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A New and Evolving Paradigm for Biocompatibility

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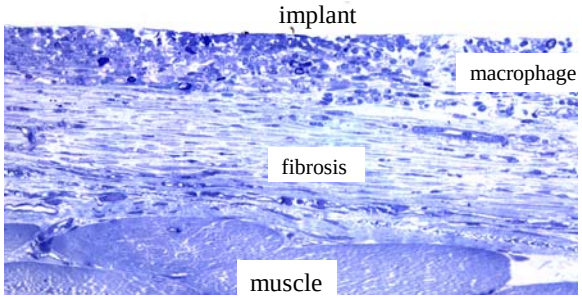
Scaffolds may position tissue and guide regeneration. In many cases, however, severe adverse reactions to the implant occur in similarity to the reactions on permanent biomaterials implants. This results in an inflammatory response followed by the formation of a fibrotic capsule around the implant shown in Figure 1.



A tissue section around an polymer implant stained with hematoxylin and showing the inflammatory macrophage response and the fibrotic capsule laid down by fibroblasts.

Research on body reactions to biomaterials has so far focused mostly on the fundamental relations between different surface chemistries i.e. various biomolecules, and the adhering cells with the perceived importance for the overall biological response placed on the implanted material. Such studies have generally pointed to large differences depending on the investigated surface in *in-vitro* cell culture experiments. It is disturbing, however, that widely different implant materials translated into *in-vivo* induce surprisingly similar tissue reactions. *Is the biological acceptance of an implant dependent on material characteristics or on other factors not directly related to the surface chemical composition?*

It is evident that interactions at the interface between tissue and material are of key importance. Little is, however, known of interfacial mechanisms leading to foreign body formation. Evidence in the literature and data from our research suggest that the structure and the absence of relative movement of the implant with respect to the surrounding tissue is the reason for the in vivo acceptance of a material. A key factor seems to be efficient stress transfer from tissue to material without causing adhesive or cohesive failures and also the overall stress on tissue close to the implant. Specifically, we are developing textured, knitted, or very soft materials that offer unique possibilities to study material factors, cellular mechanisms, and signaling pathways in order to verify our hypothesis.

Combining Drug Delivery with Medical Device Technology

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Controlled, local delivery of pharmaceuticals in vivo is recognized as an efficient and effective means of treating disease. SurModics is actively developing and testing strategies to combine local drug delivery with medical devices or other implantable platforms. The intent of this approach is to provide local delivery of bioactive agents to enhance the performance or biocompatibility of medical devices or to provide local delivery of therapeutic agents which are not readily bioavailable if administered systemically. It is believed that combining drug delivery polymer matrix technology with implantable device platforms will serve as an effective therapeutic approach.

SurModics is developing a variety of drug delivery matrix materials that span both biostable and biodegradable polymer systems. We have been successful at creating systems to control the delivery of low molecular weight pharmaceuticals and are currently expanding our capabilities in the delivery of large molecules, like peptides, proteins, and nucleic acid polymers. The time profile of drug delivery from these matrices can be controlled from days to many months with changes to matrix chemistry and composition, but also processing methods.

One example of a combined drug delivery/device platform is the drug eluting stent. SurModics developed the coating for the first-to-market drug eluting stent. The coating on this stent provides three month delivery of the drug sirolimus to the wall of a blood vessel after implantation, to control the occurrence of restenosis. SurModics is also developing and testing a helical ophthalmic implant which is designed to deliver drugs to treat retinal diseases, like diabetic macular edema (DME) and age-related macular degeneration. The current version of this device is in Phase I clinical testing for treatment of DME.

In an alternative approach to controlled delivery of a pharmaceutical agent, SurModics is developing encapsulated cell technology to allow the local or systemic delivery of cell-synthesized proteins. In the first application of this now in development, we are encapsulating islet cells with a polymer matrix so that they can be implanted in patients to treat diabetes. The polymer matrix surrounding the individual islets is designed to allow nutrients and insulin to diffuse through freely, but to keep antibodies and other proteins and cells of the native immune system from reaching the implanted islet cells. Results in animal models have been very encouraging and Phase I/II clinical implants have recently started.

SurModics is taking a variety of approaches to combine local delivery of bioactive agents with implantable devices or polymer systems. It is expected that these development activities and the resulting products will lead to novel, effective tools for improving pharmaceutical therapies.

