hydrophilicity [1-3]. This phenomenon has already

been used for the evaluation of biomaterial characteristics [4-7]. The advantage of using the peritoneal cavity as implantation site is that suspensions containing biomaterials can be injected, so that wound reactions normally occurring after implantation with more invasive surgery are largely omitted. Confusion of the interpretation of material-induced characteristics, especially during short-term evaluations, is thus avoided.

In the present study, polylactide particulates (20-80 μm in ϕ), different in molecular weight and composition were injected into the peritoneal cavity of mice. The polylactides used were poly-L-lactides (P-L-LA) 100 KD, 250 KD, 550 KD and poly-DL-lactides (P-DL-LA) 400 KD. Since the molecular weight is one of the determinants of the degradation rate, it was expected that these polylactides would degrade in a different time span [8,9]. Another determinant of degradation rate is that P-DL-LA degrades faster than the P-L-LA because of its amorphous structure, which allows water to cause hydrolysis more easily [10]. Since degradation products may also affect the cellular response, the aim of the present study was to investigate this response up to 6 months after injection of polylactide particulates.

MATERIALS AND METHODS

Materials

The polylactide particulates (20-80 μm in diameter) were obtained from Purac bv, Gorinchem, The Netherlands. Three poly-L-lactides and one poly-DL-lactides were used: P-L-LA 100 KD, P-L-LA 250 KD, P-L-LA 550 KD and P-DL-LA 400 KD. Animals were Swiss SPF female mice, weighing 22-32 g (Harlan Zeist, The Netherlands). The mice were housed under conventional conditions and allowed 1 wk to adapt to the new housing conditions before the start of the experiment.

The polylactide powders were suspended in phosphate-buffered saline (PBS) at a concentration of 10 mg ml^{-1}. The suspensions were sonificated for 3 min (Sonificator W-375, Ultrasonics Inc.) at 50 W prior to injection. Three animals per material per survival period were injected intraperitoneally with 1 ml of the suspension. Simultaneously, three animals received 1 ml PBS as a control. Survival periods were 4 h, 16 h, 24 h, 48 h, 1 wk, 2 wk, 1 month, 2 months, 4 months and 6 months.

Methods

Isolation of cells and polylactides

At the different time points the peritoneal cells were harvested according to the method described by Daems and Koerten [1]. The cells from the three animals were pooled and counted using a Bürker chamber. After harvesting the cells, the peritoneal cavity of each mouse was examined for the presence of polylactide aggregates. Special attention was paid to the ligamentum gastrolienale, the ligamentum hepatogastricum, the excavatio uteri, the mesentery and the diaphragm.

Preparation for microscopy

The isolated cells were fixed by immersion in 1.5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M cacodylate (Serva, Brunschwig, Amsterdam, The Netherlands) buffer (pH 7.4), overnight at 4°C. The cell suspensions were embedded in 2% agar (Bacto-agar, Difcolab., Detroit, Michigan, USA), dehydrated using a graded ethanol series and embedded in Epon (LX 112, Ladd Research Industries Inc., Burlington, VT, USA). Semi-thin Epon sections were used for a differential cell count in the light microscope.

The polylactide aggregates were also fixed by immersion in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at 4°C. The aggregates were divided into groups for light microscopy and electron microscopy.

For light microscopy, the aggregates were dehydrated using a graded ethanol series and embedded in Glycol Methacrylate (GMA, Technovit, Heraeus Kulzer GmbH, Friedrichsdorf, Germany).

For electron microscopy the aggregates were post-fixed using a 1:1 mixture of 2% OsO_4 (Degussa, Hanau, Germany) and 3% $K_4Fe(CN)_6$ (Merck, Darmstadt, Germany). For transmission electron microscopy (TEM) the aggregates were dehydrated and embedded in Epon. Ultrathin sections were examined in the TEM (Philips EM 201) at an accelerating voltage of 80 KV. For scanning electron microscopy (SEM), aggregates were cut with a razor blade, dehydrated, critical point dried and gold sputtered. Subsequently the specimens were examined in the SEM (Cambridge S 180) at an accelerating voltage of 15 KV.

Quantification of polylactide particulates

The particulates were characterized before injection, using a VIDAS image analysis system [11]. The particulates were spread out on a slide and the area and perimeter were determined. The obtained values were used to calculate the form-factor (Form-factor= 4π x AREA / (PERIMETER)^2). A form-factor of 1 indicates a circle. Deviations from 1 towards 0 indicates a bar shape or irregular shape.

Light microscopical sections of the aggregates at each time point were used for quantification. The percentage of area occupied by polylactide was calculated, as well as maximum and minimum diameter, perimeter and number of the particles. Detection of the polylactides was based on differences in grey values.

RESULTS

Before injection, the polylactide particulates were characterized using the image analysis system as described in Material & Methods. The maximum and minimum diameters of the four polylactide particulates were determined (Table I).

The form-factor was calculated from the area and perimeter measurements made by the analysis system (Table II).

MATERIAL	PARTICLE DIAMETERS	
	Dmax ± SD	Dmin ± SD
P-L-LA 100 KD	33.7 ± 5.3	20.4 ± 3.9
P-L-LA 250 KD	91.6 ± 31.2	32.8 ± 12.4
P-L-LA 550 KD	57.4 ± 20.9	40.3 ± 14.4
P-DL-LA 400 KD	70.8 ± 20.9	45.4 ± 12.9

Table I Maximal and minimal diameters of the polylactide particulates prior to injection (mean values ± SD)

Peritoneal cell population

The cell numbers first increased after injection of the polylactide particulates. Up to 48 hours, the cell numbers harvested from PLA stimulated animals were higher than the controls (PBS stimulated). The highest cell numbers were found after injection of P-L-LA 250 KD. At 1 wk, the cell number decreased for all four polylactides to even below the control value (Fig 1). From 2 months, there was no clear difference between the cell numbers harvested from stimulated and control animals.

The differential cell count revealed also a change over time. Initially, granulocytes were most prominent, both in number and in percentage (Fig.2). Later, macrophages were most frequently found, whereas the percentage of lymphocytes did not alter significantly.

MATERIAL	FORM-FACTOR
P-L-LA 100 KD	0.73 ± 0.08
P-L-LA 250 KD	0.53 ± 0.10
P-L-LA 550 KD	0.78 ± 0.07
P-DL-LA 400 KD	0.72 ± 0.08

Table II Form-factors (mean values ± SD) of the polylactide particulates prior to injection

Polylactide particulates

Particle aggregation in the peritoneal cavity was seen for all materials already after 4 h. Light microscopy showed that the particles were intermingled with inflammatory cells and fibrin-like threads. Initially, the neutrophil granulocyte was the main cell type present, but at 1 wk the majority of the cells consisted of foreign body giant cells, monocytes and fibroblasts. The first foreign body giant cells were observed at 24 hours and were in close contact with the particles. At 1 wk, small blood vessels appeared in the aggregates. Simultaneously, the assembly of the cells and particulates became more compact. SEM showed mesothelial overgrowth at 2 wk (Fig.3). From 1 wk to 6 months, there was no alteration in cell types. Small foci of particles and foreign body giant cells were surrounded by fibrous tissue (Fig.4). With increasing time, the amount of fibrous tissue increased. At 6 months, infiltration islands of mononucleated inflammatory cells were sometimes seen in the granulomas (Fig.5).

Particles in the aggregates were still present at 6 months and degradation was hardly visible at a light microscopical level. The DL particles seemed to be somewhat degraded

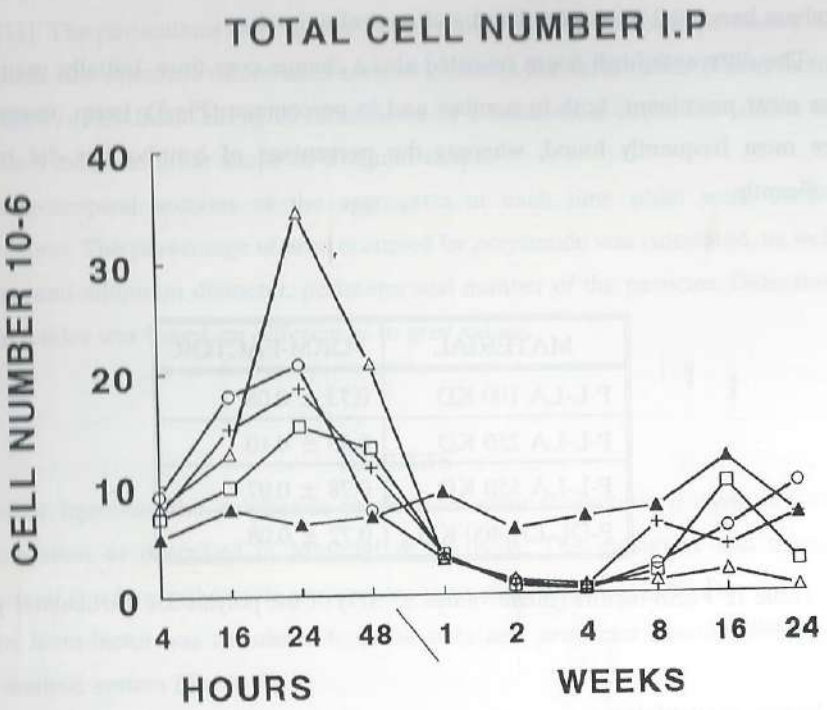

Fig.1 Number of cells present in the peritoneal cavity of mice after injection of P-L-LA 100(+), 250(▲) 550(O) KD, P-DL-LA 400(□) KD particulates and PBS (phosphate buffered saline) which served as a control (▲). Note the cell numbers at 1 to 4 weeks of the PLA injected animals.

(Fig.6), but the L particles seemed to be unaltered in size and form (Fig.5). Sometimes cellular fragments, probably cytoplasmic protrusions, inside particles were seen at the longer time periods. This phenomenon was seen for the P-DL-LA particles and P-L-LA 250 KD particles.

With TEM, degradation signs were observed for all four polylactides. Cellular protrusions in corroded areas of polylactides were seen as well as cells with intracellular particles. Sometimes, particles revealed a trabecular appearance (Fig.7). Fig. 7 also shows that the cells in contact with particles had interdigitating plasma membranes.

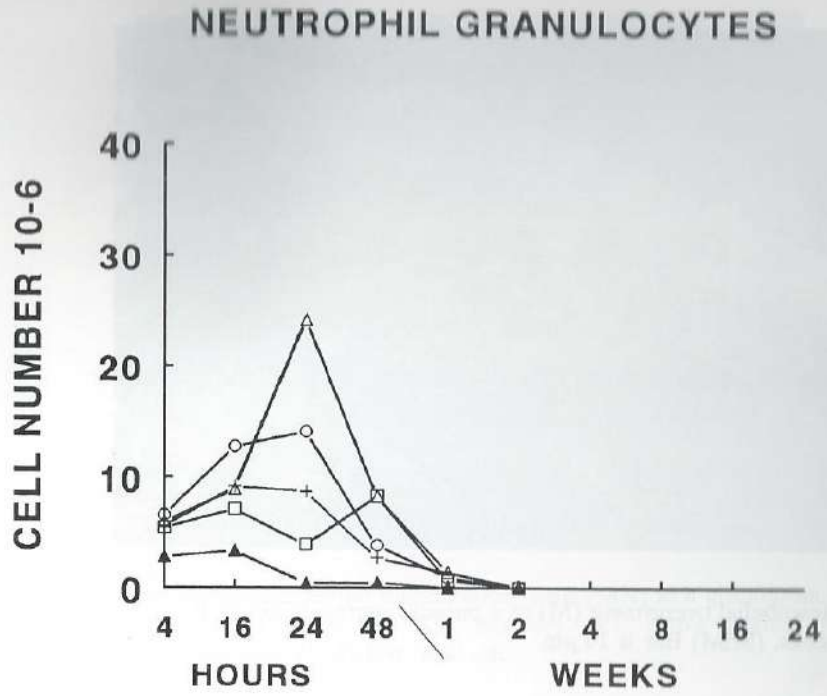

Fig. 2 Numbers of neutrophil granulocytes present in the peritoneal cavity after injection of P-L-LA 100 (+), 250(▲) 550(O) KD, P-DL-LA 400(□) KD particulates which are much higher than the control (▲) (phosphate buffered saline injection). This cell type is the main representative of the cells present shortly after injection.

Particle quantification

Light-microscopical sections of the aggregates were used for quantification of particles to establish biodegradation. The percentage of polylactide present in the aggregate sections varied roughly for all four materials between 15 and 40 %, without a clear relation to the length of the experimental period. The maximum and minimum diameters of the particles were measured. The standard deviation was large for all measurements, irrespective of the type of polylactide used (Fig.8). Up to 6 months, there was no clear change in the range of both diameters and a high correlation of both remained (r_{PLA1-4}= 0.87-0.95). The number of particles present per section seemed to vary

for the different types of polylactides used (table III).

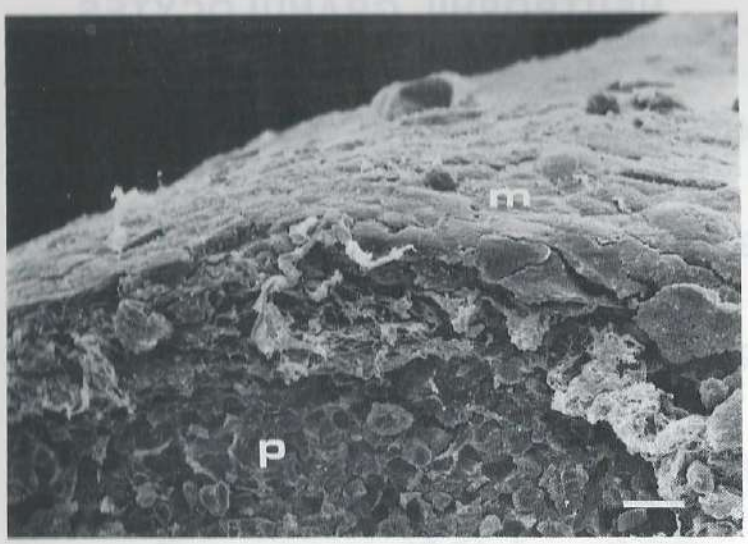

Fig.3 Mesothelial overgrowth (M) of a particle aggregate (P) of P-L-LA 250 KD at 2 weeks. (SEM) Bar is 10 μm.

