Pursuing the smaller

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INTRODUCTION: Background and origins.
Pursuit of different landscapes at different scales to discover the routes to explain how the body is built. A fortunate period during which techniques and concepts for investigating structure have improved year by year. My fortunate encounter with Michael Abercrombie and his views on the social behaviour of cells, aims for quantitation, and statistical testing. Kruyt and Vasicek's books. Varied environments – Geology, Biophysics Ph.D. in a Genetics Department, Anatomy via Zoology to Cell Biology. Immediate involvement in cell adhesion. Cell movement and cell trapping. Reaggregation phenomena, Eric Lucey and time-lapse filming An early start from the physico-chemical viewpoint. Contact inhibition of movement.

METHODS: Cell culture, Quantitative measurements of cell adhesion. Kinetics. IRM and related optical interference techniques. Micro- and nanofabrication. AFM
Polscope and photoelastic measurements.

RESULTS: Mapping the adhesion. Trying to get answers to three problems, how close do cells get.? Early data from IRM and then TIRF and then FREnergy. And the time scale – very short indeed in suspension. Differences from data re cell adhesion molecule. DLVO theory. How strong ? Why it rains. Contrast with cell adhesion molecule studies. Problems with cell adhesion molecules.

Contact guidance and Paul Weiss; explanation.
Tests with grating replicas. An area waiting for microfabrication. Force explanations, influence of Albert Harris. Nature paper with ~Mustafa Seehar. Taking microfabrication results what is best explanation. Tissue engineering ' tendon Protein adsorption explanation defects. Recent photoelastic measurements. Polscope.

Onto nanostructures. Looking at dimensions and sensing of this Why order may be important.
Collagen. How small a feature. Fortunate association with the engineers. A newish problem. Liquid crystals help, Self-assembly/

DISCUSSION & CONCLUSIONS:

The results will be discussed in terms of very recent theories of cell interaction and cell signals.

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Technologies for studying cell motility and behaviour.

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INTRODUCTION: At the Randall Centre we have developed two methods of live cell imaging for analysing cell behaviour and for studying molecular dynamics within cells. DRIMAPS (Digitally Recorded Interference Microscopy with Automatic Phase Shifting) produces maps of the distribution of non-aqueous cellular material (dry mass) within living cells. It allows us to correlate changes in distribution of non-specific material with cell locomotion and cell behaviour can be analysed automatically. FLAP (Fluorescence Localisation after Photobleaching) permits the tracking of a fluorophore after localised photobleaching by using another fluorophore as a colocalized reference. In effect, it is a new method of photoactivation with the advantage that the distribution of the non-activated molecules can also be followed. We have used it to follow both the fast relocation dynamics of monomeric (globular) G-actin and the much slower dynamics of filamentous F-actin simultaneously in transformed rat fibroblasts. We found that actin was delivered to protruding zones of the leading edge at speeds exceeding five micrometers per second. Monte-Carlo modelling confirmed that this flow cannot be explained by diffusion and may involve active transport.

Mechanosensory Function of Focal Adhesions

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Focal adhesions (FAs) are dynamic molecular complexes associated with integrin- family transmembrane receptors, which connect the actin cytoskeleton with the extracellular matrix (Fig. 1). FA assembly is induced by tension either applied to these structures externally, or resulting from myosin II-driven cell contractility [1]. Thus, FAs function as mechanosensors, "reporting" to the cell information about the physical properties of the surrounding environment [2,3]. Rho, a principal molecular switch triggering FA formation, operates by activating Rho associated kinase (ROCK) and the formin homology protein, mDia1 [3, 4]. ROCK is required for the activation of the myosin II-driven contractility, and its function can be bypassed by externally applied force. Under these conditions, mDia1 remains still necessary for FA assembly [1]. mDia1 nucleates actin filaments, and affects microtubule dynamics at both the plus and minus ends [4], which facilitate microtubule targeting toward FAs (Fig. 2). Since microtubules interfere with myosin II-driven contractility, this may create a negative feedback loop controlling FA growth [4].

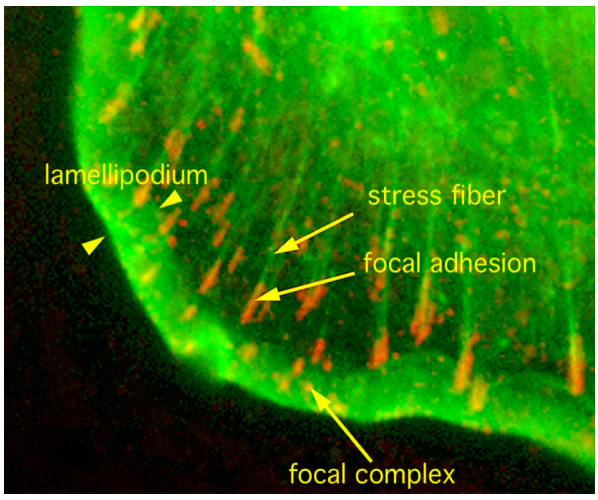

Fig. 1: Association of focal adhesions with the actin cytoskeleton. A portion of the leading lamella of a T47D cell stimulated by neuregulin is shown. Actin is visualized by phalloidin staining (green), and adhesion structures –are shown using anti-phosphotyrosine antibodies (red). The margins of the lamellipodium contain a dense actin network, marked by arrowheads. This is the site at which nascent matrix adhesions (focal complexes) are formed.

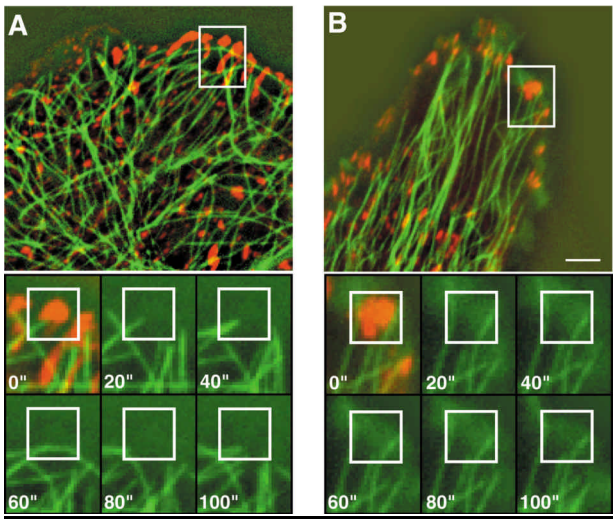

Fig. 2: mDia1 alters the mode of microtubule interactions with focal adhesions. B16F1 cells expressing tubulin-GFP (green) were super-transfected with a marker for focal adhesions (RFP-zyxin) (red) alone (A) or together with constitutively active mDia1 (B). Sequences of microtubule images presented in the lower part of the figure show that microtubules in cells expressing activated mDia1 remain in the proximity of focal adhesions for longer times than the highly dynamic microtubules in control cells

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ACKNOWLEDGEMENTS: Alexander Bershadsky holds the Joseph Moss Chair of Biomedical Research. The work of the laboratory was supported in part by grants from the Israel Science Foundation, Minerva Foundation (Germany), and the US-Israel Binational Science Foundation. We also acknowledge support from the Yad Abraham Center for Cancer Diagnostics and Therapy.

Clinical applications of surfaces: Keep it simple

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INTRODUCTION: Studying cell behaviour, morphology and adhesion on potential implant surfaces describes the surface cytocompatibility and gives a first indication as to the suitability for fracture repair applications. With long term or permanent orthopaedic implants osseointegration is vital to their success. Early soft tissue integration without liquid filled capsule formation is also important for internal fixation plates. Good vascularisation is imperative at the implant / tissue interface, especially for prevention of infection. In certain cases such as distal radius fractures where tendons have to glide over internal fixation plates, or in the cranio-maxillofacial area in orbital fractures tissue adhesion is undesirable. Bacterial adhesion to internal fixation implants is always undesirable. Microtopography is one of the fundamental factors controlling cell and bacterial adhesion and can be used simply to control this.

In this conference the participant will see a wide range of methods to improve cell reactions to surfaces *in vitro*, with some of the surfaces being tested *in vivo*. The participant will also see ways of reducing bacterial adhesion at biomaterial surfaces *in vitro*. There are several beautiful developments within this area that function well in static, non-loaded *in vitro* conditions. Not all will survive the severe test of *in vivo* situations where mechanical abrasion occurs during implantation and the time of use of the implant and the materials lie within the highly corrosive milieu within the body.

The first aim of this talk will be to show some simple examples of controlling tissue adhesion to surfaces which work both *in vitro* and are able to withstand the harsh *in vivo* conditions. Another aim of the talk will be to introduce the laboratory scientist to real examples of what happens to internal fracture fixation devices during surgical implantation.

The basic applied problem, still in 2004 eludes numerous scientists (as observed at the 7th World Biomaterials Congress in Sydney). Biomaterials are developed to help solve clinical problems. Without knowing the clinical problem how can one test a biomaterial for it's biocompatibility? One should look at the clinical problem first (the actual medical problem, repair or restoration method including surgical technique). One should then look at what material is available that may be able to be used to help this situation both biologically and mechanically, or possibly develop such a material. The material should be tested both *in vitro* (biologically and mechanically modelling the situation in which it will be used) considering it's strength for what it is to be used, and surface design. After *in vitro* biological and mechanical tests the implant design for the *in vivo* model should be considered and *in vivo* tests carried out. Many scientists are separated from the clinical problem and have a favourite material that they test *in vitro* and sometimes *in vivo* with no real idea what the clinical problem is and after several random tests that fit the current trend, look for a use it for 'their' material.

One must remember that in for example the case of orthopaedics a surface must be able to withstand the following:

- Sterilisation
- Storage time within the sterile packing material.
- Abrasion from the handling within the sterile packing material during transport etc.
- The surgeon's hands, most internal fracture fixation devices require large amounts of manipulation to the patient's anatomy.
- The highly corrosive milieu within the patient's body.
- Abrasive muscle, tendon or ligament movements upon the implant surface. Fretting particles produced should not be inflammatory.
- Time within the body to achieve the desired regeneration / repair.
- If the surface is a coating it should not delaminate from the bulk material and should not cause corrosion to the bulk material if it is to resorb.

There are many points to consider when designing a surface to help solve a clinical problem, but simple ideas usually are the most robust to the various stages of surgery and the healing process.

Significance of surface stresses for cell adhesion to NiTi

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INTRODUCTION: Many of cell functions are sensitive to the intracellular stress fields [1]. These fields arise from a complex balance of forces exerted on the adhesive contacts and the forces generated within a cytoskeleton. The dependence of forces exerted on the adhesive contacts upon the flexibility of extracellular matrix (ECM) [2] points at the important role of material surface stresses in this phenomenon. Our recent study [3] demonstrated straight quantitative correspondence between cell adhesion parameters and values of surface stresses. However, the variations in surface stresses in that study were accompanied by simultaneous changes in surface roughness. The absence of any regular relationship between the parameters of cell adhesion and surface roughness did not permit to elucidate the role of surface stresses in cell adhesion independent of surface topography. The aim of present study was to clarify the correspondence between cell adhesion parameters and surface stresses independent of the effect of surface topography.

METHODS: Material tested was hot-rolled binary NiTi shape memory alloy (Ti-44,3wt%, Ni-55,7wt%). Samples for biological tests – 6 mm-in-diameter and 5 mm-in-height disks – were simultaneously ground to avoid differences in surface topography. To elucidate the effect of surface stresses on parameters of cell adhesion the half of disks ground were annealed in vacuum ($P=10^{-4}$ mmHg) at 250°C during 1 hour. ROS-17/2.8 osteoblastic cells were used for biological studies. The procedure of biological tests and the description of methods for the measurement of surface roughness R_a and surface stress s are given in [3].

RESULTS: Results of the experiments are shown in Table 1 and Fig. 1.

Table 1. Mean ± SD of roughness R_a and surface stress s after grinding and grinding+ annealing of NiTi samples (N = 6 per group).

Surface treatment	R_a, μm	s, MPa
Grinding	0,156±0,021	183±9
Grinding+Annealing	0,155±0,020	158±6

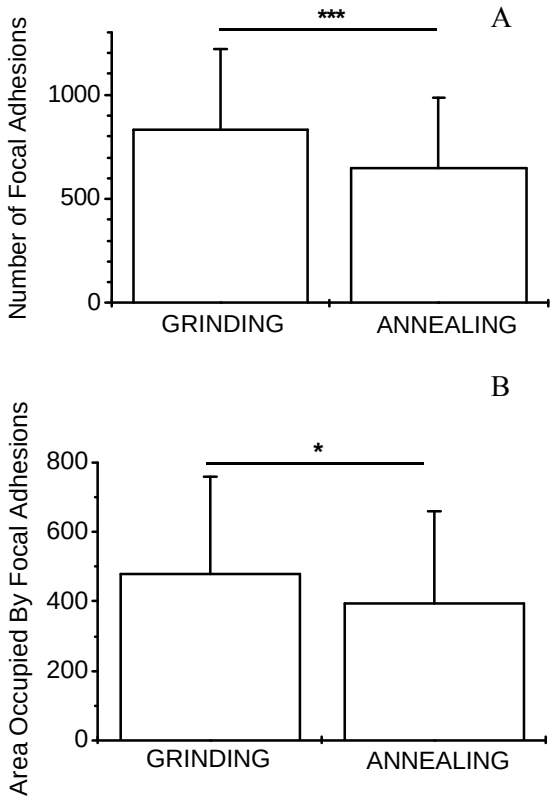

Fig. 1: Number of focal adhesions (A) and area occupied by focal adhesions (B) on NiTi after grinding and grinding+ annealing.

DISCUSSION & CONCLUSIONS: The present study confirmed the priority of stresses over roughness for cell adhesion. It also demonstrated the irregular character of correspondence between these surface characteristics in NiTi. Most probably the effect of surface stresses on cell adhesion is performed via the regulation of ECM flexibility.

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Relation of Albumin Concentration and Its Adsorbed Overlayer Thickness on Commercially Pure Titanium Using X-ray Photoelectron Spectroscopy

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Introduction: Commercially pure titanium (CP Ti) has been widely used as an implanted metal in bone surgery over many years due to its excellent biocompatibility and high corrosion resistance [1]. Protein adsorption from surrounding bone tissue is the first interfacial phenomenon of the host-biomaterials interactions therefore an implanted metal might be integrated or rejected. There are many proteins in human tissues which have different molecular weights and different degrees of adsorption on metal surfaces. In fact, albumin is the most common protein in blood and it is the first protein that covers and strongly binds to a titanium surface [2,3]. The aim of this study is to investigate the relationship of albumin concentration and the adsorbed overlayer thickness using X-ray Photoelectron Spectroscopy (XPS) on CP Ti.

Materials: CP Ti Grade 2 (As per JIS H-4600) was polished to a mirror-like finish, using silicon carbine abrasive paper down to grit 4000 specification and followed by using 1 and 0.3µm alumina. After polishing, the sample was rinsed with acetone. The study of protein adsorption was performed by mixing albumin solution with deionised water at concentrations varied from 0.000001%(v/v) to 10%(v/v). The samples were immersed in 100 ml of the albumin solution for 30 mins at room temperature, rinsed with deionised water and dried with hot air blower. The adsorption overlayer thickness was determined using the height of Ti2p XPS peak (Thermo VG Scientific Sigma-Probe Spectrometer). The adsorbed amount of albumin was determined in terms of atomic % nitrogen at a 37° take off angle.

Results: The atomic % nitrogen and the adsorbed overlayer thickness on the treated samples as a function of albumin concentrations are shown in Fig1. The amount of adsorbed albumin and the adsorbed overlayer thickness increased with increasing albumin concentrations. The albumin thickness on CP Ti was found to increase rapidly with albumin concentration from 0.000001% to 0.001%, and at 0.01% (s) it reached the saturation. At 0.0001% (m) the adsorbed overlayer was presumably in the form of monolayer.

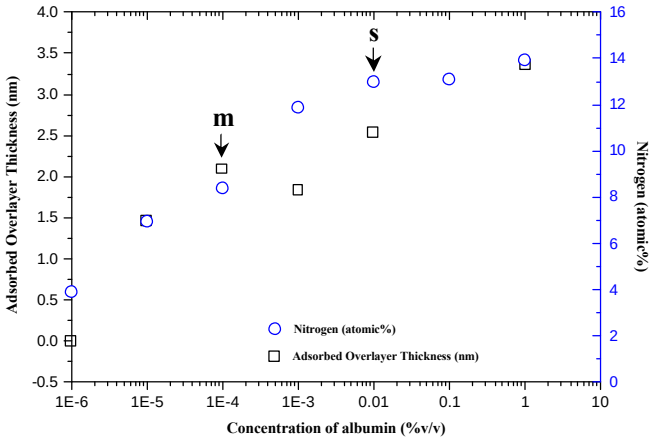

Fig1: Adsorbed overlayer thickness and Nitrogen (atomic%) on CP when Ti was immersed in various albumin concentrations.

Discussion & Conclusions: Characterisation of CP Ti using XPS indicated that albumin adsorption and its thickness were dependent on protein concentrations. At 0.01% the protein adsorption attained the saturation. Furthermore, at 0.0001% the adsorbed overlayer thickness was about 2 nm which seemed to be a monolayer of adsorbed albumin [4].

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Statistical study of attachment, adhesion and proliferation of human osteoblasts on metallic materials

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INTRODUCTION: To our knowledge, no study until now concerned the statistical analysis of the relation existing between cell attachment (or short-term adhesion), long-term adhesion and proliferation on substrates and of the effects of material composition, surface chemistry or surface topography on these cellular parameters. With this objective, we developed during the last two years an extensive study based on 30 different metallic substrates presenting 3 different compositions, 2 surface chemistries, 6 surface morphologies and 2 roughness amplitudes.

METHODS: Ti6Al4V alloy, pure titanium or stainless steel samples were processed using polishing, machine-tooling, acid-etching, electro-erosion, or sandblasting under various conditions to obtain two roughness amplitudes (Sa=0.85 or 2.35 μm). Some samples were coated by a sputtered layer of Au-Pd. Samples were inoculated with human osteoblasts and after 24 hours, 7, 14 and 21 days, the cells were enzymatically detached from the samples as previously described [1]. Each experiment was reproduced 9-fold on each surface giving 374 experiments and more than 2,000 data. The number of released cells allowed to calculate the attachment of cells after 24 hours (Initial number of cells: INC), their proliferation after 21 days (Final number of cells: FNC) and their Adhesion Power (AP) quantifying the long-term adhesion all along the time of culture and representative of the quality and evolution of interfacial properties responsible for adhesion. Next, we did proceed to two different statistical analysis: (a) the statistical correlation of INC/FNC, of INC/AP and AP/FNC on all the substrates using a bootstrap protocol and (b) the study of the effects of the material composition (material), the surface chemistry (coating), the process used to produce the surface topography (process) and the roughness amplitude (roughness) on human osteoblast behavior using multiple analysis of variance (ANOVA).

RESULTS: No statistical correlation did exist between INC and AP. On the contrary, INC was positively correlated with FNC on 26 types of substrates on 30 and was statistically asserted on 16 types of substrates on 26. AP was positively correlated with FNC on 23 types of substrates on 30, and was statistically asserted on 13 types of substrates on 23. The ANOVA demonstrated that the effects on INC, AP and FNC of "material" and "roughness" were not significant. Only "process" had a significant effect on the 3 parameters measured. Sometimes, a very high negative interaction did exist between "roughness" and "process". The "coating" influenced negatively INC and FNC.

DISCUSSION & CONCLUSIONS: This is the first statistical demonstration of the existence of a positive correlation between proliferation and short-term adhesion on one hand and long-term adhesion on the other hand. Hence, it appears that to further proliferate, cells need to adhere at short-term and to be able at longer times to elaborate a complex tissue-like cell/matrix/substrate interface. But, surprisingly, short- and long-term adhesion are not correlated. This difference could be related to the different phenomenon basing short- and long-term adhesion, on one hand physical-chemical attachment, integrin-receptor interactions, cell spreading, and on the other hand cell re-organization, migration, proliferation and 3D extracellular matrix elaboration. More, this demonstrates that short-term adhesion studies can not presume of the long-term adhesion of cells on the substrates i.e. of the elaboration of a complex tissue-like cell/matrix/substrate interface.
The ANOVA demonstrated the relative major effect of roughness versus chemistry for long-term adhesion and proliferation whereas the inverse was observed for short-term adhesion. Again, this could be related to the passive physico-chemical phenomenon occurring during the first attachment phase and logically more influenced by the surface chemistry than by the surface topography, this last having more influence on the further spreading, long-term adhesion and proliferation phases.

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Adhesion of eukaryotic cells as affected by nano-structured surfaces produced by plasma processes.

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INTRODUCTION: Different kind of polymeric surfaces can be modified by means of RF (13,56MHz) plasma enhanced chemical vapour depositions (PE-CVDs) to drastically change their attitude to promote or discourage cell-material interactions.
In this work glow discharges fed by F-containing gases (i.e. C_2F_4) were realized to deposit Teflon-like coatings with nano-features randomly distributed on the processed substrate [**1, 2**]. Different kind of eukariotic cells were cultured on samples with different density of nanostructures in order to study how the adhesion and growth of cell lines is influenced by chemical and topographic parameters.

METHODS: Glow discharges fed by C_2F_4 were realised to deposit Teflon-like coatings on PET substrates of 14mm diameters in three different experimental conditions. The utilized parameters were 6 sccm C_2F_4; 200 mTorr and100 W. Two different nanostructured surfaces were obtained by the use of modulated discharges (5%D.C.) for two different periods of time (50' and 90'). Flat fluorinated samples were obtained using a continuous discharge for 5' The three kind of samples were coated by the plasma deposition of thin homogeneous fluorinated films in order to obtain flat and nanostructured samples with the same surface chemistry. Different eukariotyc cell lines (3T3, MG63) were seeded on the nanostructured and flat samples for different time of cell culture.
RESULTS: Adhesion and morphology of cell lines representing different cell types (fibroblasts and osteoblasts) appeared to be influenced both by chemistry and topography of the modified polymers. In particular after 96 hours of cell culture the area covered by 3T3 fibroblasts adhered on nanostructured samples is much higher than the one covered by cells on flat fluorinated samples.

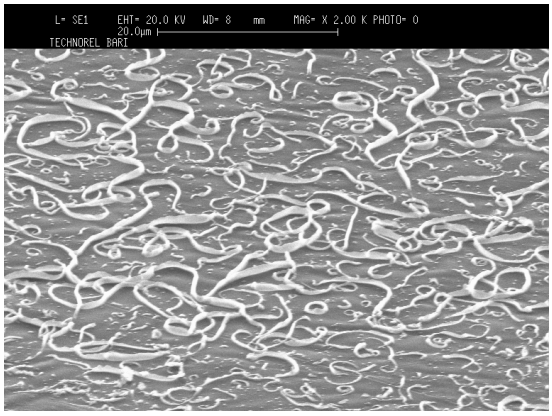

Fig. 1: Fluorinated nanostructured surfaces

DISCUSSION & CONCLUSIONS:
The used cell lines adhered in different way to nanostructured and flat samples. The fluorinated surfaces, that normally do not permit a good cell adhesion allow a better adhesion and growth of cells when is randomly nanostructured.
The surface of the so produced fluorinated nanofeatures can be changed by the plasma deposition of thin film with different chemistry in order to observe the behaviour of cells both to different chemistry and topography.

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Blood compatibility to ordered arrays of nano pits

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INTRODUCTION: It is widely accepted that surfaces presenting a nanotopography can have a profound effect on cells. In particular, highly ordered arrays of nano pits have previously shown a large reduction in the adhesion of epitenon cells [1] and fibroblasts [2]. In this study we have investigated the blood compatibility to said nanostructured surfaces. Both platelets and mononuclear cells have been investigated by image analysis. We found an increase in adhesion of both types of cells which is in sharp contrast to tissue cells.

METHODS: The nanotructured surfaces were prepared by high-resolution electron beam lithography. The generated patterns were electroplated to form a nickel shim which was used for hot embossing in poly(caprolactone) [3]. The nano pits were 35, 75 and 120 nm in diameter in an orthogonal arrangement with a centre-to-centre spacing of 100, 200 and 300 nm, respectively. We also prepared surfaces with a hexagonal symmetry with a pit diameter of 120 nm and a centre-to-centre spacing of 300 nm [4]. See Figure 1.

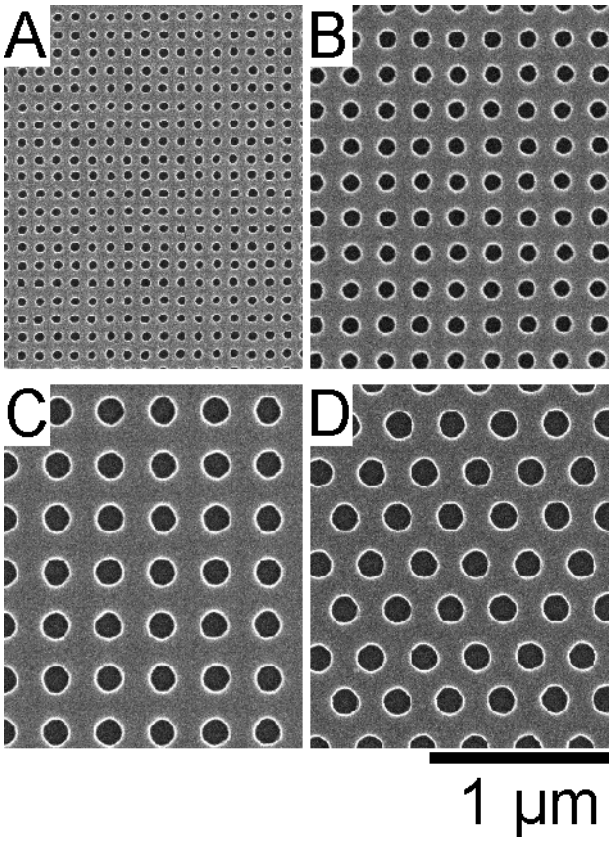

Fig. 1: SEM micrographs of the fabricated surface patterns. Ordered arrays of nano pits with (A) 35 nm, (B) 75 nm, (C+D) 120 nm diameter. The larger pits were made in different geometries (C) orthogonal and (D) hexagonal.

RESULTS: Platelets and mononuclear cells were extracted from whole blood drawn from healthy human donors. After cells were incubated on embossed substrates the samples were fixed, immuno-histo-chemically stained and optical light micrographs were acquired and analysed. We have used image analysis as a method to investigate the cell response in terms of adhesion and activation. We found that both platelets and mononuclear cells have a higher adhesion to the nano patterned surfaces as compared to a flat control. For platelets we also observed a decrease in activation for the nano pits. However, we found no difference between orthogonal and hexagonal arrays of nano pits.

DISCUSSION & CONCLUSIONS: We have found that blood cells exhibit an increased adhesion to ordered arrays of nano pits. This is in contrast to previous studies with tissue cells which have a reduced adhesion to such topographies. The increased adhesion of both cell types and decrease in number of activated platelets suggest a good biocompatibility of such nanotopographies.

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Influence of nanostructured collagen layers on endothelial cells behaviour

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INTRODUCTION: Controlling cell-material interactions is a key issue in biomaterials science and tissue engineering. The aim of this study is to examine how the nanostructure of protein layers adsorbed on a material may affect the cell behaviour.

METHODS: Type I collagen was adsorbed on polystyrene (PS) and plasma-oxidised PS (PSox) in different conditions (concentration, time) ensuring the formation of continuous layers, either smooth or presenting aggregated collagen structures (fibrils). Moreover, appropriate drying conditions allowed discontinuous collagen layers, presenting dendritic or net-like nanopatterns, to be obtained. The collagen layers were characterised using atomic force microscopy, X-ray photoelectron spectroscopy, and radiolabeling. Human umbilical vein endothelial cells (HUVEC) were grown for 4 h on these substrates, in absence or presence of serum. They were fixed, stained and observed by light microscopy. The cells were counted and their area and circularity index were calculated. In some cases, the cytoskeleton was also stained using immunolabels.

RESULTS & DISCUSSION: On the bare substrates, the number of adherent cells was high on both PS and PSox in absence of serum. If serum was added, no adhesion was observed on PS while adhesion and cell spreading were high on PSox. This is explained by the competition for adsorption, which turns in favour of adhesive proteins secreted by the cells on the hydrophilic PSox but which turns in favour of serum proteins (i.e. mainly albumin) on the hydrophobic PS.

After adsorbing a smooth collagen layer on the PS substrate, cell adhesion became high in presence of serum, and the cells adopted a needle-like shape. In these conditions, collagen thus enhances cell adhesion. However, when collagen was adsorbed from more concentrated solutions or for longer times, the formation of fibrils in the adsorbed layer provoked a decrease of cell spreading (Fig 1). The last trend was also observed on PSox. The supramolecular structure of the adsorbed collagen layer thus clearly affects the behaviour of HUVEC cells. This may be related to the accessibility of recognition sequences, which could be hidden once collagen forms aggregates.

The spreading of HUVEC cells was also enhanced on discontinuous collagen layers compared to smooth, continuous ones. This could be explained by the alternation of PS and collagen domains at the interface, which could stimulate the organisation of the cell surface receptors, and thereby of the cytoskeleton, or which could allow the coadsorption of proteins secreted by the cells. The collagen assemblies formed in this case are not arising from spontaneous self-assembly, but have been formed by dewetting. As opposed to fibrils formed in the adsorbed phase in contact with the solution, this mode of molecular association is more favourable to cell spreading. This suggests that the recognition sites are more accessible and even that their spatial organisation promotes multi-valency effects.

CONCLUSIONS & PERSPECTIVES: These results show that a better control of cell-material interactions can be achieved through a better control of the nanostructure of adsorbed protein layers. Further work will include longer adhesion experiments, enzymatic assays, as well as antibody assays to investigate the availability of recognition sites on the collagen layers.

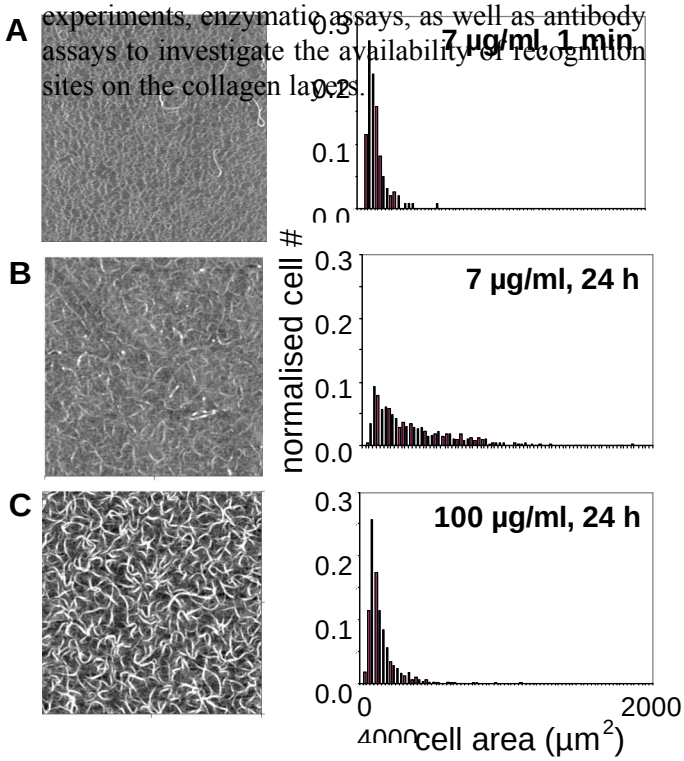

Figure 1: AFM images (5×5 µm², z = 10 nm; left) of collagen layers obtained by adsorption from a 7 µg/ml solution for 1 min (A) or 24h (B) or from a 100 µg/ml solution for 24h (C), and area of HUVEC cells adhering to these collagen layers (right).

Effect of standard orthopaedic metal implant surfaces on cell behaviour

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INTRODUCTION: The variance in surface chemistry and topography of orthopaedic materials makes for difficult characterisation and comparison. Their utilisation is widespread, but 'interface' properties relative to soft tissue cells have not been studied extensively. We present an investigation on the cytoskeletal, adhesive, morphological and molecular interaction of human fibroblasts (hTERT, Infinity™ Telomerase-Immortalized) with a set of standard implant materials: (Stainless Steel (SS), Titanium (CpTi) and titanium alloy, Ti-6Al-7Nb (TAN)). The aim was to analyse the materials and to provide extensive in vitro characterisation of human fibroblast cells on these frequently used materials.

METHODS: Atomic Force Microscopy (AFM), Laser Profilometry (LP), Scanning Electron Microscopy (SEM) (secondary and backscattered electron modes) and Contact Angle (CA) were used to characterise the surfaces. hTERT fibroblasts were cultured on implant quality EPSS, CpTi and TAN. Thermanox (polyethylene terephthalate) coverslips (Therm) were included as a control substrate. Cultured cells were fixed for SEM and Light Microscopy (LM) evaluation. The cells fixed for LM were fluorescently triple stained for F-actin and DNA as well as either Tubulin or Vinculin. Cell morphology was assessed qualitatively with SEM and quantitatively by image analysis of fluorescence images. For cDNA microarray studies 50,000 cells were seeded on the materials and cultured for 10 days before RNA was extracted. The transcribed DNA incorporated either Cy3 or Cy5 fluorescent markers and were hybridised on Human 1.7K chips (University Health Network, Toronto). The control RNA was obtained from cells cultured on Therm.

RESULTS: CA and the Roughness Average measurements describe CpTi and TAN as being similar surfaces. CpTi has an RA of 1.15μm and a CA of 80.2° while TAN returned 0.99μm and 85.2°. EPSS was considerably smoother with an RA of 0.21μm and CA of 73.1°. This indicates a positive correlation between surface roughness and contact angle. AFM and SEM demonstrate that CpTi and TAN have significantly different micro-topographies; CpTi having a rugged irregular surface while TAN displays a 'micro-spiked' topography consisting of 'electron dense' particles. EPSS appears smooth and unblemished.

Qualitatively, the cells cultured on both CpTi and TAN were restricted in spreading by the topography. Focal adhesion (FA) size was also limited for cells grown on CpTi and TAN; this is currently being measured quantitatively. The FA of cells cultured on TAN actively avoided the microspiked particles. Cells cultured on EPSS spread freely. Cells were sufficiently spread on EPSS such that we could distinguish 'dot' adhesion sites at the cell periphery, and mature 'dash' adhesions with attached actin cytoskeleton further inside the cell. None of the surfaces visibly disrupted actin filament but tubulin polymerisation was disrupted by TAN. Total RNA amounts extracted from cells cultured for 10 days on CpTi, SS and Therm substrates were between 3-4.5μg while only 0.6μg was extracted from cell cultured on TAN. RNA expression can vary due to the level of cell activation but this low number for TAN was correlated to a low cell count. Microarray studies of cells cultured on CpTi had significant up or down regulation of 213 genes from the 1700 arrayed. SS had significant up-regulations for 16 genes. 6 of these genes were commonly up-regulated in cells cultured on both substrates, in comparison with the Therm control. The most notable change was the upregulation of Matrix Metalloprotinase 7, an enzyme used to remodel extracellular matrix and associated with wound healing.

DISCUSSION & CONCLUSIONS: Difference in surface roughness correlates with differences in cell behaviour. However, CpTi and TAN elicited varying cell reactivity: CpTi topography visibly dictated cell spreading and FA development. Of the six common genes, all were upregulated more on CpTi than EPSS. Cells cultured on TAN had statistically decreased spreading, the FA formation was affected and most notably there was an apparent lack of cell proliferation. EPSS and CpTi indicated topographical variance as the mode for cell reactivity, but TAN indicated that physio-chemical material properties cannot be overlooked. The notable cell variance warrants further investigation into the cell compatibility of these 'standard' materials.

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Micro and Nano Fabrication for Cell Engineering

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INTRODUCTION: Adam Curtis has been interested in the response of cell to surfaces throughout his career. This talk will discuss the construction of exercise grounds for cells and the techniques used beginning with the first structures made for Adam by lithographic techniques in 1982 and going on to the present.

The fabrication facilities in the Department of Electrical Engineering were established in the late 70's to make nano-electronic and opto-electronic devices. Devices for biological purposes brought new challenges. The use of clean air and control over contaminates is similar. But the nature of the contaminates is different and the conditions of final use are very different – as cells like to live in corrosive liquids. However at a deeper level, the disciplines of semiconductor device engineering and cell biology have a great similarity – they are both concerned with surface phenemona (and that surface is usually an oxide).

METHODS: Interestingly the requirements of structures for studies in Cell Biology have always pushed the state of the art in microfabrication. In the 80's the masks for defining a pattern in resist using photolithography were made using photographic reduction. Typical final pattern sizes were a few millimeters square, but to get good statistics on cell behaviour, one needs a square centimeter at least. So we had to improve the narrowness of our initial lines, even inspecting the edge of the cutting tool under a scanning electron microscope. We wanted to make grooves in a bio-degradable polymer so that a sheet of polymer patterned with guiding grooves could be used to make a prosthesis for tendon repair. Such polymers often dissolve the solvents used in lithography and while a route could be found for a particular polymer, the idea of embossing was much more attractive. The die required to stamp into the heated polymer was made using conventional lithography. This an important step forward for it enables the possibility of the use of micro and nano patterning in prosthesis at a reasonable cost.

RESULTS: It was found that some cells respond to very shallow grooves – the obvious question was do they response to very small features. In the early 90's the Department acquired a large area electron beam lithography tool, so small features could be written. In the initial work 100 nm diameter pillars, 100 nm high spaced in a regular array 300 nm apart inhibited the adhesion of fibroblasts. To make this structure over a 1 cm square area requires the formation of 10^9 dots, and each dot could require requires 100 bytes of information to define it. An electron beam machine writes pattern information in a small field (1/4 mm^2) and then the fields are stitched together by moving the stage under interferometric control. Considerable development was required to develop strategies to reduce the time taken by the writing to about 1 hour/cm^2 and to ensure that the alignment of the fields was good enough that cells do not see any un-patterned area (a gap of 150 nm would suffice to cause adhesion). In the specification of a new electron beam machine, we reviewed the needs of the various groups that would use the new machine, and for alignment, the biological needs were the most demanding.

DISCUSSION & CONCLUSIONS: Lithographic techniques produce flat structures. Scaffolds for cells to be implanted in prostheses require 3-dimensional structures. The next challenge is to use the patterning techniques to give cues to the cells to align to each other as *in vivo*, to create a 3-D structure and to provide channels through this scaffold to assist in vascularisation and enervation.

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Investigating active mechanosensing with micro- and nanostructures

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INTRODUCTION:

It is widely accepted that the chemical, topographical and mechanical makeup of the environment is significantly influencing cell responses. Such environmental cues occur in a cells natural environment as well as on untreated- or surfaces specifically designed at the micro- or nanoscale. Cells react to such cues by changes in cell shape, motility, behaviour and gene expression [1]. To investigate how the responses differ between micro- and nanometric topography and mechanically yielding substrates we fabricated a variety of substrates and followed the cellular reaction by timelapse, SEM, fluorescent microscopy and gene array studies.

We hope to present evidence that a major signaling pathway relies on self-inflicted mechanotransduction.

METHODS:

Topographies were fabricated as described earlier [2] using photo-lithography, electron–beam and colloidal lithography to create master substrates with micro- or nanotopography. For regular nanotopographic features this is followed by copying first into a nickel die and then replication into polymer substrates used for experiments. Soft substrates were fabricated according to [3]. Displacement was measured using the method developed by I Toliç-Norrelykke [4] using soft sylgard with embedded fluorescent beads (modified from [5]). Cell culture, fixation and staining of hTert fibroblasts for cytoskeletal elements and chromosome painting as well as the mRNA recovery and the hybridization on a 1718 gene microarray followed procedures published previously [1].