CHARACTERISTICS AND POTENTIAL OF THE ANODIC PLASMA-CHEMICAL TREATMENT OF TITANIUM IMPLANT SURFACES

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INTRODUCTION: Anodic plasma-chemical treatment (APC) of valve metals is a cost-effective process to modify surface topography and chemistry in a single coating step[1]. The technique is suitable for coating complex three-dimensional devices and therefore an interesting alternative to conventional surface modification processes for titanium implants[2]. The project aims at developing a composite calcium phosphate/titania coating in order to accelerate the ongrowth of bone tissue on the titanium implant surface. The surface coating process presented in this paper is based on a calcium- and phosphate-containing electrolyte at high pH stabilized by organic chelating agents. The resulting coating contains partially soluble, amorphous calcium phosphate phases embedded in a titanium oxide matrix with crystalline anatase components.

METHODS: CP titanium samples (disc-shaped, diameter: 15 mm, thickness: 1 mm) or medical implants are ultra-sonically cleaned in acetone for 20 minutes, rinsed with acetone, dried in air, etched in 2%HF / 10%HNO_3 for 2 minutes, subsequently rinsed with u. p. water and dried in air.

The samples are plasma-anodized galvanostatically (between 100 and 500 mA) in aqueous electrolytes for 90 seconds. The electrolyte contains Ca^{2+}, PO_4^{3-}, EDTA as chelating agent and sodium hydroxide to adjust the pH of the solution (EP 1 372 749). All chemicals are high purity grade and supplied by Fluka, Buchs, Switzerland. After coating, the samples are rinsed with u. p. water and dried in air.

The resulting surfaces are tested by standard surface analysis techniques as well as for their bio-performance.

RESULTS: Table 1 shows the dependence of the calcium-to-phosphate ratio in the coating on the pH of the electrolyte solution. This ratio increases with increasing pH. Fig. 1 shows two different surface topographies achieved by coating at different current densities. Dissolution experiments in a model electrolyte demonstrate the partial solubility of calcium and phosphate. No disadvantageous biological reactions could be found in cell and animal studies.

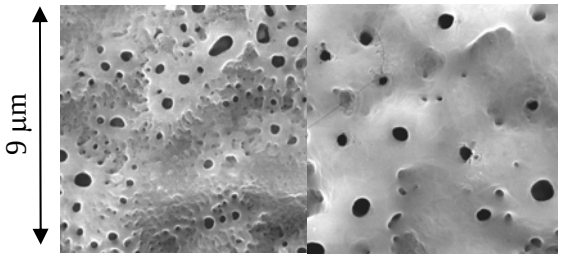

Fig. 1: Effect of current density on the topography of the coating. Low density (left), high density (right).

Table 1. XPS Ca/P-ratio [at%/at%] in the APC coating as function of pH in the electrolyte solution.

pH	6	11	14
Ca/P-ratio	0.6	0.7	1.3

DISCUSSION & CONCLUSIONS: The new high pH electrolyte system containing organic chelating agents allows us to produce coatings with substantial (>10 at%) proportions of calcium phosphate at high calcium-to-phosphate ratios. The dissolution experiments indicate that calcium and phosphate are partially dissolved without a major effect on the integrity of the anatase-based coating matrix. The animal studies showed the good biocompatibility and good ongrowth of bone tissue onto the APC-surfaces.

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PROJECT: This project is a joint project between Synthes, Dr Robert Mathys Foundation, and ETH Zurich (BioInterface Group). The animal studies were performed at the AO Research Institute in Davos.

Industry and Advanced Biomaterials Research

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Musculoskeletal conditions are among the most costly illnesses to treat, consuming on average three percent of total gross domestic product in developed countries. From arthritis to osteoporosis, fractures to dislocations, musculoskeletal diagnoses represent the primary non-psychological causes limiting activity in people of all ages worldwide[1]. Growth drivers in the orthopaedic and trauma market are demographic developments (ageing population),
population behaviour (activities), population expectations (treatment standards) and new products (new treatment methods, enhancement of clinical outcome, new technologies).

Biomaterials are part of the new technology sector and an important driving force for the growing market, valued at over $1.4 billion in 2003, the expectations are over $3.4 billion by 2008[2]. The applications of biomaterials are widely spread: from hyaluronic acid injections to substitute synovial fluid in the knee to allografts used in spinal fusions and in synthetic materials used as substitutes for bone grafts in different indications. Other very important developments in classic biomaterial are porous metals, new alloys and ceramics, in particular new manufacturing technologies. Another important trend is the merging of drugs and devices.

The increasingly short product life cycles in the medical device industry implying an urgent need for fast and effective development. There is an increasing pressure for shorter time to market. – In contrast to the time to market driven trend the complexity of the products is dramatically increasing. Most of the new product developments are based on years or decades of basic and applied research, e.g. growth factors. Furthermore the industrial exploitation of results from research is very often a time and cost consuming process. There is growing concern that many of the new basic science discoveries made in recent years may not quickly yield more effective, more affordable, and safe medical products for patients. The FDA stated that the current medical product development path is becoming increasingly challenging, inefficient, and costly[3].