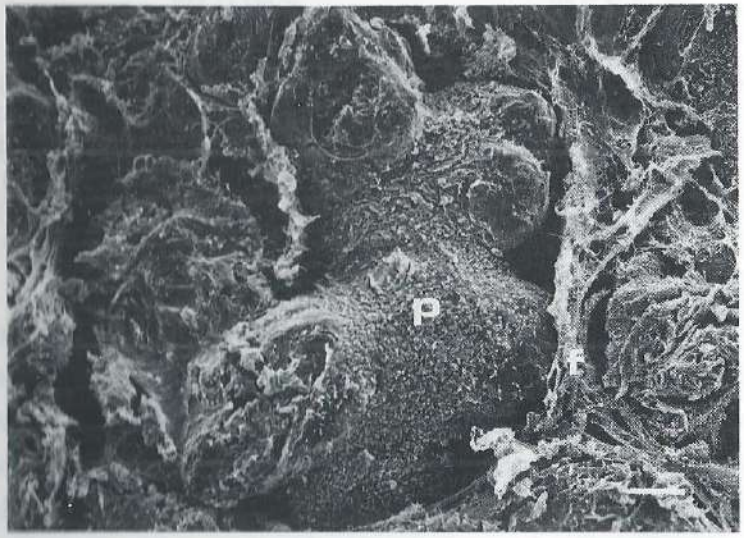

Fig.4 SEM micrograph of P-L-LA 100 KD particle(s) (P) in a granuloma surrounded by fibrous tissue (F), 1 month after injection. Bar is 12.5 μm.

Fig.5 A cluster of mononucleated inflammatory cells (MN) in a granuloma 6 months after injection. P-L-LA 550 KD particles (P), arrow points towards a multinucleated cell. (LM) Bar is 40 μm.

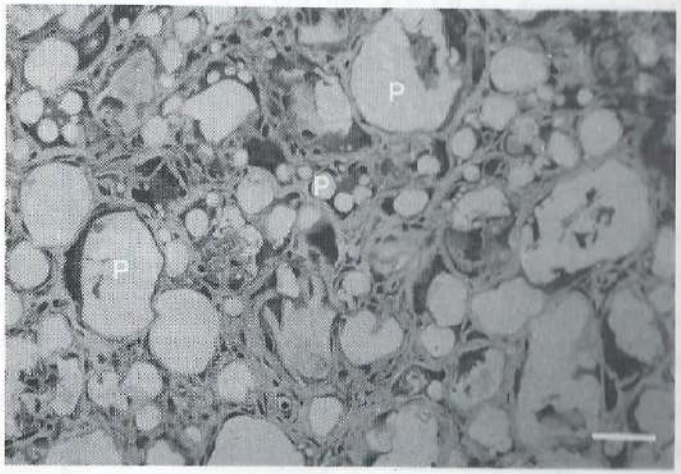

Fig.6 P-DL-LA 400 KD particles (P) 6 months after injection. The particles are rounder than those of P-L-Lactides as is seen in fig.5. (LM) Bar is 40 μm.

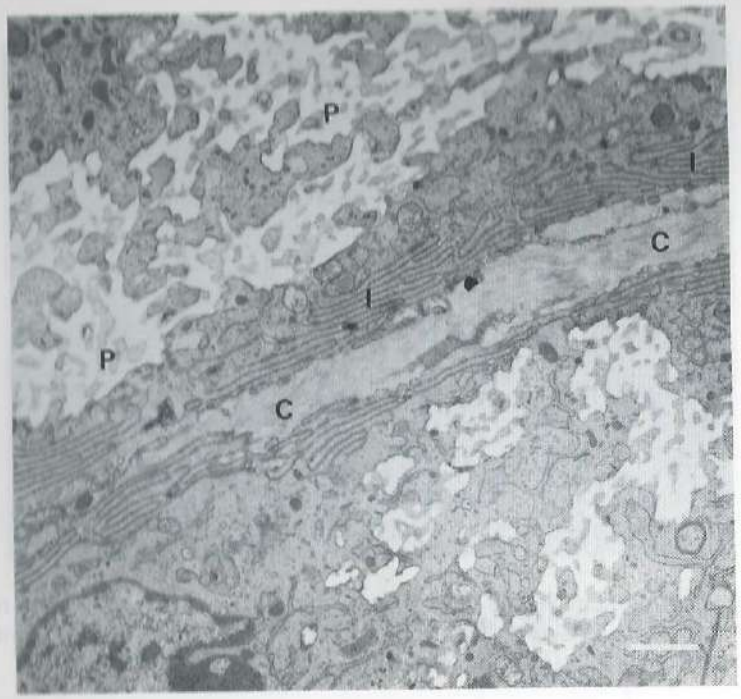

Fig.7 TEM micrograph of P-L-LA 250 KD 6 months after injection. Note the trabecular appearance of polylactide (P) and the interdigitating plasma membranes (I) of the cells. Collagen (C) is seen interposed between the cells. Bar is 0.9μm.

MATERIAL	PARTICLE NUMBER
P-L-LA 100 KD	152.1 ± 50.8
P-L-LA 250 KD	230.2 ± 65.5
P-L-LA 550 KD	316.3 ± 64.3
P-DL-LA 400 KD	152.2 ± 25.4

Table III Number of particles (mean values ± SD) in light microscopy sections of the granulomas.

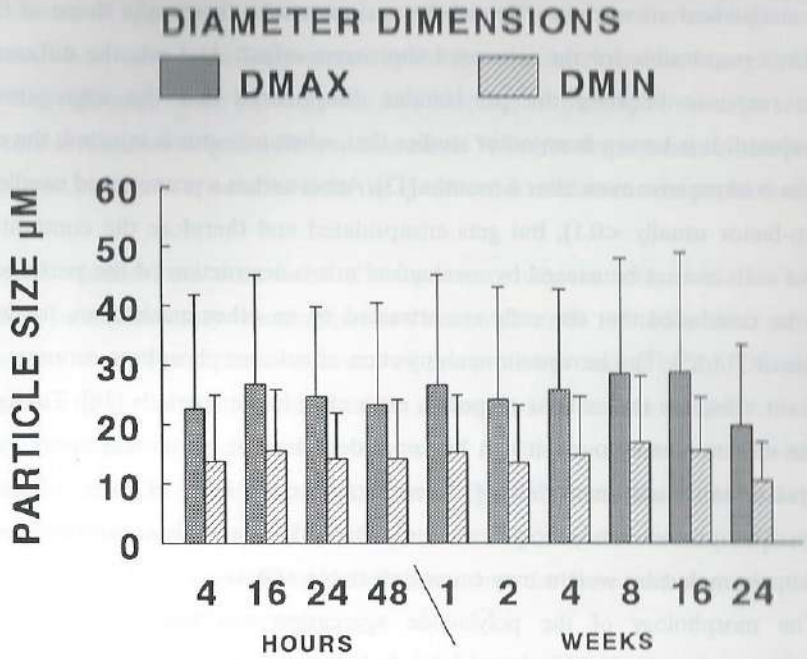

Fig.8 Maximal and minimal diameters of P-L-LA 100 KD particulates after injection. Changes in these diameters during the 6 months are not indicated.

DISCUSSION AND CONCLUSIONS

All particles were obtained as particulates with a variable size (20-80 μm). The form-factors deviated from 1, which means that none of the injected polylactides was spherical in shape. The P-L-LA 250 KD had the lowest form-factor (± 0.5) which indicates a relatively large surface area compared to the other particulates. Furthermore, P-L-LA 250 KD had by far the largest maximum diameter. Because especially this type of polylactide caused an evidently higher influx of granulocytes, it can be concluded that the inflammatory response, as judged by the number of neutrophil granulocytes, was mainly influenced by the form and the total surface area of the injected particles. It is known that shape and surface structure are important factors affecting the inflammatory response [12]. It could however not, however, be determined with certainty from the results of the present study whether the form or the larger surface area was more

important factor causing the increased cellular response. It was also unclear to what extent mechanical micro-destruction of the peritoneum by the needle shape of P-L-LA 250 KD is responsible for the increased short-term effect. At 1 wk, the differences in cellular response between the polylactides disappeared and the aggregates were encapsulated. It is known from other studies that, when asbestos is injected, the cellular response is impressive even after 6 months [13]. Asbestos has a pronounced needle shape (a form-factor usually <0.1), but gets encapsulated and therefore the constantly high influx of cells cannot be caused by mechanical micro-destruction of the peritoneum. It has to be concluded that the cells are attracted by an other mechanism, for instance chemotaxis [14,15]. The intraperitoneal injection of calcium phosphate ceramics has no significant effect on the cellular response, even after longer periods [16]. Taking these variable effects into account, it can be concluded that the peritoneal cavity model is appropriate to classify materials of different nature on basis of their inflammatory activity. The present study confirms these conclusions and demonstrates that even variations in molecular weight may cause detectable effects.

The morphology of the polylactide aggregates was similar to foreign body granulomas as described by Hirsh and Johnson [17] and Koerten et al.[4]. The proportion of foreign body giant cells and the formation of collagen by fibroblasts in these granulomas depends on physical and chemical properties such as size, chemical composition and hydrophilicity [18,19]. The higher hydrophillic properties and the more regular form of the DL particles as compared to the L particles, may be responsible for the less severe inflammatory response.

Polylactide particles were detected throughout the whole experiment. Even at 6 months, polylactide particles were present in the granulomas. Indications of biodegradation were obtained by the fact that the DL-polylactide, which was expected to degrade faster than the L-polylactides, was revealed in the light microscope sections with particles which were suggestive for rounder shapes than was seen for the L-polylactides. Some degradation of the DL particles was therefore likely. *In vitro* studies indicated that peritoneal macrophages are capable of degrading polylactide intracellularly [20]. This was confirmed by transmission electron microscopy. At larger intervals, the polylactide particles had a trabecular appearance. In a study of Brady et al. [21] who implanted DL polylactide in the abdominal wall of the rat, division of the implant into septa by capsular invagination was also seen. To quantify the possible degradation,

morphometric evaluations of the size of the particles after injection, based on measurements of aggregate sections were performed. The results showed high standard deviations, which were most likely caused by the fact that sections ran through particles, so that the real image of the particles was invisible. Even a pure sphere can be sectioned at different levels, resulting in different diameters. Whether degradation of the particles had occurred, cannot be concluded from the quantitative measurements performed in this study.

It is concluded from the results of the present study that only moderate-to-small differences in inflammatory reaction between the polylactides related to their molecular weights were seen. The differences observed are probably caused by the DL versus L polylactides and by the form of the particles rather than molecular weight.

To summarize, the peritoneal cavity model is able to demonstrate variance in inflammatory reaction in relation to different types of materials, such as asbestos, hydroxyapatite, and polylactides. The inflammatory response observed in the peritoneal cavity is related to the type of material injected, but the form and size are probably important inducers of inflammation.

In the peritoneal cavity, the particles are collected in aggregates in which material/tissue interactions without a predominant wound reaction can be studied. For quantitative evaluation of degradation, however, it is concluded from this study that standardisation of the particle form and size is essential.

ACKNOWLEDGEMENTS

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IN VITRO BONE FORMATION ASSOCIATED WITH APATITE COATED POLYLACTIDE

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ABSTRACT

Bone formation onto poly(L-lactide), which was plasma-spray coated with various quantities of hydroxyapatite (0%, 15%, 36% and 100% coverage), was investigated in an in vitro assay. Rat bone marrow cells were grown on the different coatings and the cellular response and elaborated extracellular matrix was examined at the light and electron microscopical level after 1, 2, 4 and 8 weeks of culture. Proliferation of cells into multilayers was seen on the 0%, 36% and 100%, but not on the 15% coatings. Coinciding with this was the sparse formation of extracellular matrix on the latter, and its abundant appearance on the former three coatings. Scanning and transmission electron microscopy revealed a mineralized extracellular matrix on the 36% and 100% coatings after 4 and 2 weeks respectively, and on the 15% coating after 8 weeks. Mineralization was not observed on uncoated poly(L-lactide). At the interface between hydroxyapatite and the mineralized extracellular matrix, one or more electron dense layers were frequently observed, which showed morphological similarities with structures between these two entities in vivo. The results of this in vitro study show that, in the model used, hydroxyapatite is required to obtain the elaboration of mineralized extracellular matrix on poly(L-lactide).

INTRODUCTION

An optimal bone replacement material should be bone-bonding, bioactive, mechanically strong, biodegradable and participate at least partially in the normal

turnover of bone. Currently used biomaterials however, do not fulfill all these requirements. Existing implant materials used for bone replacement can be divided into calcium phosphates, metals, polymers and composites. From the former group, hydroxyapatite is the most frequently used implant material because of its bone-bonding ability (Hench et al. 1972; Osborn and Newesely, 1980; Jarcho, 1981; van Blitterswijk et al. 1990). When implanted into bone, it can form a physico-chemical bond with this tissue. Furthermore, hydroxyapatite is degradable to a certain extent but, due to its brittleness and low fatique resistance, it cannot be used as a load bearing implant material. With regard to polymers, polylactide is an implant material that is used in bone pins, screws and fixation plates (Rokkanen et al. 1985, 1991; Bos et al. 1987; Rozema et al. 1988). It has good biocompatible characteristics (Majola et al. 1991; van Sliedregt et al. 1992) and the degradation properties and elasticity modulus can be varied with the molecular weight and the choice of L- and/or D- enantiomer (Kulkarni et al. 1971). A disadvantage of polylactide, however, is that it does not form a bond with bone tissue and is therefore not a "bioactive" material.

The combination of different biomaterials in a composite material has given novel dimensions to the development of new structural implants. Several in vivo and in vitro experiments have been described in which bone formation onto various calcium phosphate/polymer composites was studied (Nelson et al. 1977; Bonfield et al. 1986; Higashi et al. 1986; Scalzo et al. 1989; Tarrant and Davies, 1989). The potential advantage of composites made of hydroxyapatite and polylactide is that a degradable, bioactive and mechanically strong material is formed. These material characteristics will be influenced by the type of polylactide chosen and the amount of hydroxyapatite present in the composite material. The percentage of hydroxyapatite in the composite can be varied, depending on the required mechanical properties. Other studies (Verheyen et al. 1991) have indicated that >50% w/w hydroxyapatite added to polylactide resulted in too brittle materials, whereas <20% w/w hydroxyapatite added to polylactide was not expected to significantly increase the bone bonding properties. To investigate the influence of composite constitution, and particularly the amount of hydroxyapatite surface available, on its bone-bonding ability, poly(L-lactide) was coated with different covering percentages of hydroxyapatite. In order to mimic a composite material, we chose a plasma sprayed hydroxyapatite coating onto poly(L-lactide), to improve reproducibility.

The aim of this study was to examine the influence of different percentages of

hydroxyapatite covering on poly(L-lactide), on bone formation in vitro. This was examined in an in vitro bone forming system which has previously been used to study interfacial reactions with titanium, hydroxyapatite, and other biomaterials (Davies et al. 1990, 1991; de Bruijn et al. 1991, 1992a,b). The mineralization process was followed in time at both the light microscopical and ultrastructural level.

MATERIALS AND METHODS

Hydroxyapatite/poly(L-lactide) composites

For the in vitro experiments, poly-L-lactide (PLA) cylinders with a diameter of 13mm were machined from a block of high molecular weight (927kD) as-polymerized PLA (Purac bv, Gorinchem, The Netherlands), and 200μm thick discs were cut using a diamond saw (Buehler low speed diamond saw, Isomet™).