RESULTS:

Substrates of increasing softness allowed cells to spread less, show less stress fibres and less organized microtubules, this is paralleled by cells on nanotopographies which reduced cell spreading and the fibroblasts also had less organised microtubules, microfilaments and intermediate filaments. As a result of these factors, the nuclear morphology was altered in to a more spherical shape, and the positioning of chromosome 3 was altered during interphase. mRNA isolated and tested on a 1718 gene microarray has also highlighted a range of regulatory changes within the cell genome. Given cells a choice of increased mechanical feedback by placing them onto fabricated surface rigidity patterns cells would move from a soft to a hard substrate, and align to such features. Alternatively if the cells were plated onto patterned nanotopographies (patterned on a level >5µm) cells would preferentially spread on the non-nanopatterned parts of the substrate.

DISCUSSION & CONCLUSIONS:

It has been hypothesised that interphase choromosomes have a consistence of position within the nucleus. Our results indicate that by altering the positions of the chromosomes, changes in gene regulation are observed. By using nano-topography to alter the spreading and adhesion of human fibroblasts, and comparing the effects observed to results obtained on flat but mechanically varied substrates we propose that the underlying principle behind the reaction are similar, in that cells try to optimize their effective mechanical feedback according to the hypothesis put forward by Bischofs & Schwarz [6]. These changes directly relate to proliferative and phenotypical responses, and thus may be relevant to the production of materials for tissue engineering.

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Development of biocompatible functionalized superparamagnetic iron oxide nanoparticles for human cancer cell uptake.

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INTRODUCTION: The application of the different forms of iron oxides for radiological diagnostic procedures has gained wide acceptance in radiological practice [1], but therapeutic applications are still under investigation and development. Such applications are exploiting two major advantages of magnetic iron oxides: their low toxicity to human beings [2] and the possibility to exploit their outstanding magnetic properties, potentially allowing using the high magnetization of superparamagnetic iron oxide to target drugs to the tumor area through external static magnetic fields [3]. The use of SPION in drug targeting and delivery requires that these entities are delivered inside cells. Therefore the objectives of our present approach were to develop, characterize and optimize biocompatible functionalized SPION, which could be further derivatized with drugs, and to determine the ability of these particles to achieve cell uptake by human cancer cells.

METHODS: Superparamagnetic PVA coated nanoparticles were prepared by alkaline co-precipitation of ferric and ferrous chlorides in aqueous solution. The obtained particles were mixed at various ratios with different polymer solutions to obtain either SPION coated with polyvinyl alcohol (Mowiol® 3-83), (PVA-SPION) or SPION coated with PAV with attached functional groups (amino-SPION, carboxy-SPION, and thiol-SPION). Melanoma cell lines from human surgical samples (primary or metastatic tumors) were mixed either with PVA, functionalized PVA, PVA-SPION or functionalized PVA-SPION solutions. At the end of the experiment, either the MTT test was performed for the two last hours to determine cell viability or the cellular iron content was quantified

RESULTS: From the 4 functionalized SPION preparations, only the amino-PVA SPION demonstrated the capacity to be taken up by, and were not cytotoxic to, human melanoma cells. This uptake by melanoma cells was dependent on the amino-PVA to iron oxide ratio, was an active and saturable mechanism, and all cells in a culture internalized these SPION. For amino-SPION with a polymer/iron ratio of 13.8 and 18.4 by gradually increasing the pH from 2.1 to 10, the median of the size distribution shifted from 19 nm to 33 nm and from 19 nm to 54 nm (r = 18.4), respectively. The origin of this swelling can be explained by a change in the conformation of the polymer chains or by further adsorption of residual polymer. The physical comportment of SPION changed at specific PVAs to iron oxide ratios, and this ratio corresponded to the ratio of optimal cellular uptake.

DISCUSSION & CONCLUSIONS: Human melanoma tumor cells took up SPION according to an active, saturable and energy-dependent mechanism only when exposed to amino-SPION, at the exclusion of other functionalized polymer coatings. Importantly, the structure of the polymer shell of the iron oxide core determines the efficacy of cellular uptake, corresponding to a change in the physical properties of the compound.

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What can self-organisation do for you?

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INTRODUCTION: The influence of nanotopography on cell adhesion, morphology, alignment, proliferation and gene expression is becoming increasingly clear. Many studies are based on the use of electron beam lithography, often combined with replication, to create nanostructured surfaces. Here we examine the use of self-organisation to create well-defined nanotopographies and consider the potential advantages and limitations of this approach.

RESULTS: CSEM has recently begun exploring the formation of nanostructured materials, particularly the nanotopography of surfaces using self-organisation. Different methods of self-organisation have been investigated: physical vapour deposition (PVD) (Fig. 1), meniscus ordering of micro and nanobeads (Fig. 2), and thin polymer films (Fig. 3). Using all of these methods we have been able to make coatings with nanotopography.

The different methods have different pros and cons. For example, PVD nanostructures offer a wide range of different shapes, aspect ratios and structure densities, but have a low degree of order (see Fig. 1). In contrast, bead self-assembly gives highly ordered films, but with almost no choice in the form of the motif (Fig. 2).

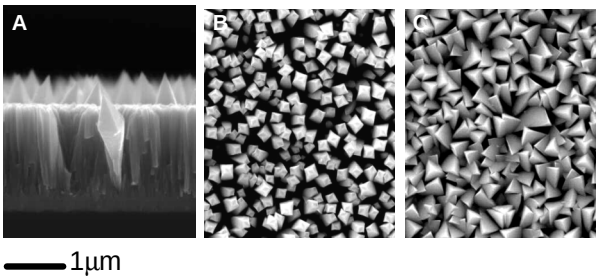

Fig. 1: *SEM images of Cr-N films with varied topographical features: (A) spikes, (B) cubes, (C) pyramids, all deposited on silicon substrates. Image A shows a cross-section, images B to C a top view of the structures.*

Using these methods we have shown good coating of large areas up to hundreds of cm2, as well as the coating of non-planar substrates such as rods and tubes.

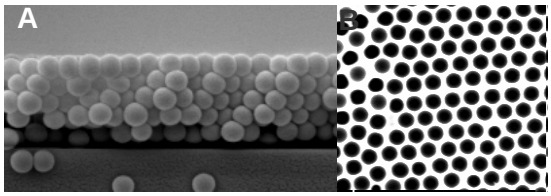

Fig. 2: SEM images of a cross-section of a silica bead thin film opal (A) and a top view of an inverse opal in silicon (B). Bead size 800nm.

Potential problems of these coatings include their possible toxicity, their softness or their brittleness. For these reasons we are now investigating nanostructure replication in plastics using embossing or casting methods. Initial results are promising.

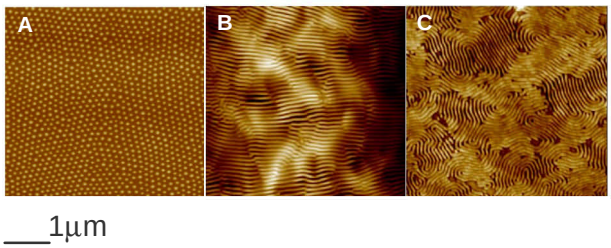

Fig. 3: AFM images of self-organised nanostructured polymer films (polystyrene-b-poly(2-vinylpyridine)) on silicon.

DISCUSSION & CONCLUSIONS: A very wide range of different structures, with very different motifs and length-scales can be produced using self-organisation. However, while a high degree of order can be obtained, the structures are clearly less well ordered than those made using top-down methods.

To date, work has concentrated on the use of coatings to structure surfaces. In future efforts will be focussed on directly nanostructuring bulk materials.

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Surface Functionalization of Biomaterials and Biosensors

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INTRODUCTION: The spontaneous assembly of multifunctional molecules at surfaces has become a useful technique to design hybrid interfaces for the biosensor field, model surfaces for cell-biological studies and drug carrier surfaces for medical application. While alkanethiol self-assembled monolayers on gold surfaces are routinely used today, there is a need for a wider range of reliable assembly systems that are compatible with oxide-based substrate surfaces. The general objective is to produce interfaces via cost-effective, robust techniques that allow the elimination of non-specific protein adsorption ("non-fouling" surfaces) and the addition of bioligands at controlled surface density and molecular conformation in order to direct the biological response to biomaterials and biosensor chips.[1,2]

RESULTS: Poly(ethylene glycol)-grafted polyionic copolymers assemble spontaneously from aqueous solutions at charged interfaces resulting in well-defined, immobilized monolayers or multilayers depending on the polymer architecture. The degree of interactiveness can be controlled quantitatively through the design of the polymer architecture. [3] If the polymer is functionalized with bioligands such as peptides (to mimic cell-interactive proteins), biotin (link to (strept)avidin) or NTA-Ni^{2+} (link to histidin-tagged biomolecules), biomaterial and biosensor interfaces with quantitative control over ligand density can be efficiently produced.

Cell-interactive peptides can be covalently coupled to polymeric self-assembly systems to achieve controlled surface density of ligands, which interact in a specific way through integrin- or heparin-mediated recognition and binding. Cell-adhesive, but bacteria-resisatnt surfaces provide a surface platform of potentially high clinical relevance. [4]

Chemical patterning of surfaces into (bio)adhesive and non-adhesive areas in the micrometer to nanometer range has become an important tool to organize biological entities such as cells and biomolecules at interfaces in a highly controlled manner (Fig. 1).[5] Two novel surface modification techniques are presented that combine conventional microfabrication (top-down) with molecular self-organization (bottom-up approach). Biologically meaningful patterns of protein-adhesive and non-adhesive areas in a size range from micrometers to as small as 50 nm could be produced with consistent quality and on comparatively large areas (e.g., whole 4-inch wafers).

Fluorescence microscopy, XPS, ToF-SIMS, Ellipsometry and AFM were used to control *ex situ* each surface modification step, while the kinetics of the interface reactions including the interaction with biological media were monitored in situ with an optical, evanescent field based sensor (OWLS) and the quartz crystal microbalance (QCM-D) technique.

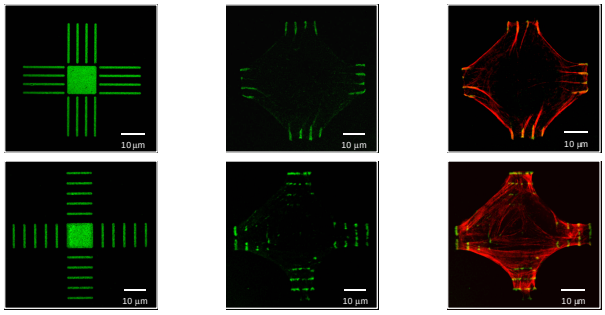

Fig. 1: *Adhesive patterns produced by the selective molecular assembly patterning (SMAP) technique. Left: Confocal fluorescence microscopy of fibronectin pattern. Middle: Focal adhesions of attached single fibroblast cell (vinculin staining in green). Right: Actin fibre network of single fibroblast cell (actin staining in red). Image size: ca. 60x60 mm^2.*

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Interactions of cells and proteins with molecularly designed surfaces

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INTRODUCTION: Protein adsorption to implant materials influences the events leading to cell-material interactions, including thrombogenesis, which is induced by a series of events, including platelet adhesion and activation processes. One of the strategies defended to improve blood compatibility is the deposition of a thin layer of albumin on the material surface in order to minimise adsorption of other proteins. The problem is the non-specific nature of such adsorption, denaturation of the protein and exchange with other proteins over time, which renders the strategy limited to short-term applications. The possibility of using highly ordered structures constituted by self-assembled monolayers (SAMs) of long alkanethiols on gold substrates offers the possibility of modelling the substrate at the molecular level. The interaction of single and multi-functional surfaces with proteins and cells is thus possible, enabling a more precise control of the role of surface properties over such interactions.
In this paper we describe the influence of the terminal functional group over adsorption of albumin and the inflammatory response. For this purpose alkanethiols with three terminal functional groups (OH, COOH and CH_3) were tested. While CH_3 produces a hydrophobic surface, the other two have a hydrophylic character. Mixed monolayers, with hydroxyl- and methyl-terminated monolayers, were also investigated to elucidate in which manner the predominance of one type over the other influences the hydrophilicity of the surface and its tendency to induce selective albumin adsorption over fibrinogen adsorption.

METHODS: The SAMs were prepared by a method described previously [1]. Esssentially, the method consists in coating gold substrates with monolayers of 11-mercapto-1-undecanol ($SH-(CH_2)_{11}OH$), 1-decanethiol ($SH-(CH_2)_9CH_3$) and 11-mercaptoundecanoic acid ($SH-(CH_2)_{10}COOH$). The monolayers we produced by immersing gold surfaces in an ethanol solution of the alkanethiol with a final concentration of 1 mM over 24h. For protein adsorption studies human serum albumin (HSA) was dissolved in PBS in a concentration normally of 0.1 mg/ml. The adhesion of human leukocytes to the SAMs was studied by seeding the samples with two subpopulations of leukocytes: polymorphonuclear (PMN) and mononuclear leukocytes, non-activated and activated with phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA). The techniques are described in detail in reference [2]. The acute inflammatory response and the adhesion of cells to these surfaces was studied *in vivo* using a rodent air-pouch model of inflammation [3].

RESULTS: The coverage of SAMs was found to be linearly dependent on the amount of HSA on the surface, which increased in the order $OH>COOH>CH_3$. The coverage took place preferentially at active sites on the surface. Blocking of these sites was very effective in terms of electron transfer processes with the solution. When mixed monolayers ($C11OH + C15CH_3$) were investigated the surface remained hydrophobic until the percentage of C11OH was ca. 70% of the total amount of alkanethiols in solution. However, the hydrophylicity was linearly related with the surface concentration of C11OH. HSA adsorption was stronger on the more hydrophobic surfaces. Moderately hydrophylic surfaces (65% C11OH) show considerable HSA adsorption, which can be easily exchanged by HSA in solution but not by fibrinogen. Therefore, this surface may possess selectivity towards HSA adsorption.
Mononuclear and PMN leukocytes have a higher affinity towards methyl-terminated SAMs than for the more hydrophylic OH- and COOH-terminated alkanethiols. *In vitro* activation of both leukocytes further increased cell adhesion. In the animal model experiments the inflammatory response was rather intense with the OH and CH_3 groups. However, the number of cells adhering to the CH_3-terminated SAMs was rather low. This high tendency for CH_3 groups to recruit a large number of inflammatory cells but to originate few attached cells is not understood.

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EFFECT OF CHEMICAL AND TOPOGRAPHICAL FEATURES OF BIOMATERIAL SURFACE ON CELL RESPONSE

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INTRODUCTION: Several methods have focused on manufacturing of biocompatible artificial materials by means of physical or chemical modification of their surface [1,2,3]. Surface chemical and physical state, its charge and morphology affect the cellular response to implant. The fiber-reinforced polymer composites seem to be the most competitive materials to metal implants. Advanced composite materials offer the potential to tailor component properties by selecting material constituents and spatially controlling composition and configuration. The major advantage of their medical applications is the possibility of specific design of their physical, chemical and mechanical properties, according to applications desired. By adding biocompatible fibrous phase, some specific physical and chemical surface and bulk properties of an artificial material, resembling natural tissue can also be designed in order to meet the demands of a living tissue**,** e.g. modification of polymer matrix with fibrous phase allows for obtaining biomimetic mechanical characteristics of a composite

METHODS: In this work, we studied the effect of fibrous phase and its arrangement in polymer matrix on cell response. Chemically treated carbon fibers were used as a tool to modify surface energy and topographical parameters of polymer samples. The fibers had the diameter similar to collagen fibers. The composite samples were prepared in such a way in order to obtain two kinds of anisotropic fibers arrangement (2D) in polymer matrices. The relationships between human cell adhesion and substratum surface parameter were investigated. HS5 fibroblasts and hFOB 1.19 osteoblasts human cell lines were used. Moreover, MG63 osteoblastic cells adhesion to the modified composite surfaces was determined. Viability of the cells, cell adhesion and collagen level were determined. Biological parameters were correlated with surface wettability and selected topographical parameters of the surface samples.

RESULTS: Figure 1 presents representative micrograph obtained in MG63 osteoblastic cell adhesion test with polymer composite sample modified in the near – surface region with anisotropically aligned (2D) carbon fibers.

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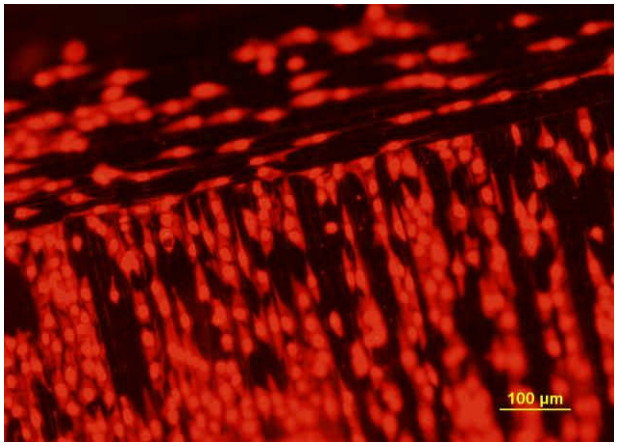

Fig.1 Effect of fibers orientation in near - surface region of composite on MG63 osteoblastic cell adhesion (fluorescence microscope, propidium iodide staining)

CONCLUSIONS

It was shown that there are specific surface parameters stimulating in selective way cell adhesion and collagen level. Cell adhesion depends upon arrangement of fibrous phase in the near – surface region of the samples.

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Is there a predictable relationship between surface physical-chemical properties and cell behaviour at interface

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INTRODUCTION: The fate of implanted biomaterials is determined by the outcome of multiple interactions with various cell populations. When encountering a foreign surface, a cell may adhere and/or spread on the surface, or depart. Adhesion may affect cell survival, proliferation, differentiation, migratory or secretory behaviour. In order to draw optimal benefit from the huge amount of available data on material biocompatibility, it would be useful to elaborate and validate a general framework for correlating cell behaviour to material properties. While this is not feasible at the present time, the aim of this communication is to discuss three basic questions as a prerequisite for addressing this general problem. For the sake of clarity and homogeneity, we shall focus on mononuclear phagocytes that may be a suitable model to illustrate various issues : indeed, when encountering a foreign structures, these cells must decide whether they will trigger and or enhance inflammatory reactions. This decision is of both theoretical and pratical interest.

PREDICTING ADHESIVE BEHAVIOUR. Will cell adhesion on an artificial surface be determined by nonspecific physical interactions or specific ligand recognition[1] ? In order to address this issue, it is necessary to consider the following questions : i) what is the difference between specific and nonspecific interactions[2] ? ii) To what extent do the nature and conformation of adsorbed macromolecules reflect the structure of underlying surfaces, or, stated in a different way, what does a cell actually see when encountering a foreign surface ? iii) Are there reliable methods for predicting the intermolecular forces between surfaces of known structure[3] ?

SPREADING OR NOT SPREADING ? Spreading is a possible consequence of cell adhesion to a surface that provides a good model for understanding the consequences of adhesion since i) this process may be fairly rapid and can be quantified very accurately and ii) there is much evidence to support the view that spreading *per se* will influence further cell behavioural responses to foreign surfaces. Much evidence suggests that cell spreading may be influenced by deformability, quantitative features of adhesion, and coordinated or random movements of the cell membrane. In view of recent experimental evidence, this process may be especially liable to quantitative modelling[4,5].

HOW DOES A CELL CHOSE BETWEEN DIFFERENT BEHAVIOURAL PATTERNS ? In order to address this problem, we need consider the following questions : i) is it warranted to assume that cell stimulation may result in a few coordinated responses, or may gene activation follow multiple independent patterns ? ii) what is the respective role of specific chemical stimulation of a combination of membrane receptors, mechanical forces, cell shape, cytoskeletal and membrane domain organization in determining cell behaviour ? (note that all aforementioned parameters are not independent). During the last years, an impressive number of biochemically oriented studies disclosed multiple intracellular signalling cascades triggered by proper receptor stimulation. However, the well known complexity of non-linear networks and multiplicity of receptor species expressed by a given cell type makes it a fairly remote prospect to find a general relationship between stimulation of a given combination of surface receptors, intracellular biochemical events and ultimate cell response.

CONCLUSION: there is presently a need for unifying concepts to draw optimal benefit form the huge number of experimental data on cell behaviour at interfaces. This probably reflects the challenge presently met by cell biologists.

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Material Surface Modification with Organized Multilayer Assemblies of Biological Macromolecules

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INTRODUCTION: Artificial polymer scaffolds are used by tissue engineering to accommodate cells and guide their growth. The cell interactions with scaffolds which themselves do not specifically promote the growth of cells but satisfy the other necessary criteria, such as, mechanical properties, shape, morphology, biodegradability, etc., can be specifically affected by modifications of the scaffold surface. The controlled successive immobilization of biological and synthetic macromolecules [1] has provided a technology for creating functional interfaces between artificial materials and biological media, e.g. blood compatible coatings [2] or biorecognition layers on optical immunosensors [3]. The technique makes possible to set up assemblies composed of a selected number of molecular layers of various macromolecules arranged in a designed order.

METHODS: The multilayer assemblies were prepared on polystyrene surfaces (PS) by successive deposition of molecular layers of laminin (LA), collagen IV (C), gelatin (G), polylysine (PL), poly(ethyleneimine) (PI), dextran sulfate (D), and bovine serum albumin (A). The first monolayer was immobilized by hydrophobic adsorption of a protein on PS. The second and third monolayers were adsorbed utilizing electrostatic interactions between molecules in solution and oppositely charged surface monolayer, i.e. interaction of LA^- negatively charged above isoelectric point (pI) or polyanion D^- with G^+ or C^+ positively charged below their pI and interaction of polycations PI^+ or LA^+ with A^- or LA^- above pI. G, C, and D were adsorbed from citrate buffer pH 4, LA, A, PI, and PL were adsorbed from phosphate buffered saline pH 7.4, (PBS). The deposition procedure was observed in real time and stability of the assemblies in PBS was tested using surface plasmon resonance (SPR) [3] on gold chips coated with a PS film. The morphology of the surfaces was observed in PBS using atomic force microscopy (AFM). The adhesion and growth of mouse embryonic stem cells line D3 was tested in PS culture dishes coated with the assemblies.

RESULTS: The molecular assemblies remained immobilized on PS without observable changes after 2-days incubation in PBS.

Table 1. Mass of differentiated (d) cells grown for 48 hours on coated PS surfaces

Arrangement of molecular layers immobilized on PS surface								
PS LA	PS C	PS C D C	PS C LA	PS G D G	PS G LA	PS A PI	PS A PL A PL	PS A PL LA
Cell culture (cell mass related to that on uncoated PS=1)								
1.5	2.3	3.6	3.0	4.5	4.2	0.9	0.8	1.6

Mass values similar to those in Table 1 were observed if the differentiation of cultivated cells was inhibited. The mass of non-differentiated (nd) cells on PI containing surface increased considerably (nd/d=14) when they were seeded in serum-free medium.

DISCUSSION & CONCLUSIONS: The stability of the coatings during the cell cultivation might be different form that in PBS. However, the results indicate, that assemblies containing extracellular matrix proteins (C, G, LA) were able to promote significantly the cell growth. Potentially, the adsorption technique allows us to prepare assemblies tailored for individual cells on scaffolds of any shape and morphology fabricated from any material on which the first protein layer can be immobilized.

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Fibronectin anchorage influences the generation of vascular- like structures by Endothelial cells

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INTRODUCTION: Control of angiogenesis within engineered tissues plays a key role for further progress in regenerative medicine since vascular networks maintain the supply of implanted cells from the surrounding tissue.
Recent studies have investigated the formation of vascular-like networks in a variety of different 3D-matrices based on proteins of the extracellular matrix like collagen and fibronectin (FN), on fibrin, or on synthetic hydrogels exhibiting a similar functionality by implemented peptide sequences [1,2].
However, for the sake of practicability, we should also ask for means to direct the generation of vascular structures in mechanically stable scaffolds without a supporting gel matrix. Therefore, we investigate the relevance of the anchorage of immobilized matrix proteins on polymer surfaces with respect to the differentiation of endothelial cells (EC) into capillary networks.

METHODS: Thin layered coatings of alternating maleic anhydride copolymers covalently linked to solid surfaces are used as a versatile platform for bio-surface engineering. By the choice of the comonomer the physicochemical characteristics of the copolymer coating can be adjusted in a very controlled way. FN was adsorbed with different anchorgae strength and in gradated amounts to the copolymer films. ECs grown in culture on these surfaces for 5 days were studied by fluorescence laser scanning microscopy.

RESULTS: The type of anchorage of the pre-deposited FN could be demonstrated to determine the remodelling of both pre-deposited und cell-secreted FN. At surfaces with a strong binding of pre-adsorbed FN the cells grow as a flat monolayer and could hardly reorganize the pre-deposited FN. Only the secreted FN is assembled in very small and flat fibrils. In contrast, weak binding of the pre-adsorbed FN leads to a formation of a 3D-network of FN and the cells can grow in a more three dimensional manner. They start to generate a vascular like network [3]. Hence, one could conclude, that the FN reorganisation characteristics determines the differentiation behaviour of ECs.
Further experiments could clarify that not only the anchorage of FN is crucial for the different cell behaviour but also the density of immobilised adhesion sites on the surface can trigger the reorganisation characteristics of FN. A lateral gradient of the amount of immobilized FN was used to show that low amounts of strongly bound FN may also permit the formation of a three dimensional FN-network. At this stage the amount of FN was found to be just sufficient to keep the cells attached and allow for the organization of the secreted FN in a 3D-network (Fig. 1, left).

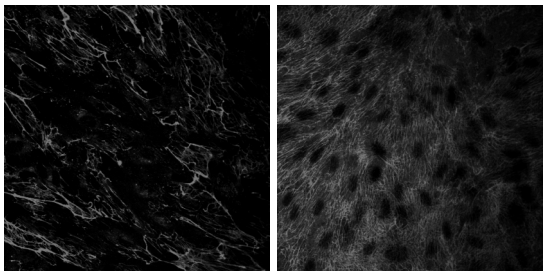

Fig. 1: Effect of the amount of pre-deposited FN. The left picture shows a small amount of FN and the resulting 3D-network. On the right, a dense FN coverage indicates to result in a flat monolayer of small FN fibrils.

DISCUSSION & CONCLUSIONS:
The immobilized amount and binding strength of FN on polymer substrates was found to control the FN remodelling and the formation of FN-networks by adherent ECs. Through this, the characteristics of polymer substrates can be adjusted to stimulate the formation of vascular like structures.

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Effect of molecular weight and concentration of Polyethyleneimine used as DNA carriers for THP-1 targeting

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INTRODUCTION: The use of plasmids containing therapeutic genes, have potential for treatment of human diseases such as cancer, infections, genetic and functional disorders. Cationic polymers, as DNA carriers in designing non-viral gene delivery systems, represent candidates with great potential use. The cationic polymers interact with and neutralize the negatively-charged DNA, thereby condensing the DNA in more compact structure. Polymer and DNA complex provide a protection against enzyme degradation and promote cellular internalization of the condensed plasmid. Our work aimed to use Chitosan-DNA (Ch-DNA) and polyethyleneimine-DNA (PEI-DNA) nanoparticles as non viral gene delivery system for macrophage targeting. Example of application could consist in targeting of anti-inflammatory cytokine or pro-apoptic genes to the macropahges and immunomodulation of inflammatory diseases.

MATERIALS & METHODS:
Polymers: Ch and PEI (Fluka Biochemika) with high, medium and low molecular weight were used for Ch-DNA and PEI-DNA nanoparticle synthesis. Ch-DNA complex formation was achieved by a complex coacervation technique[1]. PEI-DNA nanoparticles were prepared as previously described by Boussif et al[2]. Different concentrations of polymers were used for nanoparticle synthesis.
Plasmids: Different concentrations of pORF-Bax-a were used for apoptosis induction and pIRES2-EGFP for uptake and transfection efficacy.
Cell culture: THP-1 macrophages (ATCC) were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FBS (Gibco, BRL), 2 mM L-Glutamin and penicillin/streptomycin and 5.10^{-5}M of 2mercaptoethanol (Sigma). For cytotoxicity studies, the cells were seeded in 96 well culture plates at $5x10^4$ cells/well and treated with the different nanoparticles. For nanoparticle uptake, confocal microscopy was used for pIRES2-EGFP loaded nanoparticle visualization.

RESULTS: All PEI and Ch showed an efficient DNA condensation regardless to their molecular weight or concentrations as determined by agarose gel retardation assay. The use of PEI with medium and high molecular weight alone showed an evident cytotoxic effect after 24 hours. They were then discarded and PEI with low molecular weight was chosen since it did not induce a decrease in cell viability by itself. However, when complexed to Bax-a containing plasmid, it induced a higher decrease in cell viability. This effect could be related to apoptosis induced by Bax-a gene and remains to be confirmed by ongoing studies. The effect of Ch alone was similar to Ch-Bax-a. Confocal microscopy allowed the observation of GFP green fluorescent protein attesting of internalization and expression of the gene.

DISCUSSION & CONCLUSIONS: This study indicates that low, but not medium and high, molecular weight PEI is suitable for gene delivery to THP-1 cell line. Low PEI-Bax-a complex was effective in reducing cell viability, suggesting a pro-apoptic effect of these nanoparticles.

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ACKNOWLEDGEMENTS: This work was supported by the « Programme de formation des Instituts de Recherche en Santé du Canada sur les troubles de la mobilité et de la posture (MENTOR) », and the «Fonds de recherche de l'Institut de Cardiologie de Montréal».

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PLDLA Scaffolds Support the Proliferation and Differentiation of Mesenchymal Stem Cells Towards an Osteogenic Potential

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INTRODUCTION: The ultimate objective of the present study is to engineer a bone graft by expanding mesenchymal stem cells isolated from bone marrow biopsy onto PLDLA scaffolds. In a first step, we assessed the influence of fluid flow on MSC loaded onto 12 filaments PLDLA scaffolds. In a second step, we determined the kinetics of proliferation and differentiation of MSCs when cultured on 4 or 12 filaments PLDLA scaffolds for a period of 40 days in a bioreactor.

METHODS: Isolation of MSCs: MSCs were isolated from rat bone marrow and expanded in alpha-MEM + 10 % FBS supplemented with dexamethasone, Ascorbate2-phosphate and β-glycerophosphate. **Scaffolds:** Knitted 12 or 4 filament Poly-L,D-lactide (PLDLA, L/D ratio 96/4) scaffolds were used. Their characteristics are summarized in Table 1.

	4 fil.	12 fil.
Single fibre diameter	80 ±10µm	35±10µm
Distance of the fiber bundles	0.24±0.1mm	0.23±0.1mm
Distance of the single fibers	0.03±0.01mm	0.04±0.2mm
Porosity	38 %	40 %

Influence of fluid flow: At passage P3-P5 12 filament scaffolds were soaked for 1 hour in a MSC cell suspension at 10^6 cells/ml and then placed in 50 ml cell culture tube. Constructs were then cultured either on a stoval low profile roller at 6 rpm or left still. At day 28, DNA content, ALP activity and calcium content were determined.

Influence of PLDLA structure: 4 and 12 PLDLA filament constructs were prepared as aforementioned and DNA content, ALP activity and calcium content were determined every 3 days from day 0 to day 40 (n=3).

RESULTS:

Influence of fluid movement: DNA content (87000 ± 23000 versus 56000±13000cells per scaffold), ALP activity (64 ± 20 versus 2±1 UI), and calcium content per scaffold (289 ± 34 versus 21 ± 6 ng /construct), were significantly higher in dynamic culture when compared to static cultures.

Influence of PLDLA structure:

Results are summarized in Figures 1, 2 and 3.

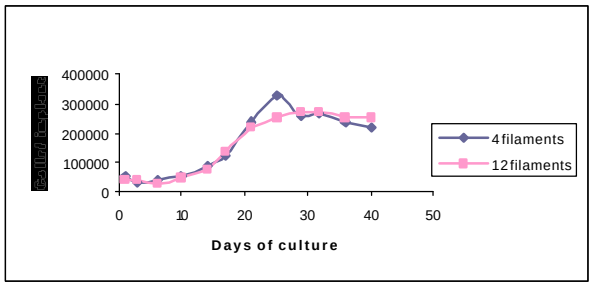

Figure 1: Kinetics of cell proliferation.

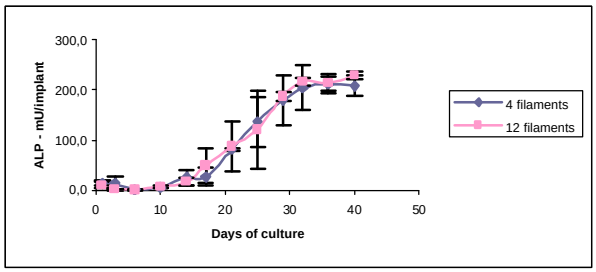

Figure 2: Kinetics of ALP activity

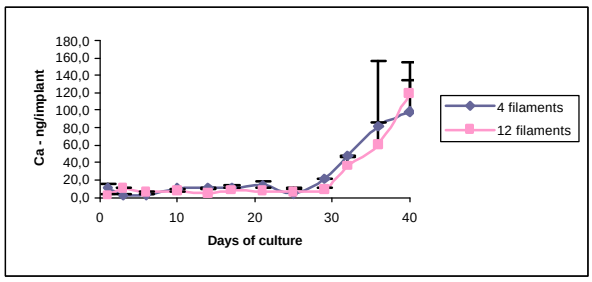

Figure 3: Kinetics of mineralization

No significant differences in DNA content, ALP activity or calcium content between the different scaffolds with different fiber thicknesses.

DISCUSSION & CONCLUSIONS:

MSCs proliferation and differentiation was significantly enhanced when fluid flow was applied to the cells. Thus, a 10 fold increase in calcium content per scaffold was observed when MSCs were cultured in the presence of fluid flow. PLDLA scaffolds were able to support MSC differentiation towards an osteogenic phenotype. Further investigations are now needed to assess the *in vivo* osteogenicity of these constructs.

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Adhesion of primary human osteoblast cells to UV/ozone modified polystyrene

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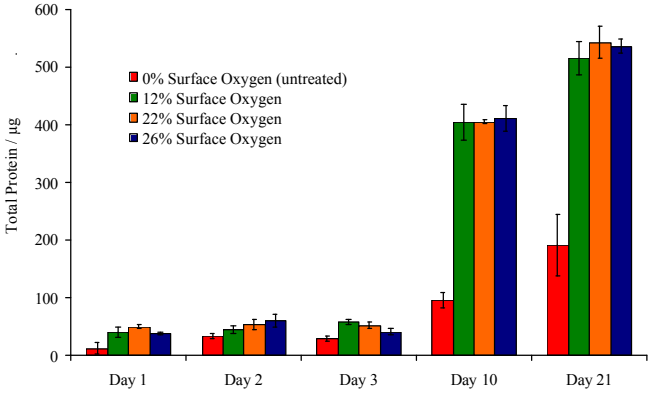
INTRODUCTION: The controlled attachment of cells to a variety of material surfaces is currently of interest for the development of a range of devices for biomedical applications[1,2]. It is generally believed that in the presence of serum, cell attachment is mediated through an adsorbed protein layer. It has been found that high-energy surfaces promote rapid cellular adhesion and spreading, whereas low energy surfaces do not[3,4]. In this research the attachment, extracellular matrix protein expression and mineralisation of human osteosarcoma (HOS) cells TE85 and human primary osteoblast (HOB) cells on UV/ozone modified polystyrene (PS) have been investigated.

METHODS: The internal surfaces of 45mm diameter PS dishes were modified by UV/ozone treatment. Surface chemical compositions of treated and untreated dishes were determined by XPS and topographic changes were examined by AFM under ambient conditions[5]. Primary human osteoblast cells were isolated from osteoarthritic and osteoporotic femur heads and both HOB and HOS cells were grown to 70-80% confluence in DMEM supplemented with 10% FCS in 5% CO_2 at 37°C, and were plated at 5000 cells/cm^2. 48hrs after plating, the media was changed to a-MEM supplemented with dexamethasone and ß-glycerophosphate to achieve mineralisation, over a 3 week period. Cell functionality was assessed by alkaline phosphatase (ALP), proliferation through Alamar blue, mineralisation, total protein and specific proteins assays.

Fig. 1: Total protein production over the 21 day time period for the different levels of treatment, compared to untreated with HOS cells ($p<0.05$ t-test with unequal variances).



RESULTS: Examination of HOB and HOS cells attached to the modified surfaces has shown that there is more total protein and ALP produced on the oxidised surfaces. There is little difference in total protein expressed on surfaces with different oxygen content, but there is a significant difference from the untreated surface (0% surface oxygen). The proliferation data from Alamar blue assays has shown a significant increase in cell number on the oxidised surfaces compared to untreated surfaces.

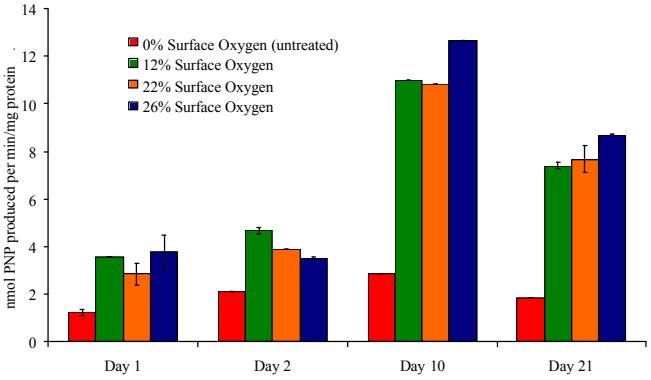


Fig. 2: ALP activity shown over the 21 day time period for the different levels of treatment with HOS cells.

DISCUSSION & CONCLUSIONS: UV/ozone treatment can be used as a simple, rapid, and cost effective method to chemically modify surfaces. This method can be used to provide a controllable chemical functionality and can be used to produce chemically patterned surfaces. These modified surfaces have been shown to increase proliferation, protein expression and ALP production in HOS, osteoarthritic HOB and osteoporotic HOB cells.

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Histological study of multifunctional bioabsorbable ciprofloxacin-releasing and plain polylactide-polyglycolide 80/20 screws implanted in rabbit cranial bone

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INTRODUCTION: Bioabsorbable surgical devices offer advantages over metallic ones. They maintain the fixation and decompose gradually. An implant removal operation is not needed. Several studies have demonstrated the biocompatibility of polyglycolide (PGA) and polylactide (PLA) and their copolymer (PLGA). The use of PLA, PGA and PLGA matrices for controlled release of therapeutic agents has also been extensively studied[1]. Local delivery of antibiotics, using a bioabsorbadable carrier, has the advantages of avoiding systemic side effects and it assures achieving high local tissue levels of the antibiotics. In this study the tissue reactions to bioabsorbable self-reinforced (SR) ciprofloxacin-releasing PLGA 80/20 screws and to plain SR-PLGA 80/20 screws in rabbits' cranial bone were evaluated in two experiments.

METHODS: In the 1st experiment, in each rabbit a plain PLGA screw was implanted on one side and titanium screw on the other side of the sagittal suture (n=21). Three animals were sacrificed after each follow-up period of 2, 4, 8, 16, 24, 54 and 72 weeks. In the 2nd experiment, in each rabbit, two ciprofloxacin-releasing PLGA screws were implanted, one screw on either side of the sagittal suture (n=20). Animals were sacrificed after 2, 4, 8, 16 and 24 weeks, four animals per group. Blocks of bone, each containing one screw were retrieved. In the 2nd experiment one block was used to assess tissue drug concentration. The other block was used for histological examination. Numbers of macrophages, giant cells, active osteoblasts and fibrous tissue layers were assessed and degradation of the bioabsorbable screws was evaluated.