To speed up the development time companies adopting a knowledge brooker philosophy. External knowledge sourcing, intellectual property evaluation and the attraction of bringing outside innovations instead of reinventing the wheel are becoming more important. Knowledge creation and basic research is being partially re-delegated to academic institutions and companies. The development success is found to be related positively and directly to how well informed and knowledgeable decision-makers are[4].

A strong collaboration between industry and advanced research focused academic institutions is essential for accelerating the development process. Important steps during industrialisation are proof of principle and proof of concept, know how transfer, up scaling and regulatory issues. It is required that research results should not be single shots and should be well documented and described in every step. What is not documented does not exist. Processes, analytical procedures and resulting data must be shown to be reproducible and should be independent from on-site findings. The biological performance of the new materials as well as any influence caused by a new technology has to be investigated.

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What is AO and AO Grant Opportunities For You

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INTRODUCTION:

The AO is a non-profit surgeon-driven organization dedicated to progress in research, development, and education in the field of trauma and corrective surgery. The goal of the AO is the research, development and approval of every aspect of osteosynthesis methods and devices and to provide the education to spread this knowledge around the globe. Using these methods and devices, the AO surgeons and OP personnel trained in the AO have successfully treated a vast quantity of patients with musculoskeletal injuries and their sequelae over the past 40 years.

AO Foundation is one of the few Foundations without an endowment to finance its activities through capital interests. It functions through the contribution of ideas and know-how as well as ongoing support in teaching and scientific activities which are made possible by its continuous incoming royalties from industrial partners. In this way a unique system of support helps to develop the latest technologies, implants and instruments based on research and development and nourished by an international family of idealistic and dedicated surgeons. The unique set-up of the organization ensures a most effective use of royalties derived from the market, thereby eliminating a potential burden on public funds.

Activities: Since its beginnings the AO has been an organization thriving on active, hands-on innovation, exploration and implementation in the field of ostheosynthesis. The AO's five main areas of activity are presented:

Research: Through the AO Research Institute (ARI) and AOCID the AO promotes investigations in all areas of trauma of the locomotor system and its diseases and helps to provide the scientific basis for new developments in AO-related fields. It investigates the performance of surgical procedures, devices and substances in order to improve patient treatment. At the same time, it renders research services within and outside the AO Foundation.

Development: The AO Development Institute (ADI) provides new concepts, operating techniques and fracture fixation products, which not only extend the existing product range but also lead on to new methods and standards. To achieve this, the ADI takes on the task of bringing together both internal and external specialists and integrating them as soon as possible into the development process.

Education: Through AO Education, the AO provides education programs in which new methods, products, and scientific findings are disseminated among orthopedic surgeons, operating personnel, and hospitals around the world. A faculty of over 1200 dedicated specialist surgeons volunteer as teachers, offering more than 150 annual courses in which in 2003 alone more than 12,000 doctors participated.

Scholarships: The AO offers scholarships through three channels:

Socio Economic Committee

The Socio Economic Committee (SEC) represents the social conscience of the AO Foundation. Its primary goal is to improve the care of the injured in developing countries by conducting or facilitating teaching events and programs as well as by supporting the training of individuals involved in the programs.

AO Research Fund

The AO Research Fund is funded by the AO Foundation, a Swiss non-profit organization. The Research commission is an independent review board chaired by Dr Adrian Sugar. The mission of the AO Research Fund (AORF) is to support basic scientific, pre-clinical and clinical research in all areas of trauma, surgery of the musculo-skeletal system and related problems. It provides predominantly seed money to individual researchers and research groups, finances pilot studies and supports investigation of new and unconventional ideas or hypotheses. It also funds projects focused on specific clinical problems as defined from time to time by the AO Academic Council. Its principal clinical areas encompass general trauma, spine, craniomaxillofacial and veterinary surgery.

Fellowships

AO Education offers 200 fellowships for surgeons and 20 for ORP every year. These provide additional experience in AO techniques for fully trained orthopedic or general surgeons and ORP with an interest in trauma.

The Interface between Nanobiotechnology and Medicine: A Life Science Perspective

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INTRODUCTION: Modern medical research and practice is becoming increasingly influenced by advances in technology, nanobiotechnology being a good example, especially in the fields of tissue engineering (TE) and regenerative medicine. The widespread acceptance of a biomimetic approach has resulted in attempts to simulate natural interfaces, one of the exciting developments being that of a biodegradable matrix or scaffold to simulate the extracellular matrix (ECM) and equipped with essential bioactive signal molecules to elicit a physiological regenerative response. Nanofabrication techniques can be part of this interface construction. The use of "intelligent" materials, able to respond to changes in the microenvironment, can also take the form of a nanoparticulate drug or gene delivery system.