Different ratios of hydroxyapatite were applied onto the PLA materials using the plasma spray method (figs 1a-c). Using scanning electron micrographs, the amount of hydroxyapatite covering was quantitatively measured with a Vidas Image Analyzing System and the percentages of coating were respectively 0%, 14.6±7.6% (15%), 36.3±14.2% (36%) and 100%. Figure 1d shows the x-ray diffraction pattern of the hydroxyapatite coatings. The molecular weight of the PLA before, and after plasma spraying was measured by Purac bv (Gorinchem, The Netherlands) and was 927kD and 570kD respectively. The samples were gas sterilized using ethylene oxide prior to the cell culture experiments.

Cell culture

An osteogenic rat bone marrow culture (Maniatopoulos et al. 1988) was used for the in vitro experiments as described previously (de Bruijn et al. 1991). Briefly, third passage rat bone marrow cells obtained from femora of young adult male Wistar rats (100-120g) were cultured on the materials at a concentration of $1x10^4$ cells/cm^2. The culture medium was composed of alpha-minimal essential medium (α-MEM, Gibco) supplemented with 15% foetal calf serum (Gibco), penicillin/streptomycin, and freshly prepared 10mM ß-glycerophosphate, 50μg/ml ascorbic acid and 10^{-8}M dexamethasone. Cultures were placed in an incubator at 37°C in a humidified atmosphere of 90% air and 10% CO_2 and refed every 48 hours. To examine the influence of culture medium on the coatings, control specimens with a 100% hydroxyapatite coating were incubated in medium without cells.

After 1, 2, 4 and 8 weeks, cultures were processed for light, scanning and transmission electron microscopy.

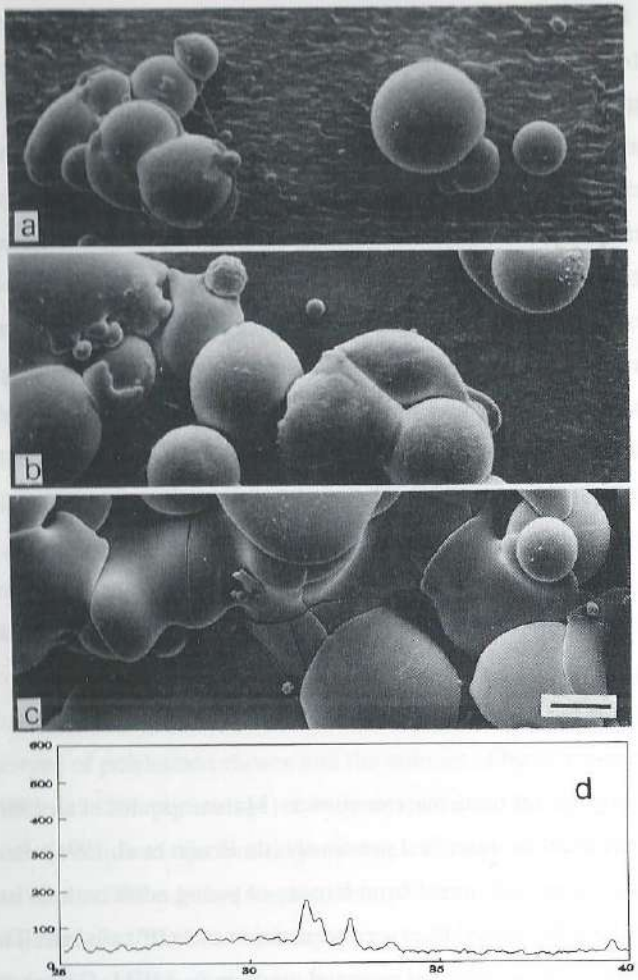


figure 1. Poly(L-lactide) discs (PLA) with an increasing amount of hydroxyapatite covering (HA); (a) 15%, (b) 36, (c) 100%. The powder x-ray diffractogram of the hydroxyapatite coating, showing low peaks and peak broadening, is indicative of an amorphous phase (d). bar= 12.5μm.

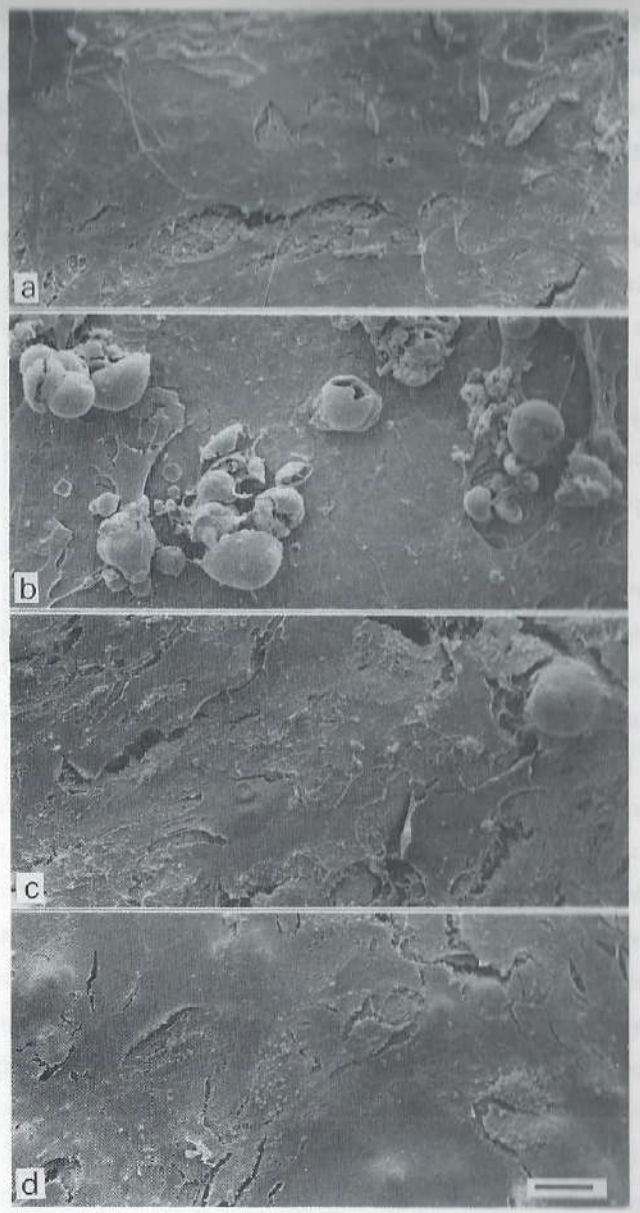


figure 2. Cells cultured for 1 week onto the various hydroxyapatite coatings as seen in figure 1. (a) uncoated, (b) 15% coated PLA, (c) 36% coated PLA and (d) 100% coated PLA. A multilayer and abundant extracellular matrix is present on 0%, 36% and 100% coated PLA. Note the plasma-sprayed hydroxyapatite particles on 15% coated PLA, that are not fully overgrown with cells. bar= 10μm (a), 23μm (b,c,d).

Light microscopy

Specimens were fixed in 1.5% glutaraldehyde in 0.14M sodium cacodylate buffer (pH 7.4, 4°C) for 30 minutes, dehydrated through a graded series of ethanol and embedded in glycol methacrylate. Semi-thin sections were cut and then stained with toluidine blue or alcian blue.

Alkaline phosphatase cytochemistry. Alkaline phosphatase activity was detected using the Azo-dye method of Gomori. The substrate solution was composed of 2mg/ml α-naphthyl phosphate and 1mg/ml Fast Blue RR salt, dissolved in 0.1M Na-barbiturate buffer pH 9.2. Fixed cells were incubated for 10 minutes in the substrate solution and then thoroughly rinsed in tap water. Specificity for alkaline phosphatase activity was determined by incubating cells with a control substrate solution in the absence of α-naphthyl phosphate.

Electron microscopy

Transmission electron microscopy (TEM). Cells were fixed according to the light microscopical procedures and after rinsing in 0.14M sodium cacodylate buffer pH 7.4, postfixation was carried out in an aqueous solution of 1.5% potassium ferrocyanide and 1% OsO_4 for 16 hours at 4°C. Specimens were dehydrated through a graded series of ethanol and embedded in Epon. Ultra-thin sections were prepared on a LKB ultramicrotome, stained with uranyl acetate and lead citrate and examined at 80kV in a Philips EM 201 or 400.

Scanning electron microscopy (SEM). Specimens were fixed and dehydrated according to the routine TEM procedure and air dried with tetramethylsilane (Merck). A layer of gold was sputter coated onto the specimens with a Balzers sputter coater model MED 010 and they were examined in a Philips S 525 SEM at an accelerating voltage of 15 kV.

RESULTS

Bone marrow culture.

Cells showed a high alkaline phosphatase activity on all materials, independent of the amount of hydroxyapatite covering. However, a difference in cell proliferation was seen on the different coatings. Figure 2 shows a SEM micrograph of the different hydroxyapatite coverings after 1 week of cell culture. The individual particles of the

hydroxyapatite coating was still visible on the 15% coated PLA, while the other materials were fully covered by a cell multilayer. In this multilayer, abundant fibrillar material was observed, while this was sparse on 15% coated PLA. The hydroxyapatite surface of all samples showed early signs of degradation after only 1 week of culture, indicated by the presence of small cavities and a porous appearance (fig 3).

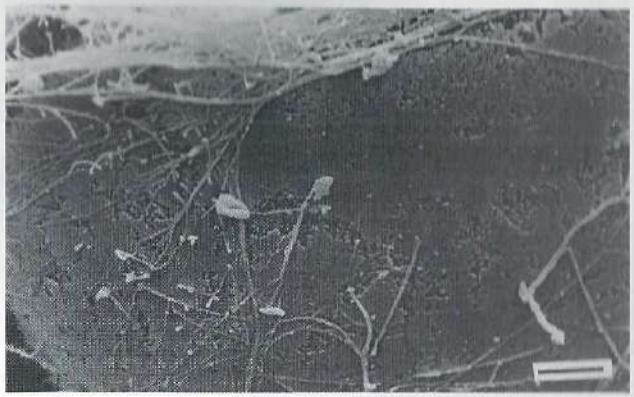


figure 3. Degrading hydroxyapatite particle from 15% coated PLA, onto which fibrillar material is deposited. Note small cavities (arrow head) in the hydroxyapatite particle, suggestive for degradation.
1 week culture, bar= 1.8μm.

To examine the time period in which mineralized extracellular matrix was formed on the different coatings, specimens were examined with LM, SEM and TEM. When stained with either toluidine blue or alcian blue, a basophilic line was observed on the hydroxyapatite coated parts of the specimens and not on the PLA. It was present after 1 week on both 36% and 100% coated PLA, but only after 8 weeks on 15% coated PLA. SEM and TEM showed a mineralized extracellular matrix from 2 and 4 weeks onwards on the 100% and 36% coated PLA respectively (fig 4), and at 8 weeks on the 15% coated PLA. An intimate contact was observed between the mineralized collagen fibres and the hydroxyapatite surface (fig 5). After 8 weeks of culture, 100% coated PLA was fully covered with globular, mineralized accretions to which collagen fibres were attached (fig 6). In addition to the formation of a mineralized extracellular matrix on the hydroxyapatite particles, calcium and phosphorous containing (as revealed by x-ray microanalysis, but not shown herein) mineralization globules were also seen on the

uncoated PLA surface of 15% and 36% coated PLA (fig 7). The formation of these globules, however, was more widespread with 36% coated, than with 15% coated PLA. Figure 8 shows TEM micrographs of mineralized extracellular matrix on 100% coated PLA after an 8 week culture. At the interface, both electron lucent and electron dense layers can be seen (figs 8a,b). Also, single or alternating electron dense layers have been observed (fig 8c). Collagen fibres were frequently seen in the mineralized extracellular matrix.

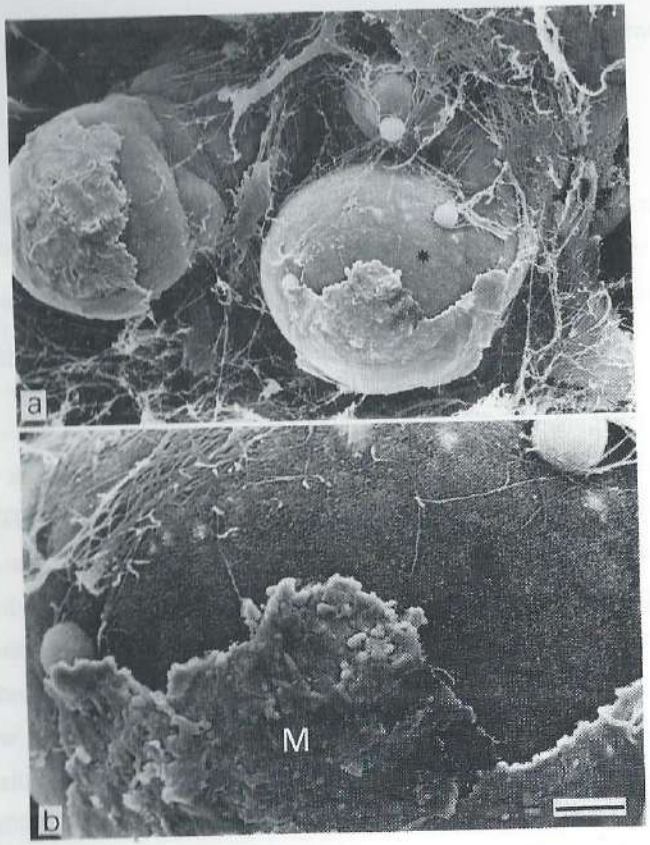


figure 4. 100% coated PLA after 2 weeks cell culture. Note the globular mineralized material (M) that is formed onto the plate-like crystals (asterisk), covering the hydroxyapatite surface. bar= 5.9μm (a), 1.9μm (b).

Cell free environment.

When stained with toluidine blue or alcian blue, a distinct basophilic line was seen on the 100% coated PLA which was similar to that seen in the cell cultures. SEM examination revealed that the hydroxyapatite surface was composed of small plate-like crystals (fig 9a,b). When examined with TEM, a gradual transition was seen between the bulk hydroxyapatite material and the outer surface, which was composed of calcium and phosphorous containing, needle-shaped crystals (fig 9c). However, neither fibrous or globular mineralized material was attached to this layer.