RESULTS: In both experiments after 2 weeks, macrophages were seen near the heads of screws. After 4 and 8 weeks, the screws were surrounded by fibrous tissue capsule that progressively grew in thickness by time. Osteoblastic activity and groups of giant cells were seen. At 16 weeks, compact fragmentation of the screw heads was seen with macrophages seen inside the screw matrices with Cipro screws. After 24 weeks, a significant change in the morphology of plain PLGA screws had occurred. In both experiments osteoblastic activity and the amount of giant cells had decreased. After one year, with plain PLGA scares: some biomaterial was still present. PLGA screws had been replaced by adipose tissue, fibrous tissue and "foamy macrophages" which had PLGA particles inside them. After 1½ years, the amount of biomaterial decreased remarkably. The particles of biomaterial were inside "foamy macrophages".

DISCUSSION & CONCLUSIONS: Plain and ciprofloxacin-PLGA 80/20 screws elicited only a mild-to moderate inflammatory reaction when implanted in rabbit cranial bone. No contraindications as regards their clinical use was found. Ciprofloxacin-PLGA screws released gradually the loaded antibiotic. They could be used clinically as infection prophylaxis and osteofixation in craniomaxillofacial surgery in nonload-bearing or slightly load-bearing applications.

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ACKNOWLEDGEMENTS: Research funds from the Technology Development Center in Finland (TEKES, Biowaffle Project 40274/03 and MFM Project 424/31/04), the European Commission (EU Spare Parts Project QLK6-CT-2000-00487), the Academy of Finland (Project 73948) and the Ministry of Education (Graduate School of Biomaterials and Tissue Engineering) are greatly appreciated.

Biocompatible phosphate glass fibre scaffolds Engineering of the hard/ soft tissue interface

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INTRODUCTION: Phosphate based glasses maintain the survival, phenotype and morphology of adherent human bone and ligament cells (1) and may prove ideal, as scaffolding material, for tissue engineering dealing with ligament-tendon/ bone attachment defects. This work assesses the biocompatibility of fibre constructs of both, ternary ($CaO–Na_2O–P_2O_5$) and Quaternary (iron containing) compositions as candidates for a contiguous osteoblast/ fibroblast seeded in vitro systems. This is achieved by firstly, identifying the optimum fibre diameter and secondly, comparing various glass compositions for biocompatibility through evaluating cell adhesion, proliferation and morphology.

MATERIALS AND METHODS:

Cells: Primary Human osteoblasts (HOB), oral (HOF) and Tendon (HTF) fibroblasts were obtained from explants derived from alveolar bone, buccal mucosa and the flexor tendon of the hand respectively.

Glass fibre production: Ternary glass compositions containing 46 and 48 mol% calcium together with quaternary forms ($CaO–Na_2O–Fe_2O_3–P_2O_5$) of 1, 2 and 3 mol% iron were utilised to generate fibres of various diameters. By keeping P_2O_5 fixed at 50 mol% Solubility of the glass was controlled as such where higher calcium or iron content resulted in lower degradation rates (2). Glass fibres where arranged in mesh and parallel monolayer constructs where a distance of 5 to 10 µm was maintained between alternating fibres. Cells were seeded at 3.2 $x10^3$ /cm^2 density.

Cell adhesion, proliferation and morphology: Cell numbers at various time points, and on various glass compositions and fibre diameters, were measured by direct counting of propidium iodide (PI) stained nuclei. Cell morphology was visualized by fluorescent immunolabeling of the intermediate filament protein vimentin. Cell count and imaging were conducted using Leica DM IRB fluorescent microscope.

RESULTS:

Fibres less than 20-25 µm in diameter were excluded from further studies due to the lack of cell attachment, faster solubility and scaffold degradation. Whilst cell survival on ternary glass compositions ceased at day 7, a clear proliferation pattern, for both cell types, was observed on quaternary glasses of 2-3 mol% iron up to 14 days in culture (figure 1). Adherent cells exhibited a well spread morphology aligned to the fibres orientation (figure 2).

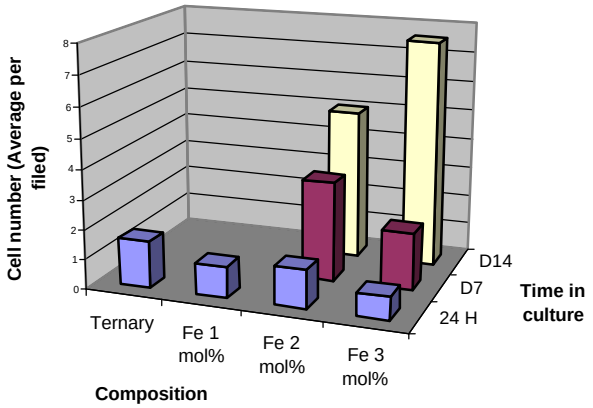

Figure 1HOF *proliferation pattern on various glass compositions at different time points. (n=3).*

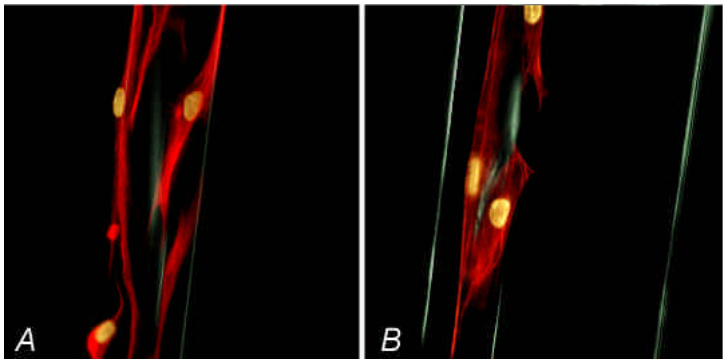

Figure 2, X20 Leica fluorescent microscope image of A, HOF cells and B, HOB cells on 3 mol% iron containing glass fibres.

Conclusion:

Phosphate based glass fibres, of quaternary glass form and containing between 2 and 3 mol% of iron are to be considered as scaffolding material for tissue engineering. These scaffolds are to be seeded with co-cultures, of both cell types, and treated with the appropriate intrinsic factors attempting to mimic the hard/ soft tissue interface in vitro.

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Combining confocal and BSE SEM imaging for bone and implant interfaces.

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INTRODUCTION: Due to structural damage in cutting, no form of sectioning can be envisaged in the investigation of calcified tissues if we wish to study cells and hard matrices in undisturbed relationships. Study of tissue remaining in a block face, discarding the damaged tissue fragments as polishing or micromilling swarf, has to be the basic approach. We therefore need to concern ourselves with means of cutting back close to the layer(s) to be studied whilst creating minimal disturbance to the remaining tissue, and choosing and optimising microscopic observation modes. Compositional mode BSE SEM of plastic embedded tissue is of key value in demonstrating differences in the degree of mineralisation of hard tissue matrices at sub-micron resolution. Sample preparation technique is critical for the success of this approach, which assumes and requires that superficial topographic relief is minimal. The same requirement is met for optimal confocal light microscopy, which has revolutionised block face microscopy in hard tissues. Here, excellent structural information is obtained in reflection-backscattering mode, albeit that such contrast is minimised in well embedded tissue and that modern commercial CSLMs are not good in this mode. However, there is usually sufficient auto-fluorescence signal in CSLM to read general histology and identify and map cell types and matrix structure. This is more so in formaldehyde and glutaraldehyde preserved material. If this is not enough, a general purpose fluorescent stain is brilliant sulphaflavine. Of course we can also utilise intra-vital mineralisation-front labels. The present studies consider practical approaches to correlating qualitative and quantitative BSE SEM imaging with confocal imaging modes and will use examples from bones embedded in PMMA, +/- tetracyline, alizarin and calcein labels. We have developed a software package tailored to the demands of our problem area. The SEM has a proper digital scan generator: we leave the BSE image unchanged, and match the CSLM image to it, rather than the reverse, because the CSLM scan mechanism is not digital, though the signal is digitised. Our overlapping program uses a linear transformation matrix which projects one system to the other, calculated by finding three corresponding points in BSE and FCSLM pictures. BSE images are empty where cells and osteoid are present. CSLM fills in these gaps. The combination images enhance our understanding of what is going on - and re-establish the need for good cellular preservation.

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Anti-Inflammatory Biodegradable Scaffolds

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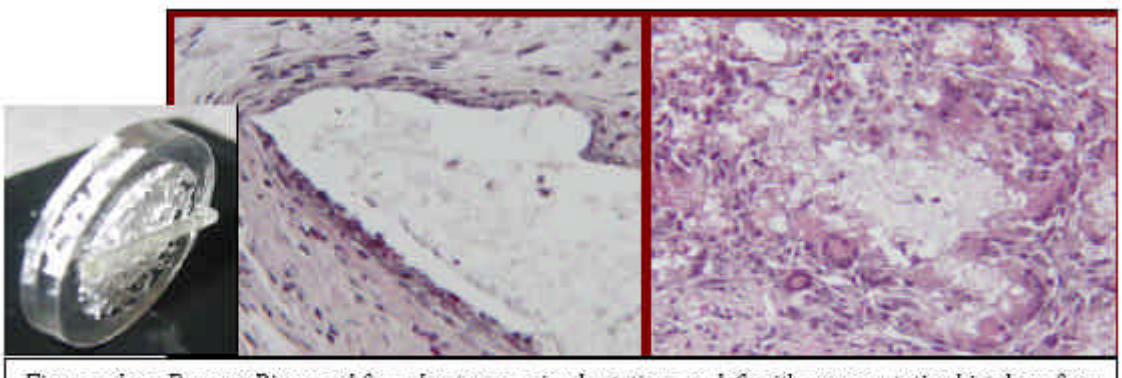
INTRODUCTION: We report that that thin calcium phosphate growth coatings can render biodegradable polymer scaffolds antiinflammatory. Thus, the foreign body giant cell response to a polymeric material such as poly(lactide-co-glycolide) (PLGA) can be abrogated by coating with a surface nucleated calcium phosphate film grown in a mixed ionic solution. This observation provides a model to examine several basic questions related to the host response to artificial materials including: Why do calcium phosphate lithomorphs not elicit a giant cell response (when particulate of the same materials do)? What causes the transition from a macrophage to a foreign body giant cell response? Does the abrogation of the giant cell response cause a long-term effects on the inflammatory response to the underlying material? We describe here some initial work with this new model.

METHODS: Macroporous, biodegradable PLGA scaffolds were coated with a thin film of CaP by immersion in 5 x simulated body fluid (SBF) for 5days. For intraosseous implantation, coated and uncoated scaffolds (2.3mm Ø) were press-fit into defects in the distal femora of male Wistar rats for 14days (n=3). For subcutaneous implantation, Perspex chamber rings either empty, or filled with half coated scaffolds (10mm Ø) were placed under the dermis of male Wistar rats up to 4 weeks (n=3). Scaffolds were disinfected with 70% ethanol prior to implantation. Recovered samples were fixed in formalin, decalcified in 10% EDTA, embedded in paraffin and 4µm thick sections stained with either Masson's Trichrome or Haematoxylin and Eosin.

RESULTS & DISCUSSION: In both intraosseous and subcutaneous sites, the coated scaffolds showed no sign of a giant cell response, while the uncoated scaffolds exhibited a florid giant cell response with additional soft tissue providing the interfacial tissue in the intraosseous site. In the subcutaneous site (see figure), the lack of giant cell response was evident, although macrophages were present, as could be expected from the initial acute inflammatory response in wound healing. In intraosseous sites this translated into direct bone apposition to the coated scaffold throughout the majority of the scaffold volume. Where the marrow cavity was exposed to the scaffold, no giant esponse was evident. These preliminary observations provide a valuable model system to not only investigate the transition mechanisms of cute into chronic inflammation, but also the role that calcium phosphates may have in this process which will inevitably contribute to their well established biocompatibility. Elucidating such mechanisms may also provide design parameters for smart biomaterials with improved regenerative capacity.

CONCLUSIONS: Part coating polymeric materials with calcium phosphate films provides a novel system to investigate the transition in cellular response

Acknowledgements: F. Sarraf (histology) ORDCF grant to JED.



Figures show Perspex Ring used for subcutaneous implantations on left with representative histology from coated (left) and uncoated (right) halves of the same scaffold. The latter shows an extensive giant cell response which has been abrogated by the calcium phosphate coating.

Stimulation of bone formation by transfer of phVEGF in a gene activated matrix

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INTRODUCTION: The early vascular response is essential for the normal progress of fracture healing.[1] VEGF (Vascular Endothelial Growth Factor) is known to stimulate therapeutic vascular growth[2]. Direct application of VEGF plasmid has been used in clinical trials on leg ischemia with positive results.[2] Furthermore VEGF is known to influence osteoclasts, osteoblasts and bone remodelling at the growth plates. We wanted to show that the application of VEGF-plasmid as a gene activated matrix is able to promote angiogenesis in bones and prevent pseudarthroses.

METHODS: A 15mm segmental defect was created in the mid-part of the radial shaft of adult New Zealand White Rabbits and filled with a collagen sponge. 60 rabbits were operated in 5 groups. The sponge was either not loaded (group 1) or loaded with 100 or 1000µg of the naked plasmid pCR3.1 (group 2 and 3) or active plasmid with $phVEGF_{165}$ (group 4 and 5). After 6 or 12 weeks animals were sacrificed and bones harvested. The amount of new bone was evaluated by µCT scan with app. 32 slices/mm. Only bone that was formed in the bed of the removed bone was counted. Postoperatively every three weeks x-rays of the operated limb were taken in two perpendicular planes and the density and amount of new bone was evaluated semiquantitative. Following fixation and decalcification longitudinal sections were taken in three predefined layers. Semi-automatic image evaluation helped counting vessels in 9 predefined ROIs.

RESULTS: No bone healing occurred in the three control groups. X-ray controls showed essential osteogenesis in the two verum groups. Complete bridging was seen in 14 of 24 animals, 10 had major ossification with transfixation to the ulnar. None of the animals in the control group achieved a bridging ossification. There was a higher degree of bridging ossification in the VEGF 100µg group than in the VEGF 1000µg group after 6 weeks.

Fig 1: x-rays 12weeks postoperative a) VEGF group b) control group

µCT scans showed a significant higher amount of new bone in the two VEGF groups. After 12 weeks slightly more bone was seen in the VEGF 100µg group than in the VEGF 1000µg group. After 6 weeks no differences were measured.

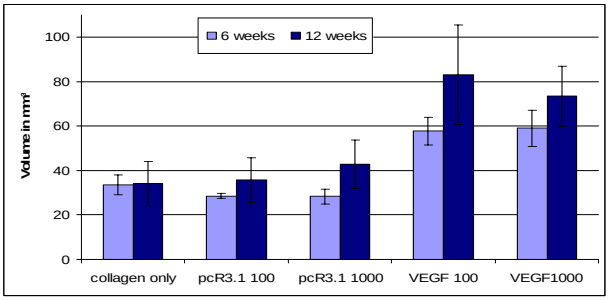

Fig. 2. Volume of new bone seen in µCT scans

More than twice as much vessels were counted in the two study groups compared to the control groups. Slightly more vessels were counted in the VEGF 1000µg group. After 12 weeks we found fewer vessels than after 6 weeks.

DISCUSSION & CONCLUSIONS: The GAM (gene activated matrix) gives us the possibility to sustain sufficient VEGF levels over the time needed.[3] Histological evaluation showed that higher amounts of VEGF plasmid led to higher vessel density after six weeks, while bone bridging was slightly higher in the 100µgVEGF group. It is possible that higher doses of VEGF reduce osteogenesis by activating osteoclasts and bone turnover. In X-ray evaluation after 6 weeks more bone bridging was seen in the 100µg group. After 12 weeks slightly more bone was found in the µCT scans. Differences between the two verum groups were not significant, whereas in all three examined criterions (amount of bone in µCT, bone bridging in x-ray and vessel formation) there were significant differences to the control groups. This study shows that VEGF has the potential to induce bone formation in situations that would otherwise lead to atrophic pseudarthroses.

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In Vitro And In Vivo Evaluation Of A Third Generation Guided Bone Regeneration Membrane Which Combines Biodegradability With Bioactivity

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INTRODUCTION: The first generation of biomedical materials developed in the 60s and 70s aimed to achieve a suitable combination of physical properties to match those of the replaced tissue with a minimal toxic response in the host (1). The second generation of biomedical materials were designed to be either resorbable (biodegradable suture or fracture fixation plates and screw) or bioactive (bioglass). The combination of both properties will lead to a third generation of biomedical materials which are biodegradable and bioactive and help the body to heal itself (2).
In the last decade guided bone regeneration (GBR) was considered to be a predictable and effective method for enhancing bone healing especially in the dental field (3). In GBR the membrane serves as a barrier for the connective tissue and maintains the open space for the time bone needs to fill it up. The only matter of controversy remains the choice of the membrane. The expanded poly-tetrafluoro-ethylene membranes are the most widely used, but require a secondary surgical procedure to remove them after they have fulfilled their task. Using biodegradable membranes a second surgery is not needed but if they are of biological origin like in the case of collagen a certain risk of the transmission of diseases remains. To avoid this problem resorbable, fully synthetic polymer membranes are an attractive alternative. The aim of this study was to evaluate a new biodegradable PLGA membrane developed for GBR, which proved to be a biomedical material of the 3rd generation because it combines biodegradability with bioactivity.

METHODS: The protocol for these studies was approved and controlled by the local authorities Rabbits were sedated and four 6 mm craniotomy defect were created (2 in the parietal and 2 in the frontal bone) with a 6 mm trephine in a dental hand piece. The surgical area was flushed with saline to remove bone debris and membranes of 7 mm in diameter were placed above and below the defect. The InionGTR membranes were used according to the recommendations of the manufacturer. After 4 weeks the calvarial bones were removed and Goldner stained histosections prepared. For each condition the percentage of defect area filled with new bone of 8 middle sections was determined. In vitro tests were performed MC3T3-E1 cells, which resemble preosteoblastic cells.

RESULTS: The evaluation of bone regeneration in standardized defects generated in the calvarial bone of rabbits showed that compared to untreated control defects (31±4%, N=17), the Osseoquest (Gore, USA) (61±5%; N=8, $P<0.001$) and InionGTR membrane (Inion, Finland) (79 ± 5%, N=8, $P<0.001$) improved the bone healing significantly. Due to the fact that the InionGTR membrane performed significantly better than the Osseoquest membrane (N=8, $P<0.048$) we determined the effect of the InionGTR membrane on different cell lines. If InionGTR membrane was applied to MC3T3-E1 cells a dose dependent increase in alkaline phosphatase activity was achieved. At 2 mg InionGTR membrane the alkaline phosphatase activity was 2,5±0.3 times (N=6, $P<0.001$) the control level. With 2 mg of InionGTR membrane mineralization of MC3T3-E1 cells determined after 4 weeks with alizarin-staining had increased 1,5±0.05 times

DISCUSSION: InionGTR membrane is a biomedical material of the third generation because it combines biodegradability with bioactivity. The increase in alkaline phosphatase activity and mineralization of MC3T3-E1 cells indicates that the InionGTR membrane accelerates the matu-ration of preosteoblasts to osteoblasts leading to an acceleration of bone healing in vivo. This extra enhancement of bone healing due to a third generation GBR membranes could make GBR a principle which can also be applied on the orthopedic field.

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SMART BIOMATERIALS ACHIEVE SMART TISSUE REGENERATION WITH CELL SHEET ENGINEERING

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INTRODUCTION: We utilize surface-modified cell culture dishes for tissue engineering and regenerative medicine. Temperature-responsive polymers are covalently grafted onto tissue culture polystyrene dishes by radical polymerization initiated with electron beam irradiation. The grafted surfaces reversibly change the cell adhesiveness depending on culture temperature. Therefore, cultured cells and cell sheets are harvested non-invasively from the dishes without need for proteolytic enzymes such as trypsin. Here, I show the reconstruction of tissue architecture *in vitro* and the clinical applications.

METHODS: A temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), was covalently grafted to surfaces of tissue culture-grade polystyrene dishes by electron beam irradiation of 0.25 MGy with using an area beam electron processing system (Nisshin High Voltage, Japan). For the non-invasive cell and cell sheet harvest, culture temperature was decreased to 20°C in a CO_2 incubator.

RESULTS: Various cell types adhered, spread, and proliferated on the grafted surfaces similarly to those on commercial tissue culture dishes. Only by reducing temperature, cells were spontaneously detached from the surfaces. Confluent cells were also recovered as a single contiguous sheet with intact cell-cell junctions and deposited extracellular matrix (ECM). The ECM beneath harvested cell sheets bound the cell sheets onto other surfaces including the other cell sheet surfaces and *in vivo* tissue surfaces.

Corneal epithelium-like tissues were prepared from limbal stem cells obtained from donor eyes on PIPAAm-greafted dishes and harvested by reducing temperature (Fig. 1). Then, the allogenic cell sheets were grafted to corneal stroma of human patients of limbal stem cell deficiencies. Cell sheet engineering technique utilizing temperature-responsive culture surfaces permitted rapid, intact corneal epithelial sheet attachment without any carriers or sutures. The grafted cell sheets containing the stem cells regenerated transparent corneal tissues and the patients recovered their sights.

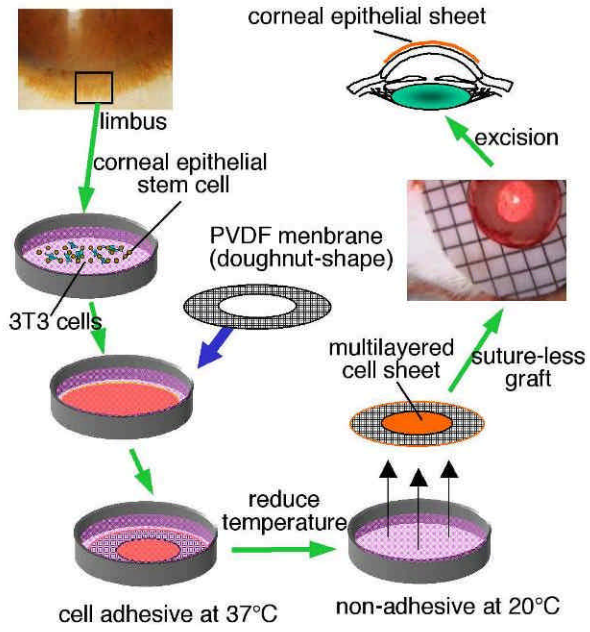

Fig. 1: Scheme of corneal epithelial cell sheet transplantation utilizing temperature-responsive culture dishes.

DISCUSSION & CONCLUSIONS: The results shown here suggest promising clinical capabilities of reconstructed tissues by cell sheet engineering for transplantation. Furthermore, the present grafting method without any carriers or suture should be applied to corneal endothelial cells, retina pigmented epithelial cells as well as other epithelial tissues. The cell sheets harvested from temperature-responsive culture dishes can achieve functional and histological integration to host tissues.

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Bioresorbable Polymers in Repair, Replacement and Regeneration of Tissues and Organs

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INTRODUCTION: Infection, tumour resection, trauma and ageing often lead to the loss of tissues and internal organs. The number of patients who suffer from these problems is increasing while the availability of autogenous tissues for transplantation remains limited. Annual healthcare costs associated with these problems exceed 400 billion dollars in the USA alone.

Genetic engineering may offer a possible solution to this problem. This is, however, still at the exploratory stage and some time may pass before it gains common clinical acceptance.

Yet another solution might be the use of structural tissue scaffolds instead of autogenous tissues. Optimally, such scaffolds implanted in place of resected or defective tissues and organs should induce their healing and/or regeneration. This, however, would only be feasible if the scaffold's biological properties approximate those of autogenous tissues. This is not yet the case for state-of-the art biomaterials technology. Therfore, the scaffold's regenerative potential needs to be enhanced, for example, by loading it with autogenous and/or synthetic growth factors or by seeding it with cells, the latter being commonly called "tissue engineering".

Structural scaffolds can function in many ways. They can fill defects, substitute tissues and organs, support attachment and proliferation of cells, enhance tissue healing, initiate regeneration, release drugs or genes.

The form and structure of the scaffold may resemble the structure and form of the tissue or organ it is intended to substitute. Thus, scaffolds used as artificial skin, artificial pericardium or periosteum would take a form of a flat microporous membrane. Scaffolds for the substitution of blood vessels or nerve regeneration would be tubular in shape, while three-dimensional spongy structures are preferred for bone substitutes and repair of articular cartilage.

Structural tissue scaffold should be biocompatible, preferably bioresorbable and porous. The scaffold's material should resorb at the required time dependent on its function. The pore structure should be interconnected allowing for a flux of nutrients, ingrowth of cells, blood vessels and tissues. The size of pores having a significant impact on the scaffold biological functionality will depend on the intended application, i.e. there is no one "universal" pore size which suits all types of tissues to be substituted.

Materials which have found an established place in the design of tissue repair and replacement implants are primarily homopolymers of lactides and glycolide, copolymers of these monomers and copolymers based on lactides or glycolide with ε-caprolactone, trimethylene carbonate or tyrosine carbonate.

Poly(hydroxybutyrate), poly(hydroxybutyrate-co-valerate), polyorthoesters, polyesteramides, and poly(propylene fumarate)-based materials can also find applications in implantable devices for tissue repair and regeneration.

Various tissues and internal organs have successfully been repaired using structural microporous scaffolds from bioresorbable polymers.

An "artificial skin" from microporous biodegradable polyurethanes promotes healing of full-thickness skin wounds. Tubular implants that form primary scaffolding for oriented migration of fibroblasts, Schwann cells and regenerating axons, facilitate healing of large defects in the sciatic nerve. Microporous 3-D scaffolds enhance the regeneration of critical size segmental long bone defects and mono-, bi- and tricortical defects in the ilium. The biodegradable elastomeric microporous vascular prostheses used to replace resected long segments of arteries in growing animals induce regeneration of a neo-artery. The cellular structure, mechanical and biological properties of the neo-artery resemble those of the native artery. Critically selected bioresorbable polymers are materials of choice for tissue repair and regeneration implants.

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Biophysical Examination of Vascular Smooth Muscle Cell Interaction with PLA and PLGA Polymer Surfaces

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INTRODUCTION: Vascular tissue engineering has yet to produce a viable vessel replacement that can be used in humans. Biocompatible scaffolds are often used, onto which cells are seeded, and grown in a bioreactor under pulsatile pressure [1]. Two polymers, PLA (polylactic acid) and PLGA (poly(lactide-co-glycolide)) are commonly used as the biomaterial scaffold. Despite recent advances in the technique, bioengineered vascular grafts do not appear to produce important components of the vessel wall, particularly elastin [2]. At present, little is known about the effect of biomaterial surfaces on cell behaviour and matrix synthesis. The aim of this project is to begin to identify cues that regulate cell attachment, migration and proliferation, and by altering the biomaterials, encourage cells to engineer their own matrix.

METHODS: 22x22 mm glass coverslips were thinly coated with 0.3% (w/v in chloroform) solutions of PLA or PLGA. The thickness and surface uniformity of the polymer films on the coverslips was determined using spectroscopic ellipsometry. Human vascular smooth muscle cells (VSMCs) were obtained using the explant technique and used between passages 2-8. The polymer coated slides were placed in 6-well plates and VSMCs seeded at a density of 1 x 10^4 cells/well, in 2ml DMEM with or without 10% bovine foetal serum. Identical conditions were used with uncoated glass coverslips or tissue culture plastic as controls. The behaviour of the cells was monitored using time-lapse video microscopy (Nikon) for a period of 24 hours. The expression of matrix genes was assessed by real-time quantitative polymerase chain reaction (RQ-PCR). The expression of Fibronectin-1, Elastin, Vitronectin, Collagen type I and IV, Versican, Fibrillin and Myosin heavy chain II (probes all assays-on-demand, Applied Biosystems) were all assessed with β-actin as the endogenous control.

RESULTS:
The VSMCs displayed marked differences in behaviour on the different biomaterials surfaces. Cells incubated with serum-containing media were active, exhibiting rapid non-Brownian motion in attachment areas on all surfaces during the first two hours. Cells on control surfaces then attached and spread, extending long filopodia and 'swirling' around each other by 24 hours (fig. 1a). Attachment on PLA was delayed until about 6 hours, but between 12-24 hours cells behaved as on control surfaces (fig. 1b). Attachment on PLGA was rapid but in contrast to behaviour on other surfaces, many long filopodia extended in a radial fashion, and even at 24 hours long filopodia were still observed (fig. 1c).

Fig 1: Appearance of VSMCs after 24-hours in culture with serum-containing media on a) tissue culture plastic b) PLA and c) PLGA

Expression of all of the target genes was identified by RQ-PCR in VSMCs in culture. Alterations in matrix gene expression on the different polymer surfaces will be presented.

DISCUSSION & CONCLUSIONS: This preliminary work provides evidence that the behaviour of VSMCs is directly affected by biomaterials surfaces. This is being followed by analysis of how these surfaces affect conformation of key extracellular proteins and cell responses to them. These, together with RQ-PCR results will allow us to begin to define how scaffolds determine tissue structure and matrix synthesis under different surface chemistry. We conclude that biomaterials may significantly affect tissue construction *in vitro*, and understanding these interactions is a key component to successful tissue engineering.

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New collagen hybrids composites devoted to cell growth and proliferation

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INTRODUCTION: Various polymers with collagen have been studied extensively for the last 20 years. Despite these efforts, there is still a need for new structure based on collagen in medical applications, including substrates for the culture of cells, control transfer therapeutic agents, and drug delivery. [1,2] The aim of this paper is to obtain and characterize a range of hybrid polymer films with various collagen content and synthetic poly-alcohols as poly vinyl alcohol (PVA) and poly glycol.(PLG). The approach of enhancing biocompatibility of biofilms was related to change chemical composition, structure and surface topography.

METHODS: Films were obtained using a "casting solution method". The collagen was stabilized by cross-linking and final cross-linking step was achieved by dehydrothermal treatment [3]. The samples include structures based on acid and neutral collagen hydrolysates or collagen gel, with and without calcium, magnesium and phosphate ions (new patent). Physicochemical, morphological properties and biocompatibility characterization of biofilms were performed using following investigations:

- Gel chromatography and viscosimetric method for molecular weight determinations;
- Spectroscopy (UV-VIS, FTIR, Specular reflection). IR spectra FTIR JASCO 620 equipment was the instrument for the structure changes;
- Surface analysis type Atomic Force Microscopy (AFM) with 2D and 3D visualization of AFM data. Software techniques for roughness and fractal dimension. For image improvement and functions for granulometric measurements and statistical analysis.
- Cytotoxicity test for osteoblast growth using direct contact method, using cultures of human bone tissues. The cell viability was checked in all cases by the standard trypan blue exclusion test.

RESULTS: The average molecular weight of the collagen hydrolysates mainly depends on the hydrolysis time. Structural characterization of samples with various collagen put in evidence the difference between them which consists in the ratio between the intensity of some band, especially the 3400 cm^{-1} band related to OH of hydrogen bonding. Concerning AFM data, these denote that collagen content of hydrolysate used in biofilms preparation and various ions are important factors in changing surface morphology. The change is a significant one as could be seen in figure 1.

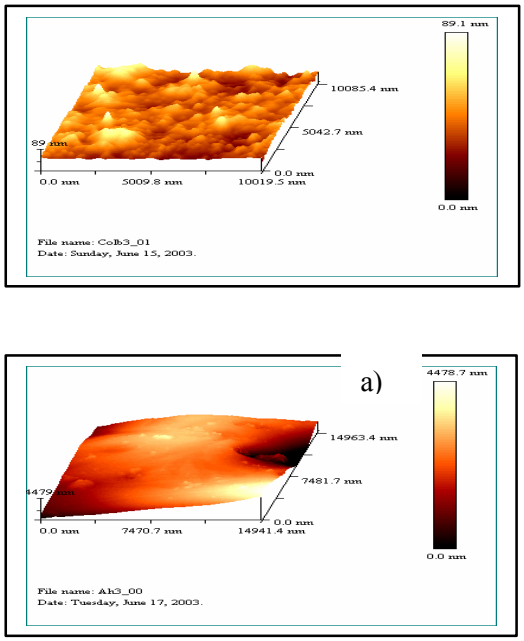

Fig.1 AFM images of films with (b) d Mg ions (a) and without (b)

DISCUSSION & CONCLUSIONS: The molecular average weight, the cross-linking, solubility, oligoelements content, roughness of bioartificial collagen / alcohol films are in a direct relationship with cell adhesion and proliferation. As roughness value is direct related with adhesion and this one is a factor of cell growth the AFM data should be a valuable criterion in biofilms selection. It is to notice that cell viability is increasing for the films based on gel structure for both components the alcohol and the collagen

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Enhancement of mechanical signals for tissue engineering bone by mechano-active scaffolds

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INTRODUCTION: Treatment of bone defects, whether non-unions, critical size defects or joint fusion due to trauma, degenerative diseases or natural aging, is a great challenge for orthopaedic surgeons. Autogenic bone grafting still remains the gold standard. However, donor site morbidity, risk of infection and limited availability are the common drawbacks of this procedure. Tissue engineering has progressed and developed into a most promising therapeutic direction for bone loss. Various strategies for bone tissue engineering are being investigated worldwide. In this study, we manipulate mechanotransduction pathway, in particular, membrane ion channels, such as voltage operated calcium channels (VOCC) and develop mechano-active scaffolds. Our results demonstrate that when the bone cells or bone marrow grown in such scaffold and subjected to mechanical stimulation, the mechanical signal is enhanced, which stimulates the cells generating more matrix comparing to the controls. Various techniques have been used to reveal the morphology and biochemical molecules in the constructs in response to mechanical stimulation.

METHODS: The mechano-active scaffolds are made from medical grade poly(l-lactide) (PLLA) with encapsulating calcium channel agonist, BAY K 8644, or FPL 64176. The resulted scaffolds have cylinder shaped (ϕ 9 x 4 mm) with 250-350 μm pore size and 90% porosity. Primary human bone cells or bone marrow were seeded into the scaffolds dynamically or statically. Collagen coated PLLA scaffolds were used as the controls. The cell-scaffold constructs were cultured for three or four weeks followed by mechanical stimulation using a perfusion bioreactor with cyclic compression for one week at 1 Hz frequency, one hour per day. Morphology analysis was carried out on cell-scaffold constructs by histological sectioning and optical coherence tomography (OCT). The productions of extracellular matrix, such as collagen type I, DNA and total protein content were evaluated.

RESULTS: Histological section and OCT images of the constructs show that bone cells and bone marrow grew into the pores and filled the pores as demonstrated in Figure 1.

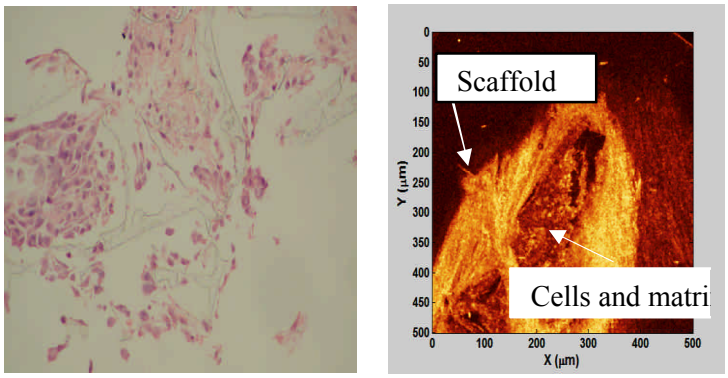

Figure 1 Histology and OCT images showing the cells and the matrix filling the pores

The collagen levels in loaded specimens were higher than in controls. This load-related elevation in collagen expression was further enhanced in the calcium agonist encapsulated scaffolds subjected to load as shown in Figure 2.

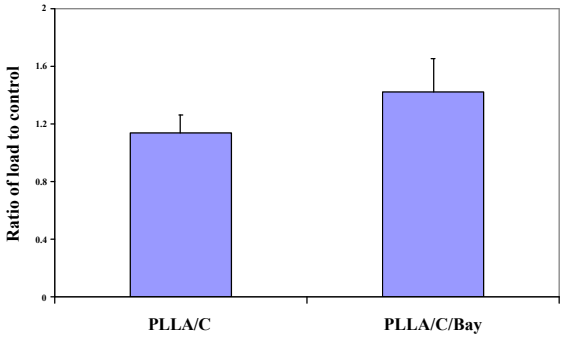

Figure 2 An elevation in collagen type I production in relation to vinculin.

DISCUSSION & CONCLUSIONS: Using mechano-active scaffolds to grow bone tissue demonstrates a feasible technique to enhance the mechanical conditioning for the constructs.

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Clinical Application of Resorbable Polymers in Guided Bone Regeneration

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INTRODUCTION: Long segmental diaphyseal bone loss often results from high energy trauma like blast injury, osteomyelitis or wide excision of malignant conditions. Treatment of these long segmental diaphyseal defects remains a difficult clinical problem. In the literature, many authors have reported that segmental bone defects longer than 2.5 cm always require bone grafting. Non-vascularized bone graft frequently fails if the defect is longer than 6-7 cm. Thus, a 2.5 cm long bone defects is probably the critical-size defect in human and a 7 cm long defect is likely the critical-size for non-vascularized bone graft.

Various treatment methods are adopted currently to address this problem, including vascularized bone graft, distraction osteogenesis and massive allograft. However, all these methods are associated with a lot of problems.

Successful guided bone regeneration has been achieved in skull bone and jaw bone using resorbable allograft. Bone regeneration in long segmental defect and relatively small defect in tumour excision has been achieved using resorbable polylactide scaffolds.

METHODS & MATERIALS: Bioresorbable scaffolds (microporous membranes and 3-D sponges) were produced from poly(L/DL-lactide) 80/20% with a molecular weight of 200 kD (PURAC Biochem; Gorinchem, The Netherlands). The membranes had interconnected open pores with an average size of 50 to 70 μm. The 3-D sponges had the average pore size in the range of 450-700 μm, and the pore-to-volume ratio in the scaffold 94%.

Ten patients with bone defect of sizes up to 6 cm due to various causes including benign tumour, osteomyelitis & fractures were treated with resorbable polylactide scaffold impregnated with marrow blood which contains stromal cells. In cases with infection, antibiotics was also loaded into the scaffold and in this situation, the scaffold also served as a drug delivery device. The patients have been assessed regularly with X-rays and clinical symptoms.

RESULTS: Serial X-ray evaluation and clinical evaluation revealed presence of bone regeneration. The limbs enjoyed satisfactory function and there was minimal donor site morbidity and major surgery can be avoided.

DISCUSSION AND CONCLUSIONS: Segmental bone defects in selected clinical cases were treated using bioresorbable porous polymeric scaffolds impregnated with autogenous marrow blood. Such cases would be treated otherwise by conventional techniques.

Vascularized bone transfer has limited supply and involves a major operation. There is always a chance of vascular complication and there is donor site morbidity. There is a limitation in bone defect size that can be lengthened using distraction osteogenesis, and in addition, this procedure requires a prolonged placement of an external fixation. There is a high chance of traction injury to nerve and other soft tissues.

Massive allograft requires a prolonged period, in terms of decades, for complete creep substitution. There is also a high incidence of disease transmission and infection.

Therefore there is a constant demand for bone substitute which can bridge long segmental defect effectively with minimal morbidity and can heal in reasonable time frame. The affected limb can be rehabilitated and bear weight for functional restoration as early as possible. If such a substitute is radiolucent this would be an additional advantage for a radiographic assessment of bone healing.

Such a new bone substitute can be for example produced from bioresorbable polymers and/or composites consisting of bioresorbale polymers and calcium phosphate ceramics which would add and osteoconductive component to the implant. Impregnation of such bone substitutes with autogenous bone marrow or marrow blood would also make osteoinductive. The early promising results of this study show that such an approach to new bone substitutes is feasible.

In vivo biocompatibility of new radiopaque polymeric microspheres

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INTRODUCTION: Polymeric microspheres find different clinical applications, for example in the treatment of stress urinary incontinence (SUI) in women [1], or in arterial embolization strategies. Use of *radiopaque* (i.e., X-ray visible) microspheres would, theoretically, offer several advantages, such as: (i), better dose control; (ii), better visualisation of the injected area; (iii), monitoring of possible migration of the injected particles. Recently, we have developed new polymeric microspheres containing covalently bound iodine. The aim of this study was to examine their biocompatibility *in vivo*.