PERSPECTIVE: It is evident from the introductory remarks that a rational approach to a successful medical application of such technologies will require a high level of sophistication on the part of any life science models adopted to test such systems. Examples will be given exclusively from the use of *in vitro* models and the discussion will centre on new developments recently initiated or being planned for the future.

The complexity of the *in vivo* system at the tissue-biomaterial interface, in which heterogeneous cell types interact with each other and with the biomaterials demands co-culture models using human cells preferably as primary isolated cells. Thus, for bone TE osteoblasts and endothelial cells (EC), the latter responsible for vascularisation of the scaffold, need to be studied in a 3D-model. This can be well achieved using immuno-cytochemical markers of cell functionality in combination with confocal laser scanning microscopy (CLSM) [1]. However, morphological studies even with sophisticated microscopical techniques and relevant phenotypic parameters (gene product) require to be complemented by parallel studies at gene transcript level. This can be readily achieved by various PCR techniques [2]. The use of a reproducible and quantifiable 3D-model of angiogenesis with human microvascular EC and pro-angiogenic growth factors offers the possibility to study how regenerative responses are regulated by various relevant factors [3].

Co-culture models of barrier systems in the body, for example the blood-brain barrier or the alveolo-capillary barrier are important in studying strategies for drug and gene delivery, using bioresorbable polymers in nanoparticulate form. An *in vitro* model of the human air-blood barrier has already been established by our group [4]. With such complex model systems it is hoped to study how nanoparticles can be transported transcellularly as opposed to being stored intracellularly. In addition, such models could also be employed to delineate possible negative effects of clinical use of nanoparticles ("nanosafety").

One of the very promising fields of endeavour for the future is that of adult progenitor cells, as this offers an autologous cell source, where the problems of disturbed immunological response should be minimal. Unraveling the lineage differentiation pathways for adult human stem cells will be vital in providing reproducible *in vitro* conditions in which to investigate possible cell adhesion ligands with which recruitment to a biomaterial scaffold could be achieved. At the moment we are studying these aspects in endothelial progenitor cells from the human peripheral blood.

CONCLUSIONS:. Further development of more sophisticated *in vitro* systems will be essential for advances in regenerative medicine, especially to test the biofunctionality of new biomaterials.

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Localized nucleic acid delivery using magnetic nanoparticles

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INTRODUCTION: We have previously developed Magnetofection™ which is nucleic acid delivery into cells guided and enhanced by magnetic fields using nucleic acids or viral and nonviral gene vectors associated with magnetic nanoparticles. These magnetic vectors can be targeted by magnetic fields in vitro and in vivo and magnetic guidance greatly improves both the efficacy and the kinetics of nucleic acid and gene delivery [1,2]. Magnetofection is suitable for overexpressing genes and for silencing gene expression (antisense oligonucleotides, siRNA). We have provided proof-of-principle for magnetically localized gene and oligonucleotide delivery upon administration in the blood circulation or local application in the gastro-intestinal tract. However, magnetic trapping against physiolgical blood flow rates is difficult to achieve, at least in major blood vessels. Solutions to this problem have to be provided in various manners. Magnetic field technology needs to be optimized for magnetic drug targeting. Most importantly, drug formulations have to be provided that are more susceptible to magnetic fields than standard magnetic nanoparticles and that are optimized for vascular administration. Therefore, we have adapted the technique of using microbubbles as drug carriers for magnetic drug delivery. Microbubbles are gas-filled spheres with a protein, polymer or lipid shell, and are currently used in the clinics as contrast agents for ultrasound imaging. Microbubbles have been used very successfully as carriers for drugs and nucleic acid. Localized delivery can be induced by application of suitable ultrasound to a target area [3,4].

METHODS: Magnetic microbubbles were prepared in glass serum vials from a mixture of surfactant-coated magnetic nanoparticles, soybean oil, a cationic lipid (Metafectene, Biontex, Munich, Germany) and fluorescence labeled nucleic acids (plasmid DNA and antisense oligonucleotides) in aqueous buffer. The gas space above the suspension was filled with perfluoropropane. The vial was shaken for 1 min at 25.000 rpm using a MiniBeadBeater (Biospec Products Inc., Bartlesville, OK, USA). Magnetic retention at various flow rates was measured using an HPLC pump, an electromagnet and microbubbles prepared with radioactive-labeled DNA. Localized nucleic acid delivery in vivo was examined by intravital microscopy using a mouse skin chamber model.