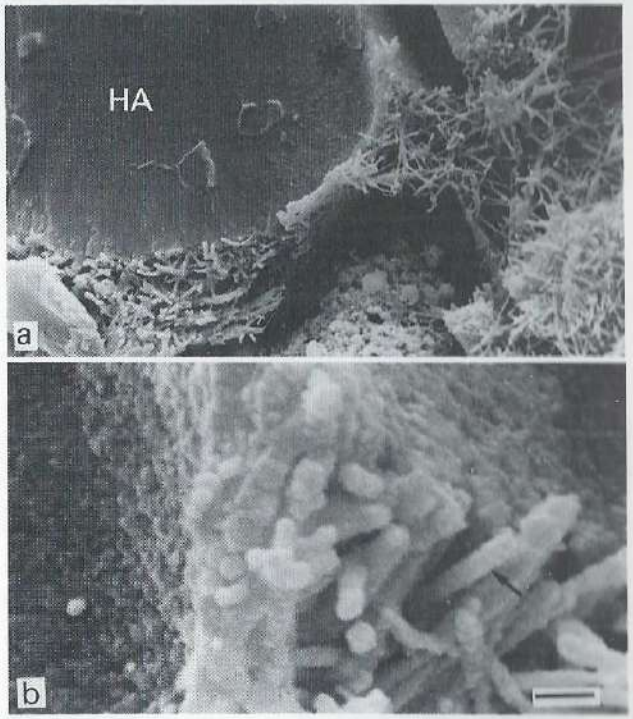


figure 5. (a) SEM micrograph of 100% coated PLA onto which a mineralized extracellular matrix is deposited. Note a gradual transition (asterisk) from the hydroxyapatite particle towards the mineralized extracellular matrix. (b) Higher magnification of (a), showing mineralized collagen fibres (arrow). bar= 3.0μm (a), 0.4μm (b).

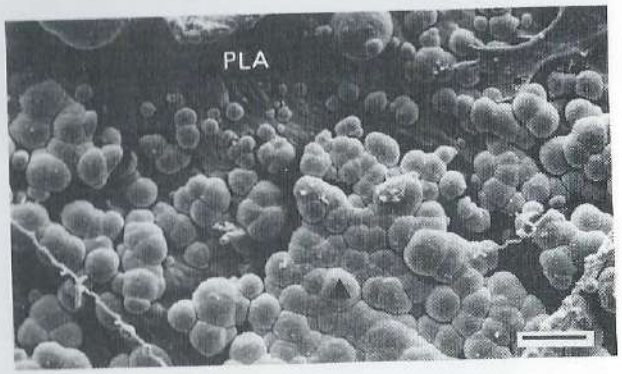
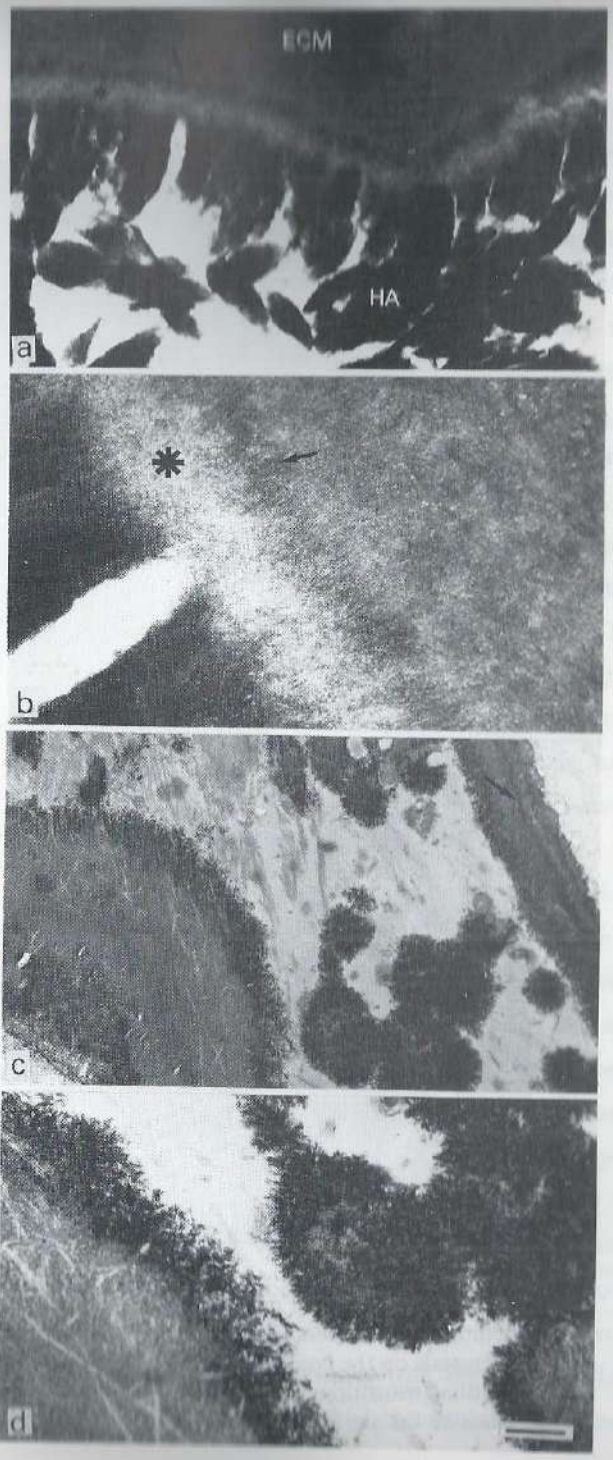


figure 6. Globular, mineralized accretions (arrow head) formed on the 100% hydroxyapatite coating in which collagen fibres are attached. 8 weeks of culture. bar= 2.94μm.

figure 7. Deposition of mineralization globules (▲) onto the PLA surface of a 36% hydroxyapatite coated sample, after 1 week of culture. bar= 6.3μm.

figure 8. TEM micrographs of 100% coated PLA after 8 weeks of culture. Direct deposition of mineralized extracellular matrix onto the hydroxyapatite can be seen and there is a gradual transition between the hydroxyapatite and the mineralized extracellular matrix. Note single electron dense layer (arrow) and electron lucent zone (asterisk) at the interface in (a). (b) is a higher magnification of (a). The electron dense layer is also present at the periphery of mineralization globules (c,d). Also note multiple electron dense layers (arrow) at the interface in (c). bar= 0.6μm (a), 0.2μm (b), 1.4μm (c), 0.6μm (d).



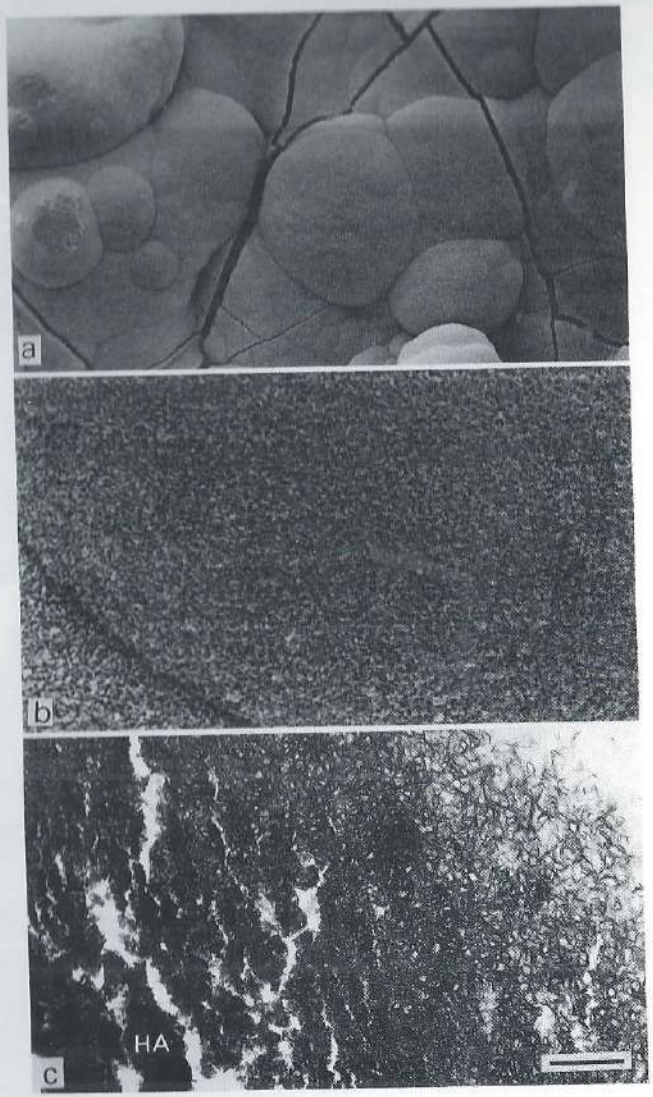


figure 9. (a,b) SEM micrograph of 100% coated PLA after 1 week in culture medium, in the absence of cells. Note the deposition of small plate-like crystals on the coating. (c) TEM micrograph of the same coating, a gradual transition is seen from hydroxyapatite to needle-shaped crystals at the periphery. bar= 11.1μm (a), 1.5μm (b), 0.4μm (c).

The results of this study indicate that a correlation exists between the percentage of hydroxyapatite coating on PLA and the time in which mineralized extracellular matrix is formed. This study has further shown that the presence of hydroxyapatite is required for the formation of a mineralized extracellular matrix on PLA. Although the interface formed was similar for all hydroxyapatite coatings, the time varied in which the extracellular matrix was produced and mineralized. A reason for this might be an alteration in cell phenotype expression induced by the amount of available hydroxyapatite in the composite (Khare et al. 1990). In addition, as the different coating percentages were achieved by plasma spraying, the resulting differences in surface topography may also have influenced cellular activity (Cherhoudi et al. 1989, Brunette et al. 1991, Curtis and Clark, 1990). A review by Curtis (1990) indeed showed that surface topography will influence cells in a wide variety of ways, which include cell attachment and cell migration. Hence, the difference in cell migration and spreading over the uncoated or 100% coated PLA and 15% or 36% coated PLA may be controlled by the surface topography of the latter two materials. The influence of ions released from the hydroxyapatite and PLA substrata cannot be the reason for the differences in cell behaviour, as far as cell migration is concerned, as cells formed a confluent layer on both uncoated and 100% coated PLA.

Interpretation of the formed mineralized layers on 15%, 36% and 100% coated PLA in cell culture is difficult, as it was also observed to a certain extent on 100% coated PLA in a cell free environment. The presence of the calcified layer on the control specimens is in contrast to earlier studies (de Bruijn et al. 1991) in which no alterations of the hydroxyapatite surface was seen, when samples were placed in culture medium in the absence of cells. A reason for this discrepancy may be the amorphous character of the hydroxyapatite used in this study (fig 1d), as opposed to more crystalline hydroxyapatite used in previous studies. Evidence for this has been given by LeGeros (1988), who showed that crystallinity or crystal size influences the degradation properties of a material and we have also observed this in a recent study (de Bruijn et al. 1992b,c); the more crystalline a material, the slower its ion release. Similar phenomena may also explain the more widespread presence of mineralization globules with 36% coated PLA than with 15% coated PLA. With the former material a higher calcium and phosphorous ion

concentration will be present in the culture medium due to the higher percentage of hydroxyapatite coating. In addition, the presence of PLA, which causes an acidic environment when degrading, will enhance hydroxyapatite dissolution (Lee et al. 1989). The above described dissolution process of hydroxyapatite will, as hypothesized by Daculsi et al. (1990), result in a high calcium and phosphate ion concentration at the ceramic surface, followed by possible reprecipitation. Together with this reprecipitation process, serum proteins may be incorporated, which may account for the distinct basophilic line on the coating particles after the light microscopical toluidine blue or alcian blue staining. Thus, part of the observed mineralization may be due to degradation of the hydroxyapatite ceramic followed by reprecipitation, without cellular activity. However, we believe that apart from this physico-chemical dissolution-(re)precipitation process, a cell mediated mineralized extracellular matrix is also formed. Evidence is shown in figure 6, in which collagen fibre containing globular accretions are deposited on the hydroxyapatite particles. With only physiological mineralization one would expect a layer that is more homogeneous in thickness. Another example which may account for cell mediated mineralized extracellular matrix formation is seen when one compares figure 9b and figure 4 of 100% coated PLA in a cell free and cell containing environment respectively; it is clear that the hydroxyapatite particles of both materials are covered with needle shaped crystals. However, in the cell containing environment, a mineralized globular matrix is formed on this crystal layer that shows similarities with early bone formation in vitro (Davies, 1991). TEM examination of this interface revealed an electron dense layer interposed with the hydroxyapatite coating and the mineralized extracellular matrix. This electron dense layer was only present at the interface in the cell containing culture, and not in the cell free culture (compare figure 9c and figure 8a,b). This is similar to earlier observations by our group (de Bruijn et al. 1991, 1992a,b) that showed an electron dense layer at the interface between hydroxyapatite and the surrounding mineralized extracellular matrix. This layer showed morphological similarities with the electron dense layer seen in vivo (Jarcho et al. 1977; Denissen et al. 1980; Tracy and Doremus, 1984; van Blitterswijk et al. 1985; Ganeles et al. 1985), between hydroxyapatite and bone tissue.

Concluding, we can state that a physico-chemical process of coating dissolution-(re)precipitation, as well as cellular activity, was responsible for the observed mineralization. Furthermore, the hydroxyapatite coating itself creates differences in

surface topography, which may alter cellular behaviour and the mineralization process. Therefore, using this model, it was not possible to determine the percentage of hydroxyapatite required in the composite to initiate bone bonding. However, it can be said that the covering rate of hydroxyapatite on PLA influences mineralized extracellular matrix formation in vitro and is expected to lead to differences in tissue response in vivo.

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HARD AND SOFT TISSUE RESPONSE TO HYDROXYAPATITE/POLYLACTIDE COMPOSITES.

A study in the rat

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ABSTRACT

The tissue reaction to four different hydroxyapatite/polylactide (50% w/w) composites with bone bonding potential were investigated using two models. The differences between the composites used, comprised variations in molecular weight of the polylactides, thus providing different degradation rates for the composites. Polylactides used were: poly-L-lactides of 100, 250 and 500 KD and a poly-DL-lactide of 400 KD. Hydroxyapatite particles were added prior to the polymerisation process providing composites with areas of hydroxyapatite mixed with polymer. In the first model, the composites were implanted in tibiae of rats and evaluated after 1, 2, 4, 12, 24, and 50 weeks. Histology of the tibiae revealed that the bone contact of the composites with the poly-L-lactides (HA/P-L-LA) differed from that of the composite with poly-DL-lactide (HA/P-DL-LA). The P-L-LA composites showed small surface areas (extending on both polymer and hydroxyapatite) covered with bone whereas the P-DL-LA composites revealed small areas of intimate contact with individual hydroxyapatite particles. This intimate contact had developed while the HA/P-DL-LA composite degraded. In contrast, the HA/P-L-LA composites remained unaltered in shape even at the 50 weeks period. In the second model, the polylactides with and without hydroxyapatite were implanted in subcutaneous tissue. All implants were encapsulated in fibrous tissue with mainly multinucleated cells at the interface. Subcutaneous degradation of the implants was not observed for the poly-L-lactides (with or without hydroxyapatite) but the poly-DI-lactide (with or without hydroxyapatite) revealed also clear signs of degradation at this implantation site.

Apart from sparse sites of bone contact, the main tissue response to all composites, both in the tibia and subcutaneously, consisted of a connective tissue encapsulation, containing mainly fibroblasts and multinucleated cells.