METHODS: The microspheres were prepared as described by Saralidze et al. [2]. They were sterilized (ethylene oxide) and mixed with an injectable collagen suspension. Injections were in subcutaneous tissue (n=9), or in musculature tissue (n=9). There was a control group of 10 animals. Euthanisia was done in two groups, one after a week (n = 14), and one after 3 months (n = 14). Microspheres and surrounding tissue were carefully excised, fixed in paraformaldehyde, and processed for histopathological analysis.

RESULTS: Microspheres were retrieved as closely packed ensembles in all cases. Histopathology revealed that the microspheres are well tolerated (Figure 1). No migration was seen. After 1 week, some inflammatory cells were encountered. Most of the macrophages were isolated, in some cases they fused to form a giant cell.

DISCUSSION AND CONCLUSIONS: We believe that radiopaque microspheres as described here may provide alternative to silicone and PTFE microspheres which are used clinically in SUI treatment. Our microspheres bring the benefit of clear X-ray visibility *in situ*. The data generated in this study indicate the feasibility of this idea.

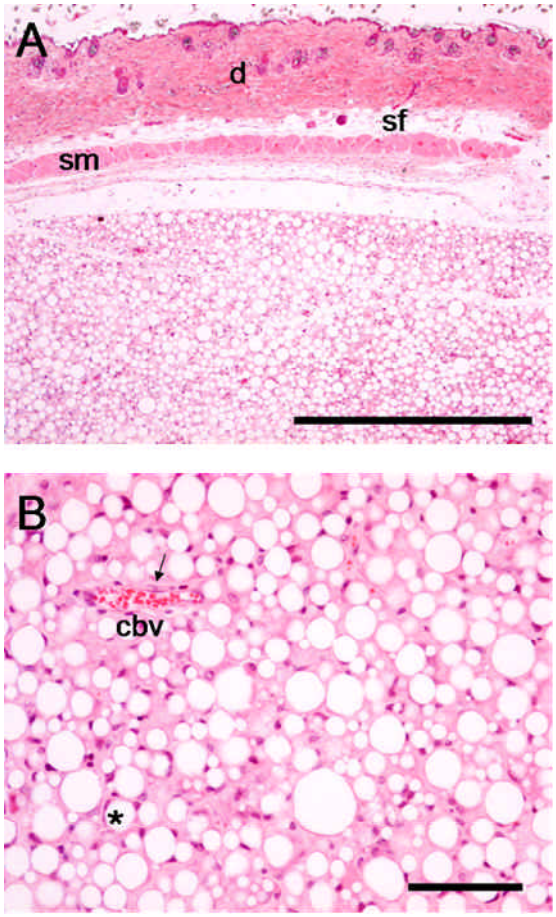

Figure 1. *Light micrographs of subcutaneous microspheres after 3 months follow-up. A: d = dermis, sf = subcutaneous fat cells, sm = subcutaneous muscle layer. The microspheres are seen in the lower half of this micrograph, scale bar = 1 mm. B: part of the microspheres in A, shown at larger magnification; cbv = capillary blood vessel, the arrow pints at an endothelial cell in the capplillary's wall. The asterisk indicates a microsphere surrounded by a giant cell. Scale bar = 100 micron.*

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Bacterial adhesion and proliferation on biomaterials. Techniques to evaluate the adhesion process. The influence of surface chemistry/topography.

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INTRODUCTION: Infection remains a major impediment to the long- term use of many implanted or intravascular devices. Bacterial adhesion to biomaterial surfaces is an essential step in the pathogenesis of these infections, however the molecular and physical interactions that govern it have not been understood in detail. Both specific and non-specific interactions may play an important role in the ability of the cell to attach to (or to resist detachment from) the biomaterial surface. This mini review reports on general aspects of bacterial infection associated with medical devices, factors that influence bacterial adhesion, techniques that are used to evaluate the adhesion process and theoretical models that explain/predict bacterial adhesion on biomaterials.

Factors that influence bacterial adhesion[1]: Bacterial adhesion is a complicated process that is affected by many factors including the bacteria (hydrophobisity, surface charge), the material surface (chemical composition, roughness, configuration, wettability) and environmental factors (serum proteins and antibiotics, temperature, bacterial concentration, time of exposure, flow conditions)

Techniques[2]: two categories of techniques used in calculating bacterial-material interactions are described: those that utilize fluid flowing against the adherent bacteria and counting the percentage of bacteria that remain adhered (parallel-plate flow chambers, radial flow chamber and rotating disc) by a number of methods such as microscopy and viable bacterial counting methods, and those that manipulate single bacteria (atomic force microscope and modified atomic force microscope[3])

Theoretical models[4]: The DLVO theory describes the Van der Waals (generally attractive) and the Coulomb (generally repulsive) interactions. The thermodynamic theory estimates the surface free energy of the bacterial and substratum surfaces and of the suspending solution. The extended DLVO theory estimates not only the Van der Waals and the Coulomb interactions but also the acid–base interactions.

RESULTS: It has been shown that both surface chemical composition and surface topography (roughness, configuration) influence bacterial adhesion. Hydrophilic materials are more resistant to bacterial adhesion than hydrophobic materials. It has been shown that large numbers of bacterial attach to hydrophobic plastics with little or no surface charge, moderate numbers attached to hydrophilic metals with a positive or neutral surface charge and very few attached to hydrophilic, negatively charged substrata. Coating substrata surfaces with proteins, such as bovine serum albumin decreased surface hydrophobisity, leading to an inhibited bacterial adhesion to the surfaces. Modifying surfaces with an antimicrobial peptide coating, Silver, DLC, nonsteroidal anti-inflammatory drug coating or amine-containing organosilicon surfaces discourages bacterial adhesion. As far as surface roughness and configuration is concerned, it has been found that the irregularities of polymeric surfaces promote bacterial adhesion and biofilm deposition. Roughening the surface of certain materials greatly increases bacterial colonisation. Moreover bacteria adhere more to grooved and braided materials compared to flat ones, probably partially due to increased surface area.

DISCUSSION & CONCLUSIONS: This review reveals that both surface chemistry and topography influence bacterial adhesion and biofilm formation. However, the relative importance of these factors has not been clearly understood yet. Refinement of the techniques for measuring bacteria-material interactions with reference materials would improve our knowledge of this important, yet complex phenomenon.

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Analysing Bacterial Adhesion to Biomaterials

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INTRODUCTION: *Staphylococcus aureus* is a major human pathogen that colonizes implant surfaces, and is of increasing importance due to the rise in antibiotic resistance[1]. It is distinct from *S. epidermidis* which is an opportunistic bacteria, associated with catheters and other indwelling medical devices[2]. *S. aureus* and *S. epidermidis* are both capable of forming biofilms which can be difficult to clinically treat because the bacteria in the interior of the biofilm are protected from phagocytosis and antibiotics[3]. Hence the need to prevent initial bacterial adhesion. This may be possible by modifying the implant surface, or coating it with an antimicrobial/protein resistant coating. Here we describe methods for the visualization and quantification of *S. aureus* and *S. epidermidis* adhering to different biomaterials.

METHODS: To visualize *S. aureus* and *S. epidermidis* adherence on different surfaces (Table 1), bacteria were cultured on the different surfaces in brain heart infusion broth (BHI) at 37°C for different times, then fixed with 2.5% glutaraldehyde in PIPES buffer, post-stained with 1% OsO_4 in PIPES, dehydrated, critical point dried, coated with Au/Pd, and visualized with a SEM using a BSE detector[4]. To quantify the amount of adherent *S. aureus*, adherent bacteria were stained with fluorescent 5-cyano,2-ditolyl tetrazolium chloride (CTC), and visualized with a Zeiss Epifluorescence microscope fitted with an Axiocam camera[5]. The density of live bacteria on the surfaces in each image were counted using KS400 software. On surfaces that autofluoresce, adherent *S. aureus* and *S. epidermidis* were detached by sonication in Tween 80, then stained with a live/dead assay (Molecular Probes). The amount of bacteria present were counted using a Partec PAS flow cytometer.

RESULTS: SEM images showed variations in the adhesion of *S. aureus* and *S. epidermidis* to the various surfaces. No differences in *S. aureus* adhesion were observed on TS, TE, and TIG, slightly more were found on TAST, but few were found on the THY (Fig 1a), and PEG and RGD surfaces (Fig 1b).

Surface	Code
Standard Titanium	TS
Electropolished TS	TE
TS coated with polymer for promoting cell adhesion	TAST
TS implanted with nitrogen	TIG
TS coated with hyaluronic acid	THY
Ti film coated with PLL-*g*-PEG	PEG
PLL-*g*-PEG functionalized with RGD	PEG-RGD
100% hydrophobic polyurethane	100%
70% hydrophobic polyurethane	70%
30% hydrophobic polyurethane	30%

Table 1. List of the surfaces studied

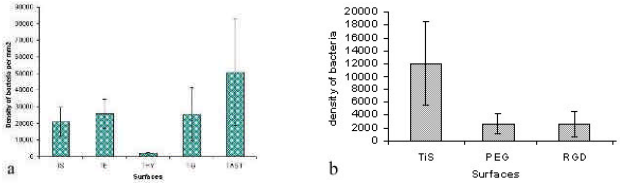

Fig. 1: Graphs showing the density of bacteria on different surfaces after 1h of culturing.

Flow cytometry results confirmed SEM observations, that more *S. aureus* adhere to the surfaces after 4h of culturing than *S. epidermidis*.

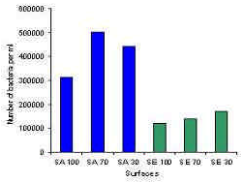

Fig. 2: Graph showing flow cytometry results. SA - S. aureus, SE - S. epidermidis.

DISCUSSION & CONCLUSIONS: These results show that different methods can be used to study the adhesion of bacteria to biomaterials *in vitro*. SEM is useful for morphology and general observations, and either CTC staining with fluorescence microscopy or flow cytometry can be used to quantify the amount of adherent bacteria.

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ACKNOWLEDGMENTS: Thanks to Kati Gorna (ARI, Davos), John Disegi (Synthes USA), Lukas Eschbach (RMS, Bettlach, CH), and Samuele Tosatti (ETH Zurich, CH) for the surfaces.

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Multiple Substrate Array, MSA ä : Miniaturized Screening of Extracellular Matrix-dependent Cell Behavior

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INTRODUCTION: Although a wealth of information about the extracellular matrix (ECM) has been generated over the past few decades, we neither completely understand the mechanisms and scope of effects of ECM on cell physiology, nor are we exploiting the power of ECM to control cell function and differentiation *in vitro*.
We have developed a biochip (Multiple Substrate Array, MSA™) and methods that allow for the miniaturized and systematic screening of extracellular matrix proteins and their effects on cell phenotype. One of the applications of the MSA™ is envisioned in ECM design for the development of artificial tissues.

METHODS: Multiple substrate arrays are generated by microspotting extracellular matrix proteins on coated glass microslides using a Perkin Elmer BioArrayer. Before spotting ECM proteins are mixed with fluorescently labeled bovine serum albumin for visualization of microspots by a fluorescent scanner. Plastic cell culture chambers are mounted on slides carrying 12 arrays such that one array is centered in each chamber. After blocking unspecific binding sites with blocking buffer cells are seeded in each chamber for adhesion and cultivation. The number of adherent cells to each microspot as well as differences in cell phenotype visualized by fluorescent markers are quantitated by automated image acquisition and analysis (Leica Qwin software).

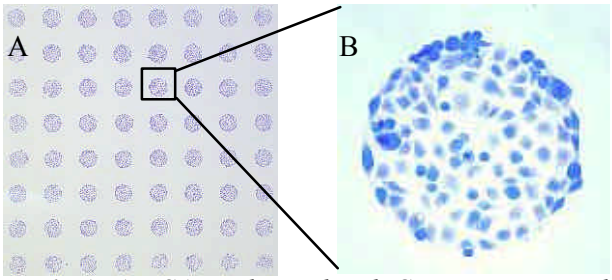
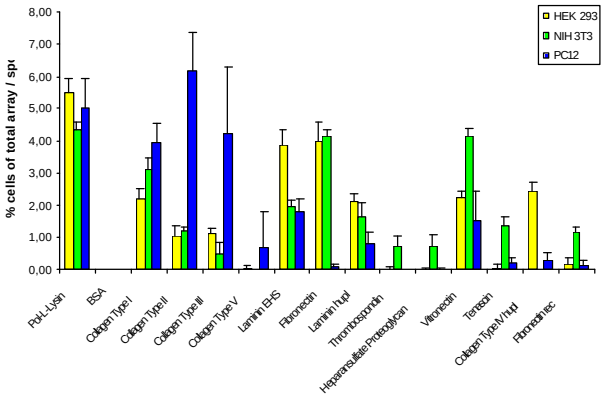


Fig.1: A: A MSAä colonized with Coomassie-stained NIH3T3 cells. B: Magnification of one microspot. The diameter of one microspot is 300µm.

RESULTS: A microarray chip and protocols were developed that can be used for the quantitative analysis of cell adhesion and cell phenotype of cells cultured on different extracellular matrix proteins. Important features of the technology have been worked out like the generation of a suitable substrate for protein adsorption and cell colonization, reproducible quantitative spotting of ECM proteins, uniform distribution of cells on arrays during colonization, and semi-automated quantitative image analysis. Extensive statistical analyses were performed to demonstrate the robustness of the technology. One example given in this presentation that demonstrates the application of the MSA™ is the generation of cell adhesion profiles for three different cell types and 15 different proteins (Fig. 2). Resulting profiles of cell adhesion indicate cell type specific preferences for particular substrates.
Fig.2: Differential cell adhesion of three cell types to ECM proteins on a MSAä: Each bar represents the mean value of 4 microspots colonized with the indicated cell type. Error bar= SD.



DISCUSSION & CONCLUSIONS: We have provided data that demonstrate the application and robustness of the MSA™, a new cell array for the rapid detection of differential cell adhesion of various cell probes to different matrix molecules. In the future this technology will be used to determine matrix-dependent cell behavior like e.g. apoptosis, proliferation, differentiation and gene expression. Main advantages compared to macro-assays in multiwell plates are the small amounts of matrix molecules necessary to perform the assays and the use of small numbers of cells which can be important when only small quantities of primary cell material is available. We predict applications of this technology in the fields of basic research, tissue engineering, and cell-based drug screening.

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Detection of Cellular Traction Forces by Microfabricated Si Force Sensors

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INTRODUCTION: To investigate mechanical cell-substrate interactions, we have microfabricated force-sensitive cell adhesion substrates by micropatterning silicon wafers. Sub-cellular forces are measured by imaging independent deflections of vertical micro-cantilevers in the 2D plane of the surface. These substrates model surfaces of different elasticity and porosity, as well as quantify direction and magnitude of sub-cellular forces.

METHODS: The silicon microchips have been patterned using photolithography and deep reactive ion etching to produce substrates of varying spatial arrays and cantilever dimensions, and therefore sensitivity. Specifically, the organisation of the substrates allows multiple sensors to be placed under each cell at any given time-point, and multiple measurements made for individual cells. SEM and lateral force microscopy have been used to characterize these cantilevers and determine their spring constants.

Time-lapse microscopy in DIC mode with an onstage incubator and immersible objective has been used to observe the behaviour of various large cells with low-seeding density on untreated substrates; including human vascular endothelial cells (EC), smooth muscle cells (SMC), fibroblasts, myoblasts, as well as the smaller adult-derived hippocampal progenitor (AHP) cells. In particular, the forces associated with cell morphology during attachment and migration-in the presence or absence of contact guidance- were investigated, together with the substrate surface.

RESULTS: Images of the eariler sensor designs and time lapse movies can be found online at http://fy.chalmers.se/~petronis/cells/. Analysis of image sequences with custom written macros has resulted in real time mapping of cantilever deflections by cells, and their force-vectors. The nanoNewton range forces are greatest in the spreading cell periphery; and the leading or trailing edges in migrating cells, where they are parallel to the direction of movement.

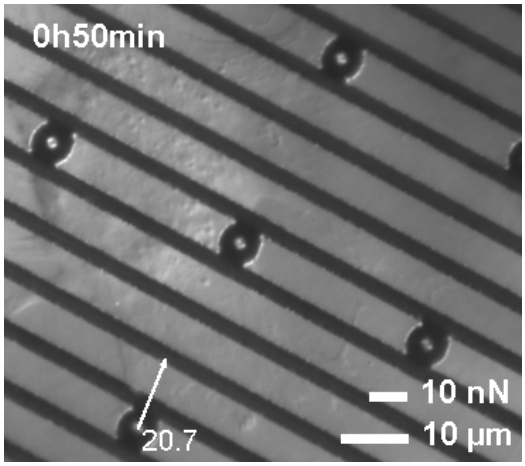

Fig. 1: Frame from time lapse sequence of endothelial cell (upper left in image) exerting a 20.7nN force on one of the senor pillars.

DISCUSSION & CONCLUSIONS: With live fluorescent membrane labels we can simultaneously image cell and substrate in two separate channels. The measurements are made in real-time, without the artefacts commonly associated with fixation. Ultimately, the aim is to use cells with enhanced fluorescent protein modified cytoskeleton components such as tubulin, actin and vinculin to correlate cytoskeleton organization and mechanics with force exertion.

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ISOTHERMAL MICROCALORIMETRY (IMC): A NEW IN-VITRO TECHNIQUE FOR DETERMINING BIOMATERIALS STABILITY & CELLULAR BIOCOMPATIBILITY

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INTRODUCTION: Some of the earliest scientific measurements were calorimetric—i.e. heat produced or absorbed during chemical reactions or changes of state. Bunsen (1811–1899) placed small animals on blocks of ice and used rate of melting as a measure of metabolic activity. Calorimetry has, of course, progressed enormously. However, not many biomaterials scientists are aware of the power of isothermal microcalorimetry (IMC) for quickly determining either rates of biomaterial degradation processes or magnitudes of cultured cell metabolic responses to biomaterials. This paper presents IMC advantages, illustrative studies, and possible future applications.

IMC ADVANTAGES: IMC has six attractive features for determining process kinetics and energetics. Sensitivity. A few grams of sample are usually sufficient for measuring even slow processes, such as oxidation of stable polymers. Rapidity. IMC can capture rate constants for slow processes in a few hours. Methods based on accumulation and quantitation of degradation products can require months. Simplicity. Studies of slow processes frequently can be carried out in sealed ampoules, with no need to monitor or replenish the environment, since there is often no significant consumption of reactants or accumulation of products. Universal Detection. Most rate monitoring techniques record a change in one property; e.g., pH, so the rate process must be understood in advance. IMC is a universal detector since heat is produced or consumed in all physico-chemical processes. Direct Determination of Kinetics & Energetics. Aggregate rates can be determined directly at one temperature using curve-fitting software to find a rate equation. Heat energy evolved or absorbed in transient processes (e.g., surface adsorption) can be obtained directly by integrating heat flow data. Re-Use of Specimens. If a slow process has been measured, the IMC specimen is little changed and thus available for other studies.

PREDICTING BIOMATERIALS STABILITY: IMC data are used routinely to quickly predict shelf life of solid pharmaceuticals. To our knowledge, IMC has not been used to study the stability of implant materials, other than in our recent work [1,2]. We have studied stability of UHMWPE, $CaSO_4$ bone void filler, and acrylic bone cement powders in air and buffered saline. One major finding was that radiation sterilization increases the oxidation rate of UHMWPE 7X-10X compared to EtO, and the rate difference persists after ~9 years (!) of post-sterilization shelf storage and/or clinical TJA implantation. Oxidation embrittles UHMWPE.

MEASURING CULTURED CELL METABOLIC RESPONSES: The first known IMC study [3] of cell metabolic response to biomaterials evaluated response of granulocytes to dialysis membranes. The order of difference in response to 4 different membrane materials correlated exactly with clinical studies of compromised granuloctye and leukocyte function. We used IMC to study response of transformed macrophages to TJA wear particles [4] (U.S. NIH-NIAMSD grant 1R41-AR44581-01). The rate of metabolic heat generated by ~$1.25x10^6$ cells ? ? RPMI was ~22 µW (~18 pW/cell). Exposure to ~$1.25x10^6$ of 1-6 µm gel-trapped particles (either Co alloy or HDPE) raised heat flow by 15-20 µW. LPS was used separately as a positive control. Also, LPS further increased metabolic response when adsorbed on HDPE, but not on Co alloy.

SUMMARY: IMC is a uniquely sensitive, rapid, direct means for quantitating both biomaterials degradation rates and responses of cultured cells to biomaterials. Planned IMC studies include surface adsorption of cells and bioactive molecules.

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Topographical Surface Effects on Cell Fate of Adult Human Mesenchymal Stem Cells

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INTRODUCTION: Bone marrow derived mesenchymal stem cells (MSCs) have the potential of self-renewal and can differentiate into various cell types like osteoblasts, chondrocytes and adipocytes. Moreover, they are among the first cells contacting the bone implant surface. The surface topography influences the adhesion, motility, proliferation and differentiation of cells in a cell-type and -state dependent way. This study investigates the effect of defined surface topographies (i.e., hemispheres with a diameter of 30 or 50 μm) on the adhesion, the morphology and the migration and further the effect on cell fate determination of adult human MSCs (HMSC).

METHODS:
Adult HMSCs were isolated from patients obtaining a total hip prosthesis. After the first passage, the cells were either plated at a density of 5000 cells per cm^2 and stained on day 7 (Fig. 2) or directly DiI labeled and plated at a density of 20000 cells per cm^2 and live monitored the following day (Fig. 1). The cells were cultivated in α-MEM plus 10% FCS and 1% antibotic mixture and investigated using the confocal laser scanning microscope (CLSM). The migration data analysis was done using visiometrics-iPS and -trace software (University of Konstanz, Germany).
The structured cell culture dishes were fabricated by injection molding. The unit of the topographies consisted of either hemispheres of 50 μm in diameter and no spacing (50/0) or hemispheres of 30 μm with a spacing of 20 μm (30/20). The surface structures were characterized using scanning electron microscopy.

RESULTS: Adult HMSCs on (30/20) exhibited a significant increased migration velocity compared

to cells on reference (flat) surface (Fig. 1) and cells were predominantly located around and less often on the hemispheres (Fig. 2, B). The cells on (50/0) showed smaller migration capacity (Fig. 1) with cells spanning across the hemispheres (Fig. 2, C). On 50/0 the focal adhesion points were located on the top of the hemispheres and frequently observed in a radial arrangement (Fig. 2, D).

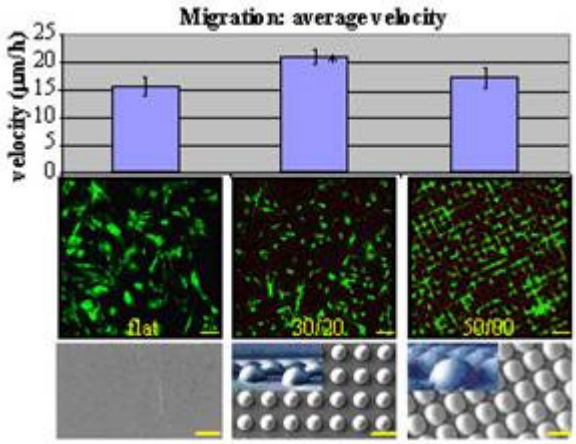

Fig. 1: Migration of adult HMSCs on the different topographies. Bar; 50 μm.

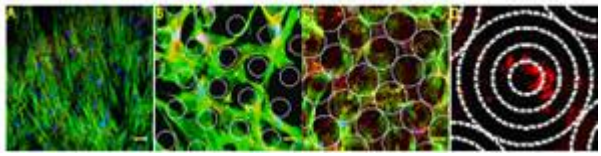

Fig. 2: Adult HMSCs were stained for F-actin (green), focal adhesion point; vinculin (red) and nuclei (blue). (A) flat, (B) 30/20, (C) 50/0. (D) single 50/0 hemisphere. Bar; 20 μm.

DISCUSSION & CONCLUSIONS:
The fact that HMSCs do not spontaneously differentiate [1] rises the possibility to guide this process *via* surface topography. The first results indicate that the surface topography influences the morphology and migration of HMSCs. In further experiments it has to be shown which effects the surface topography have on cell fate determination. The combination of the results may allow in the future the design of newly structured bone implant surfaces for medical applications.

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"Bioinspired" Interface Between – Old And New - In Medicine

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In medicine, engineered tissue substitutes (THE NEW) need to be truly "bioinspired" to have an evolutionary significance: as in the case of the evolutionary strategy, which has allowed successful vertebrate survival (THE OLD), function follows form. This involves a progressively positive role for the repetition of the cycles that make function follow form, the latter in turn following function. The methodological translation of all this is the fact that the first phase of the construction of an engineered tissue begins with the design and realisation of a metal scaffold, polymeric or mineral, serving as a substrate and interface for the growth of the cells that will give rise to the new tissue. These structures are open systems that encourage implanted cells to interact with the host tissue and form a new tissue, which after degradation of the artificial support will become structurally integrated.

"INDIRECT" INTERFACE INTERACTION

COMPUTER-AIDED DESIGN (CAD) – TISSUE-ENGINEERED INTERFACE

The development of image processing technologies (CAD/CAM) has also contributed to tissue engineering, especially to what has come to be called Computer-Aided Tissue Engineering (CATE). Magnetic resonance imaging (MRI) and computed tomography (CT) now allow computer design of the internal structures of tissues and organs (CAD/CAM System), allowing to predefine the micro- and macroscopic scaffold structure to obtain the finished product (*Fig. 1*). This is realised in the most appropriate material (polymers, metals, minerals), which after implantation in the body will promote tissue reconstitution in the desired shape (*Table 1*).

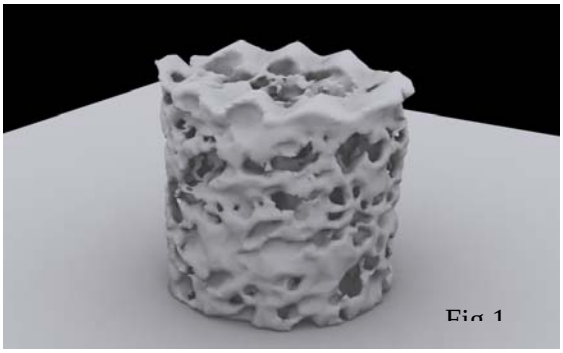

Fig.1

Table 1

MICROMORPHOMETRIC EVALUATION OF THE CONNECTIONS NETWORK WITHIN THE SCAFFOLD CAD-RECONSTRUCTED (CROSS SECTION)		
	DIAMETER	PERIMETER
Mean Value ± SD	568.9 ±146.9μm	4411.4 ±8 61.5μm

"DIRECT" INTERFACE INTERACTION

NANOTECHNOLOGICAL INTERFACE

Traditional manufacturing methods do not allow to make scaffolds with predefined 3D structures. However, in the manufacturing of devices with 2D structures surface topography, both chemical and geometrical, has a critical role in material biointegration with the tissue to be repaired. This can be achieved with recent techniques that allow to make the surface of these materials "nanostructured", i.e. characterised by high affinity for the molecular organisation of the cells that interface with it. Information on cells (morphology, doubling time, adhesion, etc.), materials (surface morphology, mechanism of degradation, contact angle, chemical composition, etc), and their interactions will be collected in a database endowed with a relational structure. The highly structured organisation of the data will allow both a user-friendly presentation of the information to improve the reasoning process and also the implementation of an artificial intelligence-based tool to indicate, in an objective way and in semiquantitative form, different degrees of compatibility between possible new materials and the cells considered.

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Cell adhesion increases with the square root of time

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INTRODUCTION: A still unsolved question in biomaterial field is how eucaryotic cells adhere on material surface and what are the main surface properties influencing cell adhesion [1,2] whereas cell adhesion is one of the crucial initial events affecting further proliferation, differentiation and survival of cells on substrate surface [1]. Here we report for the first time the mathematical modeling of cell adhesion on biomaterials taking into account simultaneously the combined influence of material nature, surface topography, and surface chemistry.

METHODS: Using an original detachment method[3], adhesion of primary human osteoblasts was quantified on 30 different substrates made of 3 different metals (pure titanium, titanium alloy Ti6Al4V, 316L stainless steel) with 5 various surface morphologies obtained by different processes (sandblasting, electro-erosion, polishing, machining, acid etching), 2 roughness amplitudes (0.85 µm and 2.35 µm), coated or not with a gold-palladium layer. As this evaluation was performed at early stage of culture (24 hours) as well as later stages (until 21 days), the measure of adhesion concerned either cells having proliferated or cells adhering since the inoculation time. Therefore a mathematical treatment of data to de-correlate proliferation and adhesion was applied.

RESULTS: Afterwards, using more than 2000 experimental detachment data, we demonstrated that cell detachment (δ) varied with culture time (T) according to a power law: $d(T)=aT^b$. *a* being independent from *b*. The exponent *b* was equal to 0.5 ±0.03 and was independent of the substrate characteristics. On the contrary, the parameter *a* we called the "adhesion power" did depend significantly on the material nature, the surface topography, and the surface chemistry of the substrate. We notably demonstrated that the adhesion power better correlated better, among 75 other surface roughness parameters, with the Order parameter which describes the morphology of the surface roughness.

DISCUSSION & CONCLUSIONS: This first attempt to model cell adhesion from experimental data allowed us to demonstrate that the cell adhesion increased with the square root of time. From this relationship, we suggest that a diffusion-based process may be involved in adhesion on materials. It could be the consequence of diffusion of proteins in the cell/material interface confirming previous hypothesis on dynamics of cell adhesion[4].This finding will be important for further optimization of implant surfaces.

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Development of hydroxyapatite-based biomaterials scaffolds for hard tissue regeneration

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INTRODUCTION: Hydroxyapatite (HA, $Ca_{10}(PO_4)_6OH_2$)[1] has been widely used in tissue engineering specially in bone and cartilage regeneration. Sol-gel technology offers an alternative technique for producing bioactive and osteoconductive HA. Because HA can be described as a multisubstituted calcium-phosphate apatite, the presence of small concentration of ions, such as silicon, play an important role in the biochemistry of the hard tissue. The aim of this research project is to improve HA biocompatibility and bioactivity and to develop a methodology able to tailor HA scaffolds appropriate for any specific biomedical application through controlling composition, impurities concentration, crystal size and morphology.

METHODS:
HA was synthesized by sol-gel method using $PO(Et)_3$ and $Ca(NO_3)_2$ as P/Ca precursors respectively. Silicon was introduced into the HA matrix using TEOS as reactant[2]. The obtained gel was washed in water overnight and freeze-dried to achieve the desired porosity. In order to obtain the HA crystalline phase, samples were calcined for 4 hours at 400ºC. HA was tailored into tablet shape using a 5 tones press and bioactivity studies were performed by soaking HA tablets into simulated body fluid (SBF). Cell attachment assays were performed by culturing human mesenchymal stem cells on top of HA and silicon-modified HA tablets.

RESULTS:
HA obtained by the sol-gel method was observed as a porous structure under a SEM.
HA tablets modified with 11% silicon were immersed in SBF for one week and analysed by XRD every other day. Diffractograms showed cristallity peaks increased from day 1 to day 7. SBF samples were also analyzed by electrophoresis to detect Ca release and an increase in Ca concentration was observed due to a Ca delivery from the HA tablet matrix to the SBF. Cell attachment assays showed higher cell density when cultured on silicon modified substrates in all percentages studied (Figure 1). Samples containing a smaller percentages (4%, 11%) of silicon showed a 4 fold increase on cell attachment compared to non-modified HA, while higher amounts of silicon (28%) showed a 2 fold increase.

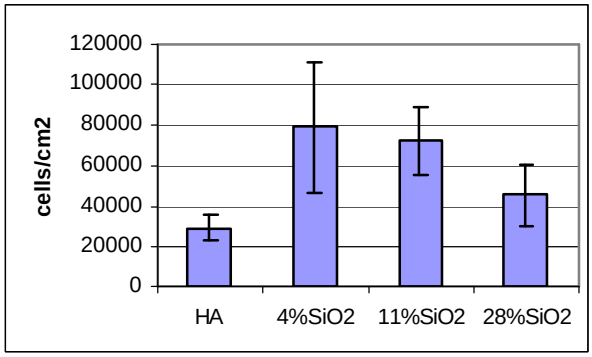

Fig 1: Cell attachment assay on different HA substrates. Cells were counterd 90 minutes after seeding.

DISCUSSION & CONCLUSIONS:
It is possible to obtain different HA porous structures for different applications demonstrating the sol-gel versatility. XRD of silicon modified HA after immersion in SBF showed an increase in HA cristallinity. On the other side, Ca release observed in electrophoresis results showed that the increase in cristallinity may be due to a stoichiometric reorganization in the HA matrix. In addition, the interaction between HA tablets and the SBF is indicative of HA bioactivity. Preliminary cell culture results demonstrate that HA obtained by sol-gel methodology is a biocompatible matrix. The addition of SiO_2 in the matrix may modify biological properties as observed the increase of cell attachment in the performed cell culture assays. Further experiments will be addressed in order to modify HA based biomaterial surfaces to obtain a total integration betwen the tissue and the scaffold.

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Polymer and carbon fibers with HAp nanopowder: properties and biocompatibility of degradation products

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INTRODUCTION: Biomimetic fibrous biomaterials were used in reconstructions and replacement of ligaments, tendons, hard tissues and cartilage defects. Biomaterials made from polymers and carbon phases implanted into bone tissue are generally encapsulated with fibrous tissue and become insolated from surrounding bone. Among most of biomaterials only bioactive ceramics (Bioglass, hydroxyapatite) spontaneously bonds to bone and strongly integrates with the living tissue. The goal of this work was to manufacture and to investigate bioactive carbon fibers obtained from polyacrylonitrile polymer precursor containing nanoceramic particles of HAp [1-5].

METHODS: Morphology, microstructure and chemical analysis of polymer, carbon and ceramic nanopowder (HAp) were characterized by SEM, EDS microanalysis and FTIR method. *In vitro* bioactivity test of fibers containing HAp nanoparticles was performed in SBF solution buffered at physiological pH=7.4, at 37 ^{0}C. The samples of powdered carbon fibers modified with HAp were implanted into gluteal muscles of rats. Resulting tissue reaction to implant degradation products were estimated by studying the activity of three enzymes as a function of time, acid phosphatase, NADH dehydrogenase and cytochrome c oxidase. The presence of cells involved in immunological response (macrophage, mast cells, eosinophils, neutrophils) and cells of granulation tissue was assessed in sections stained by the Pappenheim method.

RESULTS: As a result of *in vitro* activity test indicates that the surface of HAp – modified carbon fibers stimulates biomimetic growth of apatite. With *in vivo* examination we have not observed toxic reactions from rat cells and tissues resulting from the presence of degradation products of HAp- modified carbon fibers.

CONCLUSIONS

It was shown that biomimetic material in the form of ceramic modified fibers is bioactive, and its degradation products are biocompatible.

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The effect of AdBMP-2 on ovine osteoblasts and BMSC including a comparison to human and murine cells

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INTRODUCTION: Osteoinductive cytokines like Bone Morphogenetic Protein-2 (BMP-2) are able to accelerate fracture healing by stimulating differentiation into the osteoblastic lineage. However the rapid proteolysis and the need for a carrier matrix are relevant disadvantages for clinical application of recombinant proteins. Gene transfer offers an attractive technique that allows in situ production of growth factors. Direct administration of adenoviral vector encoding hBMP-2 showed enhanced bone formation in small animal models, but failed to accelerate fracture healing using a sheep model.

Human BMP-2 enhances osteoblastic activity and differentiation of bone-marrow derived mesenchymal stem cells (BMSC) into osteoblasts in human, murine and lapine cells, but there is no data available for ovine osteoblasts and BMSC.
This study evaluates the effect of an adenoviral vector encoding hBMP-2 on ovine osteoblasts and BMSC including a comparison to human and murine cells with respect to osteoblastic differentiation.

METHODS: For primary culture of osteoblastic cells, trabecular bone was harvested from iliac crests of adult White Mountain Sheep, from the calvaria of Swiss Wistar rats and from human femur head (after approval by the Swiss Ethic Committee). The cells were isolated enzymatically. The BMSC were isolated from bone-marrow taken from ovine iliac crests or murine femora followed by Ficoll-gradient.
Cells were cultured to confluence and then subcultured in 24-well plates, 30000 cells per well. After 24h, cells were transfected with a recombinant, first generation type 5 adenoviral vector encoding human BMP-2 (AdBMP-2) using a concentration of 10^7 to 10^8 particles per well (50 pfu/cell). For control, cells were treated with recombinant BMP-2 (50ng/ml), were transfected with an empty vector or left untreated. After 3days an osteogenic medium containing 0,1mM ascorbic acid and 1mM β-gycerophosphate was added.
For evaluation BMP-2 was measured in the media (ELISA, R&D), alkaline phosphatase activity (ALP) (Sigma) was determined at day 4, 8 and 14, mineralization was tested by incorporation of radioactive calcium (Ca^{45}) at day 21. Total DNA was measured 4 and 14 days after transfection by fluorometric method to normalize the results. All analyses were performed in triplicates.

RESULTS: Four days after transfection with Ad.BMP-2 ovine osteoblasts and BMSC produce 138.4±106.8ng/ml and 65.2±2.5ng/ml BMP-2 respectively. After Ad.BMP-2 treatment the alkaline phosphatase activity increases over time with highest values at day 14 (osteoblasts: 6.1-127.8-fold, BMSC: 2.7-42.8-fold increase compare to control) and an increase of calcium incorporation (osteoblasts: 1.1-163.2-fold, BMSC: 0.9-6.4-fold). The total DNA content decreased after transfection (Ad.BMP-2 and empty vector), in the BMSC more than in the osteoblasts, representing a lower cell number. No significant changes in ALP and Ca45 were observed when cells were treated with empty vector or rhBMP-2. Large interindividual differences were found in all parameters. ALP did not correlate to age of donor or amount of BMP-2 in the media.
The human and murine cells showed the same tendency in ALP, calcium incorporation and DNA content. The amount of BMP-2 produced after transfection was lower in the murine and human cells compared to ovine cells.

DISCUSSION & CONCLUSIONS: The present study used an adenoviral gene transfer approach to show that human BMP-2 enhances the activity of ovine osteoblast and differentiation of ovine BMSC into the osteoblastic lineage. Decreased cell number after transfection might indicate vector toxicity. No effect was seen after treatment with recombinant BMP-2 albeit the added amount of BMP-2 was similar to the concentration seen after transfection. This might be due to higher biological effectiveness when a gene transfer approach is used. The comparison between the three different species did not show significant differences after treatment with Ad.BMP-2 or rhBMP-2, although the comparison is limited due to difficulties to culture cells identically. However, human BMP-2 is able to stimulate bone formation in species different to humans.

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Interactions between Cell and Calcium Phosphates Depend On Chemistry: Theoretical Considerations

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INTRODUCTION: In the last 3 decades, the number of publications devoted to calcium phosphate (CaP) materials has exploded. This is partly due to the discovery of CaP cements which allows the synthesis of granules and blocks with chemical and physical properties similar to the CaP crystals present in bone. These CaP crystals are octocalcium phosphate (OCP), dicalcium phosphate dihydrate (DCPD), and nanocrystalline apatites, such as calcium-deficient hydroxyapatite (CDHA), hydroxyapatite (HA) or carbonated apatite (CA).

So far, there is still a poor understanding of the mechanisms of dissolution-degradation-resorption occurring when these materials are implanted *in vivo*. The goal of the present communication is to elucidate these mechanisms. For that purpose, the solubility of calcium phosphate phases in serum is calculated. Results are then discussed and compared to *in vivo* results.