RESULTS: We have developed magnetic microbubbles in that we incorporated a high load of magnetic nanoparticles in microbubble shells. These bubbles can easily be loaded with nucleic acids or cytostatics. Importantly, the magnetic retention of these bubbles at a given flow rate is tremendously improved compared with the same quantitiy of magnetic nanoparticles in liquid suspension. Using a skin chamber model in mice, we were able to demonstrate by intravital fluorescence microscopy that nucleic acids can be locally delivered to the vasculature and surrounding tissue within the chamber by applying a suitable magnetic gradient field to the target area in combination with ultrasound of 1 MHz frequency. Without magnetic field or without ultrasound, no localized delivery was feasible.

DISCUSSION & CONCLUSIONS: These results indicate that magnetic microbubbles are highly promising drug carriers that can be remote-controlled within the blood circulation by a combination with two independent physical forces.

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Stimuli-sensitive Biomaterial System for Regenerative Medicine

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Most polymers used in medical applications today are materials that have been developed originally for application areas other than biomedicine. Not all potential biomedical applications can successfully be realized using the existing materials. This finding resulted in a targeted development of new implant materials.

An actual trend in polymer science is the design of materials which show multifunctionality meaning an unexpected combination of material functionalizations like the combination of biofunctionality, hydrolytic degradability, and shape-memory functionality. The introduction of biodegradable implant materials as well as minimal invasive surgical procedures in medicine has significantly improved health care within the last decades.

This paper describes a group of degradable polymers, which are able to change their shape in response to an increase in temperature or a irradiation of a specific wavelength. The shape-memory effect results from the polymer's structure and morphology in combination with a certain processing and programming technology. A substantial development in this context is the introduction of polymer systems in which macroscopic properties can be tailored over a wide range for a specific application. Their mechanical properties and degradation rate can be tailored by variation of molecular parameters. Their shape-memory capability enables bulky implants to be placed in the body through small incisions or to perform complex mechanical deformations automatically. A smart, degradable suture visualizes performance and potential of these shape-memory plastics in biomedical applications.

Superparamagnetic iron oxide nanoparticles (SPIONs) as non-viral vectors for gene delivery in *vitro*

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INTRODUCTION: The treatment and control of acute and chronic inflammatory processes of the bone and cartilage remain a major goal in orthopedic research. Gene therapy would be an attractive alternative to chemotherapy which is accompanied often by various side effects that are detrimental to the patient. The true benefits of these approaches can only be realized when delivery methods are perfected or new ones developed.

METHODS: Superparamagnetic nanoparticles (SPIONs) were synthesized according to Chastellain et al.[1] SPIONs were coated either with polyvinyl alcohol (PVA) (Mowiol® 3-83, Clariant) and further functionalized with amino-groups and fluorochromes Cy3.5 or Texas red, or alternatively coated with 25 kDa polyethylenimine (PEI) (Aldrich). Intracellular uptake of PVA-SPIONs into cells was evaluated by confocal microscopy and flow cytometry. The feasibility of using SPIONs coated with PVA (PVA-SPIONs), for the delivery of a DNA expression plasmid encoding the green fluorescent protein (GFP) gene (pEGFP-C1 plasmid, Clontech), into different cell lines within a magnetic field, was subsequently explored, and evaluated using fluorescent microscopy and flow cytometry. The gene expression after delivery with PVA-SPIONs was further compared with that of particles coated with polyethylenimine (PEI), PEI-SPIONs.

RESULTS: Flow cytometric analysis showed that the PVA coated particles were taken up, resulting in more than 80% of the synovial cells being labelled with Cy3.5. Gene delivery was also achieved using PVA-SPIONs, and 17.7% of 293T cells expressed GFP. In contrast, in cells transfected with PEI-SPIONs, 43.5 % of 293T cells expressed GFP. The green fluorescence in cells transfected with PVA-SPIONs was faint and seen clearly after 48 hrs of incubation, while in cells transfected with PEI-SPIONs the fluorescence intensity was high and seen clearly after 24 hrs of incubation. This indicated a difference in either particle uptake by the cells or gene release from these polymers. Although PVA-SPIONs were less toxic to the cells, these results showed that PEI-SPIONs were more efficient gene vectors. This was confirmed in synovial cells where up to 96.2% of the cells expressed GFP after transfection with PEI-SPIONs. Gene transfection using of PEI-SPIONs in presence of magnet for 5 min resulted in significantly higher proportion of cells expressing GFP when compared with conventional transfection system with lipofectamine, calcium phosphate and PEI alone.

DISCUSSION & CONCLUSIONS: Our results show that PEI-coated SPIONs are very efficient for non-viral gene delivery, resulting in high transfection efficiency *in vitro*. High transfection efficiency was achieved within minutes and the transfection rates achieved were significantly higher than those achieved with conventional transfection methods. The PVA-coated particles were less efficient in gene delivery, however their efficient uptake and low toxicity is to be explored for protein delivery. Preliminary studies by our group have shown that the use of PEI-coated SPIONs is also feasible in gene delivery *in vivo*. Further studies will evaluate the toxicology and *in vivo* efficiency of these transfection systems. This study will serve as a basis for further studies with plasmids, peptides and proteins targeted at inhibiting joint inflammation and cartilage matrix degradation.