INTRODUCTION

Hydroxyapatite ceramic is generally characterized by a satisfactory biocompatibility and it possess bone bonding properties [Rosen 1990, Blitterswijk 90, Alexander 87]. It is clinically applied as a coating on implants used in orthopaedic and dental surgery [Geesink '90, Pilliar'91] as well as it is used in bone replacement [Jarcho '81, van Blitterswijk '90]. Due to its mechanical properties, it can only be applied in non-load bearing implant materials. Polylactide, a degradable polymer, has been investigated for many purposes and is for instance applied clinically in sutures and in bone repair materials [Rokkanen '85 '91, Bos '87, Eitenmuller '88]. It lacks however bone bonding potential. Composites of these two materials might thus potentially have a favourable mechanical properties and biocompatibility, be resorbable and possess bone bonding abilities.

Prior to verification of these characteristics in animal studies, various *in vitro* studies have been performed. These studies comprised different polylactides and hydroxyapatite/ polylactide composites (HA/PLA). The *in vitro* degradation rates of HA/PLA composites have been investigated by Verheyen '92 The results showed that the degradation rate of composites with 30% and 50% w/w hydroxyapatite, was related to the degradation rate of the polylactides. The breakdown of HA/PLA composites resulted in the release of degradation products into the surrounding buffers. Consequently, the effect of degradation products on tissue was investigated. For this purpose cells were cultured in the presence of degradation products obtained by a forced degradation of polylactide films providing an unknown mixture of small polymers, oligomers and monomers. Cells were also cultured in media containing known concentrations of the end products of degradation, represented by the L- and D-monomers [van Sliedregt '92]. These *in vitro* experiments showed a satisfactory biocompatibility of the degradation products of polylactide, although some negative influence was caused by concentrations of 0.5% or higher L-and D-monomers.

Subsequently, HA/PLA composites were tested *in vitro* using a bone marrow culture. A positive correlation between the amount of hydroxyapatite present on the surface of polylactide and the *in vitro* bone formation was demonstrated [de Bruijn].

These *in vitro* studies led to the following postulations:
I: the amount of hydroxyapatite in the composite is crucial for its bone bonding properties,

II: the degradation rate of the composite can be controlled by variations in molecular weight and composition of the polylactide part.

Four different HA/PLA composites evolved of this in which the HA percentage was maintained on 50 % w/w because mechanical tests indicated that above this percentage the brittle properties of hydroxyapatite prevailed [Verheyen '92], whereas lower HA percentages were not expected to contribute to the bone bonding properties of the composite. The other 50% was composed of polylactides which varied in molecular weight and composition.

In the present study, composites were implanted in the tibia and subcutaneously in rats to investigate the bone bonding abilities, degradation and connective tissue response.

MATERIALS AND METHODS

Implant materials

Poly-L-lactides and a poly-DL-lactide (Purac bv., Gorinchem, The Netherlands) were polymerized either with or without the addition of hydroxyapatite powder. The molecular weights of three poly-L-lactides (with or without hydroxyapatite) were 100, 250 and 500 KD and the molecular weight of the poly-DL-lactide (with or without hydroxyapatite) was 400 KD. Hydroxyapatite powder (CAM-implants, Leiden, The Netherlands) with a particle distribution of 1-40 μm, was added in a 50 % w/w ratio prior to the polymerization process. Small blocks (1 x 2 x 1 mm) were machined from the as-polymerized blocks. The polylactide and composite blocks were sterilized in ethylene oxide vacuum 9 KPa, water vapour 5 KPa, for 2 hours at 52 °C and aerated for 72 hours.

Experimental design

In the first model, the HA/PLA composites were implanted in the tibiae of rats to investigate its bone bonding properties. In the second model, the HA/PLA composites and the polylactides without hydroxyapatite were implanted subcutaneously to study the general tissue response. The investigation period lasted 1, 2, 4, 12, 24 and 50 weeks for both models. For each composite, 8 tibiae were operated per survival time and for each material (both composites and pure polylactides) 8 blocks were implanted subcutaneously per survival time.

Surgical procedure

A total of 120 male Wistar rats weighing about 300 g received the implants under Hypnorm anaesthetics (0.1 ml/100 g body weight). For the first model, both tibiae of each rat were operated and similar implants were inserted. The periosteum was scraped from the proximal part of the tibiae, distal of the diaphysis and holes of ± 2 mm were drilled up to the marrow cavity attended with extensive saline cooling. After placement of the blocks, which protruded into the marrow cavity, the muscle and skin were closed with vicryl 3-0. For the second model, subcutaneous pockets were created at the lateral side of the abdomen by an incision and blunt preparation. Generally, two implants were inserted per pocket which were subsequently closed with vicryl 3-0.

Histology

At the various evaluation times, the tibiae and subcutaneously implanted blocks were fixed by immersion in 1.5 % glutaraldehyde (Merck, Darmstadt, Germany) in 0.14 M cacodylate (Serva, Brunschwig, Amsterdam, The Netherlands) buffer (pH 7.4) at 4 °C. The tibiae were either dehydrated in a graded alcohol series, embedded in Spurr resin, scoured and polished for backscatter image analysis or they were decalcified in 10 % EDTA (pH 7.4), dehydrated, embedded in glycol methacrylate (GMA, Technovit, Heraeus Kulzer GmbH, Friedrichsdorf, Germany) and sectioned for light microscopy. The subcutaneous implants were dehydrated, embedded in GMA and sectioned for light microscopy.
Toluidine blue was used to stain the light microscopical sections.

RESULTS

Composites in the tibia

The four types of HA/PLA composites were implanted in the tibia. Their bone bonding and degradation properties were evaluated using light microscopy.

At 1 week after surgery the composites were surrounded by exudate, mainly composed of granulocytes and macrophages, and fibroblast-like cells.

At 2 weeks the exudate had predominantly disappeared and was largely replaced by fibroblasts and macrophages (Fig.1). Close to the implants, small islands of new bone

formation could be observed intramedullary.

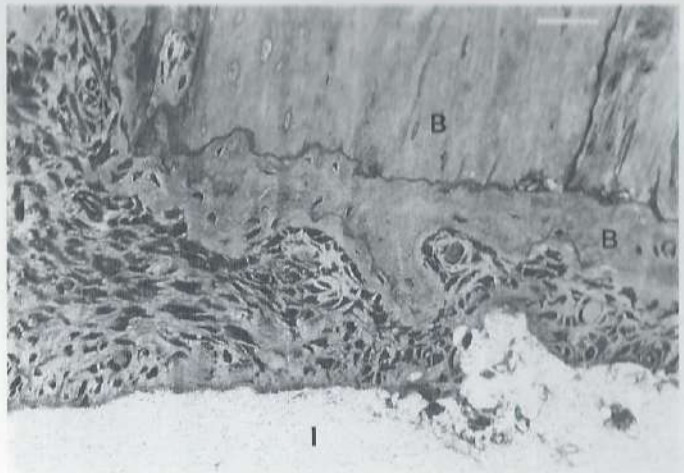


Fig.1 HA/P-DL-LA composite 2 weeks after implantation in the tibia. Note the fibrous tissue between the implant (I) and bone (B). bar is 50 μm .

At 4 weeks, the intramedullary bone formation was lining the implant. Between the bone and the implant, a layer of fibroblasts and macrophages remained which was attended with multinucleated cells at the implant interface. Seldomly, direct contact of bone tissue and implant was seen. Some tibiae, irrespective of the type of implant, revealed small clusters of chondrocytes near the implant surface (fig.2). A similar tissue response was observed at 12 weeks. The HA/P-DL-LA composites revealed an increased irregularity of the surface. Only one tibia showed some chondrocytes at the implant interface.

At 24 weeks, clear differences between the three HA/P-L-LA composites and the HA/P-DL-LA composite were seen. The HA/P-L-LA composites remained unaltered in shape irrespective of the initial molecular weight, whereas the HA/P-DL-LA composite had changed in shape. These clear signs of degradation were accompanied by an increased number of multinucleated cells and fibrous tissue extending into the irregularities of the composite (fig.3).
At some sites hydroxyapatite particles were in intimate contact with newly formed bone.

This was not observed for composites with poly-L-lactide, which revealed small bone contact areas extending over both polymer and hydroxyapatite (Fig.4).

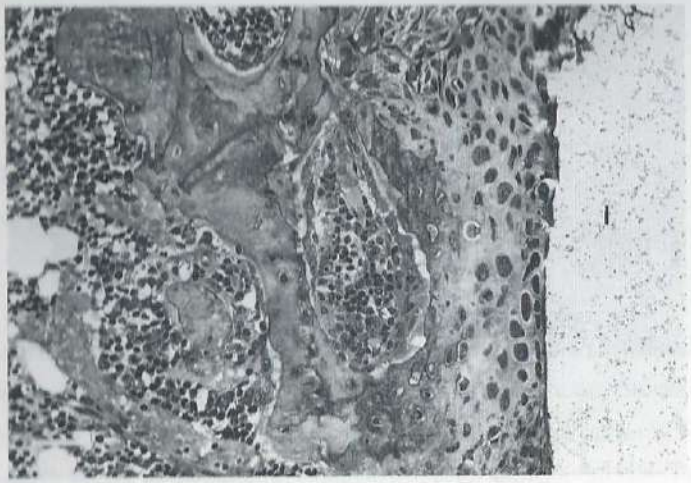


Fig.2 HA/P-DL-A composite 4 weeks after implantation in the tibia. A cluster of chondrocytes (C) is located near the implant (I). bar is 50 μm

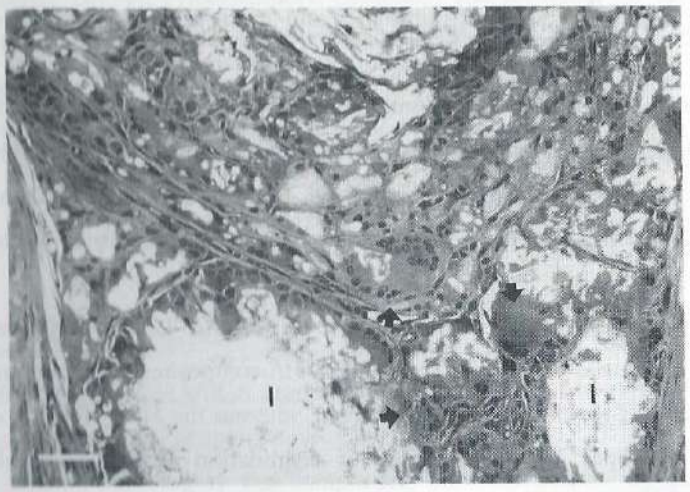


Fig.3 HA/P-DL-LA composite 24 weeks after implantation showing clear signs of degradation. Implant (I), arrow multinucliated cell, bar is 50 μm

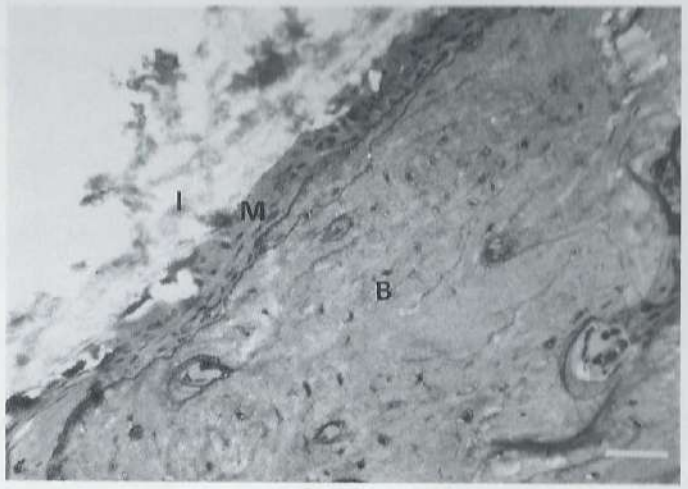


Fig.4 HA/P-L-LA 100 24 weeks after implantation in the tibia. A layer of multinucleated cells (M) is present between the bone (B) and implant (I). bar is 50 μm

At 50 weeks, the process of degradation of the HA/P-DL-LA composites had proceeded although remnants of the implants were still present. Again, sites of intimate contact between bone and hydroxyapatite particles were found (Fig.5). The composites made of P-L-LA however, had remained their shape as well as the tissue response which was composed of multinucleated cells and fibroblasts at the implant interface, and at some sites bone tissue was directly lining the implant.

Two implants of each type were evaluated for bone contact. For the estimation of the percentage of contact between implant and bone, the total length of an implant was studied. The sites of bone/implant contact were summed and thus an indication of the percentage of bone contact was obtained. Using this method, a contact area varying from about 10 % at 4 weeks to about 20 % at 50 weeks was established. However, variations were observed when similar implants were compared.

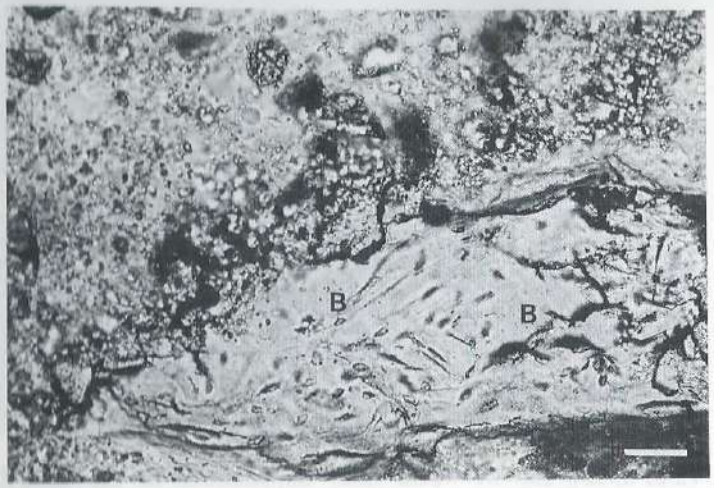
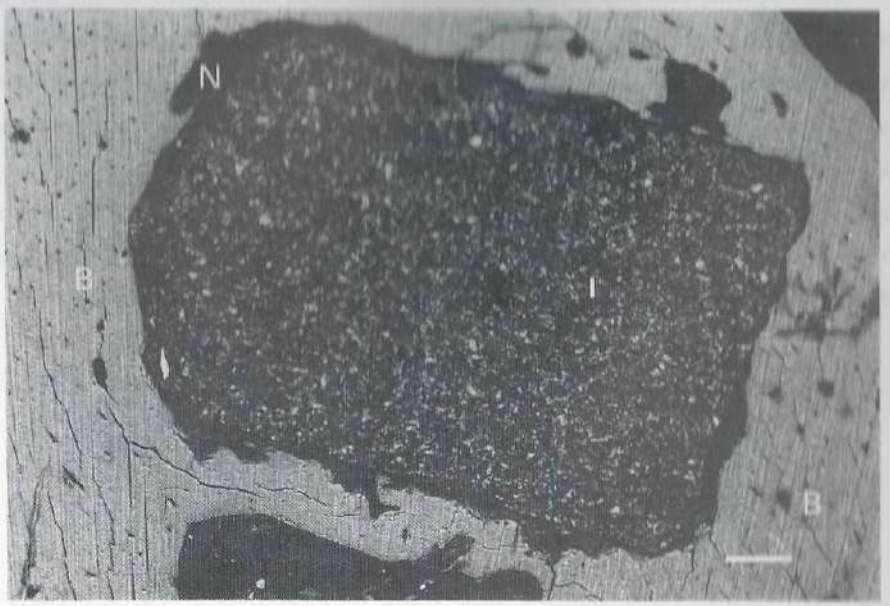


Fig.5 HA/P-DL-LA composite 50 weeks after implantation in the tibia. Hydroxyapatite particles are in close contact with the bone (B). bar is 50 μm

Backscatter images

Scanning electron microscopy (SEM) was used to reveal back scatter images of the bone tissue, composite and soft tissue. As was seen with light microscopy, bone tissue had developed around the implants (Fig.6), thereby separating the marrow cavity from the implant. A non-calcified layer was present between the implant and the newly developed bone. The location and thickness of this layer was indicative for the fibrous tissue and multinucleated cells as observed at the light microscopical level. At 24 weeks a difference was observed for composites with poly-L-lactide and composites with poly-DL-lactide. Backscatter SEM showed that some individual hydroxyapatite particles of the HA/P-DL-LA composites were in intimate contact with newly formed bone (Fig.7). The portion of hydroxyapatite particles seemed to have increased at the interface as compared to the initial composition. A variation in the portion of HA particles in the HA/P-L-LA composites was not observed.