METHODS: To perform the solubility calculations, the following conditions were chosen (see also [1]): (i) The equilibrium of 42 inorganic species that are present in serum were considered [2]. (ii) The ionic strength and the pH of serum were assumed to be 0.15M and pH 7.4 respectively. (iii) The activity of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HPO_4^{2-} and HCO_3^- was varied until the total concentration of sodium, potassium, calcium, magnesium, chlore, phosphate and carbonate corresponded to the concentrations present in serum [3] (iv) Based on the concentrations of the different ions and the phase equilibria, the saturation of each calcium phosphate phase was determined. As a concentration range is given for calcium and phosphorus in serum [3], calculations were made with the minimum and maximum concentrations of calcium and phosphorus.

RESULTS: Solubility calculations show that serum was saturated toward DCP, OCP, β-tricalcium phosphate (β-TCP), CDHA, CA and HA (Table 1), indicating that these compounds do not spontaneously dissolve *in vivo*. The opposite is true for α-tricalcium phosphate (α-TCP). Finally, DCPD is a special case, since it is soluble in a serum containing a low concentration of calcium and phosphorus and insoluble in a serum containing a high concentration of Ca and P.

Saturation	DCP	DCPD	OCP	β-TCP
Low Ca & P	0.05	-0.11	0.21	0.52
Av. Ca & P	0.13	-0.02	0.29	0.59
High Ca & P	0.20	0.04	0.35	0.66
Saturation	α-TCP	CDHA	HA	CA*
Low Ca & P	-0.14	0.31	1.31	0.58
Av. Ca & P	-0.07	0.38	1.38	0.64
High Ca & P	0.00	0.45	1.44	0.70

*$Ca_{10}(PO_4)_6(CO_3)_{0.04}(OH)_{1.92}$

Table 1: saturation of serum towards various CaP phases. A positive value indicate that serum is saturated, hence preventing spontaneous dissolution.

DISCUSSION & CONCLUSIONS: Based on these observations, we can conclude that due to their solubility in serum, DCPD and α-TCP are expected to be dissolved *in vivo*, whereas the other calcium phosphates are expected to be removed by cells such as osteoclasts and macrophages. Interestingly, CDHA, CA and β-TCP have similar saturations in serum, suggesting that these three compounds should be resorbed at the same rate.

These conclusions are coherent with experimental *in vivo* data except for DCPD [4] and α-TCP. These two phases tend to be converted into nanocrystalline apatite, hence reducing the apparent resorption rate. However, in the presence of inhibitors of apatite growth, conversion can be postponed [5].

The conversion into an apatite is expected to occur at constant pH for α-TCP and at decreasing pH for DCPD. As DCPD solubility increases at lower pH values, DCPD conversion is autocatalytic. However, as apatites do not precipitate below pH 7.0, conversion proceeds at a controlled rate.

In conclusion, solubility calculations can be used to predict and understand the *in vivo* behavior of calcium phosphates.

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TRANSFORMING GROWTH FACTOR BETA-3 (TGF b-3) ACTIVITY IS RELEVANTLY DEPENDANT ON CARRIERS DIFFERENCES WHEN USED FOR A CRITICAL MANDIBULAR BONE DEFECT REPAIR IN A *BEAGLE* DOG MODEL.

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INTRODUCTION: For mandibular bone regeneration, osteogenic growth factors might be a good alternative to the *gold standard,* autologous bone grafts. They have to be carried and properly delivered to the appropriate site and biological and biomechanical properties of new bone have to be similar to normal bone. Transforming growth factor β (TGF-β1, TGF-β2 and TGF-β3) and their receptors are found in every ossification site. TGF-β3 is the main isoform in intramembranous bones, as the mandible. Tricalcium phosphate β (β-TCP) and polylactide acid (PLA) derivates are osteoconductive substances used as carriers.

AIMS: To evaluate TGF-β3 as an osteodynamic substance in a critical size mandibular bone defect, in a *Beagle* dog model. To evaluate Tricalcic phosphate (TCP) granules and Poly(L/DL-lactide) sponges 70/30 (PLA) as TGF-β3 carriers.

METHODS: 30 Beagle dogs, both sexes, 21-24 months old, randomly distributed to five groups (n=6). Six months after edentation a 30 mm long complete unilateral mandibular bone defect, including the periosteum, was surgically created. Stabilization with AO titanium 2.4 mm locking mandibular reconstruction plates. The surgical bone defect filling: 1) autologous cancellous bone; 2) TCP alone; 3) PLA alone; 4) TCP+TGF-β3 and 5) PLA+TGF-β3. A thermoplastic (70ºC) custom-made poly(L/DL-lactide) 80/20 biodegradable membrane for surrounding of reconstructed area. Specimens harvest at 26th week. Qualitative and quantitative bone evaluation: 1) contact radiography; 2) Quantitative Computed Tomography; 3) nondestructive mechanical tests (four point bending); 4) histomorphological evaluation (Giemsa-Eosin; intravital fluorochromes; *permortem* staining with India Ink for vascular evaluation); 5) Scanning acoustic microscopy of new bone impedance, density and elastic modulus.

RESULTS: The cancellous bone graft group presented 3 mandibles (50%) with a structure that can be considered adequate for normal use, 2 with incomplete bone formation and 1 with minimal bone formation. PLA or TCP groups showed minimal bone formation. TCP plus TGF-β3 group presented a partial bone bridging. The PLA plus TGF-β3 group showed a minimal bone formation and an exuberant cellularity and angiogenesis. Membranes delimited osteogenesis and anatomical contour to the shape of a normal mandible.

DISCUSSION & CONCLUSIONS: TGF-β3 had an osteogenic effect but amount and quality of the regenerate was not equivalent to cancellous bone grafting. The experimental growth factor used, TGF-β3, seems to induce high mesenchymatous cell stimulation at the angiogenic and/or osteogenic level, depending on significant differences between different carriers. Further investigation for establishment of the appropriate carrier seems to be necessary. Osteopromotive membranes are recommended for anatomical contour and osteogenesis delimitation in this *Beagle* dog mandibular reconstruction model.

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Evaluation of bioactive glass 13-93 fibre-based scaffolds for healing of large bone defects

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INTRODUCTION: Restoration of large bone defects or construction of new bone structures during reconstructive surgery requires the use of autologous bone transfer or the use of bone-supporting bioactive material. In this study we investigate bioactive glass (BaG) 13-93, poly-L/DL-lactide (PLDLA) 70/30 and their combinations for their suitability as artificial bone in craniofacial plastic surgery

METHODS:

In vitro analysis: Rabbit osteoblast-like cells (OBs) were obtained trypsin-collagenase treatment of bone explants, expanded in vitro using DMEM supplemented with 10% FCS, induced for 1-2 weeks with dexamethasone, glycerophosphate and vitamin C and characterized by determination of alkaline phosphatase (AP) activity. Human osteogenic sarcoma cell line SaOS2 was transduced with a retrovirus expressing green fluorescent protein (GFP) to monitor cell growth and density within three-dimensional scaffolds. Cells were grown and monitored by microscopic inspection.

In vivo analysis: Biopsies were taken from the iliac crest of a 6 to 8 week female rabbits. The harvested bone samples were digested using collagenase/trypsin and cells expanded in vitro for about 3 weeks. After characterisation of the expanded cells using a commercial AP kit, and AP staining, OBs were transferred onto the scaffolds and incubated for 48 h prior to implantation. A critical sized bone defect of 1 cm^2 diameter was made in the calvaria of the cell donor on both sides of the head. Group 1 was treated with BaG 13-93 fibrous scaffold attached to thin PLDLA 70/30 plate. Group 2 received BaG 13-93+PLDLA70/30 (as group 1) plus cultured OBCs. In Group 3 the defect was left untreated. The experimental setting comprised 9 animals, 3 in each group. The observation period was 3 wks and the healing process was assessed using serial weekly radiography and histology.

RESULTS: In vitro cultivated cells derived from rabbit iliac crest were found to be AP positive (Fig.1). Rabbit as well as human sarcoma cells grew on three dimensional scaffolds to high cellular densities (Fig. 2) indicating that BaG 13-93-derived material supports the growth of OBCs in vitro.

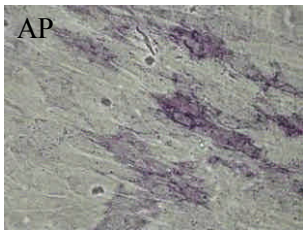

Fig. 1: In vitro cultivated bone-derived rabbit cells stained for AP activity (400x).

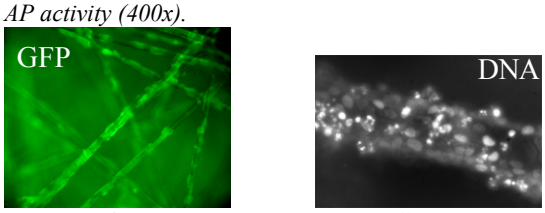

Fig. 2: Left: GFP expressing SaOS2 cells grown on BaG 13-93 fibrous scaffolds (100x). Right: 400x magnification of DNA stained SaOS2 cells.

In contrast, when the three-dimensional scaffolds were implanted into rabbits to cover a large bone defect, they induced a strong inflammatory response (Fig. 3).

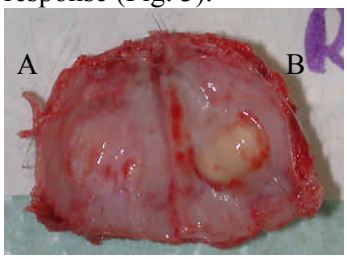

Fig. 3: Bone explant showing the dura mater covering the operation field. (A) 2-D PLDLA 70/30 plate, (B) 3-D BaG 13-93 fibrous scaffold with bulging white mass

This response was not caused by the pretreatment regimen of the 3-D scaffolds as also observed when scaffolds were pre-incubated with phosphate buffered saline. The 2-D PLDA 70/30 plates did not initiate an inflammatory response. (Fig. 2A).

DISCUSSION & CONCLUSIONS: BaG 13-93-derived 3-D scaffolds support the growth of OBs in vitro but induce a strong inflammatory response

in vivo. In addition, due probably to, the rapid degradation of bioactive material in vivo.

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In Vitro Neuron Interactions with Oriented Electrospun Fibres

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INTRODUCTION: Current electrospinning collection techniques typically result in random 'mats' of material [1], while for neural tissue engineering, orientated structures are of interest as guidance scaffolds [2]. Electrospun fibres have a high surface area to mass ratio; potentially providing significant surface-mediated signals for therapeutic cells while minimizing the quantity of degradation products. The ultimate aim is to orient, functionalize and determine their efficacy as a scaffold for neural tissue engineering.

METHODS: Poly(ε-caprolactone) (PCL) was dissolved in a mixture of chloroform and methanol (75/25 v./v.) to make a 9 wt% solution. The polymer solution was pumped to a 16-gauge, flat-tipped, stainless steel spinneret at a rate of 0.1 mL/h. Two stainless steel collection rings (35 mm OD, 25 mm ID) were positioned and a voltage of 15 or 25 kV was applied for 30 s to create an electric field of 1 or 1.67 kV/cm. The collected array of fibres was deposited on a glass slide and sputter-coated with gold. Telencephalon neurons were isolated from day 6/7 chick embryos and cultured on gold-coated electrospun fibres and slides in serum-free media for 8 days.

RESULTS: An array of electrospun fibres was collected between the dual ground rings after electrospinning (Fig. 1). Fibre splitting was controlled by voltage, with 15 kV producing single fibres with lengths of 8 cm and diameter of 1.26±0.19 μm and 30 kV resulted in web-like structures due to extreme fibre splitting (Fig. 2).

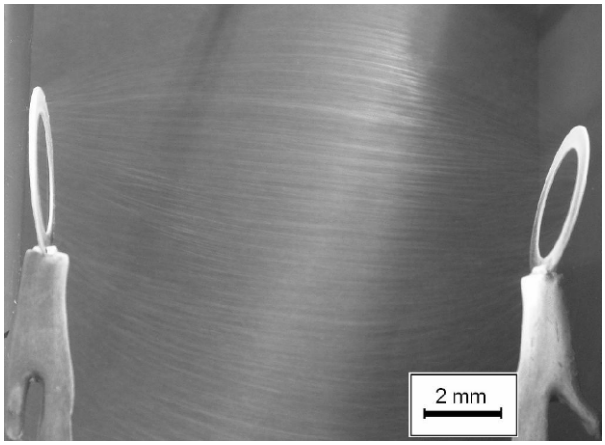

Fig. 1: An array of collected electrospun fibres.

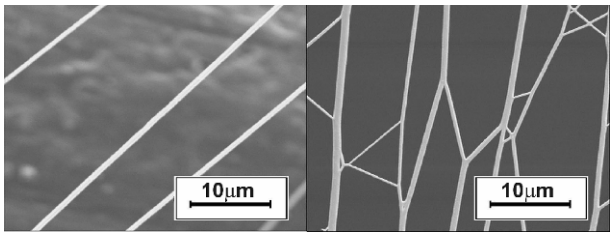

Fig. 2: Effect of voltage on electrospun fibres: 15 kV (left) and 25 kV (right).

As would be expected on gold surfaces, cell adhesion was poor; however neurites that extended from telencephalon neurons were influenced by the shape of the electrospun fibre, which is of similar dimensions to the neurite itself, and were guided along the length for some distances.

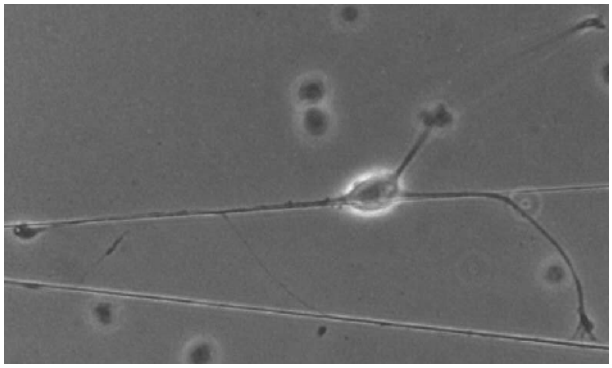

Fig. 3: A neuron after culturing on an electrospun fibre.

DISCUSSION & CONCLUSIONS: We have been able to produce an array of oriented electrospun fibres that are suspended between two collection plates. Lower voltages resulted in less fibre splitting and interconnectivity, with single electrospun fibres with lengths of up to 8 cm, and a diameter of 1.26±0.19 μm. These fibres, when placed upon flat surfaces, had a guiding effect on the extended neurites.

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Comparative study of *in vitro* colonization of different biomaterials by rabbit stem cells and macrophages of rabbit and humans

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INTRODUCTION: Different biomaterials have been able to function as osseal implants. Between them Hydroxyapatite, marine corals and polymethylmethacrylate are widely used. The interaction of cells with a biomaterial is a prerequisite for tissue regeneration.

The aim of this work was to follow the interactions between bone marrow cells and peritoneal macrophages of New Zealand Rabbits and human monocytes THP-1 with several biomaterials: hydroxyapatite (HA), a marine coral specie *Porites astroide* and polymethylmethacrylate (PMMA) by *in vitro* studies. The process was followed by optical, scanning and transmission electron microscopy and by bioactivity tests.

METHODS: Peritoneal macrofages (Mϕ) were extracted from New Zealand rabbits and were cultured in plates of 24 ponds with a density of 2.10^6 cell/pond in 500 µL of RPMI-1640 supplemented with fetal bovine serum 10%, 1mM L-glutamin, 50 U/mL of penicillin/streptomycin. The human monocytes THP-1 were also incubated by the same procedure. These cells were stimulated with the different biomaterials seeding them onto each one during 24, 48 and 72 h.

Cellular activity was followed by optical microscopy and scanning electron microscopy using a Hitachi S-4500 field emission SEM and transmission electron microscopy (TEM) using a Phillips CM10 operating at 80 keV. Also tumoral necrosis factor (TNF) determination was carried out. Experiments using rabbit bone marrow cells were also performed. The cells were incubated in minimum essential medium supplemented with 10 nM of β glycerophosphate, 80 µg/mL of L-ascorbic acid 12000 unit/mL of penicillin, 12 mg/mL of streptomycin and 30 µg/mL of anphotericin B. The cells were placed in a 24 pond plate with a density of 2.10^4 cell/pond in 500 µL. After 15 days fibroblast were observed by optical microscopy. These were stimulated with the different biomaterials under the same conditions used for the Mϕ for 24 h. After this period the alkaline phosphatese activity test was performed and also samples for transmission and electron microscopy were prepared.

RESULTS: The cells stimulated with hydroxyapatite presented abundant vacuoles and small deformations compared to the control cells, as observed by SEM. The observation by SEM showed morphological changes consisting of lost of the spheroidal shape characteristic of this type of cells, also a slight increase in the formation of cytoplasmatic digitations or filopodia which is expected as a typical cell reaction to an antigen presence.

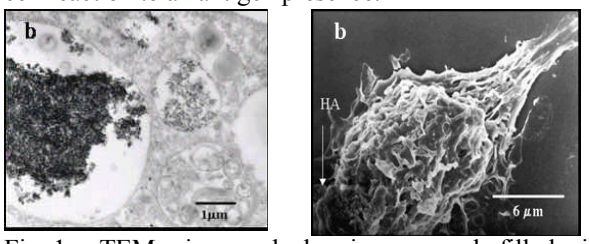

Fig. 1 a. TEM micrograph showing a vacuole filled with HA. b. Scanning electron microscopy image of a macrophage after 24 h activation with HA showing formation of filopodia.

The peritoneal macrophages (Mϕ) stimulated with HA also presented vacuoles, and some morphological changes were observed by SEM, ridges on the surface and an increase in size. TEM observation showed HA particles inside the vacuoles showing that a phagocytic process has occurred after only 24 h (Fig.1) The values of TNF concentration obtained from the macrophages cell culture were significantly high indicating that HA activates the macrophages cells. A similar behavior was observed for the cells stimulated with marine coral material. A very different response was observed on the cells seeded to PMMA. The material does not seem to cause any activation to the cells. No morphological changes and very low TNF levels were observed.

CONCLUSIONS: The coral specie *Porites astroide* showed the highest level of activation to macrophages and monocytes. The fibroblast cells phagocytated the HA and the coral material indicating the initial stages of the mineralization process.

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Lipid coating influences cell adhesion and differentiation

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INTRODUCTION: The use of metal implants is appropriate for a broad range of applications in bone and cartilage repair or substitution. For a better acceptance of the implant, coating of the implant surface is one of the main interests in current research. The aim is to minimize the influence of the artificial material on the cells of the surrounding tissue. A promising but so far not thoroughly investigated approach is the covering of the surface by lipids. By mimicking the surface to which the cells adhere with a model membrane, the artificial character of the implant is hidden and the implant recognition by the surrounding tissue avoided [1].

METHODS: We used porous and compact Ti-6Al-4V (composition in mass percent) plates as metal carrier. Covering of the surfaces was done with lipid layers, by applying 30 µl of palmitoyl-oleoyl-phosphatidyl-ethanolamine (POPE) in chloroform:methanol (80:20) on the surface of the plates and evaporation of the solvent. The characterization of the lipid layers was done by epifluorescence microscopy and SEM (Leo Gemini 1550 VP).

Human chondrocytes were proliferated up to seven passages with bFGF (basic fibroblast growth factor). Cell adhesion was investigated by applying the chondrocytes onto the carrier, where they were left for two days in medium with 10% fetal calf serum (FCS). Afterwards the number of adhered cells was measured indirectly by DNA-content; cell morphology was investigated by low-voltage SEM. The chondrogeneic potential of the cells was examined by applying chondrocytes on the carrier, where they were proliferated in a medium containing FCS and bFGF for seven days and afterwards "switched" to a medium supplemented with insulin-like growth factor-I (IGF-I), transforming growth factor ß1 (TGF-ß1), Interleukin-4 (IL-4) and human serum for three weeks to induce chondrogenesis. The tissue was analyzed biochemical for its amount of glycosaminoglycans, DNA and collagens.

RESULTS: All cells adhered to native surfaces show a flattened, elongated morphology resembling the adhesion to tissue culture plastic. During proliferation on the native carriers chondrocytes tend to assemble. On materials coated with POPE the cells show a completely different behaviour. The major parts of chondrocytes develop a more rounded shape and show no assembling tendency (figure 1). The chondrogeneic potential is influenced in a passage-dependent manner. In early passages matrix secretion of chondrocytes is higher on coated surfaces (up to 130% of the native carriers), while severely passaged cells secrete only a small amount of cartilage-like tissue, but significantly more on the native carriers.

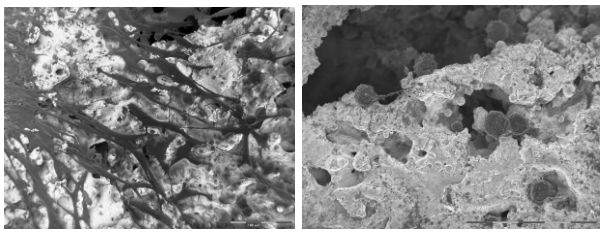

Fig. 1: Morphology of adhered chondrocytes on native (left) vs. POPE-coated carriers (right). Scale bar: 100 µm; Low-voltage field-emission SEM, 0.3 kV.

DISCUSSION & CONCLUSIONS: We used a simple system to modify implant surfaces with lipid layers. The simplicity of this system allows us to test the influence of modifications easily on different materials. The first results with this model have clearly shown that the lipid layer alters cell adhesion behaviour and chondrogenesis. The mechanism of this alteration is not yet known, but we propose a similarity between the POPE-layer and a natural cell membrane, leading to a *in vivo*-like cell adhesion with the chondrocytes remaining in a differentiated phenotype. Also, the influence of the underlying material can be excluded by this coating. Further experiments will focus on the effects of the lipid layers on other cell types, such as osteoblasts or mesenchymal stem cells.

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ACKNOWLEDGEMENTS: The authors wish to thank Peter Adamietz from the IMBM 2, University Hospital Eppendorf and Reinhard Bredehorst, Department of Biochemistry and Molecular Biology, University of Hamburg for expert technical assistance and helpful discussions.

Mineralisation and Cross-Linking Techniques to Improve the Mechanical Properties of Collagen-Calcium Phosphate (Coll-Cap) Composites to be used as Bone Analogues

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INTRODUCTION: Bone is subjected to such widely varying stress conditions that no one material can effectively handle them all (Currey 1984). A composite material is nature's answer to this problem. Bone as a material can be thought of as a polymer-ceramic composite, made up of a fibrous protein matrix (collagen) that is stiffened by a mineral phase (carbonated hydroxyapatite) with water acting as a plasticizer and porosity/matrix structure essential for nutrient transport and mechanical response. Trying to engineer an adequate replacement for bone - fulfilling all the roles of natural bone - is a tall order. However an adequate temporary replacement, whose primary role is that of support, is within grasp.

METHODS: Collagen-Calcium Phosphate composites (Coll-CaP) are novel materials that have the potential to be used as bone analogues. These composites can be made in a number of ways we produce Coll-CaP composites by the precipitation of calcium phosphate from aqueous solution into a collagen matrix (Lawson 1998). A collagen sheet (20 - 200 µm thick) separates reservoirs of calcium (30 mM) and phosphate ions (50mM). The pH of the precipitation reaction is kept at a constant value of pH 8 by computer controlled auto-burettes. The reaction is kept at the physiological temperature of 37 ^{o}C using a water bath and terminated after 12 hours of precipitation. Precipitation conditions; pH and ion concentration are controlled so as to maximize the weight percentage mineral in the composite and to achieve the most intimate mixture of collagen and hydroxyapatite.

RESULTS: Strengthening bone analogues by cross-linking techniques and mineralisation is well documented (Thompson and Czernuszka 1995; Kikuchi, Itoh et al. 2001). We investigated the possibility of a superposition additive effect by combining different strengthening techniques. From our experiments we observed that the mineral phase (30-40 wt%) significantly stiffened the collagen matrix (Young's Modulus: Dry – 1.20 GPa) better than the cross-linking agents did.

Table 1: Young's Modulus of Collagen Matrix strengthened by cross-linking (X-GTA/ DPPA), mineralisation (Ppt) or a combination (XP).

	Young's Modulus (MPa)
Pure	972
PPT	1209
X-GTA	1060
X-DPPA	1115
XP-GTA	852
XP-DPPA	1320

However on comparing the response to dynamic stresses it was the cross-linked samples that showed better dynamic properties when tested on the Dynamic Mechanical Analyzer (DMA 7e).

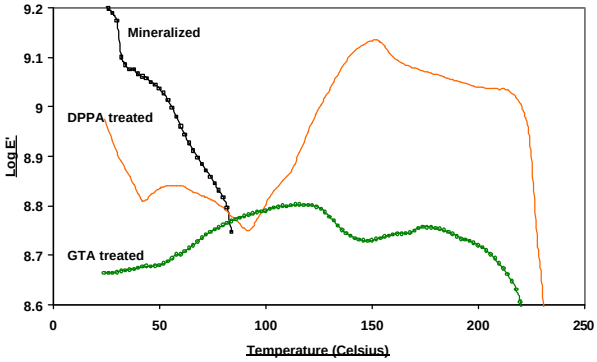

Fig. 1: Dynamic Mechanical property -: Storage modulus variation of strengthened matrices

DISCUSSION & CONCLUSIONS: The introduction of artificial cross-links using the agents Diphenyl phosphoryl azide (DPPA) and Glutaraldehyde (GTA) into the composite before precipitation, further improves the composites Young's Modulus to 1.34 GPa (Dry). However the strengthening effects do not superimpose. Also as bones are under dynamic stresses, it is not enough to base bone analogues mechanical design on static loading conditions.

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Development of a Waveguide Excitation Fluorescence Microscope for Micro and Nanoscale Characterization of Bio-Interfaces

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INTRODUCTION: The ability to investigate interactions occurring between a biological system and a surface, be it a native biological surface or a synthetic surface, is of critical importance for the elucidation of the biological response toward surfaces and, thus, to the design of new and improved biomaterials for a variety of applications ranging from biosensors to tissue engineering. Correspondingly, a number of useful techniques have been introduced into the field either for imaging or for quantification of mass absorbed. In this paper we present yet another technique for the biological surface science toolbox that has the unique advantages of being able to provide both imaging and quantitative information of a bio-interface in-situ, in real time, and with very high sensitivity (femto-molar). The technique is based on the evanescent excitation of fluorescence using a waveguiding substrate and is thus known as the Waveguide Excitation Fluorescence Microscope (WExFM).

METHODS: The WExFM is in essence a combination of Optical Waveguide Lightmode Spectroscopy and Total Internal Fluorescence Microscopy. Although the final design of the system remains in the development stage, a series of feasibility studies have successfully demonstrated the power and potential of the technique. Our current test system is based on the streptavidin-biotin biding event. Biotin is immobilized onto the waveguiding (WG) substrate through a covalent bond to a graft co-polymer which is itself electrostatically bound to the WG. The polymer used, poly(L-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) [1], exhibits resistance to protein adsorption when left unfunctionalized and thus provides a good background for patterning specific binding areas. Results shown here are for 60 x 60 micrometer pattern of biotin labeled PLL-g-PEG in a background of unlabeled PLL-g-PEG during the flow of a 10 ng/mL solution of Alexa-488 labeled Streptavidin at a rate of 0.2 mL/min. Other streptavidin concentrations (down to 1 ng/mL) and colours (Alexa-633) have also been investigated.

RESULTS: The diffusion limited binding kinetics of this system can be seen in *Fig.1*. In *Fig.2*, a WExFM image is compared to a normal FM image of the same system illustrating the improved sensitivity to the surface interactions.

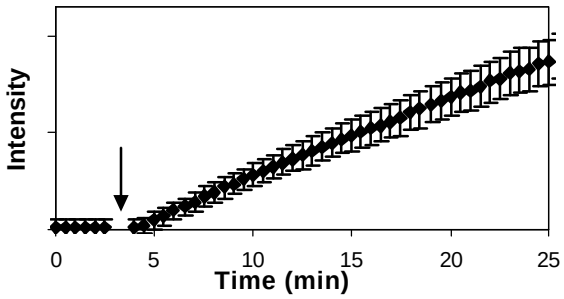

Fig. 1: Fluorescence Intensity versus time for the streptavidin-biotin binding kinetics as described in text. Arrow indicates start of streptavidin flow.

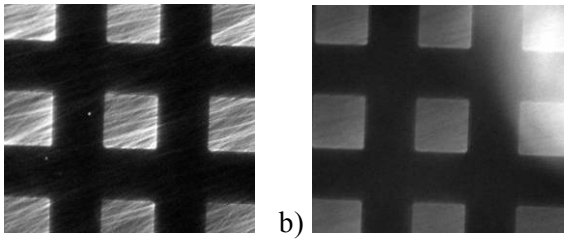

Fig. 2: a)WExFM and b) Normal FM images after 22 and 44 min of Streptavidin flow, respectively.

DISCUSSION & CONCLUSIONS: A new technique for the in-situ study of the bio-interface with both spatial and temporal resolution has been developed: the WExFM. Single molecular adsorptions should be detectable with this technique as a sensitivity in the sub-picomolar range has already been achieved. The high surface sensitivity and multi-colour detection capability make this an ideal technique for quantitative and dynamic studies of protein interactions, cell adhesion sites and more.

REFERENCES: [1] N.P. Huang, N.D. Spencer, *et. al.* (2001) *Langmuir* **17**:489-498.

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Osteocalcin and CbfA1-expression in human osteoblasts following low molecular weight heparin or Fondaparinux (Arixtra®) treatment

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INTRODUCTION:

Heparin preparations have been used in the prevention and treatment of venous thromboembolism for decades. Despite several new anticoagulant drugs currently in development, low molecular weight heparins (LMWH) remain one of the most frequently used drugs in surgical practice. Side effects include bleeding, heparin-induced thrombocytopenia, skin reactions and heparin-induced osteoporosis. The osteoporotic effect of LMWH has been demonstrated *in vitro*[1], but the underlying mechanism is unclear. Fondaparinux (Arixtra®) is a synthetic antithrombotic drug that has the ability to specifically inhibit factor Xa[2]. Because of the known interactions of anticoagulatory drugs on bone, we analysed both drugs in an *in-vitro* model. We focus our study on osteocalcin, the osteoblast specific protein, and its transcription gene Cbfa1, which are key regulators of osteoblast differentiation and function[3].

METHODS:

Experimental in-vitro study. Primary human osteoblast cell cultures were incubated every 24h with the LMWH Dalteparin (Fragmin®) in concentrations of 30, 300 and 900 µg/ml or with Fondaparinux (Arixtra®) in concentrations of 25, 50, 100, 150, 200 and 250µg/ml. Doses were chosen according to clinical application. Proliferation rates (MTT- test), protein synthesis (BioRad® Assay), Osteocalcin-, Cbfa1- and alkaline phosphatase gene-expression (Reverse-transcription polymerase chain reaction (RT-PCR)) were measured. Vital fluorescence stain was performed after 7 days.

RESULTS:

Proliferation rates and protein synthesis were significantly inhibited by LMWH in doses of 300 and 900 µg/ml ($p<0.05$) while Fondaparinux showed no inhibitory effect. RT-PCR analysis showed significantly depleted expression of Cbfa-1 and Osteocalcin in heparin treated cultures only ($p<0.05$) (*figure 1*). Vital fluorescence showed typical osteoporotic changes exclusively in cultures treated with LMWH but not after Fondaparinux application (*figure 2*).

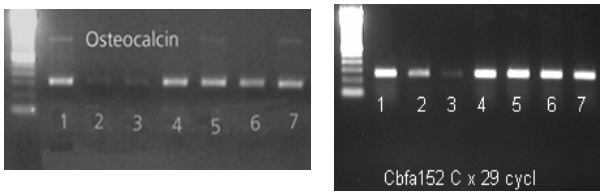

Figure 1: *Inhibited Osteocalcin and Cbfa1-expression by Dalteparin 300 and 900 µg/ml (2),(3). No-effect with Fondaparinux: 4-6, Control: 7*

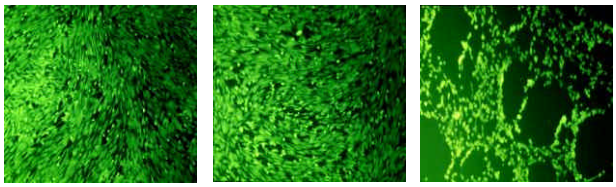

Figure 2: *Vital Staining (Fluorescein Diacetate) of primary human osteoblasts after 7 days of fondaparinux incubation (250µg/ml) and dalteparin incubation (900µg/ml, 7 days).*

DISCUSSION:

Heparin osteoporosis is a serious complication of long-term and high dose heparin therapy and may cause impaired fracture healing[4]. The pathomechanism seems to involve changes in gene-regulation and impaired osteoblast function, both necessary for strict balance of the bone remodeling process. Fondaparinux lacks the inhibitory *in-vitro* effect on osteoblasts of LMWH and may have less adverse effects on bone in vivo.

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Assessment of migratory and proliferative behaviour of osteogenic cells on type I collagen vs. BSA using a method for continuous single cell observation.

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INTRODUCTION: Besides proliferation and differentiation, migration of osteogenic cell populations plays a crucial role in bone remodelling and osseointegration of dental implants. Various ECM proteins like type I collagen have been proofed to affect bone repair ("guided tissue regeneration"). The exact assessment of dynamic migratory cell pattern is difficult, as established experimental set-ups display discontinuous observation methods, thus only take net changes of the whole cell population into account.

METHODS: For the continuous observation of individual cells, the *Time-lapse Individualcell Migration-Assay* (TIM-Assay) was established, recording the migratory and proliferative behaviour of up to 12 vital cells over a period of 24 hours or more[1]. For quantification of the migratory activity, consecutive positions of nuclei in all cells are tracked using an image analysis program (NIH-Image). These coordinates are used to calculate and visualise the following parameters: migration velocity, migration path and proliferation index.

An osteogenic cell culture was obtained from a routine surgical procedure at our Department of Oral and Maxillofacial Surgery and characterised by AP and osteocalcin staining. Using the TIM-Assay, we investigated the migratory and proliferative pattern of 31 cells, having coated the culture plates with type I collagen. For reverence values we observed cells on bovine serum albumin (BSA; n=22) and on untreated glass (n=18) to assess intrinsic migration and proliferation attributes.

RESULTS: The investigated cells stayed vital over the whole observation period. They showed a constant migration pattern, no acceleration or deceleration was observed. On BSA and on glass, cells migrated 5,00 ±1,94 µm/h and 4,06 ±2,43 µm/h respectively. Proliferation rates were 36% for BSA and 50% for glass. On collagen, migration velocity was increased significantly (8,88 ± 3,39 µm/h) while proliferation decreased (16%).

Fig 1: Inverse phase-contrast-microscope equipped with a styrofoam isolated lucite cube to maintain hemostasis over a longer period.

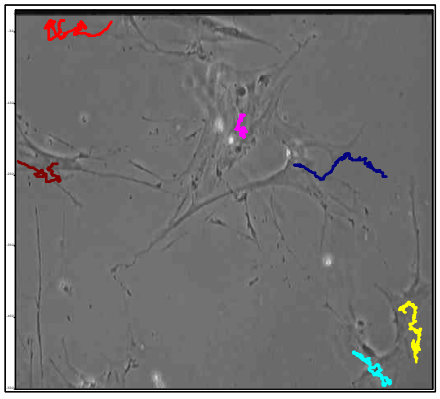

Fig 2: Start - image of osteogenic cells in the TIM-Assay with projection of the individual pathways over the next 24h on type I collagen. An oscillating rather than a straightforward migration pattern is observed.

DISCUSSION & CONCLUSIONS: The TIM-Assay might serve as a tool to analyse dynamic cell responses to distinct ligands. In our study, type I Collagen enhances migration and inhibits proliferation of osteogenic cells ("go vs. grow?"). One limitation of the method is the fact that only a small subpopulation of the very heterogenous osteogenic cells can be observed by cycle. In further studies with larger cell populations, cell differentiation and cell-cell interactions have to be considered as well.

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Modification of polysulfone by means of UV irradiation and H_2O_2 plasma treatment

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INTRODUCTION: Polysulfone is frequently used as a material for filtration membranes (eg., for haemodialysis) and medical implants of various types**.** The hydrophobic character of polysulfone surface, resulting from its chemical structure, is the reason of membrane fouling with proteins, but this effect can be diminished by making the surface more hydrophilic. The effect of the irradiation of polysulfone with the UV light absorbed by the polymer and the effect of plasma H_2O_2 treatment was studied. The interactions of the cells with polysulfone and modified polysulfone samples were monitored.

METHODS: Materials: polisulfone (PSU) (Aldrich, M_n = 16000); the samples were prepared in the form of homogeneous films or compsites consisting of monofilament polypropylene (PP) mesh (Bard) covered with PSU thin layer (PPSU/PP). The spectra of PSU samples were recorded with 8452A Hewlett Packard (UV-VIS), EQIUNOX 55, Bruker (FTIR), FTIR Excalibur (ATR) and Renishow 2000 (RS) spectro-photometers. SEM observations of the polysulfone surface were performed with JSM-5410 Jeol instrument. Contact angles of water on the investigated samples were measured using the sessile drop technique with a Krüss DSA 10 apparatus. The samples were irradiated with the UV light absorbed by the polymer (?=254 nm) with the aid of ASH 400 medium pressure mercury lamp (PSU/irr) and subjected to plasma H_2O_2 etching in a Sterrad 100 system (PSU/pl). The samples were immersed in a bovine serum albumin solution and the evaluation of protein adsorption was done with a spectrophotometric method at 280 nm. Cells: human osteoblastic line HFOB 1.19, human fibroblastic line HS-5, human macrophagous line KMA. Cell cultures were run in an incubator in the atmosphere of 5%CO_2/95% air. The viability of the cells was determined using the method based on MTT (Sigma) dye metabolism in living cells mitochondria. The concentrations of collagen type I and IL-1ß were evaluated by means of ELISA tests (DSLabs Inc., USA).

RESULTS: The modified surfaces present a much rougher morphology than the initial PSU films, which was observed in SEM micrographs. The decrease of sulfone end ether bands connected with the degradation of polysulfone as well as the formation of carbonyl and hydroxyl groups and polyphynyl structures was confirmed by spectroscopic measurements carried out for UV irradiated samples. The amount of bovine serum albumin adsorbed on the modified surfaces was lower than that taken off from the original films. The contact angle of water on the investigated samples visibly decreased after UV irradiation and plasma treatment (e.g., PSU: 82^o, PSU/irr: 44^o, PSU/pl: 63^o).

The viability of fibroblasts and osteoblasts cultured with the modified samples was lower than in the presence of the unmodified ones (e.g., osteoblasts - PSU: 82%, PSU/irr: 60%, PSU/pl: 66%; fibroblasts - PSU: 78%, PSU/irr: 65%, PSU/pl: 75%) while macrophages were unaffected by irradiated PSU (PSU: 84%, PSU/irr: 86%, PSU/pl: 92%). The level of cell's activation was estimated by the determination of the amount of collagen type I secreted by fibroblasts and osteoblasts as well as IL-1ß by fibroblasts. The yield of collagen type I production by living osteoblasts and fibroblasts was enhanced after the UV and plasma modification of the surface. The level of IL-1ß is low (100-140 pg/ml for PSU samples, 125 pg/ml for fibroblasts cultured without polymer sample).

DISCUSSION & CONCLUSIONS: The irradiation of polysulfone with the light absorbed by the polymer (254 nm) and the etching with H_2O_2 plasma change the polymer surface making it more hydrophilic due to the introduction of polar groups containing oxygen (carbonyl, hydroxyl). These modifications resulted in the diminished ability to absorb albumin and the decrease of the contact angles of water. The cellular response to the various samples is differentiated and depending on the kind of the cells, but the biocompatibility of the investigated samples is satisfactory.

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On the way to novel nanostructured bone replacement materials:

First cytological investigations of mesoporous solids

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INTRODUCTION: Bone is an organic-inorganic composite material that combines high strength with special elastic properties. Materials currently in use as bone replacement materials include inorganic phosphates, ceramics, glasses, metals and polymers, none of which has the characteristics of a composite. The surface structure of an implant material can affect cell attachment, growth and differentiation. Specifically, it has recently been shown that nanostructured surfaces can influence these types of cell behaviour.