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Targeted Drug Delivery to Solid Tumors by Thermally Responsive Polypeptides

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This talk will describe thermal targeting of cancer therapeutics to solid tumors by two different classes of thermally responsive recombinant elastin-like polypeptides (ELPs) that exhibit a lower critical solution temperature transition slightly above 37 ˚C. The first generation of ELPs that we have designed as thermally triggered molecular actuators for drug delivery are pseudorandom copolymers of the VPGXG repeat where the mole fraction of X and the polymer chain length were precisely specified so the polypeptide would undergo its phase transition between 37 and 42 ºC. In vivo fluorescence videomicroscopy of human tumors implanted in nude mice demonstrated that the phase transition of this thermally responsive ELP occurs in heated tumors resulting in the formation of micron-size aggregates of the thermally responsive ELP within the heated tumor. The phase transition results in a ~two-fold increase in tumor localization compared to the same polypeptide without hyperthermia even for heating periods as short as one hour. We have observed that thermally cycling the tumor can further increase the uptake of the ELP within the tumor by five-fold compared to the same polypeptide without hyperthermia. Doxorubicin was conjugated to this first generation ELP carrier via an acid labile hydrazone bond to enable release of the drug in the acidic environment of lysosomes. The ELP-doxorubicin conjugate was endocytosed by squamous cell carcinoma cells and trafficked into lysosomes, as observed by the colocalization of the doxorubicin with a lysosome-specific dye by confocal fluorescence microscopy. The ELP-doxorubicin conjugate and free drug exhibited equivalent cytoxicity in cell culture. These results suggest that thermal targeting of a soluble macromolecular carrier may be useful for the delivery of cancer therapeutics.

A second generation of diblock ELPs will also be described that function as temperature triggered polymer amphiphiles. Two classes of ELP amphiphiles have been synthesized: the first class form monodisperse, ~60 nm diameter micelles in the range of 37-42 ºC, a range approved for clinical hyperthermia of solid tumors, which will allow the multivalent presentation of tumor specific ligands only in tumors, thereby enhancing their accumulation in tumors. The second class of diblock ELPs are designed to undergo their monomer to micelle transition at room temperature to enable thermally triggered loading of drugs or imaging agents into the core of the micelle, followed by release of their contents upon undergoing their micelle- aggregate transition in heated tumors.

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The Bone Inducing Principle: Paving the Way Between Bench Top and Bed Side

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INTRODUCTION: Bone morphogenetic proteins (BMPs) are the key cytokines in bone formation and repair. Since the cloning of the first members of the BMP superfamily in 1988 recombinant BMPs failed to substitute autologous bone as gold standard in clinical treatments for bone repair, mainly because the first clinical trials showed, that milligram doses of rhBMP-2 are required for effect [1]. Therefore, BMP-presentation and release kinetic mainly determined by the delivery system have to be optimized for an efficient clinical application of rhBMP. Another strategy to decrease the BMP dose in clinical applications is to combine the cytokine with enhancers of BMP activity. Here we show that NMP (N-methylpyrrolidone) is an enhancer of BMP activity and can be used to generate biomaterials of the 3rd generation, where biocompatibility, biodegradability, and bioactivity are combined.

METHODS: MC3T3-E1 pre-osteoblastic cells were tested for different cell maturation responses: ALP (Alkaline phosphatase activity) and Alizarin Red mineralization assay. At the molecular level, cell extracts were analyzed by Western Blotting for Smad 1,5,8 phosphorylation, as well as by quantitative real time PCR (qRT-PCR) for specific osteoblastic markers (Osteocalcin-OCN, Bone Sialoprotein-BSP). Histological and histomorphometric analysis of bone repair *in vivo*: non critical size 6 mm defects were created in the rabbit calvaria and subsequently treated with three different membranes, namely PLGA, Osseo quest and NMP-PLGA, or left untreated (control). Membranes were placed both on top and underneath the defects, and the regenerated bone within the defect was determined 4 weeks after the operation.

RESULTS: NMP increased ALP activity of MC3T3-E1 cells concentration dependent. Compared to controls 2.5 mM NMP increased ALP 2.5±0.3 times (N=6, P<0.001) and mineralization of MC3T3-E1 cells determined after 4 weeks by Alizarin-red staining was enhanced 1.5±0.05 times (N=6, P<0.001). NMP action depended on extracellular bone morphogenetic protein (BMP), because in the presence of 2.5 mM NMP it was reduced below control levels (67+/-10%) by the addition of the BMP antagonist Noggin (1 µg/ml). In combination with rhBMP-2 NMP showed a synergistic effect on ALP activity, mineralization and Smad 1,5,7 phosphorylation. The *in vivo* results in the guided bone regeneration model showed that in the presence of NMP healing of the defect was 79.17±5.61% compared to 49.31±8.75 % without NMP.