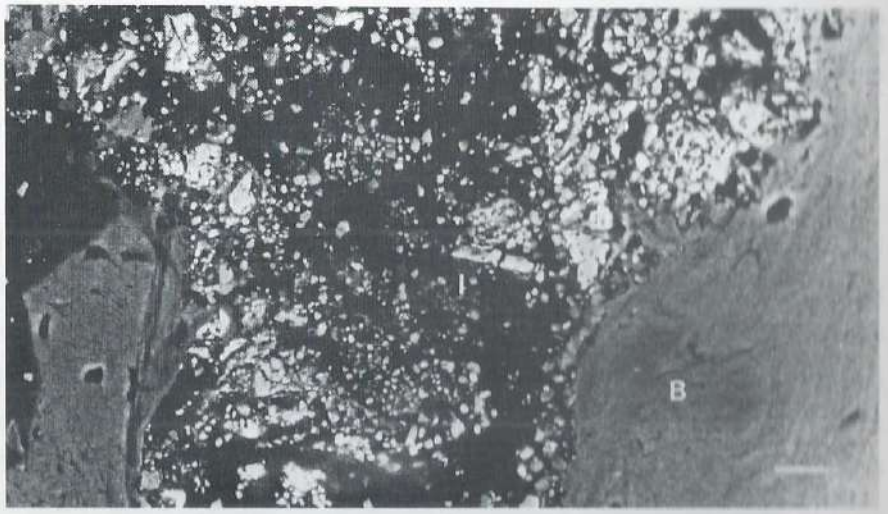


Fig.6 HA/P-L-LA 500 composite 24 weeks after implantation, backscatter graph. The composite is surrounded by bone, although a non-calcified layer (N) is present between bone (B) and implant (I). bar is 140 μm

Fig.7 HA/P-DL-LA 400 composite 50 weeks after implantation, backscatter graph. At the bone(B) implant (I) interface, hydroxyapatite particles are more abundantly present. bar is 25 μm

Subcutaneous response to HA/PLA composites and the polylactides

The implants with and without hydroxyapatite were investigated for the soft tissue response. The evoked tissue response was characterized by the formation of a capsule composed of fibroblasts, macrophages and multinucleated cells, similar to that observed in the tibiae. The degradation of the composites in the tibiae and in the subcutaneous tissues corresponded as well. Throughout the implantation period of 50 weeks no light microscopical signs of degradation were seen for the poly-L-lactides and the composites composed of poly-L-lactides (Fig.8). The poly-DL-lactide, with or without hydroxyapatite, showed clear signs of degradation starting at 12 weeks.

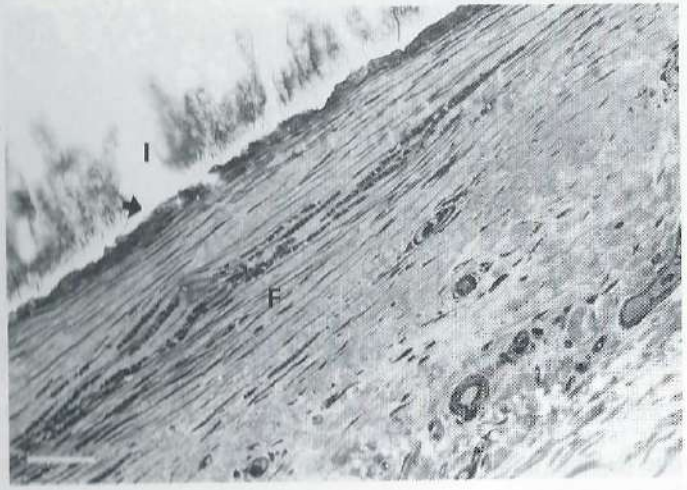


Fig.8 P-L-LA 500 50 weeks after subcutaneously implantation. A layer of darker stained cells is present at the material interface. F is fibrous tissue. bar is 50 μm

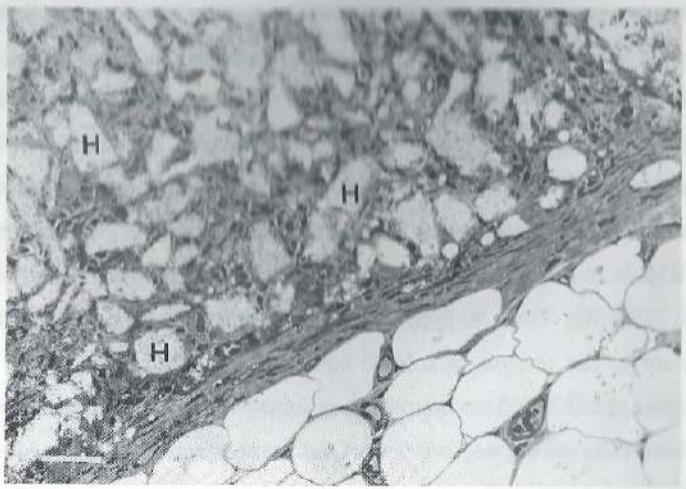


Fig.9 HA/P-DL-LA composite 50 weeks after subcutaneously implantation. The initial location of hydroxyapatite particles (H) remained and is surrounded by cells instead of polylactide. This was not often observed, mostly a mixture of cells and remnants, both hydroxyapatite and polylactide, was present like in fig.3. bar is 50 μm

DISCUSSION

The HA/PLA composites implanted in the tibiae evoked a tissue response very similar to that of a subcutaneous reaction. Bone bonding indicated by a direct contact between bone and implant was rarely observed. At 50 weeks a maximum bone/implant contact of about 20 % was observed. An explanation for the lack in bone formation might be the implantation procedure. This procedure implied the creation of a hole in the tibia which differed in shape as compared to that of the composites; round versus square. This resulted in a loose-fit implantation through which a lack of initial bone contact arose. Fibroblasts and multinucleated cells filled this area between implant and bone throughout the experimental period of one year, although the newly formed bone was deposited close to the implant. Verheyen et al. implanted 30 % and 50 % w/w HA/P-L-LA composites in femora of goats and he observed an initial bone contact of about 50 % which decreased to about 30 % after 24 weeks. The initial high percentage of bone

contact is probably caused by the almost press-fit implantation procedure. Bone remodelling at the composite/bone interface might be responsible for the decrease in bone contact.

The presence of multinucleated cells at the polylactide interface has been described by others as well, although the intensity of the reaction seemed to vary per experiment [Schakenraad '91, Bostman '90, Olsen , Majola '91, Manninen '92]. Such differences might relate to the stage of degradation, as proposed by Schakenraad. The precise mechanism and rate of degradation of these polymers is not fully understood but a relation with the initial molecular weight and composition is clear [Chawla '85, Leenslag '87, Christel '83]. *In vivo* degradation is characterized by a relatively steady initial chain scission phase [Pitt '81, Tunc '83], followed by an increased tissue reaction which is thought to relate to the release of soluble compounds and the removal of small solid fragments [Schakenraad '91]. This hypothesis is supported by the increase in the number of multinucleated cells during degradation of the composite HA/P-DL-LA and the pure P-DL-LA. The cellular response to P-L-LA (with or without HA) did not alter during the investigation period of one year, therefore it can be concluded that this composite did not reveal clear signs of degradation.

The bone bonding ability of the composites was thought to relate to the portion of hydroxyapatite in the composite. Hydroxyapatite is capable to bridge defects up to one millimetre by new bone formation [Soballe '91]. Thus, the loos-fit implantation could have been compensated by the ability of hydroxyapatite to bridge the bony defect. Since this was not observed, the amount of hydroxyapatite in the composite might have been insufficient to express this ability. During the degradation of the HA/P-DL-LA composite however, its bone bonding abilities were sometimes expressed by the formation of an intimate contact between newly formed bone and hydroxyapatite particles. These particles were probably exposed at the surface while the degradation of the matrix proceeded.

This phenomenon of proceeding bone contact was also described by Higassi [], who used different HA/P-DL-LA composites of which the molecular weight of the polylactide varied from 11,500 to 45,000 Dalton. He observed bone contact with the hydroxyapatite particles of HA/P-DL-LA of 11,5000, especially when the polylactide part degraded. Bone contact was absent for a composite consisting of P-DL-LA 45.000 Dalton. This composite was still present after 1 year. The results of Higassi's study and the present study are very similar although the initial molecular weights of the polylactides varied enormously. In

both studies, bone tissue was lining the implant without direct contact when signs of degradation were not observed. It seems that the bone bonding/ forming abilities are initially suppressed by the polylactide and that the bone bonding capacities are disclosed when the polylactide part degrades. The increased bone bonding properties are most likely related to the relatively enrichment of the hydroxyapatite due to the degradation of polylactide.

The sites of bone contact observed for the HA/P-L-LA composites, irrespective of molecular weight, extended over polylactide and hydroxyapatite surface areas and were not limited to the hydroxyapatite particles as seen for HA/P-DL-LA composite. Therefore bone contact of the HA/P-L-LA composites was most likely caused by a very intimate lining of the bone since bonding osteogenesis has not been described for polylactide [Matsusue '92].

An other explanation for the lack in bone contact in the present study is the possible occurrence of micro-movement since the implant extended into the soft tissue. Micro-movement is a known cause of fibrous capsule induction [Soballe '92]. The fibrous capsule in the present study was reduced at 12 months and the bone contact was increased as compared to the earlier evaluations.

The poly-L-lactides, with or without hydroxyapatite, were still manifest 12 months after implantation and hardly changed in shape. The poly-DL-lactide had however degraded remarkably. This difference in degradability was analogous for the subcutaneous implanted composites and the pure polylactides. The composition (amorphous versus crystalline) of the polymer seems to have a dominant impact on the degradation rate.

It can be concluded from this study that composites of hydroxyapatite and poly-L-lactides do not possess the expected bone bonding abilities, but the HA/P-DL-LA composite has some bone bonding potential, possibly due to its degradable property.

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GENERAL DISCUSSION

Hydroxyapatite/polylactide composites, as candidate materials for maxillofacial reconstructive surgery, were tested upon their biocompatibility using both *in vitro* and *in vivo* experiments. *In vitro* tests are considered to precede *in vivo* tests concerning the cytotoxicity/biocompatibility of materials. The importance of relevant *in vitro* experiments is represented by the numerous directives proposed by ISO (International organisation for standardization). These directives are however, "..not intended to be a set of definitive statements to be followed by individuals not qualified by training and experience; it should be applied with interpretation and judgement by the appropriate professionals qualified by training and experience taking into consideration the factors relevant to the device/material, its intended use, and the current knowledge of the material/device provided by scientific literature and previous clinical experience." [ISO 10993] This statement implies the importance of common sense and the development of new tests and methods where necessary.

in vitro testing of polylactides

The aim of testing the polylactides *in vitro* was to gain insight into the possible short and long-term toxic effects they might have on cells. Therefore, the polylactides were presented to cells in several ways, representing different stages of degradation. These experiments are described in chapter II, III and IV. All tests indicated satisfactory biocompatibility characteristics for the polylactides, although some remarks need to be made. Different types of epithelial cells, fibroblasts and osteosarcoma cells were used, which all showed the same proliferation characteristics on the non-degraded polylactide substrates. Some parts of the non-degraded polylactides remained uncovered and, since the cause is not yet clear, it should be considered as a potentially unfavourable characteristic of the polylactides.

To obtain the degradation products of polylactides, an accelerated degradation procedure was performed according to the method described by Homsy and Bakke

(chapter II). Addition of these products to different cell types revealed no significant alterations in morphology and in general the proliferation was unaffected, although occasionally minor deviations were observed. In retrospect, the amount of polymer used for extraction was approximately one third of the minimum amount advised by the ISO [fall 1992 ISO/TC 194]. The relevance of the present results is therefore reduced, although it is indicated in chapter IV that 0.1% of degradation products, as represented by the lactic monomers, does not affect the cells. A concentration of 0.5 % lactic monomer, however had an adverse effect on the cells. The relevance of these concentrations for the *in vivo* situation is difficult to address.

The relevance of *in vitro* studies for *in vivo* biocompatibility

The new generation of polymers which are intended for use as biomaterials, are often degradable. These materials will release more degradation products during the extraction procedure than so called inert polymers. The possibility that an effect is caused by these materials will be greater because even essentially non-toxic substances might be toxic when present in large amounts. A biased result may be obtained when, for instance, the local osmolarity is increased to non-physiologic levels, thus causing negative effects on cells. Due to the increased osmolarity, the material may be classified as cytotoxic, but the result is in fact a false positive. A further difficulty is the fact that requirements for cells in culture are defined experimentally instead of analytically and information about the tolerance of cells for variations in culture conditions is not available. The amount of material which can be added to culture medium, without influencing the basic requirements, is therefore limited (chapter IV).

Considering the above, the predictive value of *in vitro* tests for the *in vivo* biocompatibility of a material should not be overestimated. Too often the results obtained in *in vitro* experiments are extrapolated to the *in vivo* situation, without verifying whether the same underlying mechanism is as important *in vivo* as it is *in vitro*. Although several studies have indicated that a correlation exists between *in vitro* and *in vivo* toxicity [Homsy '70, Rice '78, Ziats '88], the *in vivo* process is far too complex to conclude that *in vitro* toxicity implicates *in vivo* toxicity or vice versa. It may very well be possible that certain materials prove to be toxic in a specific *in vitro* test while the *in vivo* properties appear to be favourable. This possibility should be considered to avoid rejection on the basis of *in vitro* toxicity without further research on the *in vivo* effects.