Materials for the replacement of the middle-ear ossicular chain must satisfy certain specific requirements. In particular, the audiological and vibrational properties should preferably be similar to those of bone in order to generate a similar sound transmission behaviour. The bioactivity of the material must be adjusted so that formation of a thin layer of cells on the surface of the implant is favoured. Extensive ossification must be suppressed in order to avoid fixation of the implant.

In our approach we engineer novel bone replacement materials in a biomimetic approach which is based on nanostructured solids. Synthetic organic-inorganic composite structures should possess similar sound transmission behaviour as natural bone. Nanostructure engineering will be used to control the attachment and proliferation of cells. The interactions of cells with the engineered materials are investigated in cell culture assays. The acoustic properties are tested using Laser Doppler vibrometry. As a first step, we have synthesized mesoporous silica films and have investigated the interaction of cells with these materials.

METHODS: To prepare samples for structural analysis and cell culture testing, thin films of nanostructured silica materials on standard glass slides were prepared by dip-coating. Non-ionic surfactants (octaethyleneglycolmonodocecylether, $C_{12}EO_8$, or amphiphilic triblock copolymers as Pluronic P-123®, $EO_{20}PO_{70}EO_{20}$ or F-127® $EO_{106}PO_{70}EO_{106}$, EO: ethylene oxide, PO: propylene oxide) served as structure-directing agents. Together with a silica precursor, these structure-directing agents form organic-inorganic hybrid nanostructures. The organic part of the hybrid structure can then removed by calcination to yield silica nanostructures. A control sample of amorphous silica was obtained by applying a similar synthesis procedure, but omitting the organic material.

RESULTS: X-ray diffraction shows that the as-synthesized film obtained with $C_{12}EO_8$ possessed a nanostructure with a characteristic repeat of ca. 7 nm, decreasing to ca. 5.2 nm after calcination. The corresponding values for the films obtained with Pluronic P-123 were 8 nm before and 5.7 nm after calcination. In case of Pluronic F-127 the corresponding values were 8 nm before and 7 nm after calcination.

First results in cell culture assays using the C3H10T1/2 human mesenchymal precursor cell line indicated a reduced cell adhesion on the as-synthesized composite materials. However, after removal of the organic matrix by calcination, highly efficient cell adhesion was observed that was indistinguishable from cell adhesion to standard cell culture plastic material. The same results were obtained when using the human kidney carcinoma cell line 293. After initial attachment, the cells showed continued adherent cell proliferation until confluency was reached. The results were similar for the unstructured sample, i.e., the nanostructures did not influence the cell behaviour. In conclusion, the mesoporous materials exhibited an excellent in vitro biocompatibility after calcination.

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An attempt to correlate the characteristics of micro structured surfaces of Titanium implants and biological parameters of adhesive cells

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INTRODUCTION

One of the premises for biocompatibility studies of implant materials is the determination of morphological characteristics of their modified surfaces. Our investigations here were focussed on the question if the physical and chemical parameters used for the description of the surface of commercially pure titanium with different roughness can be utilised for the prediction of the cellular behaviour of osteoblastic cells.

MATERIALS & METHODS

The surface structure of cp-titanium samples was modified in a range of roughness average R_a from 0.19µm to 48.59µm by polishing (P), machining (NT), blasting with glass balls (2.7 bar) (GB), blasting with corundum particles (2,5 bar and 6 bar) (CB) and vacuum plasma spraying (VPS fine/medium/high). For the physical characterisation of the surface morphology the roughness average R_a and the fractal dimension D_F were used [1]. The R_a-values were obtained by surface profiling. The D_F-values were calculated by Digital Image Processing (DIP), Electrochemical Impedance Spectroscopy (EIS) and Linear Sweep Voltammetry (LSV) [2,3].
Cellular investigations were carried out in human primary osteoblasts [4] and MG-63 osteoblastic cells. Cells were cultured in DMEM with 10% fetal calf serum (FCS) and 1% gentamycin (Ratiopharm GmbH, Ulm, Germany) at 37°C and in a 5% CO_2 atmosphere. In general, cells were seeded with a density of $3x10^4$ cells/cm^2 onto the titanium materials and into control dishes. Following cellular parameters were investigated to evaluate the correlation to physico-chemical properties of the titanium: Adhesion, spreading (area), proliferation (cell cycle), integrin expression, length of integrin contacts, vinculin contacts in living cells (length) and their dynamics (GFP-vinculin), fibronectin synthesis, and mineralization.

RESULTS & DISCUSSION

The correlation between the biological results and the values of roughness parameters R_a and D_f was attempted with methods of PEARSON and of SPEARMAN.
Our trial showed not a trend to correlate between roughness parameters and length of integrin contacts, vinculin contacts in living cells (length) and their dynamics (GFP-vinculin) and spreading (area). But we could found a good correlation (coefficient of correlation r=0.8) between roughness parameters and fibronectin synthesis, mineralization and proliferation (cell cycle).
Interestingly, that the correlation between the fractal dimension D_F and the cell biological parameters is mostly better than with roughness average R_a.

CONCLUSIONS

The physical parameter fractal dimension D_F seems to be suitable for the correlation of morphological surface characteristics of biomaterials with cell biological properties and could be an indicator of later cell behaviour. We suggest that a more organized surface with pronounced D_F enhance cellular functions concerning adhesion.

ACKNOWLEDGEMENTS

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Tissue reactions to poly-L/D-lactide (PLDLA) 96/4 scaffolds implanted subcutaneously in rats

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INTRODUCTION:
Different materials have been used as scaffolds for tissue engineering purposes. Polylactide has been widely studied as plates, screws, tacks and also as a monofilament[1,2,3]. Material is totally bioabsorbable, in soft tissues it is eventually replaced by fibrous tissue. Fibrous tissue formation can be of benefit in certain situations, such as in finger joint replacement in rheumatoid arthritis patients. For that purpose hundreds of patients have been operated over the years[4].
Aim is also to follow the biodegradation of the scaffold material and also its tissue reactions. Another aim of this study is to look also at scaffolds with various pore sizes, is there difference with the tissue ingrowth speed or in the amount of formed fibrous tissue at 1 year post-operatively.
We made scaffolds of Poly-L/D-lactide (PLDLA) 96/4 (96/4, molar ratio of L/D lactide) for study the PLDLA scaffolds in vivo in the subcutaneous tissue of rats.

METHODS: Cylindrical knitted mesh scaffolds were made of PLDLA 96/4 fibers, with each fiber made of 8 PLDLA filaments. All implants were sized 15 x 3.5 mm. Three types were evaluated: Dense (weight 30 g), ordinary (25 g) and loose (20 g). Four scaffolds (2 ordinary, one dense and one loose type) were implanted in the dorsal subcutis of each of 32 Sprague-Dawley male rats. The implants were retrieved after 3 days, 1, 2, 3, 6, 12, 24 and 52 weeks postoperatively and they were removed with 5 mm of surrounding tissues. The samples were examined histologically for tissue reactions and ingrowth.

RESULTS: No postoperative complications occurred. Tissue ingrowth reached the innermost part of the implants within three weeks. Fibrin was the first to fill in the scaffold followed by the cells and at last collagen fibers were found in the structure. The orientation of the collagen fibers inside the implant changed from non-oriented to highly oriented fibers making septae. The amount of macrophages increased over the follow-up period of 52 weeks. The material was not fragmented at 52 weeks.

DISCUSSION: Upon implantation in rats, fibrous tissue ingrowth proceeds from all sides of the scaffold filling it completely at 3 weeks. The collagen fibers get more organized by time. Increasing number of macrophages by time is most probably due to increasing degradation of implant material. Single fibers of the material were not fragmented at 52 weeks, thus for determining the total bioabsorption time for this kind of implants, longer follow-up time is required.

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Bioabsorbable poly-L/D-lactide vs. silicone sponge scleral suckling implants can induce sufficeintly perisistant indentation in rabbits

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INTRODUCTION: In the treatment of rhegmatogenous retinal detachment, scleral buckling operation has been used for decades. The sclearl buckling implant creates an inwards impression for attaching the layers of retina and chorioidea to each other. In simple rhegmatogenous detachments, after the chorioretinal scar has developed and matured, the supporting buckling implant would no longer be required[1]. Various materials for scleral buckling have been used. Non-absorbable materials are commonly used, such as silicone, either solid rubber or silicone sponge. Now also bioabsorbable synthetic materials are available for scleral buckling in cases when only temporary buckling effect is needed. Because polylactides have been extensively studied in surgery, we chose to fabricate a scleral buckling implant made of poly-L/D-lactide 96/4 (PLA96) as raw material[2].
The aim of this study was to measure the amount and duration of indentation depth achieved with biodegradable poly-L/D-lactide 96/4 and silicone sponge implants.

METHODS: Thirty rabbits underwent a scleral buckling procedure. A PLA96 buckling implant was used in 15 and a silicone sponge buckling implant in 15 rabbits. A circumferential scleral buckling implant was sutured episclerally on the left eye of each rabbit, just temporal to the superior rectus muscle and 7 mm posterior to the limbus. CT scanning was performed at one week, and three and five months postoperatively.

RESULTS: The PLA96 buckling implant (implant diameter 3–3.5 mm) used in this study created lower indentation than the silicone sponge implant (implant diameter 4 mm). The indentation created by the PLA96 implant decreased over time compared with the silicone implant. There were no macroscopically detectable complications related to either kind of implant.

DISCUSSION: The indentation achieved with bioabsorbable implant was lower than indentation with silicone sponge implants, probably due to difference in the original sizes of the implants. Both silicone sponge implants and PLA96 implants caused indentation that decreased in a comparable manner over the follow-up period of 5 months.
With non-absorbable implants there is always a risk for long-term complications, such as infection, extrusion, intrusion, pain or persistent diplopia[3]. By using bioabsorbable implants, the long-term complications can be avoided.
The recommended time period for determining the functional success of operation, measured as the best corrected visual acuity, is six months[4]. At that time period, in most of the cases of simple rhegmatogenous detachments, the retina has reattached and chorioretinal scar is strong enough to keep it attached. In the current study it was proved that with PLA96 implants the indentation lasted for the follow-up period of 5 months.

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ADHESION AND PROLIFERATION OF OSTEOBLASTIC CELLS SEEDED ON CHITOSAN – HYDROXYAPATITE POROUS SCAFFOLDS

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INTRODUCTION: Bone and Cartilage generation by three dimensional scaffolds is one of the promising techniques in the biomedical engineering. Osteoblasts and mesenchymal cells can be seeded on these 3D polymeric scaffolds, which can eventually degrade slowly and resorb as tissue structures grow in vitro or in vivo. These scaffolds provide the necessary support for cells to proliferate and maintain their differentiated function, and its architecture defines the ultimate shape of the new bone and cartilage [1]. The aim of this work is to produce a cell seeded mineralized polymer scaffold, intended for bone tissue application. A biopolymer, chitosan was used, and the polymer scaffold was mineralized to form hydroxyapatite crystals in porous network by biomimetic method.

MATERIAL AND METHODS: Interconnected porous chitosan scaffolds were prepared by freeze drying method and were mineralized by calcium and phosphate solution by double diffusion method [2]. The mineralized 3D chitosan scaffold had hydroxyapatite crystals on the surface and also within the pore channel of the scaffold. Primary human osteoblast (SAOS-2 cell line) was used for cell cultivation study. As control a pure chitosan scaffold was used. About $1x10^6$ cells were seeded on to the scaffolds over the pores and total volume of scaffold was filled by cells and incubated for period of 1 and 3 weeks. After harvesting, scaffold sections were stained by different stains like von Kossa technique to show calcification, Masson–Goldner stain to assess collagen accumulation. Alkaline Phosphatase (ALP) activity was measured using a commercially available kit (Sigma Diagnostics, USA). Total protein content was determined spectro photometrically using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Germany).

RESULTS AND DISCUSSION: Human osteoblast (SAOS-2) cells were cultured on the composite scaffolds for up to 21 days, and the cell growth and function were analyzed. The cell growth is much faster on the chitosan/HA scaffolds than on the pure chitosan scaffold. The total protein content and ALP activity of cells were quantified and increased over time in composite after 7 days of culture. The cells on chitosan/HA scaffold also expressed a distinct ALP activity of the osteoblasts seeded on the prepared scaffolds compared to the control.
Most of the outer macropores became sealed off by a continuous layer of cells. While some of the cells adhered on the macropore surface of scaffold, others spanned the pores in a three dimensional fashion, surrounded by a fibrous extracellular matrix (Fig. 1). It could be demonstrated by von Kossa staining that such areas had already been started to mineralize. Sometimes cells were surrounded by lacunae. Masson-Goldner stains showed clear collagen biosynthesis.

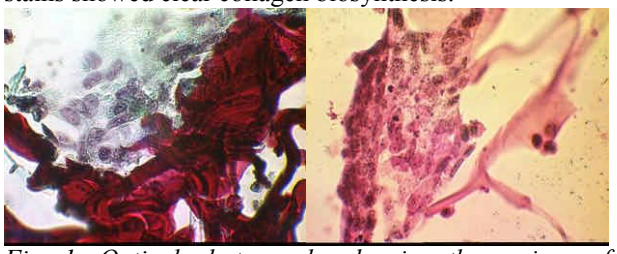

Fig. 1: Optical photographs showing the regions of chitosan/HA scaffold seeded with osteoblast and stained with Masson-Goldner and Von-Kossa stains. (x400)

CONCLUSIONS: Bone regeneration applying novel techniques of tissue engineering requires biocompatible and preferably resorbable materials providing a framework for cells to adhere, proliferate and create extracellular matrix.
This study demonstrated an excellent biocompatibility of the fabricated scaffolds and their potential to serve as biodegradable matrices for bone tissue engineering.

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Mesenchymal stem cells on plasma-coated materials: an "acrylic interface" in vitro study

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INTRODUCTION: Human mesenchymal stem cells (MSCs) are multipotent œlls also present in the adult marrow that are able both to replicate as undifferentiated elements and to differentiate as progenies of mesenchymal tissue, including bone. The aim of the present work was to evaluate a heterogeneous range of bio-functional and modulatory performances of MSCs grown onto two plasma-deposited Acrylic Acid substrates with different COOH contents.

METHODS: Two plasma-deposited Acrylic Acid (pdAA) coatings [1] were compared with a different COOH/R component of 4 +/- 1% (**A coating)** and a COOH/R of 9 +/- 1% component (**B coating)**. The proportion of COOH/R groups was calculated with respect to the area of the carbon peak recorded by X-ray Photoelectron Spectroscopy (XPS) analysis. Coating A (WCA = 61 +/- 1°) was less hydrophilic than coating B (WCA = 50 +/- 1°). Human MSCs isolated from iliac bone marrow [2] were seeded in triplicate ($5x10^4$ cells/cm^2) onto the two coatings and onto TCP (polystyrene wells) as control. Viability (MTT), morphological (SEM), biochemical (ALP) and cell movement (Time Lapse Video Microscopy, expression of Cdc 42) analyses were performed.

RESULTS:

Table 1

	SEM	MTT	ALP
TCP	Layer of spindle-shaped cell with cells overlapping	100%	11.7± 2.3 IU/l
coating A	Spread spindle-shaped cells with cells overlapping	92%	17.6±2.9 IU/l
coating B	Spread spindle-shaped cells with cells overlapping	81%	18.5±2.6 IU/l

Table 2

	Time–Lapse	Focal Cdc 42 expression	
		1	**2**
TCP	Cells with no random walk but devoid of evident aggregation features	50%	65%
coating A	Greater motion than on TCP random walk, no cellular aggregation	70%	40%
coating B	Greater motion than on TCP no random walk cellular aggregation pattern	60%	10%

1)% cells with a polarised shape
2) % polarised cells with localised cdc 42 expression

DISCUSSION AND CONCLUSIONS: Carboxyl-terminal groups are important cell-binding domains in both IIICS and Hep II fibronectin regions. The interaction with these domains through alpha4beta1 and other beta1 integrins bindings can support cell adhesion and migration [3]. Cell activation induced by COOH group interactions with the cell surface is sustained in particular by our time-lapse results. In fact, both acrylic acid coatings (A and B) induced a greater migration pattern of MSCs than controls (Tab. 2). An interesting feature was also the formation of rounded multicellular aggregates of MSCs 6 hours after seeding onto coating B. Presence of these aggregates on B samples may be due to low-shear stress microenvironmental culture conditions, probably related to the higher concentration of COOH/R inducing cell association by means of cadherin [4].
MSCs thus represent an interesting precursor cell population for growth onto substrates coated with plasma-deposited acrylic acid, obtained and controlled by means of plasma processes.

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Human fibroblast proliferation and cytoskeleton reactivity to characterised metal implant surfaces

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INTRODUCTION: Orthopaedic implant manufacturers now supplement the choice of stainless steel (SS) and titanium (CpTi) with titanium alloys. Ti-6%Al-7%Nb (TAN) is an 'a+ß' microstructured alloy that is utilised in nailing systems (CpTi has a pure 'a' microstructure). It has been used extensively in the hard tissue environment, but utilisation in more biologically complex areas with soft tissue and hard tissue exposure requires further investigation. The aim of this study was to investigate *in vitro* soft tissue reactivity, by means of human (hTERT) fibroblasts, towards SS, CpTi and TAN. The materials were produced and finished identically to their clinically used counterparts, providing 'standard' types of the materials.

METHODS: Atomic Force Microscopy (AFM), Laser Profilometry (LP), Scanning Electron Microscopy (SEM) were used to characterise the surfaces numerically and visually. hTERT fibroblasts were cultured on the materials and fluorescently triple stained for F-actin and DNA and either Tubulin or Vinculin. Cell proliferation was assessed by quantifying DNA from cells cultured at 24 hours, 5 and 10 day timepoints. DNA was harvested from cells cultured directly on the substrates and from cells cultured in the presence of the materials.

RESULTS: Both numerically and visually SS is a smooth surface (Roughness Average (RA) -0.21µm). CpTi and TAN are both rougher surfaces compared to SS (RA's of 1.11 and 0.99µm respectively). While TAN is numerically similar to CpTi, visually it differs. The AFM of TAN displays a 'microspiked' topography that SEM demonstrates to be ß-phase particles protruding from the surface.

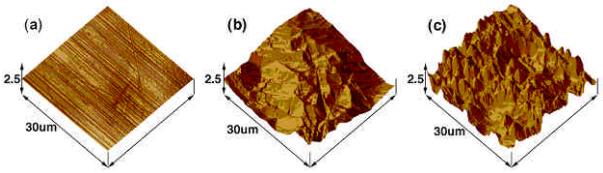

Fig. 1: AFM images of (a) CpTi, (b) TAN and (c) EPSS

Cells cultured on SS were unrestricted in spreading and formed 'mature' focal adhesion sites. Cells were sufficiently spread on EPSS such that we could distinguish 'dot' adhesion sites at the cell periphery, and mature 'dash' adhesions with attached actin cytoskeleton further inside the cell. The rougher topography of CpTi dictated the spreading of cells cultured. Focal adhesion morphology was different to the cells on SS with a tendency for small 'dot-like' focal adhesion sites. Neither SS nor CpTi visibly disrupted the microtubule network.

Cells cultured on TAN were restricted in spreading with small focal adhesion sites that avoided the ß-phase particles. Microtubule formation was also impeded by the ß-phase particles. Cells cultured on TAN demonstrated suppressed cell proliferation, while the remaining samples had an increase in DNA at 5 and 10 day timepoints. Cells cultured in the presence of the materials demonstrated similar growth curves for all, indicating that the surface of TAN suppresses proliferation.

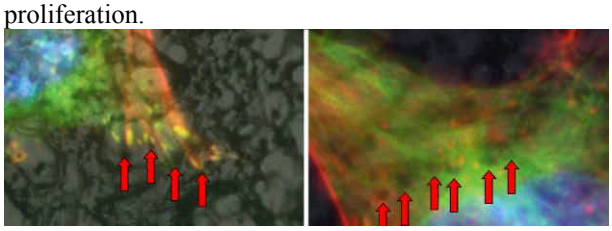

Fig 2: Avoidance of Focal Adhesions and Microtubules to the ß-phase particles of TAN

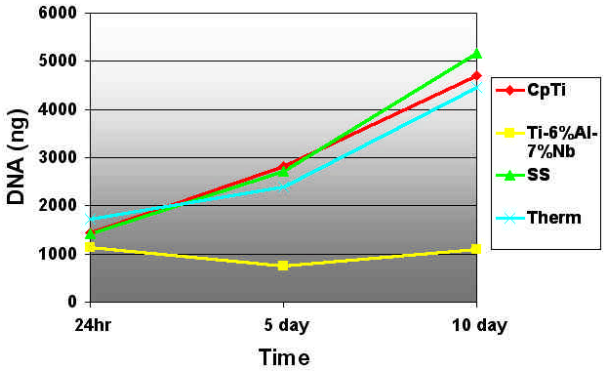

Fig 3: Cell proliferation on the materials

DISCUSSION: The presence of ß-phase particles, or the microtopography produced by the presence of ß-phase particles on TAN produces a non-cytocompatible substrate for human fibroblasts. Caution should be taken when utilising TAN in contact with soft tissues.

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Geometry Modifications of a New Three-dimensional Bioactive Glass Fibre Matrix with a Feasible Structure for Tissue Engineering Applications.

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INTRODUCTION: A suitable matrix scaffold is one of the essential factors for bone formation. Scaffolds have been characterized taking into account their physical, chemical, biochemical, and lately also geometrical properties [1]. It is nowadays accepted that in order to achieve good vascularization and thereby bone formation, the scaffold needs to have a highly interconnected porous structure and a feasible geometry. The manufacturing of a three-dimensional matrix with the requested properties is, among all, material dependent. Bioactive glasses have been difficult to process thermally because of their tendency to devitrify, and have been mostly used in particulate form. From the middle of the 1990s [2] new bioactive glasses with large working range have been available and they have provided new processing possibilities. In this work a three-dimensional scaffold with a tailored structure has been produced and characterized structurally.

METHODS: Three dimensional scaffolds have been manufactured by sintering glass fibres 3 mm long and 75 um in diameter with the following composition in wt%: SiO_2=54, Na_2O=12, CaO=11, P_2O_5=2, K_2O=15, B_2O_3=1, MgO=5. The sintering process has been carried out at different temperatures as to evaluate the effect of the sintering parameters on the scaffold geometry. The sintering time has been kept fixed at 45 minutes and the temperature has been varied from 590 to 620C by 10 degrees intervals. The evaluation of the geometry has been performed qualitatively by SEM. To characterize quantitatively the scaffold geometry, the collected images obtained have been further processed. Porosity and sintering neck radius have been chosen as parameters.

RESULTS: Thermal processing of chopped bioactive glass fibres allowed the manufacture of three-dimensional networks with diverse characteristics. The modification of the scaffold geometry is emphasized by the following pictures. The temperature increment causes the structure to shrink and to change its conformation because of solid state sintering. The measured values of porosity and sintering neck radius as a function of sintering temperature are shown below.

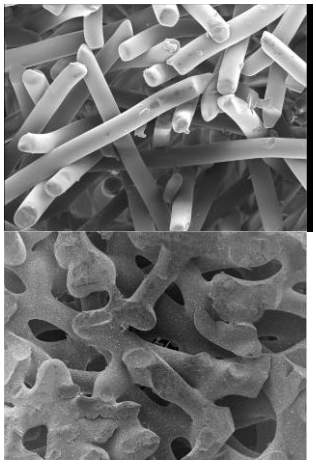

Fig. 1: Effect of processing on scaffold geometry: lower (left) vs. higher (right) sintering temperature.

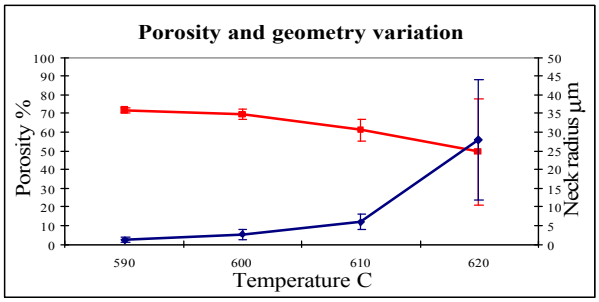

Fig. 2: Variation of sintering neck radius (blue) and porosity (red) as a function of sintering temperature.

DISCUSSION & CONCLUSIONS: The sintering of bioactive glass fibres at different temperatures allowed obtaining three-dimensional net-shaped porous scaffold with diverse internal architecture. Solid-state sintering is responsible for changes in pore size and porosity but also in pores shape and structure morphology. Convex and concave surfaces have been shown to be related to bone growth [1]. By this processing technique not only structural, by means of porosity and pore size [3], and mechanical properties [4] can be tailored, but also the geometric features.

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3D Plotted Scaffolds for Tissue Engineering: Dynamical Mechanical Analysis

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INTRODUCTION: One of the main issues in tissue engineering is the fabrication of scaffolds that mimic as close as possible the biomechanical properties of the tissues to be replaced [1]. Nevertheless, no systematic research on the effect of mechanical properties on tissue re-growth has been conducted. Moreover, conventional fabrication techniques do not seem suitable to control scaffolds' structure, in order to modulate mechanical properties. Within novel scaffold fabrication processes, 3D plotting is quite promising, because of the precision of the obtained scaffold architecture and for the 100% interconnecting pores [2].

In this study, we focused on dynamical mechanical analysis (DMA) of 3D plotted PEGT/PBT copolymer scaffolds. We investigated the influence of pore geometry and architecture on viscoelastic properties of copolymeric scaffolds with different soft/hard segments ratio.

METHODS: PEGT/PBT block copolymers were obtained from IsoTis S.A. (The Netherlands). Following the aPEGTbPBTc notation, different initial PEG molecular weight (a) and PEGT-PBT ratios in the copolymer (b and c) were used to fabricate 3D scaffolds. A bioplotter device (Envisiontec, Germany) was used to produce the samples [2]. A flow of polymer comes through a heated nozzle (T=190°C) under high pressure (p=5 bars) and is plotted on a stage as a fiber (Fig. 1).

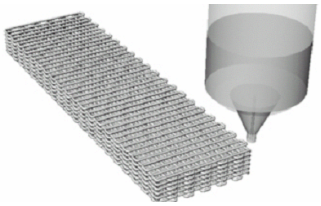

Fig. 1: Bioplotter: scheme of fabrication process.

Multi-stress, creep-recovery and frequency scan experiments were achieved with a DMA Perkin Elmer 7e. Viscoelastic properties of 3D scaffolds were evaluated in terms of dynamical stiffness, equilibrium modulus, damping factor and creep unrecovered strain.

RESULTS: 3D plotted scaffolds experienced a decrease in stiffness with an increase of the porosity. By varying the layer configuration from single to double layer and from 0°-90° to 0°-45° fiber orientation (Fig. 2), viscous properties were enhanced, although the porosity remained constant.

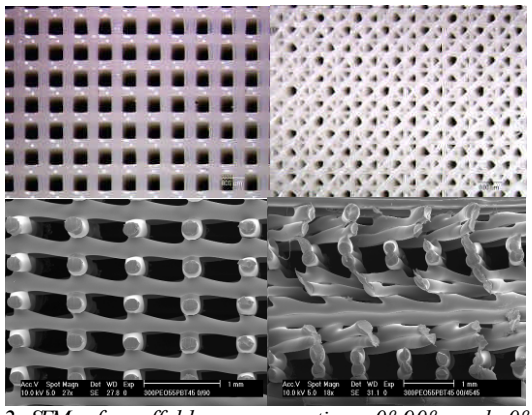

Fig. 2: SEM of scaffolds cross section. 0°-90° and 0°-45° configurations were printed in single and double layer versions.

Stiffness also decreased with more hydrophilic copolymers, by increasing PEG molecular weight and PEGT ratio over PBT (Fig. 3).

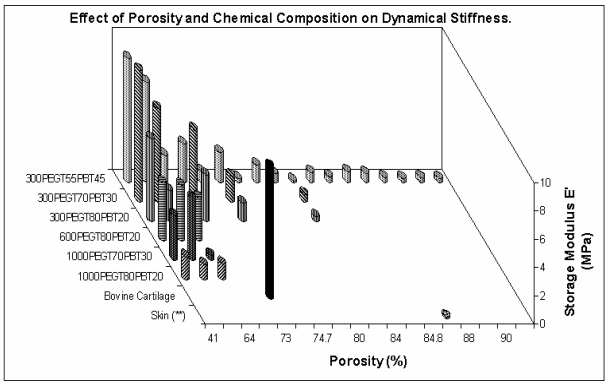

Fig. 3: Dynamical stiffness matrix shows mechanical modulation of 3D scaffolds from cartilage to skin values. Skin E' is taken from Dirillidou et al. Skin Res Tech, 2000.

DISCUSSION & CONCLUSIONS: This study shows how viscoelastic properties of 3D plotted scaffolds could be modulated to accomplish mechanical requirements for tailored tissue engineered applications. 3D plotting offers a wide variety of solutions in terms of geometrical and architectural features. Furthermore, DMA is a powerful technique to establish whether mechanical properties are crucial for specific tissue repair and to tune fabrication parameters to optimize scaffold constructs.

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Bacterial adhesion to surfaces of defined polarity in relation to previous protein adsorption

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INTRODUCTION: The biocompatibility of implant materials is mostly defined by their surface properties which cause a certain response by the surrounding tissue. It is widely accepted that infections at the implant site result from bacteria competing with tissue cells in adhering to the biomaterial surface [1]. This initial attachment of cells is mediated by a conditioning film that forms itself on the biomaterial surface immediately after contact with the surrounding biological fluid [2]. In our study, we investigated the influence of the surface properties on protein adsorption and subsequent adhesion of cells and bacteria. As a model system silicon wafers were used, because they allow the preparation of surfaces with a large variety of chemical functions and roughnesses. In this paper, the first results of bacterial adhesion to surfaces of various polarities are presented.

METHODS: Surfaces of defined polarity were prepared by chemical modification of silicon wafers. The surface parameters were determined by XPS and water wettability. Acid-modified wafers showed a contact angle of 16,2 $\pm$ 0,9 degrees (hydrophilic surface), and wafers modified with a fluorocarbon silane (HFS) gave 104 $\pm$ 6 degrees (strong hydrophobic surface). A coating with a hydrocarbon silane (OTS) resulted 67 $\pm$ 7 degrees in the formation of hydrophobic surfaces.

Parts of the modified silicon wafers were pre-coated with BSA in a phosphate buffered solution (PBS) for 1 hour. The surfaces were incubated with bacteria (10^8 bacteria/ml) for various incubation times. Bacterial adhesion was visualized either by culture of imprints from the wafer surface on agar plates or by fluorescence labeling of the bacteria.

RESULTS: Surfaces that were not pre-coated with BSA were rapidly colonized by all types of bacteria independently of the surface modification. Pre-coating with BSA inhibited bacterial adhesion significantly. Here, a dependence on the surface modification and on the bacterial strains were found. The hydrophilic surfaces allowed faster bacterial colonization in the presence of BSA than both types of hydrophobic surfaces. *Streptococcus aureus* and *S. gordonii* DL1 were found to adhere faster to the different surfaces than *S. mutans*.

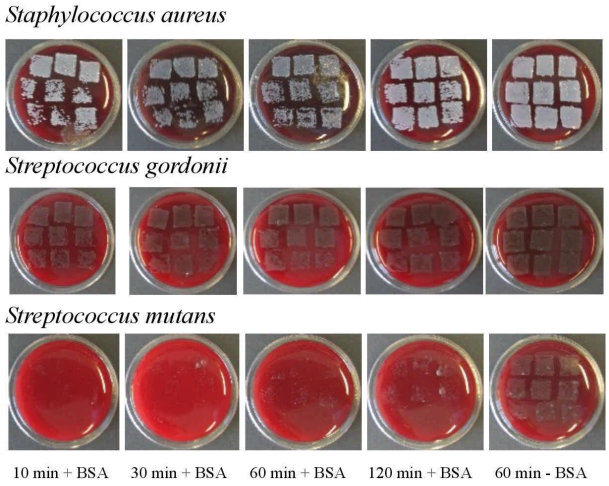

Fig. 1: Determination of bacterial adhesion after imprint on agar plates. Wafers are arranged in the same manner as it is shown in Fig. 2.

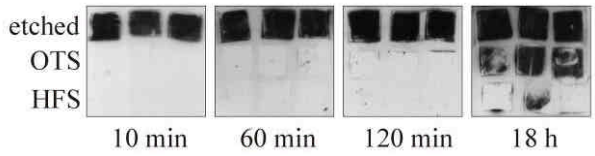

Fig. 2: Determination of S. gordonii DL1 adhesion using fluorescence labeling techniques.

CONCLUSIONS & OUTLOOK: Preliminary, we showed that initial bacterial adhesion is dependent on both the polarity of the material surfaces and the presence of albumin. Further investigations will include the pre-coating of surfaces with fibronectin and with body fluids such as serum and saliva.

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The Role of Extracellular Matrix in Biocompatibility of NiTi Shape-Memory Metal Implant Material

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INTRODUCTION: Shape-memory alloys (SMAs) are unique class of metals that after a large deformation can recover their original shape. SMAs can be used in various practical applications. The most commonly used material is NiTi (nickel-titanium alloy). In the field of medicine, NiTi is considered biocompatible and its special properties offer a wide variety of interesting applications e.g. in orthopedics. Since biocompatibility is a surface related property, the interaction between the implant material and the surrounding tissue can be altered by altering the implant material surface characteristics. The first aim of this study was to determine if different properties of NiTi alloy (crystal structure, phase state, internal stresses etc.) are reflected to cells grown on its surface. The altered behavior of cells in different areas of one sample was investigated by observing the changes in cell adhesion and spreading. The second aim was to test the hypothesis that the surface of a biomaterial does not directly influence the cells in contact, but a layer of extracellular matrix (ECM) is formed between the surface and the cells. The ECM acts as a guideline, affecting cell behavior (e.g. spreading, proliferation and survival). The biomaterial–cell interaction depends on the adsorption and conformational changes of ECM proteins over various surfaces. The material surface–ECM–cell interactions are yet poorly understood.

METHODS: A set of specifically fabricated hybrid discs of hot-rolled binary NiTi (Ti 44,3 wt%, Ni 55,7 wt%) were used. The 6 mm discs had a 3 mm martensitic core surrounded by tightly adjusted austenitic shell. As a result austenite was tensiled and martensite compressed. NiTi disks of 40 mm-in-diameter were also made either in austenitic or in martensitic state (Ti 45 wt%, Ni 55 wt%). These large samples were coated with FITC-labeled fibronectin (FITC-Fn; 20µg/ml). Osteoblast-like cell lines ROS-17/2.8 and MC3T3 E1 were chosen for experimental procedures. In the experiments, cells were allowed to grow 2 or 48 hours before staining with a5-integrin or paxillin monoclonal antibodies. Confocal microscope was used for imaging the fluorescently labeled cells and Fn-substratum over metal samples.

RESULTS: The results clearly show that cell behavior was affected by the physical state of the metal alloy instead of its chemical composition. The differences in cell shape and adhesion formation were distinct and depended on the cell location in the hybrid sample. A positive effect of internal stresses (high in austenite) towards these cell characteristics was observed. The appearance of FITC-Fn coated NiTi surfaces showed striking differences after 2 hour cultivation. These differences between austenite and martensite samples might be related to fibronectin fibrillogenesis on austenite. This demonstrates that the underlying NiTi substratum had an influence to the organization of the Fn-coat, most likely to the conformation of adsorbed fibronectin. The organization of the artificial Fn-ECM (and other proteins adsorbed among Fn) is reflected to cell behavior.

DISCUSSION & CONCLUSIONS: It can be concluded that variations in the phase state of NiTi alloy can be reflected to cells in the vicinity of its surface. These material properties are mediated to nearby cells through the ECM, which construction and organization can be altered by altering substrate material characteristics. This “chain reaction” - from implant material to cell behavior - should be taken for consideration, for example, when investigating reasons for loosening of bone implants.

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Development of a new tool to study protein adsorption in relation with hemocompatibility

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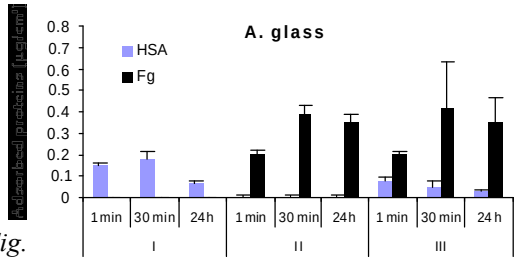
INTRODUCTION: Plasma protein adsorption is the first step which occurs when a biomaterial comes in contact with blood. This adsorption influences subsequent reactions, such as platelet adhesion or thrombus formation. The aim of this work is to better understand hemocompatibility through the influence of the surface properties of a biomaterial on the competition of proteins for adsorption. Human serum albumin (HSA), the most abundant plasma protein, and human serum fibrinogen (Fg), a key factor in the coagulation pathway, were labelled using distinct radioisotopes, allowing their adsorption to be monitored simultaneously.

METHODS: Model substrates were used: clean glass and polystyrene spin-coated on glass (PS). HSA and Fg were labelled by reductive methylation using sodium[^{3}H]borohydride and [^{14}C]formaldehyde, respectively. The proteins were dissolved in phosphate buffered saline (PBS, pH=7.4) at a concentration of 200µg/ml for HSA and 20µg/ml for Fg. The samples were incubated at 37°C in a solution of a single protein or of the two proteins for the desired period of time, then rinsed with PBS. Adsorbed amounts of ^{3}H-HSA and ^{14}C-Fg were quantified simultaneously using a liquid scintillation counter in two energy areas.

RESULTS: Figure 1 shows results of protein adsorption on glass (A) and PS (B). Simple adsorptions and adsorptions with a competition between HSA and Fg were carried out. Adsorption of HSA on glass reaches a peak after 30 minutes of incubation and then decreases until 24 hours. On the other hand, Fg adsorption reaches a plateau after 10 minutes. In the case of competition, the amount of adsorbed Fg remains similar, but the HSA adsorption is reduced. The behaviour on PS is different. When proteins are incubated separately with the substrate, the adsorbed amount is higher on PS compared to glass. However, the competition between HSA and Fg leads to a strong reduction of the Fg adsorbed amount, while the HSA adsorbed amount is not appreciably affected.

DISCUSSION & CONCLUSIONS: On glass, the higher amount of adsorbed HSA at short period of time, compared to 24 hours, is explained by the combination of the reversibility of initial bonds



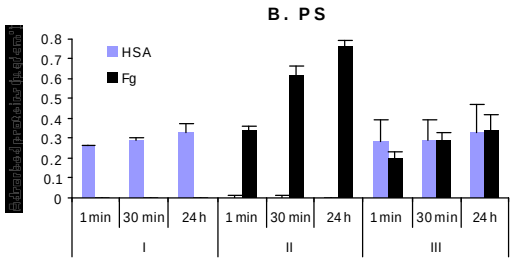


Fig. 1: HSA and Fg adsorption, as a function of time, on glass (A) and on PS (B), in a HSA solution (II), in a Fg solution (II) and in a mixture of HSA and Fg (III). Light and dark columns correspond to the adsorbed amounts of HSA and Fg, respectively.

formed with the surface and irreversible structure rearrangements of HSA at the surface[1]. When the support hydrophobicity is higher (PS compared to glass) : (a) the amount of adsorbed HSA reaches quickly a high value and does not decrease at longer incubation times, (b) the preferential adsorption of Fg with respect to HSA is lost due to the fact that Fg adsorption is lowered in presence of HSA while the HSA adsorption is not significantly affected by the presence of Fg. This work shows that it is possible to monitor the adsorption competition of proteins in single assays. The mild radiolabelling procedure used is expected to bring less modification to the proteins compared to commonly used procedures. In the future, protein competition in human serum or in medium containing other plasma proteins will be studied, and potentially hemocompatible surfaces will be tested.