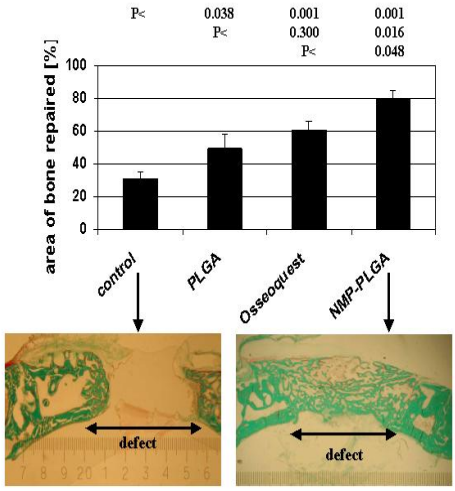

Figure 1: Enhancing of bone repair by guided bone regeneration and NMP: Defects of 6 mm in diameter created in the calvarial bone were treated with three different membranes (PLGA, Osseo quest and NMP-PLGA) or left untreated (control). The upper panel shows the percentages of the area where bone regeneration in the defect has occurred in relation to the original defect area.

DISCUSSION & CONCLUSIONS: The results show that NMP improves the biological activity of BMP *in vitro* and *in vivo* by enhancing the kinase activity of the BMP-BMP-receptor complex. In the end, our finding could translate into novel treatment strategies for bone regeneration under the influence of autologous BMP for non-critical size defects and recombinant human BMP for critical size defects. Since NMP can be delivered by PLGA-based materials these materials become bioactive and are turned into materials of the 3rd generation.

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GUIDED CELL GROWTH AND TISSUE REGENERATION

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INTRODUCTION: Spinal cord injury is a devastating disorder of the central nervous system, which does not spontaneously regenerate but has the capacity to regenerate. In an attempt to promote regeneration after traumatic injury to the spinal cord, we have been investigating the stimuli critical to axonal guidance and testing these in biomimetic strategies of repair. Specifically, since axons are guided to their targets by a combination of attractive and repulsive, short-range and long-range cues, we have been investigating ways to incorporate these signaling molecules into tissue engineering constructs and then test their guidance potential in vivo.

To test the importance of the short-range, contact-mediated cues, we created a 3D hydrogel scaffold that had biochemical volumes of cell-adhesive ligands (i.e. RGD) separated by non-adhesive volumes of agarose, thereby mimicking the attractive and repulsive cues found in development.

To test the importance of long-range, diffusible cues, we designed immobilized concentration gradients of neurotrophins (i.e. NGF) and tested their regenerative capacity.

METHODS: Three-dimensional (3D) agarose gels were modified with RGD peptides by a combination of photochemistry and focused single photon laser as previously described.[1]

Neurotrophin concentration gradients of nerve growth factor (NGF) were immobilized in poly(2-hydroxyethyl methacrylate) macroporous scaffolds using a gradient maker, as previously described.[2] Primary rat dorsal root ganglia neurons were used to test the guidance potential of both the RGD-agarose patterned gels and the NGF-concentration gradient gels.

RESULTS: Fluorescein-isothiocyanate (FITC)-labeled GRGDS was immobilized in agarose gels and visualized by confocal microscopy to have biochemical volume channels of approximately 170 um in diameter. These channels had almost identical rheological properties to non-RGD modified agarose. Dorsal root ganglia neurons aggregated on the RGD-channels and extended cell bodies and neurites into the channels and not into the agarose that separated the RGD-channels. Scrambled RDG peptide channels, identically synthesized, had no cells or neurites growing within, demonstrating the specificity of the interaction between neuronal cell integrin receptors and RGD.

A series of linear NGF-concentration gradients were immobilized in PHEMA scaffolds as determined by ELISA. Dorsal root ganglia neurites were guided by the NGF concentration gradient at 310 ng/ml/mm. When a second neurotrophin concentration gradient of neurotrophin-3 (NT-3, at 200 ng/ml/mm) was applied, neurite guidance was observed at a lower NGF gradient of 200 ng/ml/mm. Interestingly, both tyrosine kinase receptors, TrkA (for NGF) and TrkC (for NT-3) were shown to be co-localized on the dorsal root ganglia neurons studied, demonstrating a synergistic effect in terms of neurite guidance observed. The importance of the dual concentration gradient was demonstrated through a series of controls with constant concentrations of either NGF or NT-3 while the other was presented as a gradient.