The *in vitro* tests of the present study indicated that the polylactides used should be considered as non-toxic materials. The implications of these studies for the *in vivo* situation, however, is difficult to access. An increase in osmolarity and a possible pH shift close to the degrading implant is likely to occur, but a possible effect depends on the draining capacity of the tissue. A consequence of *in vivo* bulk degradation in tissue which lacks a good vascular system might be swelling of the implantation site due to exudate formation. Rokkanen stated, in his review article on degradable implants for orthopaedic use, that 6.5 % of the patients developed a transient fluid accumulation when treated with self-reinforced polyglycolide or self-reinforced polyglycolide / polylactide copolymer rods. These materials degrade at a relatively fast rate (weeks) and it was suggested by Rokkanen that the complications might be due to local accumulation of polymer debris, in combination with a low elimination capacity of the tissue. Similar findings for the same material were described by Böstman who found an incidence of 7.9% of a clinically manifest foreign-body reaction, producing a fluctuant swelling at the implantation site. A case report of severe aseptic synovitis of the knee after biodegradable internal fixation is described by Fridén '92. Polylactides degrade, at least initially, at a slower rate than polyglycolide or polyglycolide/polylactide polymers. However, similar complications may eventually arise when the degradation rate finally increases due to collapse of the bulk material. This will subsequently give rise to the release of large amounts of degradation products. The exact mechanism for the unwanted symptoms related to the implantation of alpha-hydroxyacids is not fully understood and is probably caused by many factors.

Inflammatory response of polylactides

Like all materials, polylactides will evoke a tissue response when implanted. The physio-chemical characteristics of the material determine the type of cells that will be attracted, as well as the duration and severity of the reaction [Deams '78, Pizzoferato '87, Williams '87, van Blitterswijk '89]. The inflammatory response of polylactides was studied by using the peritoneal injection model as described in chapter V. Suspensions containing particulates of polylactides were injected into the peritoneal cavity of mice, thus avoiding a wound reaction, and a cellular response was established. Initially, this response was high but it was followed by a significant decrease which was probably associated with the clustering of particulates and cells into aggregates which later developed into granulomas

The abundance of multinucleated cells in these granulomas is indicative of a severe reaction and although their presence is desired for the removal and elimination of degradation products, the particles did not reveal clear signs of degradation. The (re)appearance of inflammatory cells at 6 months might be indicative for a sensitivity reaction. Although polylactides lack antigenic sites, impurities of the polymer might act as an immunogenic sensitizer. However, trace elements could not be detected by x-ray microanalysis [unreported observation], therefore it is possible that another mechanism is responsible for the new attraction of inflammatory cells after longer implantation times. The fact that a change in pH can result in the attraction of granulocytes is described by Remes and Williams '92 in their review article on immune response in biocompatibility. The appearance of inflammatory cells at 6 months might therefore be indicative of the start of degradation of polylactides, since degradation is associated with a pH decrease.

The results of the study, as described in chapter V, were compared with a recently published study on the establishment of the inflammatory activity of bioceramics by using the mouse peritoneal cavity model [Koerten '92]. It was reported that the number of neutrophil granulocytes that appeared in the peritoneal cavity, after injection of the particles, was used as a measure of inflammation. Using the same parameter, the present study showed a much higher (5-fold) influx of inflammatory cells for polylactide particulates than described by Koerten for calcium phosphate particles. Therefore, it is concluded that polylactide is a relatively strong inflammatory stimulus.

***In vitro* testing of the hydroxyapatite/polylactide composite**

The hydroxyapatite in the composite was believed to be responsible for the bone bonding properties of the material. The amount of hydroxyapatite that could be added to the composite was, however restricted to a maximum of 50% w/w, because higher amounts of hydroxyapatite would negatively affect the mechanical properties. To investigate the effect of the amount of hydroxyapatite on bone formation, a polylactide surface was covered with different amounts of hydroxyapatite using the plasma spray method. These surfaces were tested upon their bone bonding potential using the *in vitro* bone forming system of Maniatopolous as is described in chapter VI.

The results revealed that mineralization of the extracelluar matrix was related to the amount of hydroxyapatite on the surface. Mineralization of the extracelluar matrix was not observed on the uncovered polylactide surface. It was concluded that hydroxyapatite

was, at least *in vitro*, a necessary component for mineralization to occur. Electron microscopic evaluation revealed that the interface formed was similar for all the hydroxyapatite coatings (15%, 36% and 100%). It is possible that the amount of hydroxyapatite present in the composite will have an effect on the rate of bone formation after implantation.

***In vivo* testing of the composites**

The hydroxyapatite/polylactide composites tested differed in polylactide proportion. Since three poly-L-lactides with different molecular weights were used, the composites were expected to degrade at different rates. The composite with poly-DL-lactide was expected to degrade faster than the composites with poly-L-lactide. The initial bone bonding properties were expected to be similar as all the composites contained the same amount of hydroxyapatite. The experiment in which these composites were implanted in bone tissue is described in chapter VII. The results revealed that all composites were initially encapsulated by fibroblasts and multinucleated cells. Very few areas of bone contact were observed with the poly-L-lactide composites, but the poly-DL-lactide composite revealed increasing bone contact sites, with time. The bone contact of the latter was seen with the hydroxyapatite particles. This is certainly a promising result for the development of bone bonding composites based on hydroxyapatite and poly-DL-lactide. The degradation of poly-DL-lactide most likely caused a relative enrichment (%) of hydroxyapatite particles at the composite/bone interface, which in turn triggered the development of bone bonding.

The initially insufficient amounts of hydroxyapatite at the implant interface and the lack in bone bonding potential of polylactide resulted in minimal bone contact. This phenomenon was in agreement with an vivo experiment [Woodard '88] in which porous hydroxyapatite was dip-coated in polylactide, resulting in a covering of the pores. Both the porous hydroxyapatite and the dip-coated porous hydroxyapatite were implanted in the femur of rabbits. Initially, the hydroxyapatite coated with polylactide revealed less bone ingrowth as compared to the bone ingrowth in hydroxyapatite without the coating. Apparently, polylactide decreased the bone bonding capacity of the composite, at least temporarily. As soon as the polylactide part had degraded, the bone bonding characteristics of hydroxyapatite prevailed.

The bone bonding mechanism is thought to be related to the dissolution of

hydroxyapatite and the precipitation of carbonate apatite (introduction & chapter VI). Since the dissolution of hydroxyapatite is most likely affected by pH changes caused by degradation of the polylactide, the precipitation will be altered as well. The observed bone formation on the hydroxyapatite/poly-DL-lactide composite might relate to this also.

Degradation

The *in vivo* experiment described in chapter VII revealed no clear signs of degradation for the poly-L-lactides, independent of the presence of hydroxyapatite. The major drawback of this experiment was the fact that the evaluation time was relatively short as compared to the time necessary for the composites to degrade. In contrast to the composites made of poly-L-lactide, the poly-DL-lactide/hydroxyapatite composite did reveal clear signs of degradation, although remnants of the composite were still present after 12 months of implantation. The higher degradation rate is most likely due to the amorphous character of poly-DL-lactide, which will probably degrade completely. In contrast, the poly-L-lactide might leave crystallites as remnants of the degradation. Generally, the poly-L-lactides receive more attention in research because of the superior mechanical characteristics prior to degradation. The mechanical properties are closely related to the regular arrangement of the polymer chains at the molecular level, which is also reflected in the crystallinity [Chawla' 85/86]. The crystalline areas are expected to be very resistant to hydrolysis and to remain in the tissue as small sharp polylactide particles [Rozema '91].

The observed composite degradation is mainly due to the degradation of poly-DL-lactide, but hydroxyapatite is also subject to degradation [Fellows '88]. The exact degradation rate for hydroxyapatite is difficult to determine since many factors are involved. The degradation rate of hydroxyapatite in the composites might be enhanced since the solubility of hydroxyapatite is 40 times greater in an acidic environment (pH 5.2) [Lee '89].

The degradation of the poly-DL-lactide matrix might even result in the detachment of hydroxyapatite particles although this was not observed for the composites tested in the present study. It is possible that detached particles will be phagocytosed by cells and subsequently transported from the implantation site, or that they may give rise to a locally increased inflammatory effect.

FINAL REMARKS

From the results of the *in vitro* tests it has to be concluded that the polylactides tested possess satisfactory biocompatibility characteristics. The *in vivo* results concerning the biocompatibility, bone bonding ability, and degradability of hydroxyapatite/poly-DL-lactides are promising, therefore further research should be focused on this combination. A major disadvantage of poly-DL-lactide is the insufficient mechanical potential of the material. If further research is focused on poly-L-lactides, one should realize that several factors involved in the *in vivo* biocompatibility can not be investigated in *in vitro* experiments. In the present *in vivo* experiment it was not possible to investigate the long term effect of poly-L-lactide, which might comprise crystalline remnants at the implantation site. The amount of hydroxyapatite present in a hydroxyapatite/polylactide composite determines whether the composite has bone bonding potential. A relative enrichment of hydroxyapatite due to the degrading polylactide matrix, may provide bone bonding potential, but the amount of hydroxyapatite must also be sufficient to ensure early bone bonding. This is desirable as it will prevent fibrous tissue encapsulation and subsequently, replacement of the degrading composite by bone tissue is possible.

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SUMMARY

Hydroxyapatite/polylactide composites as candidate materials for application in reconstructive surgery of the head and neck region, were evaluated upon their biocompatibility using both *in vitro* and *in vivo* experiments. The biocompatibility of hydroxyapatite is generally known to be satisfactory and this ceramic is commended for hard tissue replacement, in non-load bearing situations, due to its bone abilities. Although polylactide is applied successfully in different medical disciplines, its biocompatibility is open to debate, due to the lack of agreement between the results of several studies.

Polylactides with different degradation rates, poly-L-lactides 100, 240, 500 kDa and a poly-DL-lactide 400 kDa, were tested *in vitro* using five cell types, which are also found in the head/neck region. The proliferation of the cells was investigated by culturing on polylactide films and by culturing with media containing degradation products of artificially aged polylactides. Two of the five cell types were also cultured with media containing different concentrations of L-or D-monomer. These monomers represented the ultimate degradations products of polylactides. The results, as described in chapters II and III, revealed that the morphology of the cells was not affected by culturing on films, although some parts of the films remained uncovered. The morphology also remained unaffected when the cells were cultured with media containing the degradation products of the artificially aged polylactides. The proliferation of cells cultured with these media showed some minor alterations, although not consistently with a particular polylactide. Cells cultured with media containing the different monomer concentrations revealed that high (1%) monomer concentrations had a clear effect on the proliferation as well as on the morphology. Fibroblasts cultured with media containing 1% L- or D-monomer were larger and the nasal septum epithelium showed more signs of terminal differentiation with the 1% D-lactic monomer than with the L-lactic monomer, regardless of the osmolarity of the medium. Further research was therefore needed before final conclusions could be drawn, the results are described in chapter IV.

Both L- and D-lactic monomer were added to culture medium in concentrations of 0.01% and 0.5 %. The 0.5% monomers were also added to a modified medium with an initially lower osmolarity. Evaluation showed that fibroblasts were not affected by 0.01 % lactic monomers (1mg ml^{-1}), but that the addition of 0.5 % monomer reduced the metabolic activity of the cells by about 40% as compared to the control, regardless of the

osmolarity of the medium. Cell proliferation and morphology were only negatively affected when 0.5% monomer was added to standard medium, thereby increasing the osmolarity. The addition of sucrose, however, resulted in an increased proliferation and acid phosphatase activity, as well as an altered morphology.

The impact of these results for the *in vivo* situation can not be made, but these concentrations are unlikely to occur in the body. It can be concluded however, that the osmolarity, as well as the nature and concentration of the added material, can exert an influence on proliferation, morphology and cellular activity. Thus, the osmolarity of test media should be determined for *in vitro* biocompatibility assays.

The *in vitro* experiments indicated favorable biocompatibility properties of the polylactides used. However, *in vitro* experiments lack the complexity of the *in vivo* reaction, therefore the inflammatory response of polylactides was determined by injecting particulates in the peritoneal cavity as is described in chapter V.

Four types of polylactide particulates, P-L-LA 100, 250, 550 kDa and a P-DL-LA 400 kDa were injected into the peritoneal cavity of mice. The inflammatory reaction showed an increase in cell number (mainly neutrophilic granulocytes) up to 48 hours; after this time the cell numbers decreased to a level below the control (phosphate buffered saline). All four polylactide particulates aggregated and intermingled with inflammatory cells. The aggregates remained present throughout the investigation period of 6 months. Quantitative measurements showed that standardisation of the particle form and size is essential. Based on the results of this study and of other experiments in which calcium phosphates and asbestos were injected intraperitoneally, it is concluded that the inflammatory response observed in the peritoneal cavity is related to the type of material (polylactide gave rise to a higher (5-fold) reaction than was seen for calcium phosphates) injected and most probably also to the form and size of the individual particles.

Thus, both *in vitro* and *in vivo* experiments indicated that the polylactides used could be considered as candidate components of the hydroxyapatite/polylactide composites. Prior to the *in vivo* investigation of the composites, *in vitro* bone formation was studied on a poly-L-lactide which was plasma-spray coated with various quantities of hydroxyapatite (0%, 15%, 36% and 100% coverage) as described in chapter VI. Rat bone marrow cells were cultured on the different coatings and the cellular response and elaborated extracellular matrix was examined at the light and electron microscopical level after 1, 2, 4 and 8 weeks of culture. Proliferation of cells into multilayers was seen on the

0%, 36% and 100%, but not on the 15% coatings. Coinciding with this was the sparse formation of extracellular matrix on the latter, and its abundant appearance on the former three coatings. Scanning and transmission electron microscopy revealed a mineralized extracelluar matrix on the 100% and 36% coatings after 2 and 4 weeks respectively and on the 15% coating after 8 weeks. Mineralization was not observed on uncoated poly-L-lactide. At the interface between hydroxyapatite and the mineralized extracelluar matrix, one or more electron dense layers were frequently observed, which showed morphological similarities with structures between these two entities *in vivo*. The results of this *in vitro* study show that, in the model used, hydroxyapatite is required to obtain the elaboration of mineralized extracelluar matrix on poly-L-lactide.