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ACKNOWLEDGEMENTS: This research has been partially supported by the Région Wallonne.

Characterisation and cell response of chitosan and alginate derivatives blend membranes with different crosslinking methods

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INTRODUCTION: Chitosan is a cationic polysaccharide, the second most abundant natural polysaccharide after cellulose. It has been thus far broadly used as a biomaterial in various applications because of its biocompatibility in these applications and the fact that it is relatively simple to process and modify. [1] It is readily soluble in dilute organic acids in which it exists as a cationic polyelectrolyte. Alginate is an anionic polysaccharide of similar structure to chitosan proved as well to be biocompatible in a range of uses including food industry and drug delivery. When mixed in acidic conditions they form a polyelectrolyte complex. Nevertheless, when chitosan is solubilised in the form of an anionic polyelectrolyte derivative it is possible to achieve a homogeneous blend with sodium alginate in an aqueous environment.

METHODS: In this work, two types of membranes are being studied. The first is a membrane of chitosan water soluble anionic condensation product with sodium formaldehyde bisulfate (CNaFBS) blended with sodium alginate and crosslinked with Glutaraldehyde and Calcium Chloride (blend1). The second is a blend of (CNaFBS) with Ammonium Alginate without any other crosslinking step (blend2). In both cases two blend ratio were prepared, 50% CNaFBS and 70% CNaFBS. The materials have been characterized using Fourier Transform Infra Red spectroscopy (FTIR), X-ray Diffraction (XRD) and Differential Scanning Calorimetry (DSC). Initial in vitro assessment of cytocompatibility of these membranes was carried out using 3T3 fibroblasts. Neutral Red Assay and SEM microscopy were used.

RESULTS: FTIR analysis suggested that formation of hydrogen bonds between -COO, -OH in NaAlg, and -OH in blend1 occurred. Other interactions are suggested by the FTIR spectra of the blend 2.
X-ray diffractograms showed that the blend1 membranes exhibit a crystalline form due to the effect of calcium ion induced egg box structure while blend2 samples are rather amorphous. This is supported by the DSC data where blend1 has increased stability.
Initial in vitro assessment of cytocompatibility of these membranes was carried out using 3T3 fibroblasts. Neutral Red assay results showed good cell response in terms of cell viability. However the SEM images demonstrated poor cell attachment in both cases. The tests were repeated for samples treated with Fibronectin (FN) which improved the cell attachment on blend1 but not on the blend2 membranes.

DISCUSSION & CONCLUSIONS: Both systems present a great interest in both chemistry and biomaterials field. Their initial in vitro biocompatibility in combination with their anionic character can possibly address the coagulation problem related with chitosan surfaces which makes it a good candidate for blood contacting applications.

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Interaction of Porphyrin Covalently Attached to Poly(Methacrylic Acid) with Liposomal Membranes

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INTRODUCTION: Porphyrins are being used as photosensitizers in the photodynamic therapy (PDT), but they are known to aggregate readily in the aqueous solution. The aggregation of the dye lowers the efficiency of PDT. In this study we describe the results of the model studies on the interactions of a porphyrin (5-(4-acryloyloxy-phenyl)-10,15,20-tritolylporphyrin) covalently attached to poly(metacrylic acid) chain (PMA-Po) with liposomes. PMA-Po is well soluble in water so the aggregation of the porphyrin chromophores can be avoided.

METHODS: PMA-Po with no more then one porphyrin chromophore (Po) at the end of the polymeric chain was synthesized by the anionic polymerization, as reported previously [1]. Liposomes were prepared by extrusion using the Nucleopore Membrane Whatman filters with the 100 nm pore size. For each pH value, a separate stock solution of liposomes was prepared. Determination of liposome-binding constants (K_b) was done using the spectroscopic titration technique [2]. UV-Vis absorption spectra and fluorescence spectra were measured using a Hewlett-Packard 8452A spectrophotometer and an SLM-AMINCO 8100 Instruments spectrofluorimeter, respectively.

RESULTS: Effect of pH on the conformational changes of the polymer was studied using pyrene as a molecular probe. At pH values lower than 4 the ratio of the intensities of the third and the first vibrational bands (I_3/I_1) in the emission spectrum of pyrene was 0.83 indicating that polymer chain forms defined, hydrophobic microdomains. In the pH range between 4.5 and 6.0 the reduction of that ratio was observed to the value characteristic of the water environment. The finding suggests that above pH 6 the polymer chain adopts expanded conformation. The partitioning of Po chromophores between the lipid vesicles and aqueous phase was studied as a function of pH in the range from 6.5 to 9.2 (the ionic strength was 0.1). At acidic pH values the solution becomes cloudy after the addition of the liposome suspension to the solution of PMA-Po and a precipitate appears with time. PMA-induced flocculation of lipid vesicles dispersed in acidic solution was observed earlier by Oto *et al.* [3]. The emission spectra characteristic of Po chromophores were measured in the presence of lipids at various concentrations ([L] varied from 0 to 0.2 mg/mL). The binding constant, K_b, was determined by fitting the experimental data to the following formula:

$$F = \frac{F_{\text{init}} + F_{\text{comp}} K_b [L]}{1 + K_b [L]} \qquad (1)$$

where F_{init}, F and F_{comp} is the fluorescence intensity of the dye that is measured without lipid, with lipid at concentration [L] and that characteristics of complete binding, respectively.

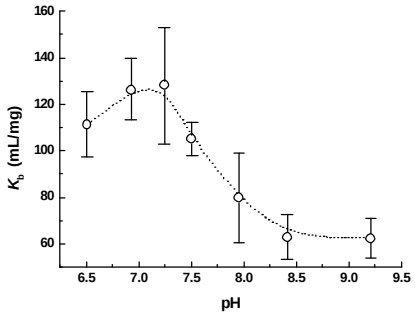

Fig. 1: Depdendence of the binding constant on pH.

DISCUSSION & CONCLUSIONS: Fig. 1 depicts the dependence of K_b on the pH. Similar bell-shaped curve was found previously for dicarboxylic porphyrins [4]. The values of K_b decrease with increasing pH of the solution, which can be attributed to the ionization of the polymeric chain. The ionization of PMA results in the formation of polyanion, which is repulsed by the liposome vesicle (also negatively charged under these conditions). Our results show that PMA-Po binds effectively to the liposomes. The value of K_b is quite high even at pH = 9 when more than 90 % of the carboxylic groups on the polymer chain are ionized.

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Micropatterned polysaccharides: a useful tool for cell guidance

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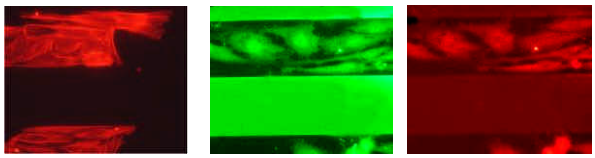
INTRODUCTION: Micropatterned surfaces containing geometrically defined bioadhesive and non-adhesive domains are useful tools to study cell attachment and growth for the creation of highly orientated cell pattern that mimic a functional tissue [1]. An open question is whether the cell behaviour is influenced by the surface chemistry or topography or both. The aim of the work is to find a correlation between the chemistry and/or topography of the different domains and the cell response. Micropatterned surfaces with different chemical and topographical heterogeneity have been realised by the photoimmobilisation of hyaluronic acid (Hyal), one of the main components of the extracellular matrix [2].

METHODS: The photoimmobilisation technique allows the surface-grafting of the polysaccharide previously functionalised with a photoreactive moiety (4-azidoaniline) by the formation of an amidic bond. A precise volume of a 1wt/v% aqueous solution of the photoreactive polysaccharide was spin coated onto a glass substrate and then the surface irradiated with UV light. Using a photomask containing domains of suitable dimensions and geometry, stripes in the microscale with different chemistry and topography have been obtained on the substrate surface. The entire process is simple and inexpensive. The effect of the UV radiation on the Hyal surface chemistry and the surface chemical distribution were analysed by ToF-SIMS. The micro-stripes morphology was investigated by AFM.

RESULTS: Homogeneous Hyal/glass patterns (stripes 50μm wide) have been prepared. By controlling the deposition parameters, microstripes with thicknesses ranging from 20 up to 70nm have been obtained.

The ToF-SIMS analysis evidenced that the UV irradiation changed the polysaccharide surface chemistry. In particular, the peak at 147.07m/z assigned to the molecular ion $C_9H_9NO^+$ of Hyal was not revealed after UV irradiation, while the peak at 242.28m/z belonging to the $C_{15}H_{34}N_2^+$ was detected only after the irradiation. Furthermore, the ToF-SIMS analysis confirmed that the pattern chemistry consisted of well defined Hyal and glass alternating microstripes.

Lymphatic endothelial cells (LEC), as other cell types, aligned along and grow on the glass stripes. The presence of integrin clusters along the cell borders and at the migrating leading edge together with actin stress fibres polarised along stripe directions (Fig 1a), indicated that the cell adhesion is integrin-mediated and the cell migration and orientation guided by the Hyal/glass micropatterns.



a) *b)* *c)*

Fig. 1: a) Actin staining (TRITC); b) αv integrins (FITC); c) FAKs (TRITC).

The co-localization of the αv integrins and the focal adhesion kinases (FAK) showed that the signal transduction occurs from outside into the cell (Fig. 1b, 1c). Different types and amounts of serum proteins were detected on Hyal and glass by 2D-electroforesis. The different proteins that interact with the substrate play a key role in modulating the cell behavior and may explain why the aminosilanised glass is a so cell adhesive substrate [3].

DISCUSSION & CONCLUSIONS: This work demonstrates that the Hyal/glass patterns are suitable substrates for cell guidance, since they are able to direct the LEC growth. The control of surface protein interactions is a key element to drive all subsequent events leading to cell biomaterial response.

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Hydroxyapatite growth induced by extra cellular matrix deposition on solid surfaces

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INTRODUCTION: Biological systems have a remarkable capability to produce perfect fine structures such as seashells, pearls, bones, teeth, corals, etc. These structures are composites of interacting inorganic (calcium phosphate or carbonate minerals) and organic (molecules) parts. Which part has the primary role is difficult to say unambiguously.

In this work the growth of hydroxyapatite (HA) by two methods is reported: (i) a simple soaking process in a simulated body fluid (SBF) and (ii) a laser-liquid-solid interaction (LLSI) process which allows interaction between a scanning laser beam and the substrate immersed in SBF. Metallic and non-metallic materials are used as substrates. Their surfaces are modified by a deposition of extra cellular matrix (ECM) proteins. The deposited layers are investigated by FTIR Spectroscopy and Scanning Electron Microscopy.

METHODS: Samples are prepared by coating of ECM on AISI 316 stainless steel (further named ECM/SS), (100) n-type silicon (ECM/S), and silica glass Herasil (ECM/SG). The osteoblast-like cell line SAOS-2 is allowed to synthesize and assemble its own ECM on the substrates under standard cell culture conditions. Cells are then selectively removed resulting in surfaces coated with a thin film of ECM. To evaluate the ability of the as-modified samples to precipitate HA two methods are applied: (i) a simple soaking process in SBF for 4 and 24 h in which the samples are immersed simultaneously in horizontal and vertical positions and (ii) a laser-liquid-solid interaction process which allows laser beam-substrate interaction in the fluid and subsequent soaking in SBF for 4 and 24 h. Some samples are taken out immediately after the laser interaction to study the instantaneous effect of the laser energy on the HA nucleation and growth (named ECM/SS-LLSI 0h). CuBr vapor pulsed laser (λ= 578.2 nm, 330 mW) equipped with a scanning system is applied. The laser beam is focused on the substrate surface and the interaction time is approx. 2 min. A container with 400 ml SBF kept at physiological conditions (temperature 37^{0}C and pH 7.4) is used in the experiments.

RESULTS: FTIR shows the formation of HA layers on all materials, modified by the two methods (Fig. 1). An estimation of the layer thickness shows that the applied LLSI leads to a thicker layer in comparison to the soaked samples, as well as to an effective formation of nuclei within few minutes for facilitation of further layer growth. Such fast formation of nuclei is not observed in the case of the simple soaking. The vertically soaked samples induce a HA layer on their surfaces, which shows that the deposition is not simply a question of gravity.

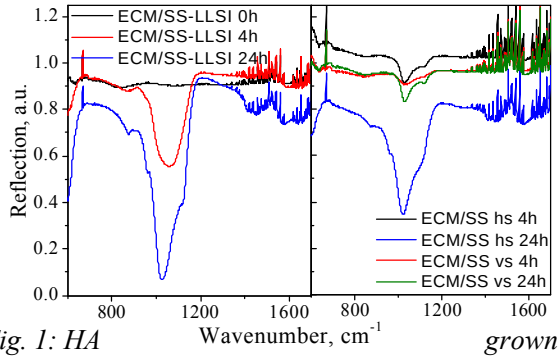

Fig. 1: HA grown on ECM-stainless steel samples modified by a laser-liquid-solid interaction in SBF (left) and by a simple soaking in SBF (right)

HA morphology on the samples modified with surface ECM proteins presents regular sphere-like particles, grouped homogeneously in a network and embedded in the ECM structure as in a matrix. The latter observation is ascribed to the ability of the organic molecules to control and define the crystallization process.

DISCUSSION & CONCLUSIONS: It was found that (1) the surface deposited ECM proteins serve as a matrix for a homogeneous HA growth and influence the size of the HA spherules; (2) the HA nucleation and growth on as-modified materials, prepared by two methods is strongly influenced by the application of laser energy; (3) the layer growth in the case of the soaked samples is not simply a question of gravitational forces.

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Fibroblast Interaction with Different Material Surfaces

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INTRODUCTION: The assessment of biomaterials compatibility relies heavily on the analysis of macroscopic cellular responses to material interaction. The cells are highly sensitive to the substrates having certain topography. The substrates that show this topography may be covered by other cells, extracellular matrix (ECM), or single soluble matrix components, such as fibronectin (FN). The initial steps of cellular interaction *in vitro* may be approximated with the process of cell adhesion and spreading. These early cellular events are particularly well pronounced in fibroblasts.

In this study, the initial human fibroblast interaction with three different types of materials, including stainles steeel (SS), silicon (S) and silica glass (SG), which were further coated with ECM or with ECM and hydroxyapatite (HA) was investigated. The particular effects of FN were evaluated.

METHODS: Human dermal fibroblast was suspended in serum-free medium and left to adhere for 2 h at 37^{0}C under an atmosphere of 5% CO_2 onto three types of substrates: (1) standardly treated SS, S and SG materials, (2) SS, S and SG materials, pre-coated with a native ECM, synthesized and assembled by the osteoblast-like cell line SAOS-2 under standard cell culture conditions and (3) samples from the second type, covered with a porous HA layer through immersion in a simulated body fluid (SBF) for 4 days at 37^{0}C at pH 7.4 (further considered as composite structures). The samples from the three groups were cut in flat 8x8 mm pieces, placed in 12 well TC plates and studied plane or after coating with FN (20 μm/ml for 30 min). The overall cell morphology and spreading were visualized via fluorescein diacetate (FDA) in living cells and viewed on fluorescent laser scanning confocal microscope (LSCM) at low magnification.

RESULTS: The difference in the overal cell morphology of fibroblasts adhering to FN-coated SS samples from the three groups is demonstrated in Fig. 1. It was observed that the number and the shape of the cells were strongly dependent on the type of material used, as well as on the way of their pre-coating. It was found that the FN-coated substrates of the type 'ECM-coated' exhibit the best cell morphology. The 'substrate-ECM-HA' composites showed lower biological activity. The cells adhered well but remain smaller and developed a star-like morphology. From the three classes of materials, stainless steel was found to induce the best cellular reponse in terms of adhesion and spreading of fibroblasts, especially in the presence of FN, followed by SG and Si.

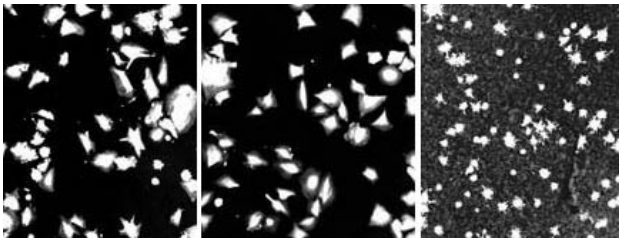

Fig. 1: Overall cell morphology of fibroblasts adhering on three types of FN-coated structures: stainless steel substrate (left), the same substrate coated with ECM proteins (middle) and 'stainless steel-ECM-HA composite (right).

DISCUSSION & CONCLUSIONS: Fibroblast behavior on different solid substrates depends on the nature of the materials and the amount of surface-associated ECM proteins. The best result, however, was obtained on naturally deposited ECM. The composite structures of the type 'substrate-ECM-HA' possess lowered biological activity presumably inserting specific topographical effects. The lowered fibroblast adhesion to these composites was ascribed to a different way of absorption of FN to the cells contacting the upper porous HA. FN pre-coating principally improved the cellular interaction to all tested materials and might serve as a positive control. Thus, fibroblasts were regarded as an appropriate model for studying the initial cell-material interaction. We consider that application of natural ECM matrix components on the different materials is an efficient way to improve their initial interaction with living cells and thus to contribute for their *in-vivo* tissue compatibility.

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Influence of surface patterning on cell migration and spreading

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INTRODUCTION: Recent advances in cell-material interaction have demonstrated that both chemical and topographical properties of the material surface can regulate and control cell shape and functions [1]. Several cell parameters, including cell orientation, motility, adhesion and shape, can be modulated by a specific surface micro- and nano-topography [2]. In this work the influence of a micro-patterned surface topography on all these parameters has been studied, using hydrogenated amorphous carbon (a-C:H) grids with various interspace and height deposited on glass substrate. It is shown that cells are able to recognize specific dimension of the surface topography and to be guided on material surface presenting well defined grid interspace. In particular, cells recognize topographic surfaces patterned with periodicity of 20 to 40µm by showing a better adhesion and spreading. Furthermore, on surface with grid of 40µm interspace, cells preferentially migrate along the carbon paths, exhibiting a persistence length much longer compared to non-patterned surfaces. The cell movement on these patterned surfaces is well described by percolation theory.

METHODS: Patterned surfaces were obtained in two steps: (1) a homogeneous film of a-C:H. was deposited by plasma enhanced chemical vapour deposition (PECVD) on glass substrates [2], and (2) a patterned layer was deposited using metal masks of different dimensions positioned over the sample to realize grids with a depth of around 30nm, measured by AFM analysis (Fig. 1). Therefore topographical patterned surfaces showed the same chemical composition. NIH 3T3 mouse embryo fibroblasts (ATCC CRL 1658) were seeded on the patterned surface at two different cell densities ($1x10^3$ cells/grid and $2.5x10^3$ cells/grid). Cell adhesion and morphology on the patterned surfaces were observed using an optical transmission microscopy. Cell movement was followed with time-lapse cinematography, using an Image Analysis Software (Metamorph, USA).

RESULTS: The results indicate that cell adhesion and motility are significantly dependent on surface topography. On flat surfaces cells spread randomly while on patterned surfaces cells are elongated and aligned along the edges (Fig. 2). When the grid dimensions are too small or too large compared to the cell size, cell orientation is not affected by surface topography. Cell migration on patterned surfaces does not follow the 2D Stokes-Lauffenburger [4] behaviour. Since cell migration occurs preferentially along the patterned carbon grooves, the description of the cell motion is best interpreted by a percolation model. The fractal dimension evaluated by the analysis of the cell motion well correlates with the surface fractal dimension.

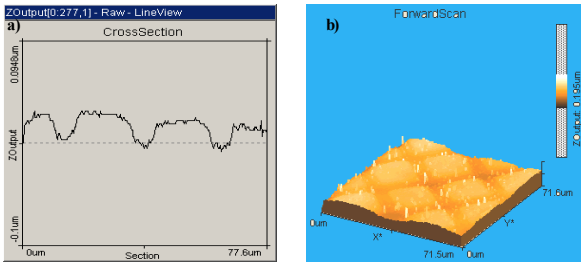

Fig. 1: AFM images of patterned surface of a-C:H: (a) Cross section line view, (b) 3D topography.

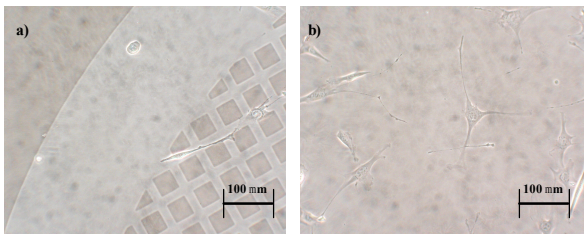

Fig. 2: Light microscope images (20x magnification) of NIH 3T3 on (a) patterned surfaces with square length of 40 mm an spacing of 20 mm.

CONCLUSIONS: Results show that the length of the square, the spacing and depth of 30 nm affect fibroblast morphology and migration. By appropriate surface patterning is potentially possible to pilot and guide cell spreading and direction of motion.

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Immune response to cobalt-based alloy wear particles increased by third-body wear from metal-on-metal THR

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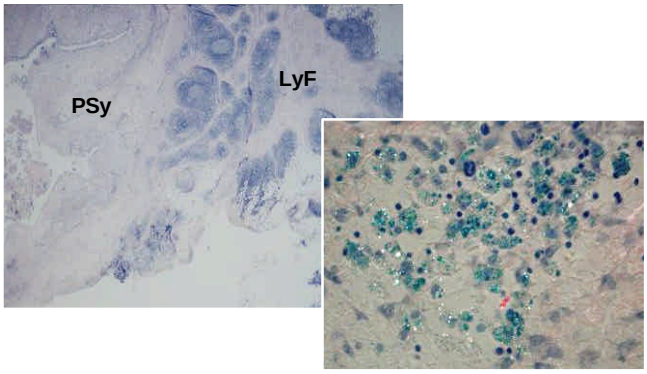
INTRODUCTION: Cobalt-based alloy bearing surfaces for total hip replacement endoprotheses (THR) seemed a promising alternative to other articulating surfaces because reduced wear could avoid the so-called polyethylene particle disease. However, the biological reactions to cobalt-based alloy wear particles are still observed with great cautions [1], and immune cells have been identified in periprosthetic tissues of loosened all-metal THRs [2]. Own findings of such adverse tissue reactions to metallic wear particles from seven retrieved THRs [3] are now expanded in this study to eighteen metal-on-metal THRs, including an equal number of so-called low carbide and high carbide cobalt-based alloys from two different manufacturers.

MATERIAL and METHODS: From 18 patients all-metall THRs (n=9 "SL-Plus", Plus-Endoprothetik, A; n=9 "Alloclassic", Sulzer AG, CH) were retrieved with surrounding tissues at reoperation due to pain and/or radiographic loosening 4 to 75 months after cementless implantation. Surfaces of alumina-blasted implants (cpTi sockets, TiAlNb stems) and bearing surfaces (Co-based alloys) were analysed by SEM, EDXA, and tribology, respectively. Neocapsules and periprosthetic tissues were analysed by LM and SEM/EDXA of undecalcified microtome and ground sections, as well as by ICP-AES elemental analyses for Co,Cr,Mo,Ti and Al.

RESULTS: Alumina particles, attached/impacted from blasting, can be demonstrated on all implant surfaces. Alumina particles are found in high concentrations in periprosthetic tissues (Fig.1b). Alumina particles led to metallic Ti and Ti-alloy wear at loosened implant interfaces. Alumina particles are transported into the metallic articulation, and cause increased third-body wear (Fig.2). The tissue response to the Co-based alloy particles is characterised by basophilic granules (=phagolysosomes) within the macrophages of the pseudosynovia and the neocapsules. Even more impressive are extensive lymphocyte and plasmacell infiltrates in the periprosthetic tissues which could accumulate to lymphatic follicles (Fig.1a).



Figs.1: a: Neocapsule, 13 mos p.impl. showing lymphatic follicles (LyF) underneath the pseudo-synovia (PSy). b: Macrophages with basophilic granules (containing metallic wear particles) and birefringent (alumina) particles.

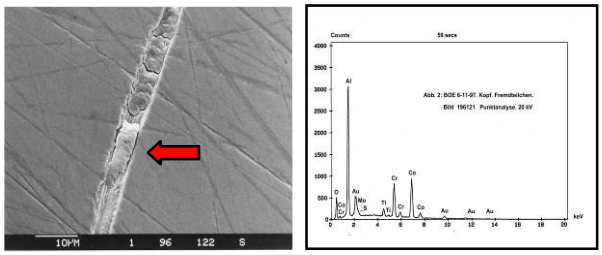


Fig.2: SEM of a Co-based alloy bearing surface with deep scratches, in which alumina-particles are identified by EDXA (arrow).

DISCUSSION & CONCLUSIONS: Co-based alloy wear particles are obviously not as inert as other materials used for articulating surfaces, and apparently cause immune responses, at least in certain patients. Metallic wear is increased by alumina particle contaminations from the currently used blasting procedures for cementless implant surfaces.

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ACKNOWLEDGEMENTS: This study was supported in 2001 by a research project and analytical contributions (G.Reinisch) from Biomech.Forschg.GmbH, Vienna, A.

Aspects of Biocompatibility of Various Thin Films on Titanium Surface

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INTRODUCTION: This paper is an approach of biocompatibility evaluation of various thin film on titanium surface using electrochemical and cytotoxicity procedures, taking into account that the corrosion of biomaterials is an aspect of their biocompatibility. The substrates were tested after different procedures of enhancing Ti resistance, by modifying surface properties of film stability at the biointerface. This is a built of a new friendly interface of metallic implant-living tissue which will induce osseointegration. [1]

METHODS: The specimens were made from pure Ti and were tested in various biofluids. Methodology of surface change in order to achieve desired host response has involved physicochemical treatments and biochemical technique. The first type of methods is illustrated by anodic oxidation and the second type involves dipping the electrode in collagen solution and treatment with collagen gel.
The evaluation of biocompatibility was done using followings investigations:

- electrochemical methods including monitoring of the variance of electrode potential in open circuit in time and cyclic voltametry. In open circuit experiments curves potential versus time for a short and medium time was obtained and simultaneously the dependence pH-time was recorded. Cyclic polarization experiments were performed with the help of a Par 179 potentiostat with computer interface
- cytotoxicity test using direct contact method, with secondary cultures of human skin fibroblasts (HSF), obtained, grown, and subcultured at 37°C in a humidified incubator equilibrated with 5% CO_2. The cells were seeded on the films at a density of 1 $\times 10^5$ cells/ml and cultured for up to 7 days. Fibroblast proliferation was assessed by measuring the mitochondrial dehydrogenase activity.
- complementary technique as microscopic analysis, infrared analysis type FTIR, MedCalc for the statistical treatment and image analysis.

RESULTS: The evolution of open circuit potential in all studied cases reveals a trend to steady state. The difference consists in the time when the equilibrium is reached, depending on the surface film. It is to point out that the potential values are more electronegative in the case of surface electrode untreated with collagen. Fig. 1 presents voltamogrames of Ti.

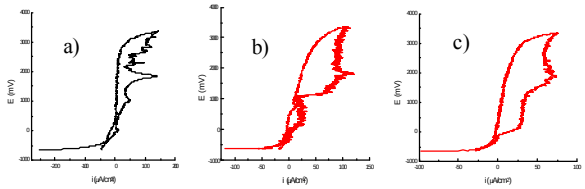

Fig.1 Cyclic curves for Ti: a) untreated; b) and c) immersed 1/2h respectively 2h in collagen solution

In all cases (including the anodized electrode and the one with collagen gel) no breakdown was noticed and the passive domain is larger for the sample with gel. The stability of films was in direct relation with the amount of adhesion for fibroblasts. [2,3] The cell viability (checked by the standard trypan blue exclusion test) is more intense in the case of gel treatment.

DISCUSSION & CONCLUSIONS: The corrosion resistance of Ti system is due to a protective stable mixture of oxides film. [4] If the film is damaged some corrosion will occur, this depending on film reformation. The controlled altering of the surface by suitable treatment (anodic oxidation, collagen treatment) increases corrosion resistance of implant material and the stability of the film. The specimen roughness is direct related to fibroblast growth and proliferation. All processes could be interpreted in 3-D image of an interfacial model and the role of collagen adsorption in formation of a characteristic interfaciale layer should be a part of an empirical model of osseointegration. Concluding inorganic and, organic thin films on Ti surface offers a way to produce a better interface biomaterial-living tissue

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Electrochemical Studies of the Adsorption of Fibrinogen and Bovine Serum Albumin on a Charged Pt Surface

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INTRODUCTION: Most proteins have a strong tendency to adsorb at a solid / liquid interface. This phenomenon plays one of the major roles in both biocompatibility of medical implant devices and biosensors development, due to problems related to surface fouling, bacterial growth, metal dissolution and interface charge distribution. Despite a large number of investigations performed using various experimental techniques, the process is not yet completely understood because of the intrinsic complexity of the process itself. The current study discusses the thermodynamics and kinetics of adsorption of fibrinogen (Fib) and bovine serum albumin (BSA) on a platinum electrode using *dc* and *ac* electrochemical techniques.

METHODS: An oxygen-free phosphate buffer solution pH 7.0 has been used as an electrolyte. A standard three-electrode glass cell comprising high purity Pt (99.99%) working and counter electrodes and mercury sulfate reference electrode (MSE) has been used. Cyclic voltammetry (CV), differential electrochemical capacitance (DEC), electrochemical impedance spectroscopy (EIS), and electrochemical quartz crystal nanobalance (EQCN) techniques have been used in research.

RESULTS, DISCUSSION & CONCLUSIONS: CV and EIS measurements have shown that the surface charge density (Q_{ads}) and charge-transfer admittance (Y^{ct}) resulting from Fib and BSA adsorption are directly proportional to the amount of adsorbed protein, *i.e.* surface concentration (Γ), indicating that adsorption is accompanied by the transfer of charge from the carboxylate groups on the protein[1]. EIS and DEC measurements have shown that the protein adsorption results in the blockage of the Pt surface. The adsorption of the proteins has been found to be reversible under the conditions applied. The dependence between the surface and bulk protein concentration has been found to be of a Langmurian type (Fig.1). High negative values for the Gibbs free energy of adsorption indicate strong adsorption of the proteins onto the Pt surface. Although the adsorption process of both proteins has been found to be endothermic, the change in entropy appears to be the driving force for the protein adsorption. The experimentally obtained saturated surface concentration of Fib and BSA has been shown to increase linearly with the increase in temperature, corresponding to the formation of up to a monolayer of side-on adsorbed Fib layer [2], and several monolayers of BSA at high temperatures.

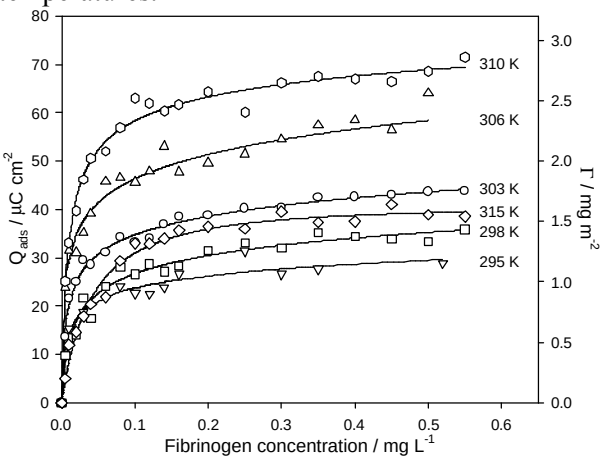

Fig. 1: Dependence of surface charge density (Q_{ads}) and surface concentration (G) on the fibrinogen bulk concentration at various temperatures.

At anodic potentials the adsorption of the proteins has been found to be a competitive process with the surface oxidation. This has been verified by EQCN measurements where the mass changes calculated from the recorded frequency changes correspond to the adsorption and reduction of the OH_{ads} species in the presence of Fib and BSA[3]. The kinetics measurements have shown that the adsorption of BSA occurs with the subsequent rearrangement of the protein at the surface.

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Iron and Alumina Grit Blasted TMFZ Alloys Differentially Regulate Bone Formation *in Vitro*

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INTRODUCTION: Orthopaedic implant surface roughness has been shown to enhance osteoblast adhesion and differentiation [1-2]. Many uncemented hip implants now use modified surfaces to promote implant fixation and osteointergration. In this study a titanium, molybdenum zirconium and iron (TMZF) alloy was prepared with different surface roughness and contrasted for their ability to support osteogenesis in vitro.

METHODS: Four different TMZF surfaces were produced: a highly polished surface (features <0.3um), a finish of moderate roughness (<3um) and two highly roughened surfaces (>10um), produced by blasting with either iron grit giving a contamination-free surface or alumina - grit giving a surface contaminated with embedded alumina. SEM was used to observe surface topography and the chemical composition of the surfaces monitored by EDX ion beam SEM.
Primary rat osteoblasts were seeded on to the surfaces (at least 6 duplicates of each) and grown for three days to confluence. The growth media was then supplemented with L-ascorbic acid, dexamethasone and β-glycerol phosphate to induce bone formation. After a further 25 days, cells were fixed and Von Kossa staining was used to identify bone nodule formation. The percentage area of osteogenesis was determined by image analysis.

RESULTS: (sizes, annotation)

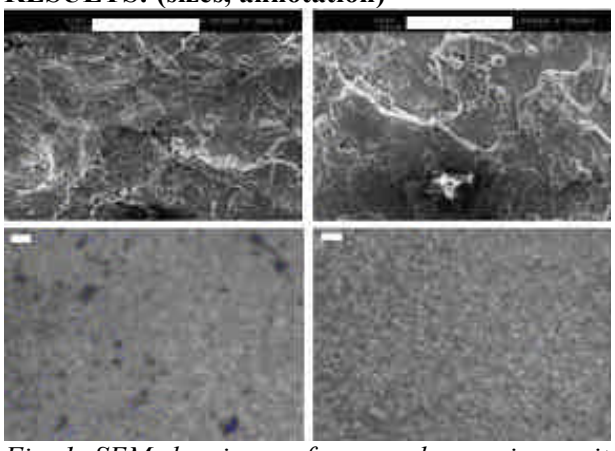

Fig. 1: SEM showing surface roughness, iron grit blasted (top left) vs. sand blasted (top right),Von Kossa staining shows bone nodule formation on respective materials (shown underneath),white bar= 50 mm.

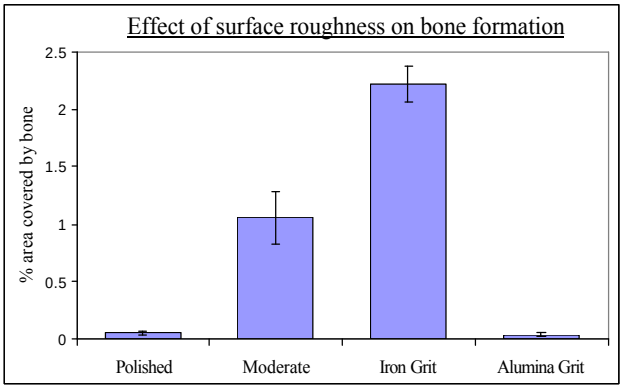

Fig 2. Bone nodule formation on implant surfaces. All surfaces, apart from polished and alumina grit, were statistically significantly different by ANOVA (p>0.05).

DISCUSSION & CONCLUSIONS: In agreement with the published literature on other materials, surface roughness increases bone nodule formation on the TMZF alloys used here. Although the levels of surface roughness achieved with the grit blasting techniques are similar (Fig1.), alumina particles embedded in the surface appear to result in inhibition of bone nodule formation (Fig 2.). In contrast the iron grit blasted surfaces (with no embedded impurity) gave the best bone formation consistent with a surface of this roughness. Indeed, in a separate series of experiments the iron grit roughened surface gave superior results to hydroxyappatite coatings, a surface modification often employed in implant manufacture. In summary the surface produced by iron grit blasting (contamination – free) supported greater bone formation in these *in vitro* assays and could be used to increase bone formation resulting in superior integration of implants into the patients skeleton.

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Immunohistochemical Characterization of the Bone-Implant Interface on Mma-Embedded Cutting and Grinding Sections

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INTRODUCTION: When investigating the tissue reaction on metallic implants, the cellular activity at the interface between the host tissue and the implant *in situ* is of special interest. The preparation of undecalcified bone specimens with MMA leads to a better preservation of bone trabeculae and more information about mineralization and recent bone formation than routine paraffin embedding [1]. However, the technical workup is demanding and few reports exist on the immunohistochemical characterization of orthopedic implants *in situ* [2]. We present a reliable method for immunohistological staining of cutting and grinding sections embedded in Technovit 9100N of rat, sheep and human bone.

METHODS: The following specimens were used:

1. Titanium Kirschner wires [3] inserted as intramedullary nails into the rat tibia [Fig. 1]
2. Schanz screws (orthopedic steel) implanted as external fixator into the tibia of sheep
3. Human bone from heterotopic ossifications and tumor metastases

Briefly, specimens were fixed immediately in 1.4% paraformaldehyde or 4% neutral buffered formaldehyde, dehydrated with increasing concentrations of alcohol, then infiltrated with xylene and polymierized with MMA-based Technovit 9100N (Heraeus-Kulzer, Friedrichsdorf, Germany). The hardened specimen blocks were cut with Exact cutting and grinding equipment (Exact, Norderstedt, Germany). Grinding sections of 10 to 15µm thickness were obtained and polished. The mounted sections were deacrylated in three steps with 2-methoxy ethyl acetate for 24h, rehydrated, and pretreated with microwaves and citrate buffer at pH 6.0. Endogenous peroxidase was blocked with 10% H_2O_2 in methanol and the electrostatic protein charging with dry milk and normal sera (rabbit, swine). Specimens were incubated with the respective primary antibodies (see Table 1). Biotinylated secondary antibodies (anti-mouse, anti-rabbit) were added, followed by an avidin-biotin-complex (ABC from Vector, Burlingame, VT, USA). The peroxidase activity was visualized with Romulin or diamino benzidine. Control procedures without adding primary antibodies were performed.

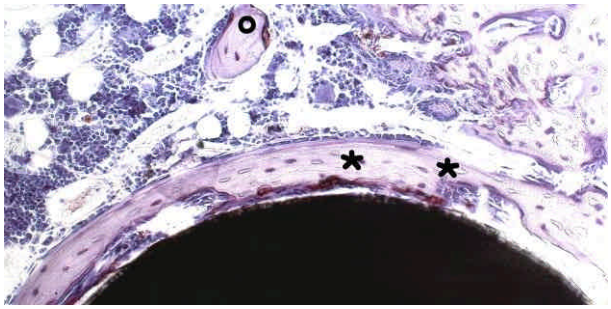

Fig. 1: Staining against ED1 macrophage antibody around the rat tibia implant (stars) and local bone (circle). Original magnification x200.

RESULTS: The following antibodies could reliably and reproducibly be detected specifically:

Rat	Sheep	Human
osteopontin	osteopontin	osteopontin
osteonectin	osteonectin	MNF118
Cathepsin D	Cathepsin D	CD 3
ED 1		CD 68
v. Willebrand factor		v. Willebrand factor

DISCUSSION & CONCLUSIONS: Reliable detection of immunohistochemical markers of bone resorption, bone formation, inflammation and angiogenesis at undecalcified bone sections with the implant *in situ* appears promising in enhancing our understanding of the cellular activity and cell- matrix interactions at the bone-implant interface.

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Copolymerization of HEMA with N-Containing Monomers in Order to Produce a Protein Resistant Biomaterial Surface

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INTRODUCTION: A biomaterial is a material designed to fulfill a purpose and to exist at a physiological interface without being rejected. The most important problem, in the use of most biomaterials is their biocompatibility. It is essential for an implanted device to be able to avoid physiological rejection at the biological interface to which it applied. A number of polymers are known which have a wide range of properties ranging from hard and glassy plastics, through hydrophobic rubbery materials, to soft water containing hydrogel matrices.