Subsequently, the tissue reaction to four different hydroxyapatite/polylactide (50% w/w) composites with bone bonding potential was investigated as described in chapter VII. The differences between the composites under study, comprised variations in molecular weight of the polylactides, thus providing different degradation rates for the composites. Polylactides used were: poly-L-lactides of 100, 250 and 500 kDa and a poly-DL-lactide of 400 kDa. Hydroxyapatite particles were added prior to the polymerisation process, thus providing composites with areas of hydroxyapatite mixed with polymer. The composites were implanted in tibiae of rats and evaluated after 1, 2, 4, 12, 24, and 50 weeks. Histology of the tibiae revealed that the bone contact of the composites with the poly-L-lactides (HA/P-L-LA) differed from that of the composite with poly-DL-lactide (HA/P-DL-LA). A close contact between hydroxyapatite particles and bone had developed whereas the HA/P-DL-LA composite degraded. The shape of the HA/P-DL-LA composite was clearly changed, which was in contrast to the unaltered shape of the HA/P-L-LA composites, even after the 50 weeks period. The polylactides with and without hydroxyapatite were also implanted in subcutaneous tissue. All these implants were encapsulated in fibrous tissue, with mainly multinucleated cells at the interface. Subcutaneous degradation of the implants was not observed for the poly-L-lactides (with or without hydroxyapatite) but the poly-DI-lactide (with or without hydroxyapatite) also revealed clear signs of degradation at this implantation site.

Apart from sparse sites of bone contact, the main tissue response to all composites, both in the tibia and subcutaneously, consisted of a connective tissue encapsulation, containing mainly fibroblasts and multinucleated cells.

It can be concluded from the described studies that the composite of hydroxyapatite

and poly-DL-lactide has some bone bonding potential and is most likely degradable, without leaving crystalline remnants in the tissue. In this study, the initial amount of 50% w/w hydroxyapatite present in the composite was insufficient to establish bone bonding directly (within 6 weeks) after implantation. Thus, further research on its bone bonding, mechanical and application properties is justified, but also necessary, prior to clinical use. Care should be taken in the use of crystalline high molecular weight polylactides, with or without hydroxyapatite, until their complete degradation has been demonstrated.

SAMENVATTING

Hydroxyapatiet/polylactide composieten zijn, met behulp van *in vitro* en *in vivo* testen, onderzocht op hun biologische geschiktheid om binnen de reconstructieve chirurgie van het hoofd/hals gebied gebruikt te kunnen worden. Van hydroxyapatiet is bekend dat het een goede biocompatibiliteit bezit en aan bot bindt. De biocompatibiliteit van polylactide is nog onderhevig aan twijfel omdat de resultaten van verschillende studies niet overeenkomen, alhoewel polylactide reeds succesvol wordt toegepast binnen verschillende medische disciplines.

Er zijn vier polylactiden met verschillende degradatie snelheden, poly-L-lactiden 100, 240, 500 kDa en een poly-DL-lactide 400 kDa, *in vitro* getest met behulp van vijf verschillende celtypen. De proliferatie van de cellen is onderzocht door deze op polylactide films te kweken en in medium dat de degradatie produkten bevatte van kunstmatig verouderde films. Twee van de vijf celtypen zijn ook in medium gekweekt dat verschillende concentraties van L- of D-monomeer bevatte. Deze monomeren stelden de uiteindelijke degradatie produkten van polylactiden voor. De resultaten beschreven in de hoofdstukken II en III, toonden aan dat de morfologie van de cellen niet was aangetast door het kweken op de polylactide films; er bleven echter wel bepaalde stukken van de films onbedekt. De morfologie werd ook niet aangetast door de cellen te kweken met media die de degradatie produkten van de kunstmatig verouderde polylactiden bevatten. De proliferatie van de cellen vertoonde soms een afwijking ten opzichte van de controle, maar hierin kon geen consistente lijn worden ontdekt, waardoor een bepaald polylactide mindere biocompatibele eigenschappen zou bezitten. Het experiment waarin de cellen gekweekt werden met verschillende concentraties L- en D-monomeer toonde aan dat hoge (1%) concentraties een duidelijke afname in de proliferatie veroorzaakten en een verandering in de morfologie. De fibroblasten waren veel groter en de neusseptumepitheelcellen vertoonden een toename in de terminale differentiatie voor de D-monomeer, zelfs wanneer de osmolariteit van het betreffende medium was bijgesteld. Diepgaander vervolgonderzoek was echter nodig om hierover definitieve conclusies te trekken. De resultaten van dit vervolg onderzoek staan beschreven in hoofdstuk IV.

Zowel de L- als de D- monomeer zijn hiertoe in concentraties van 0.01% en 0.5% aan kweek medium toegevoegd. De 0.5% monomeren zijn ook toegevoegd aan een gemodificeerd medium met initieel een lagere osmolariteit. Evaluatie van de proliferatie,

morfologie en cellulaire activiteit van de fibroblasten toonde aan dat ze niet werden aangetast door 0.01% melkzuur monomeer, maar dat toevoeging van 0.5% monomeer leidde tot een reductie in de metabolische activiteit van ongeveer 40% vergeleken met de controle, ongeacht de osmolariteit van het medium. De proliferatie en morfologie werden alleen aangetast wanneer 0.5% monomeer was toegevoegd aan normaal medium, waarvan de osmolariteit dus verhoogd was. De toevoeging van sucrose aan normaal medium (wat eveneens tot een hogere osmolariteit leidde) zorgde voor een toegenomen proliferatie en zure fosfatase activiteit alsmede een veranderde morfologie.
De waarde van deze bevindingen voor een *in vivo* situatie kan helaas niet gegeven worden, mede omdat zulke concentraties zullen waarschijnlijk niet *in vivo* voorkomen. Op basis van deze studie kan wel de conclusie worden getrokken dat zowel de osmolariteit als de aard en concentratie van het toegevoegde materiaal een invloed kunnen uitoefenen op de proliferatie, morfologie en cellulaire activiteit. De osmolariteit van testmedia moet dus worden gestandaardiseerd voor het gebruik in *in vitro* biocompatibiliteitsassays.

Alle *in vitro* experimenten hebben de goede biocompatibiliteit van de onderzochte polylactiden aangetoond. *In vitro* experimenten missen echter de complexiteit van *in vivo* reacties. Daarom is de ontstekingsreactie van de polylactiden ook bepaald door polylactidepartikels in de buikholte van proefdieren te injecteren zoals beschreven in hoofdstuk V.

Vier typen polylactidepartikels, poly-L-lactide 100, 250, 550 kDa en een poly-DL-lactide 400 kDa zijn in de buikholte van muizen geïnjecteerd. De inflammatoire respons bestond uit een toename van het aantal cellen (voornamelijk neutrofiele granulocyten) tot 48 uur na injectie waarna het celaantal afnam tot onder het niveau van de controle (fosfaat gebufferd fysiologisch zout). Alle vier de polylactidepartikeltjes gaven aanleiding tot aggregaatvorming samen met ontstekingscellen. Deze aggregaten bleven gedurende de hele onderzoek periode van 6 maanden aanwezig. Kwantitatieve resultaten hebben aangetoond dat standaardisatie van de partikelvorm en -grootte essentieel is om betrouwbare metingen aan bv. degradatie te kunnen uitvoeren. Op basis van de resultaten uit deze studie en andere experimenten waarbij calcium fosfaten en asbest werden geïnjecteerd in de buikholte, kan de conclusie worden getrokken dat de inflammatoire respons zoals deze is waargenomen in de buikholte, gerelateerd is aan het type materiaal (polylactide geeft een veel (5-voudig) hogere reactie dan calcium fosfaten)

en mogelijk ook aan vorm en grootte van de individuele partikels.

Zowel de *in vitro* experimenten als het *in vivo* experiment hebben aangetoond dat de polylactiden beschouwd kunnen worden als kandidaatcomponenten van het hydroxyapatiet/polylactide composiet. Alvorens deze *in vivo* te testen is de botvorming *in vitro* onderzocht (hoofdstuk VI). Hiertoe zijn poly-L-lactide substraten gecoat met verschillende hoeveelheden hydroxyapatiet (0%, 15%, 36%, en 100%) m.b.v. de plasma spray techniek. Hierop zijn beenmergcellen van een rat gedurende een, twee, vier, en acht weken gekweekt, waarna de cellulaire respons en de vorming van een extracellulaire matrix werden bestudeerd op zowel licht- als elektronen microscopisch niveau. Het ontstaan van meerdere cellagen werd waargenomen op de 0%, 36% en 100% coatings, maar niet op de 15% coating. Bovendien was bij de 15% coating een matige vorming van een extracellulaire matrix te zien in tegenstelling tot de andere coatings waar de extracellulaire matrix rijkelijk gevormd was. Met behulp van scanning- en transmissie elektronen microscopie kon een mineralisatie van de extracellulaire matrix na respectievelijk twee en vier weken worden aangetoond op de 100% en 36% coatings en na acht weken op de 15% coating. Er werd geen mineralisatie waargenomen op het ongecoate poly-L-lactide. Aan de interface van hydroxyapatiet en de gemineraliseerde extracellulaire matrix werden vaak een of meerdere elektronen dichte lagen waargenomen, zoals deze ook *in vivo* kunnen worden waargenomen. De resultaten van deze studie tonen aan dat, in het gebruikte model, hydroxyapatiet nodig is om een gemineraliseerde matrix op poly-L-lactide te bewerkstelligen.

In hoofdstuk VII is vervolgens de weefselreactie op vier verschillende hydroxyapatiet/polylactide (50% w/w) composieten na implantatie beschreven. De gebruikte polylactiden waren poly-L-lactiden van 100, 250 en 500 kDa en een poly-DL-lactide van 400 kDa. De hydroxyapatietpartikeltjes zijn vlak voor het polymerisatieproces toegevoegd waardoor uiteindelijk composieten zijn verkregen met afwisselend gebieden van hydroxyapatiet en polylactide. Deze composieten zijn geimplanteerd in de tibias van ratten en onderzocht na een, twee, vier, twaalf, vierentwintig en vijftig weken. Histologische evaluatie van deze tibias toonde aan dat het botcontact van de composieten met poly-L-lactide verschilde van dat van composieten met poly-DL-lactide. Een nauw contact tussen hydroxyapatiet partikels en bot had zich gevormd tijdens de degradatie van het hydroxyapatiet/poly-DL-lactide composiet. De vorm van het HA/P-DL-LA composiet was daarbij duidelijk veranderd, dit in tegenstelling tot de vorm van de HA/P-L-LA

composieten die onveranderd bleef, zelfs na twaalf maanden implantatie. Het botcontact dat voor deze composieten werd waargenomen beperkte zich tot kleine gebiedjes gelegen tegen zowel polylactide als tegen hydroxyapatiet. De polylactiden met en zonder hydroxyapatiet zijn ook subcutaan geimplanteerd. Deze implantaten werden ingekapseld in fibreus weefsel met voornamelijk meerkernige cellen aan de interface. Degradatie van de poly-L-lactiden (met of zonder hydroxyapatiet) werd niet waargenomen, maar degradatie van poly-Dl-lactide (met of zonder hydroxyapatiet) vertoonde ook op deze implantatieplaats duidelijk tekenen van degradatie. Naast het sporadische botcontact werd de voornaamste weefselreactie van zowel in de tibia als subcutaan geimplanteerde composieten gevormd door een bindweefselinkapseling die grotendeels bestond uit fibroblasten en meerkernige cellen.

Het composiet van hydroxyapatiet en poly-DL-lactide heeft enige bot bindende eigenschappen en is waarschijnlijk degradeerbaar zonder dat er kristallijne restanten achterblijven in het weefsel. De hoeveelheid van 50 gewichts procenten hydroxyapatiet die initieel in het composiet aanwezig was, bleek in deze studie te weinig om snel (binnen 6 weken) een botbinding te bewerkstelligen. Verder onderzoek naar de verbetering van de botbindende en mechanische eigenschappen in de toepassingsgebieden is gerechtvaardigd en noodzakelijk alvorens klinische toepassing kan plaatsvinden. De toepassing van hoogmoleculaire kristallijne polylactiden, met of zonder hydroxyapatiet, moet met enige scepsis beschouwd worden zolang de volledige degradatie nog niet is aangetoond.

Curriculum vitae

Alice van Sliedregt was born on March 25th 1962 in Voorburg. After graduating (V.W.O.) from high school (Erasmuscollege Zoetermeer) in 1980, she worked for 18 months in the University Hospital Leiden as a student nurse. In 1982 she started studying Biology at the University of Leiden and graduated in 1988 with a specialization in Medical Biology. During her studies, she performed practical work (12 months) at the Institute for Experimental Gerontology (IVEG-TNO, Rijswijk). At the Biology Department of the University of Leiden she performed practical work (6 months) on the purification of proteins for monoclonal antibody production. In 1988 she started as a Ph.D.student under the combined supervision of the Laboratory for Otobiology and Biocompatibility (Head: Dr.C.A. van Blitterswijk), the ENT Department (Head: Prof.Dr. J.J. Grote) and the Biomaterials Department (Head: Prof.Dr. K. de Groot), all of which are participants of the Leiden Biomaterials Research Group. She worked on the biocompatibility of hydroxyapatite/polylactide composites.

Yvonne Bovell is greatly acknowledged for english text corrections of the Introduction and Discussion.

James W. Hackett, The Zen Haiku and Other Zen Poems of J.W. Hacket, Tokyo: Japan Publications, Inc., 1983

STELLINGEN

1 De osmolariteit van kweekmedia die extracten van biomaterialen bevatten dient te worden vermeld in publikaties.

dit proefschrift

2 Het gebruik van meerdere celtypen teneinde de cytotoxiciteit van een materiaal te testen leidt tot onnodige kostenverhoging.

dit proefschrift

3 Kwantitatieve evaluatie van *in vitro* cytotoxiciteit testen dient te worden aangevuld met kwalitatieve evaluatie.

dit proefschrift

4 De toevoeging van 50 gewichtsprocenten hydroxyapatiet aan polylactide is, althans initiëel, onvoldoende om van een botbindend materiaal te spreken.

dit proefschrift

5 De relevantie van *in vitro* cytotoxiciteit testen kan niet worden ontleend aan de hypothese dat *in vitro* toxiciteit een afspiegeling is van *in vivo* toxiciteit.

6 In het geval van fractuurfixatie zijn langzaam degraderende poly-L-lactiden een alternatief voor patiënten die overgevoelig zijn voor metaalsubstraten, zelfs als het polymeer na enige tijd verwijderd moet worden.

7 Het achteraf zoeken naar de statistische test die het gezochte resultaat bevestigt doet afbreuk aan de waarde van de statistiek en aan de waarde van het gezonde verstand.

8 De localisatie van 3-methylcholanthreen induceerbare P450-isoenzymen in de rattelever, wordt bepaald door de dosis van de stimulator.

A. van Sliedregt and CFA van Bezooyen, Biochem. Pharmac. 39(11): 1703-1708, 1990

9 Vliegvakanties ten behoeve van de eco-toerist vragen om een zodanige milieu heffing dat slechts weinigen die reis kunnen maken.

10 Gezien de vrije keuze in het (wel) hebben van kinderen is een equivalent van het ouderschapsverlof voor kinderlozen gerechtvaardigd.

Stellingen behorende bij het proefschrift:

Hydroxyapatite/polylactide composites
for reconstructive surgery
An *in vitro* and *in vivo* biocompatibility study

Alice van Sliedregt
Leiden, 24 maart 1993