METHODS: In this piece of research work, three different nitrogen containing monomers (Fig. 1) were used for copolymerisation with HEMA. The polymerization was carried out as bulk polymerisation at 60° C for three days. The produced polymers were hydrated in distilled water with frequent changes for a period of two weeks. The cross-linker was 1% ethylene glycol dimethacrylate (EGDM). The quantity of protein deposited after incubation in protein solutions, was measured by U.V. at 280 nm.

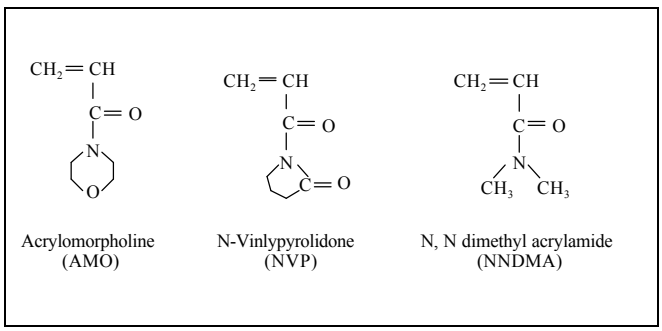

Fig. 1 The chemical structures of co- monomers.

RESULTS: A typical adsorption profile is presented in Figure 2.

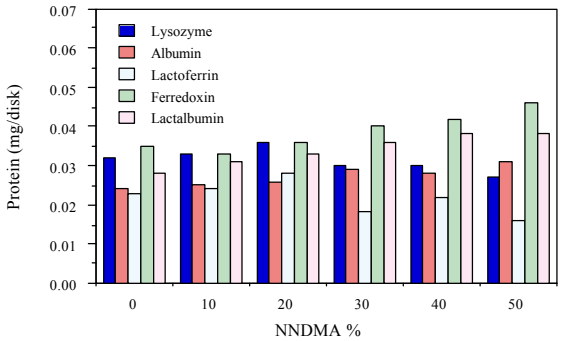

Fig.2 Protein adsorption on HEMA/NNDMA.

DISCUSSION & CONCLUSIONS: It was shown that in this case, NVP produced a copolymer that highly resist the biological environment, i.e. protein adsorption. NVP carries a high positive charge, while NNDMA has a slightly positive charge due to the mesomeric effect of the methyl groups on the nitrogen. On the other hand, AMO is a hydrophilic monomer with low positive charge. The results presented here are the mean values of three similar spoilation experiment carried out under the same conditions (± SD). In this case, although NNDMA has introduced some positive charge on the polymer matrix, but lysozyme (the positively charged protein, MW=14KD) shows high adsorption which may be due to its small size and compact structure.

Ferredoxine (negatively charged with molecular weight similar to lysozyme) showed the highest adsorption on this positively charged copolymer.

REFERENCES: [1]H. Shirahama, J. Lyklema, W. Norde (1990) *J Colloid Interfcae Sci* **139(1)**: 177-87. [2]R. Sariri (1997) *Iranian Poly J* **6(2)**: 135-143. [3]F. Fang and I. Szleifer (2001) *Biophys J*. **80 (6)**: 2568-89.

ACKNOWLEDGEMENTS: We would like to appreciate the scientific advice given by Prof. Tighe.

Selective Cell Delivery for 3D Tissue Culture and Engineering

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INTRODUCTION: A contact-less nano-dosing technique has been investigated for direct delivery of mammalian cell suspensions. The method is based on drop-on-demand piezoelectric ink-jet printing and allows for selective deposition of multiple droplets according to digitally stored patterns, under full computer control. A variety of cell types and jetting parameters were evaluated to determine optimum printing conditions.

METHODS:

Human fibroblasts were obtained from the UK Centre for Tissue Engineering (University of Manchester, UK). Two types of fibroblast cells were used, primary cells (Type 1) and HT1080 sarcoma cells (Type 2). Human primary osteoblasts were isolated from femoral heads after total hip replacement surgery. Bovine chondrocytes were isolated from metacarpalphalangeal joints obtained from a local abattoir.

Cells were harvested using a standard trypsination procedure. The harvested cells were re-suspended in the required quantity of fresh media. To ensure uniform distribution and to disrupt cell clumps the final solution was gently agitated using a pipette. Cells were re-dispersed in fluid at different desired concentrations prior to printing trials.

Printing experiments were carried out using single nozzle tubular piezoelectric jets (Microfab Inc., Plano TX, USA). The nozzle orifice diameters used were of 30 micron for fibroblast suspensions and 60 micron for both osteoblast and chondrocyte suspensions. All suspensions were printed at a rate of 13000 droplets per second, with piezoelectric driving voltages ranging from 30 to 80 V onto well plates. The latter were standard tissue culture plastics made of non-pyrogenic polystyrene (Costar®). The cells were incubated at 37ºC - 5% CO2; and monitored over a period of 6 days using a light microscope.

RESULTS: Figure 1 shows a montage of results for both fibroblasts and chondrocytes after printing at various voltages. Cells appear to be able to survive the conditions of drop formation and impact without harm, as shown by their ability to attach, spread and proliferate over time, almost to confluency in 144 hours.

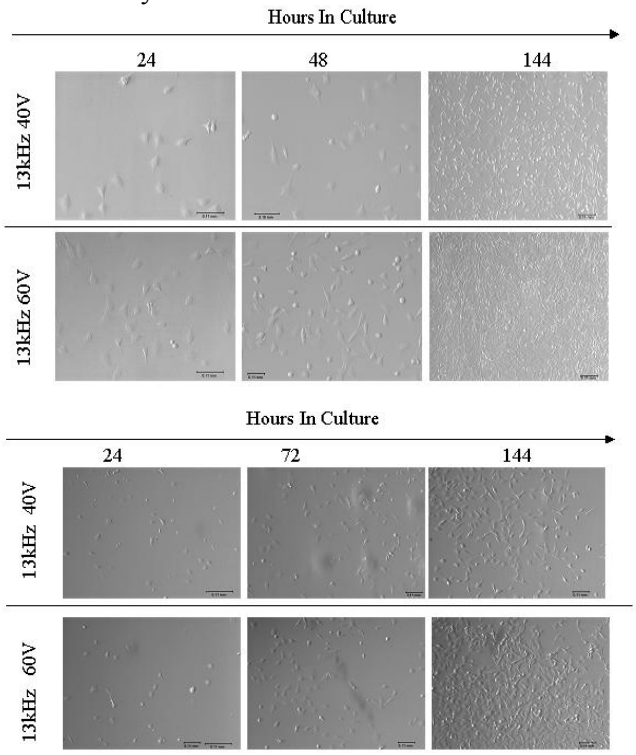

Figure 1 – Montage of results of bovine chondrocytes (above) and fibroblasts - type 2 (below) after ink-jet printing.

DISCUSSION & CONCLUSIONS: Despite the high transient shear rates in excess of 10^3 s^{-1} observed during ink-jet printing, tests with mammalian cells (bovine chondrocytes, human fibroblastic cells and primary human osteoblasts) have shown cellular viability after printing. The combination of direct cell delivery with layer-wise delivery of a compatible substrate or matrix material allows for internal seeding of 3D scaffolds with control over each individual volume element regarding cell type and/or biochemical factor. Hence it is a useful technology for 3D culture studies which are known as better models for studying biological activity in living

organisms and could be a key technology for tissue engineering.

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Designing Metallic Biomaterial Surfaces by Anodic Immobilization of DNA-Single Strands Utilizing Hybridization for Attachment of Biomolecules

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INTRODUCTION: Bio surface-engineering, i.e. coating of implant surfaces with biologically active molecules, offers a high potential for accelerating the healing process and improving the long term behaviour of implants, especially in locations with low bone quality and/or for patients with systemic diseases like diabetes.

Aim of this work is to use the self-organising principle of DNA double strand formation for the immobilization of bioactive molecules [1] on implant surfaces. This system allows for (i) the immobilization of a number of different biomolecules with (ii) defined release kinetics and (iii) the possibility of adapting the implant surface properties to a specific patient immediately prior to surgery.

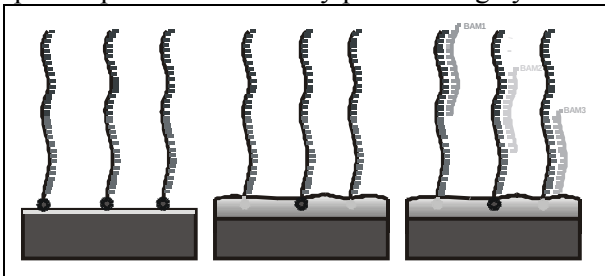

Fig. 1: Scheme of the immobilization method.

METHODS: Samples of $TiAl_6Nb_7$ were turned, ground down to P600 (26µm) and subsequently etched in a mixture of 0.4 M hydrofluoric acid and 1 M nitric acid at room temperature.

All nucleic acids used were single stranded oligodeoxyribonucleotides (ODN). Synthesis and modification (phosphorylation) were done by Thermo Electron Corp., radioactive labelling using ^{32}P-NTPs by Hartmann Analytic GmbH.

The anchoring strand (AS1) was a 5'-phosphorylated 60mer, the complementary (CS1) and non-complementary (NS1) strands were non-phosphorylated 31mers.

Immobilization was performed in acetic acid buffer (pH = 4.0) by (i) physisorption of AS1 followed by (ii) anodic polarization to potentials out up 14.5 V_{SCE}. Hybridization and desorption experiments were performed in TRIS-HCl-buffer (pH = 7.5).

RESULTS: Results indicate that a threshold potential for a stable immobilization of AS1 exists of about 4 V_{SCE}. Increasing the potential does not result in a significant increase of the immobilized amount. As shown in figure 2, CS1 can be hybridized effectively to AS1. The amount of counter strand (normalized to the amount of AS1) decreases with an increasing amount of immobilized AS1, possibly due to sterical hindrance and electrostatic repulsion. Optimal surface coverage seems to result from AS1-concentrations of 400 nM in the immobilization medium.

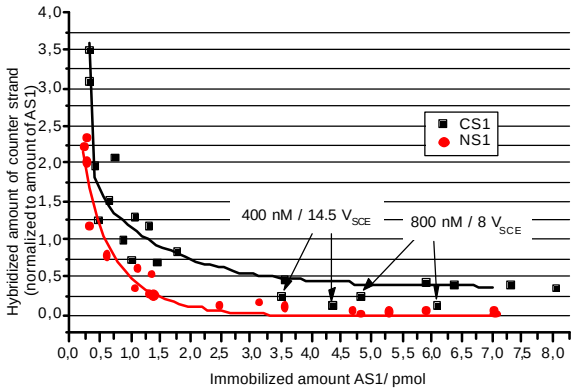

Fig. 2: Hybridized fractions of AS1 with CS1 and NS1 as function of AS1 surface coverage. Effects caused by polarization potential and higher concentrations of AS1 marked by arrows.

DISCUSSION & CONCLUSIONS: Based on initial electrostatic interactions, modified DNA single strands can be immobilized on titanium materials by partial incorporation into anodic oxide layers via their termini in a stable and regio-selective manner. Complementary DNA strands could be successfully hybridized with kinetics similar to the behavior in solution. Further experiments will focus on further development of the described system by using complementary strands conjugated with biologically active molecules (Fig. 1) and ensuing first cell experiments [2].

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Micropatterning of DNA-tagged vesicles

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INTRODUCTION: A new approach for the creation of vesicular micro-arrays using Molecular Assembly Patterning by Lift-off (MAPL) [1] in combination with the immobilization of DNA-tagged intact vesicles is presented [2]. (See Figure 1)

METHODS: In order to combine these two concepts a multistep surface modification was developed. Nb_2O_5 coated substrates were coated with PLL-*g*-PEGbiotin. BiotinDNA - neutrAvidin complexes were immobilized to this modified surface. Complementary cholesterol DNA-tagged vesicles were then specifically coupled to the surface through the hybridization of the DNA strands. Optical Waveguide Lightmode Spectroscopy (OWLS) and Quartz Crystal Microbalance/Dissipation (QCM-D) were used for the *in situ* monitoring of the multistep surface modification process.

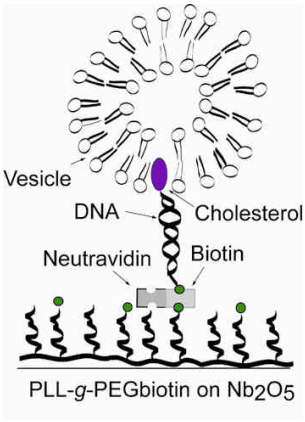

Fig. 1: The immobilization of DNA-tagged vesicles requires a multistep surface modification. Nb_2O_5 coated substrates were coated with PLL-g-PEGbiotin. BiotinDNA - neutrAvidin complexes were immobilized to this modified surface. Complementary DNA cholesterol - tagged vesicles can then be specifically coupled to the surface through the hybridization of the DNA strands.

RESULTS: The amount of adsorbed biotinDNA - neutrAvidin complexes was found to decrease with increasing biotinDNA to neutrAvidin molar ratio in solution (R_b) because of charge effects.

In order to allow for the sorting of the vesicles, the cholesterol cDNA and the POPC vesicles were mixed in solution. It was found that the amount of adsorbed cholesterol cDNA - POPC vesicle complexes depends on the surface density of biotin and on the ionic strength of the buffer. It was found that increasing the number of anchor points to the surface results in the deformation of the vesicles.

Well defined microarrays of vesicles were prepared on the MAPL modified surfaces using the optimized immobilization protocol. (See Figure 2).

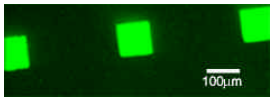

Fig. 2: Fluorescent microscopy image of labeled, cholesterol cDNA-tagged vesicles which have selectively hybridized to the surface immobilized single stranded biotinDNA on the squares while the background remained resistant to the non-specific adsorption of vesicles.

DISCUSSION & CONCLUSIONS: We present a novel platform technology for the localized surface immobilization of intact DNA-tagged vesicles in a controlled, non-destructive environment. The combination of this surface chemistry with the MAPL patterning technique provides a unique possibility for the creation of enzyme and/or membrane protein microarrays..

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ACKNOWLEDGEMENTS: The EC STREP-NANOCUES project (FP6-NMP-2002-3.4.1.2-1) is acknowledged for the financial support.

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Woven P(L/D)LA Scaffolds for Cartilage Tissue Engineering

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INTRODUCTION: The autologous chondrocyte transplantation (ACT) technique has been used successfully to treat defined cartilage lesions. However, so far the treatment of degenerated cartilage has not been possible due to the lack of suitable scaffolds. In this study we developed P(L/D)LA-scaffolds which might enable the treatment of osteoarthritic lesions with ACT. Since the differentiation state of the transplanted chondrocytes is an important factor determining the success of this procedure is, we investigated if chondrocytes differentiation was affected by scaffold geometry.

METHODS: *Scaffold production:* Polymer used in this study was medical grade polylactide L- and D-copolymer with L, D-monomer ratio 96 to 4 (P(L/D)LA 96/4, PLA96) (Purac biochem b.v., Gorinchem, The Netherlands). Multifilament yarns were melt-spun using a Gimac microextruder ϕ 12 mm (Gimac, Gastronno, Italy) equipped with a spinneret with orifices each ϕ 0.4 mm (4 and 8 filament) or ϕ 0.2 mm (12 filament yearn). The yarns were oriented by drawing freely in a three-step process at elevated temperatures to the draw ratio around 4.3-4.6 depending on the batch used. All yarns were knitted to tubular single jerseys. Parallely or anti-parallely aligned layers were used to produce a total of 12 different scaffolds (ϕ 6 mm h 3 mm). All samples were sterilized by γ-irradiation.

Chondrocyte seeding: Scaffolds (n=4) were seeded with 1.10^5 porcine chondrocytes and cultured for three weeks. After embedding in Technovit 8100 (Hereus-Kulzer), 4µm sections were cut using a Leica microtome. Haemotoxylin and Eosin- or Safranin O-stained sections were then analyzed using stereological methods.

RESULTS: Three weeks after seeding, all scaffolds were covered with a layer of shiny white cartilage-like material. Histological analysis showed that most of the cartilage formation occurred on the surface of the scaffolds, while deeper in scaffold, large areas were devoid of cartilage. The varying of the scaffold geometry had only minor effects on cell seeding and chondrocyte distribution.

Interestingly, clear differences could be observed between the distribution of round chondrocytes with matrix and flat fibroblast-like cells (these probably represent dedifferentiated chondrocytes) (fig 1). At the surface of the scaffold, only chondrocyte-like cells could be found, while deeper in the scaffold, increasing numbers of dedifferentiated cells were present. The cells penetrating the deepest into the scaffold basically all had a fibroblastic phenotype.

DISCUSSION & CONCLUSIONS: The preferential location of cartilage-like tissue at the surface of the PLDLA-scaffolds, might be caused by two factors: firstly, it appears that the woven structure of the scaffolds prevents the efficient seeding of the chondrocytes. Secondly, reduced diffusion of nutrients to the inner parts of the scaffold might prevent the optimal formation of cartilage proteins. This lack of nutrients might also induce chondrocytes in the zones farther removed from the surface to dedifferentiate. This would explain why a large part of the cells in the deeper zones have a fibroblast-like phenotype. On the other hand this distribution might simply be explained by the fact that dedifferentiated cells are more mobile than differentiated chondrocytes and therefore move deeper into the scaffolds. Changes in scaffolds structure should improve the seeding of cells within these scaffolds and improve the distribution of differentiated cells within the scaffold.

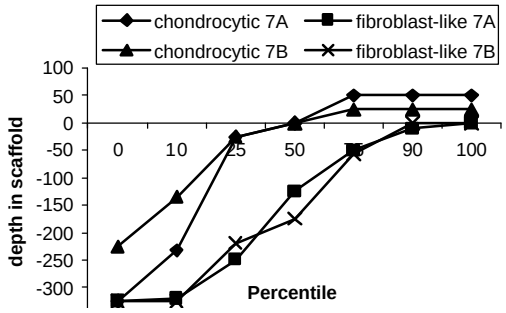

Fig 1. Distribution of chondrocyte- and fibroblast-like cells within P(L/D)LA scaffold with parallely (7A) and anti-parallely (7B) aligned layers.

ACKNOWLEDGEMENTS: Financial support for this study was provided by grant QLK6-CT-2000-00487 from the EU fifth framework program.

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Bacterial delivery of nanophase hydroxyapatite and tricalcium phosphate as a novel coating method for complex architectures.

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INTRODUCTION: Hydroxyapatite (HA) and tricalcium phosphate (TCP) are used as synthetic bone graft materials and as coatings on metallic implants. Coatings are most commonly applied by the plasma spray procedure but implants with complex architectures are difficult to coat evenly because areas not in the direct line of sight are obscured. Alternative methods, such as gel coating, require additional steps to remove the gel to obtain a layer of HA.

The bacterium *Serratia* N14 forms nanoscale (25-28nm) extracellular crystals of HA when grown as a biofilm and subsequently challenged with glycerol-2-phosphate (G2P) and calcium chloride. The reaction occurs due to enzymic cleavage of G2P by acid phosphatase, present in the bacterial periplasmic space and within the extracellular polymeric matrix[1] After sintering, calcium phosphate (CaP)-encrusted biofilm grown on polyurethane foam yields a TCP scaffold with porous microstructure that supports growth of bone cells *in vitro*.[2] In this study the ability to produce CaP on polyurethane foam of smaller pore size and on titanium discs, was investigated, as a potential "non-line-of-sight" method of coating materials with HA and TCP for dental and orthopaedic applications.

METHODS: Bacteria were cultured in an air-lift fermenter on approximately 1cm^3 polyurethane cubes (pore diameter 1.0 or 0.5mm) or on grit-blasted titanium (Ti) discs for 6 days to establish the biofilm, under conditions of carbon limitation. Cubes were then transferred to a 25cm long glass column, through which the challenge solution, (TAPSO buffer, pH 8.6, $CaCl_2$ and G2P) flowed upwards so that the liquid reached all areas, for approximately 14 days. Ti discs were coated with biofilm either in flasks (batch culture) or in an air-lift fermenter. They were challenged either in the flasks, the fermenter or the column. Na citrate was added to some experiments to inhibit spontaneous precipitation of CaP. Cubes and discs were air-dried and sintered at 1100 ºC for 2h.

RESULTS: Crystal-encrusted biofilm formed evenly throughout the labyrinth of foams of both 1mm and 0.5mm pore sizes and when sintered yielded reticulated scaffolds with interconnected, pores and links of even thickness *(Fig1a,b)*. Biofilms formed more evenly on titanium discs from the air-lift fermenter than in flasks and thickest, most uniform coatings were obtained from biofilm grown and challenged in the fermenter in the presence of citrate *(Fig 1c,d)*.

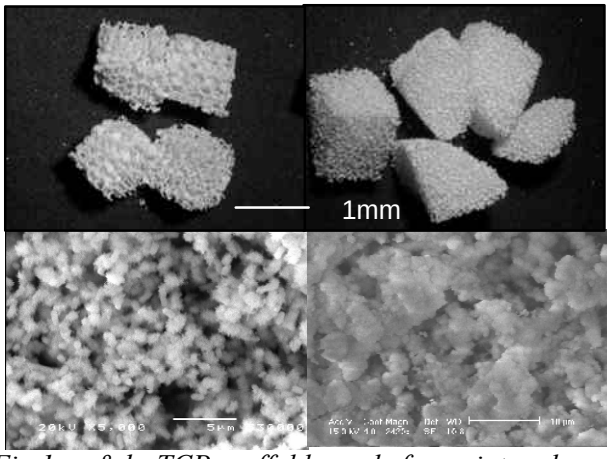

Fig.1: a & b: TCP scaffolds made from sintered (a) 1mm and (b) 0.5mm CaP-encrusted foam. (c) unsintered biofilm on Ti disc from flask showing individual CaP-coated bacteria. (d) closely packed biofilm on disc from fermenter.

DISCUSSION & CONCLUSIONS: This study showed that *Serratia* N14 could form a biofilm on polyurethane foam of 1 and 0.5mm pore sizes and on Ti. TCP scaffolds produced after sintering could have applications as synthetic resorbable bone graft materials, the 0.5mm pores permitting ingress of blood capillaries. *Serratia* N14 can form biofilms on a range of substrata including other polymers, glass and stainless steel. The bacterial delivery system is thus a potentially useful "non line-of sight" method of obtaining an even coating of HA and/or TCP on different materials and structures of complex architecture. Further work is required to optimize culture conditions for biofilm formation and to improve mechanical properties of the products.

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Leptin regulates human osteoblast proliferation and phenotype marker expression in vitro

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INTRODUCTION:

Leptin, known as the obesity gene, is a small polypeptide that controls body weight and gonadal function. In addtion, leptin has been advocated as an important signal to modulate bone metabolism. In animal models, leptin knock out mice (ob/ob) show an increased bone density, mineral content, and total bone area following systemic leptin administration[1]. In vitro, continuous leptin exposure promotes collagen synthesis and mineralization[2]. However, other experimental studies failed to identify leptin receptors in osteoblasts, implying a central, neuroendocrine, and indirect leptin-regulation of bone metabolism[3]. Because of these contradicting results we wanted to elucidate the effect of leptin on bone cells. We used two different types of osteoblasts: primary osteoblast cultures originating from the iliac crest and osteoblasts from posttraumatic heterotopic ossifications (HO) into soft tissue. These ectopic bone formations occur especially after traumatic brain injury[4] .

METHODS:

Experimental in-vitro study: Semiquantitative RT-PCR was used to analyse the expression of human leptin receptor of the osteoblasts. Primary human osteoblast cell cultures (n=6) and osteoblasts from HO (n=6) were incubated every 24h with recombinant human leptin (Calbiochem ®) at concentrations of 0 (control), 25, 50, and 100 ng/ml. After 7 days, we analysed cell proliferation rates using a MTT-test. The expression of the phenotype marker osteocalcin was analysed in both groups (ELISA).

RESULTS:

Human leptin receptor expression was strongly positive in both type of cells. (Figure 1). Cell proliferation rates (MTT) in human osteoblasts from iliac crest showed an increased proliferation under leptin stimulation with 100ng/ml. (Figure 2). HO cells showed no enhanced proliferation under leptin influence (Figure 2.) Osteocalcin expression was depleted in HO-cells but not in primary human osteoblasts.

DISCUSSION:

Present knowledge on leptin indicates both a direct stimulatory effect on bone growth and an indirect suppressive effect on bone through the hypothalamus [1-3]. Our data suggests a possible peripheral action of leptin on bone growth via an osteoblast receptor. The role of leptin in the pathogenesis of certain bone-diseases, such as HO has to be further investigated.

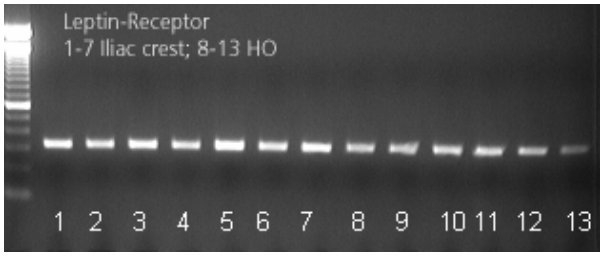

Figure 1: Leptin-Receptor Expression in primary human osteoblast (1-7) and heterotopic ossifications (8-13) (RT-PCR) sense: 5TGCGCT GTAAGAGGCTAGAT-3`antisense: 5TG GCC AGA ACC GTA ACAGT-3`) (NM002303).

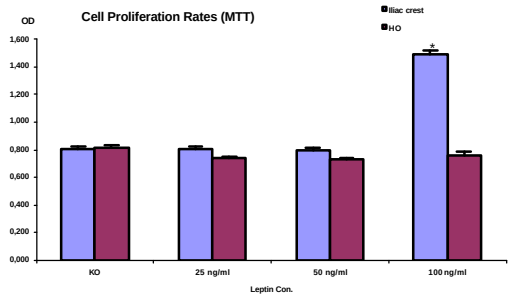

Figure 2: Increased proliferation (MTT) under exposure of recombinant leptin in human osteoblasts (100ng/ml)

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Osteoblasts viability, alkaline phosphatase production, collagen secretion and cell apoptosis in the presence of glasses with novel formulations containing mica and wollastonite structural components

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INTRODUCTION: Osteoblasts support formation, secretion and mineralization of extracellular bone matrix in tissue regeneration process. Biomaterials made of glasses have attracted wide interest in biomedicine, largely in applications for bone substitution [1]. In the framework of *in vitro* studying glass-tissue interactions [2], this work investigates osteoblasts' behaviour, viability, secretion of collagen and alkaline phosphatase as well as the apoptosis rate in the presence of two glasses with novel formulations which contained mica and wollastonite structural components.

MATERIALS: The glassed contained SiO_2, Al_2O_3, B_2O_3, MgO, CaO, Na_2O and CaF_2, in a ratio which corresponded to the co-presence of wollastonite and mica, while the second glass was doped with P_2O_5. Glasses in bulk form were produced by melting at 1400ºC for 1 h, casting and annealing (close to T_g). For the biological experiments, fine glass powders were obtained by milling (grain size <30 μm).

BIOLOGICAL TESTS: Primary cultures of osteoblasts were prepared from sequential enzymatic digestion of calvarie obtained from 1-5 days old Wistar rats. Briefly, after cut into small pieces, the calvarie bone was digested with trypsin 1% and four times with collagenase 2%. The supernatant of the three last washes were centrifuged at 1400 g for 5 min. The pellet re-suspended in culture medium and plated. After confluence the cells were replicated and used in passage 2. Culture medium containing glass powder (separately for each glass) was put in contact with osteoblasts that have been plated in $5x10^4$ cell density at passage 2. After 72 h of incubation the viability and proliferation was measured by MTT method, which is based on the capacity of viable cell to metabolize tetrazolium to formazan crystals, a purple dye that can be solubilized and measured by optical density. Alkaline phosphatase production was analysed by NBT-BCIP assay. Nitroblue-tetrazolium react with the alkaline phosphatase secreted by osteoblasts spreading purple crystals that can be solubilized and measured by optical density. Collagen secretion was measured using SIRCOL method. This assay is based on the capacity of Syrius red dye to bind to the end of collagen molecule and precipitate it. After solubilization, collagen concentration was quantified using linear regression from samples of known concentrations of collagen. Apoptosis was observed by incubating the cells with propidium iodide, a fluorescent dye that stains the apoptotic nucleus. Data were statistically analysed by variance test ANOVA using Bonferroni's post-test.

RESULTS: With respect to the control, the presence of both glasses caused no perceptible changes in the morphology of osteoblasts. Neither osteoblasts' viability and proliferation nor alkaline phosphatase production were altered in the presence of the glasses when compared to control. However, collagen secretion significantly increased in the presence of both glasses when compared to control. Similar apoptosis rate was observed in all cases.

DISCUSSION & CONCLUSIONS: It has been reported that the composition of biomaterials can influence the temporal sequence of matrix protein secretion [3], while ceramics structure and topography can alter osteoblasts' function [4]. The increased collagen secretion may be due to the silicon content of the glasses. Earlier studies have demonstrated that silicon enables enhancement of collagen secretion by osteoblasts [5]. The present results indicate both the investigated glasses for further *in vivo* testing since there was no evidence of significant negative effect with regards to cell proliferation, viability, alkaline phosphatase production and apoptosis rate.

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Biocompatibility evaluation of four different Bioglass™-HA using primary culture of osteoblasts

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INTRODUCTION: Hydroxiapatite (HA)-based materials are considered to be potentially useful as bone implant materials, particularly those reinforced with glass to improve mechanical strength (1). However, the effects of glass-reinforced HA on bone cells depends on the material composition and need further investigation. In the present study we examined the response of rat primary culture osteoblast to HA, reinforced with 5% and 10% of Bioglass™ and sinterized at 1200^{0}C and 1300^{0}C.

METHODS: Primary cultures of osteoblasts were prepared from sequential enzymatic digestion of calvarie obtained from 1-5 days old Wistar rats. Culture medium containing each of the four different BG-HA powders was put in contact with osteoblasts that were plated in 5 x 10^4 cell density at passage 2. After 72 h of incubation cell viability and proliferation was evaluated by MTT assay. The alkaline phosphatase production was measured by NBT-BCIP assay and secreted collagen by SIRCOL assay. The apoptosis index was investigated by nuclei staining with propidium iodide (2).

RESULTS: The viability and proliferation of osteoblasts cultured in the presence of reinforced HA were similar to control. Alkaline phosphatase production decreased around 5% in the presence of all BGTM-HA, when compared to control. On the other hand, collagen secretion enhanced approximately 20% above control in all conditions (Fig 1). A slight increase in apoptotic osteoblasts was observed when cells were in the presence of the biomaterials (Table 1).

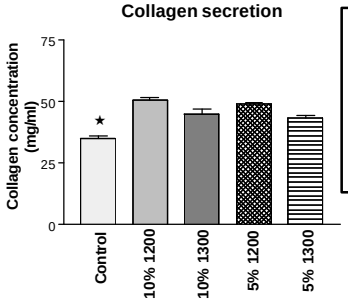

Fig 1. Increase in collagen secretion observed in osteoblasts cultured in the presence of biomaterials, (triplicates from 3 different experiment, P< 0.05)

BG™-HA	Apoptotic cells (%)
10% 1200	18 %
5 % 1200	19,5 %
10% 1300	19 %
5% 1300	20%
CONTROLE	16%

Table 1. Apoptosis. After staining osteoblasts with propidium iodide, apoptotic cells were counted as well as the total number of the cells in the field. Comparing to control, a slight increase was found on the proportion of apoptotic cells, when in the presence of BG™-HA. (Results reflect counting six fields in three different experiments.)

DISCUSSION AND CONCLUSION: Bioactive glasses and calcium phosphates have a recognized good material-tissue interaction (3). Our studies of viability and proliferation corroborated this statement. The observed increase in collagen secretion may be due to the presence of BioglassTM contents, since we already demonstrated that bioactive glass enhance the collagen production (4). Previous studies showed that ceramics particle induces macrophage apoptosis (2) and that 1,5% more apoptotic osteoblasts were present in ceramic perimplant region compared to other regions *in vivo* (5). These findings are in accord with our results of a slight increase in the percentage of apoptosis. Together our data indicate that BGTM-HA would be an interesting material for medical application, and substantiate its *in vitro* studies.

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Resorbable coatings with controlled antibiotic release for porous ceramics and bioglasses

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INTRODUCTION: Infections still represent a serious problem in bone graft procedures. Because of the poor accessibility of the infection site by systemically administered antibiotics, the local administration of antibiotics is preferred to treat or prevent bone infections. Unfortunately, most of the common antibiotics are water-soluble salts and their release from inert or slowly degradable carriers is mainly controlled by diffusion processes. Therefore, it is often difficult to generate a desired release profile with regard to a special indication. In this context we report on the design of new biodegradable antibiotic coating systems in which the release profile is modulated in a simple way by a complex formation of the antibiotic.

METHODS: Three different porous bone substitute materials were used as substrates for coating experiments: a resorbable phosphate glass (P_2O_5-CaO-MgO-Na_2O, porosity: 65%), a synthetic hydroxyapatite (HA) ceramic (Synthacer®, Heraeus Kulzer), and a ß-tricalcium phosphate ceramic (Syntricer®, Heraeus Kulzer). Gentamicin pentakis (dodecylsulfate) (GPD) was prepared as described in the literature [1,2]. Aqueous solutions of gentamicin sulfate (GS) and sodium dodecyl sulfate (SDS), resp., and a methanolic solution of GPD were used as coating solutions. The antibiotic concentration in the coating solutions was systematically varied. The material specimen were soaked with the coating solutions and carefully dried. *In vitro* release studies were performed in phosphate buffered saline (pH = 7.4) at 37°C. Drug concentrations were determined using a conventional microbiological agar well diffusion assay.

RESULTS: The impregnation of porous phosphate glasses and ceramics with aqueous solutions of GS and SDS or a methanolic solution of GPD gave stable and well adhering coatings. It was shown by scanning electron microscopy that in all of the tested samples the open porosity of the bone substitutes is preserved during coating.
In vitro studies on the antibiotic release from the coated bone substitutes show an initial burst release of gentamicin for all materials during the first day after the incubation followed by a sustained release of small gentamicin amounts up to several weeks (see Tab 1). The amount of gentamicin released during the initial phase can be properly controlled by the molar SDS/gentamicin ratio in the used coating. The total duration of gentamicin release depends on the content of GPD in the coating. The antibiotic containing coating is completely dissolved during the release period.

Table 1. Release of gentamicin from coated syntricer (SC-1 and SC-2,resp.) specimen in phosphate buffered saline (molar SDS to gentamicin base ratio given in brackets).

	Release of gentamicin base [mg]					
Time [d]	**1**	**2**	**7**	**14**	**21**	**28**
SC-1 (2.0)	4.10	1.30	0.30	0.10	0.05	0.02
SC-2 (1.6)	16.70	4.22	0.60	0.25	0.14	0.19

DISCUSSION & CONCLUSIONS: A completely degradable antibiotic coating based on a gentamicin dodecylsulfate complex has been developed. The described coatings show excellent film forming and adhesion properties on various bioglass and ceramic materials. The use of additional film forming agents or polymeric carriers is not necessary. Porous bone substitutes can be coated without sealing macropores or disturbing inter-connective pore structures.
The release profile of the antibiotic from the coated implant can easily be modulated by varying the molar gentamicin to SDS ratio. Instead of SDS, fatty acid salts forming water-insoluble complexes with gentamicin like sodium palmitate or laurate can be used leading to the corresponding gentamicin pentakis (palmitate) and gentamicin pentakis (laurate) complexes, resp. In these cases a similar release behavior was observed as described for the GPD system.

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Cytometric Viability of Human Corneal Epithelial Cells Grown on a Biomaterial

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INTRODUCTION: *In vitro* tissue culture studies were used to compare the viability and possible inflammatory response of human corneal epithelial cells, SV 40 transformed (HCE-T), grown on tissue culture plastic, (TCP), and a polymeric hydrogel biomaterial (BioM).

METHODS: Cell viability was by flow cytometry using Molecular Probes, Live/Dead –Cell Viability kit (LDCV). HCE-T cells were grown in UltraCulture™ media w/o antibiotics (1.5 X 10^5cells/ml), removed from tissue culture flasks and seeded to 24 well flat bottomed plates and to the biomaterial surface. Supernatants were collected and frozen for cytokines evaluation. HCE-T cells were trypsinized, rinsed, spun and characterized by double staining with calcein (Ca) and ethidium homodimer -1 (EthD). The LDCV endpoint was flow cytometric analysis carried out at 488 nm on a BDFACSCailbur™ 4 color unit cytometer. Subpopulations were identified and sorted by regions for live (green fluorescent) and dead (red fluorescent) cells. Dead cell controls were treated with BAC (50ppm) for 15 minutes before analysis. Representative HCE-T cells populations from TCP and BioM were counted. Cytokine stimulation was by PMA at 100ng/ml. Cytokine analysis was done using the BD CBA assay for human pro-inflammatory cytokines.

RESULTS: Cell morphology was examined by phase microscopy. Cell populations were 5x 10 ^{4}cells/ml-BioM and 8 x 10 ^{4}cells/ml-TCP (Fig 1). BioM flow cytometry were 95% live cells (LR) and 4% membranes (UR) after 4 days growth. TCP cells were 98% (LR) and 2% (UR). Only BAC treated cells exhibited 98%(UL) for dead cells (Fig 2). Cytokine levels were normalized for cell counts, supernatant volume and TCP final pg/ml concentrations were then used for normalization. TCP and BioM PMA HCE-T cells were stimulated for TNF-a, IL-8 & IL-6 (Table 1).

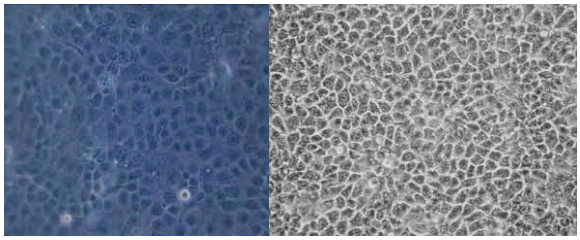

Fig. 1: HCE-T morphology after 4 days growth: (left) HCE-T monolayer grown on BioM. (right) HCE-T monolayer grown on TCP.

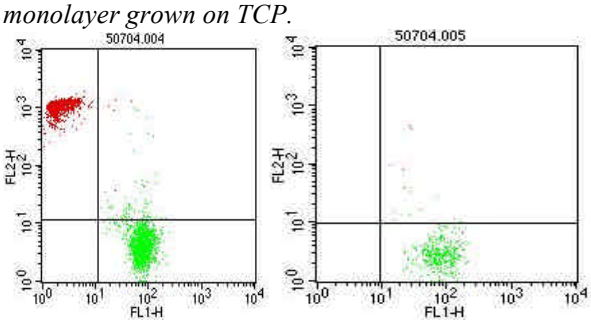

Fig. 2: Flow cytometry of HCE-T stained with LDCV: (left) HCE-T mixed live- dead (BAC) treated population grown on TCP. (right) HCE-T grown on BioM with gated live/dead regions.

surface	IL 1ß	TNF a	IL10	IL8	IL12 p70	IL6
TCP	1	1	1	1	1	1
TCP/ PMA	1	26	4.5	13	1	4
BioM	0.8	0.8	1.7	1.2	0.5	1.3
BioM/PMA	0.5	14	0.5	6	0.9	2

Table 1. Cytokine ratios of HCE-T normalized to TCP. Signf. at a ratio >2.0

DISCUSSION & CONCLUSIONS: HCE-T cells are viable BioM as compared to TCP. BioM is a viable surface for HCE-T cell growth and attachment. Short term exposure to BAC killed HCE-T cells. Surface material differences may attribute to the approximate 38% growth inhibition of BioM to compared TCP. HCE-T cytokines can be stimulated on both surfaces with PMA. BioM did not up-regulate pro-inflammatory cytokines compared to TCP HCE-T cells

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