DISCUSSION & CONCLUSIONS: Primay neurons were shown to be guided by both contact mediated, extracellular matrix analogs and long range, neurotrophin concentration gradients. These systems demonstrate the fundamentals that are important to guidance and are being tested in a nerve guidance channel for their potential applicability in an in vivo model of repair of the spinal cord.

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ACKNOWLEDGEMENTS: This project was financially supported in part by the Natural Sciences and Engineering Research Council of Canada.

Engineering Osteophilic and Osteoinductive Surfaces on Metallic Implant Scaffolds

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Surfaces of medicinal implant metals like cp titanium, 316L steel or cobalt chromium alloys (CoCr29Mo), generally possess contact angles of 60-80°, thus displaying effective hydrophobic surfaces (for review see [1]). In 1972 Baier [2] suggested a model, in which a correlation exists between biocompatibiltiy, bioadhesion and the critical surface tension of solids. In this model Baier postulated a good bioadhesion on hydrophilic surfaces. Several years ago we discovered a novel wet chemical etching method with chromosulfuric acid (CSA) at 200-240 °C for the preparation of extremely hydrophilic surfaces on transition metals like titanium, steel (316L) [3] and cobalt chromium (CoCrMo) alloys [4]. It was demonstrated that these metals with surface roughness values of Ra > 1 µm were nano-structured and exhibited ultra-hydrophilic properties i.e. dynamic contact angles < 10° with absent contact angle hysteresis [5], a phenomenon which we have called "Inverse Lotus Effect" [6]. The development of ultra-hydrophilicity on titanium surfaces follows a typical time course. The advancing and receding contact angles respectively decrease from ca. 70°/60° at zero time to 1°/1° at 60 minutes and then increase again to 15°/3° or more at 120 minutes of heating in chromosulfuric acid. This typical minimum-function behavior of the dynamic contact angle was found for electropolished, anodically oxidized, SLA- (sand-blasted surface etched) and PVD- (plasma vapor deposited) titanium surfaces, inspite of the fact that the surface roughness (Ra value) varied between 1 µm (electropolished) and ~ 40 µm (PVD-surface). In pilot animal experiments such ultra-hydrophilic surfaces show an enhanced bone growth (osteophilicity) versus controls.

Based on the above CSA-surface as a priming coat, we have developed a method for immobilizing bone morphogenetic protein 2 (rhBMP-2) on metal surfaces [3-6]. In this way chemotactic-juxtacrine surfaces may be produced: chemotactic by way of a slow controlled release of rhBMP-2 and juxtacrine by simulating juxtacrine secretion in the form of a 2-dimensional layer of immobilized rhBMP-2 for solid phase interactions with the receptors of osteoprogenitor cells. 125I-rhBMP-2 can be immobilized in amounts between 0.1-5 µg/cm2 on different titanium surfaces in clinical use. The half-life of rhBMP-2 released from such surfaces is in the order of bone growth depending on the immobilization procedure and varies between 30 and 100 days [4]. In vivo and in vitro experimenters show that the immobilized BMP-2 is biologically active, thus opening the possibility of rationally engineering osteoinductive implant surfaces.

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Bacterial Reactions to Modified Biomaterial Surfaces

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INTRODUCTION: Infections are serious complications associated with osteosynthesis implants [1-3]. Prolonged hospitalisation with systemic antibiotic therapy, several revision procedures, possible amputation, or even death may occur with implant associated infections. *Staphylococcus aureus* and *S. epidermidis* are both associated with implant infections, and have increasing resistance to various antibiotics [4-5]. The need to minimize their initial adhesion is great. A possible solution may be to modify the implant surface topography and/or chemistry, or by using an antimicrobial or protein resistant coating. This study describes the visualization and quantification of *S. aureus* and *S. epidermidis* adhering to a variety of different treated/coated titanium surfaces, including polymer coatings impregnated with an antiseptic.

MATERIALS AND METHODS: To visualize *S. aureus* and *S. epidermidis* adherence on the different surfaces, the bacteria were cultured on the surfaces in brain heart infusion broth (BHI) for 1h at 37°C. For SEM study, samples were fixed with 2.5% glutaraldehyde in PIPES buffer for 5 min, post-stained with 1% OsO_4 in PIPES for 1h, dehydrated, critical point dried, and coated with Au/Pd, and visualized with an SEM. To quantify the amount of bacterial adherence on surfaces, bacteria were cultured as before, then stained with fluorescent redox dye, 5-cyano,2-ditolyl tetrazolium chloride (CTC) for 1h, and visualized with a Zeiss Axioplan 2 Epifluorescence microscope fitted with a Axiocam camera [6]. The density of live bacteria adhering to the surfaces were counted using KS400 software. On surfaces that autofluoresce, adherent bacteria 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. Statistical analysis was performed using a one-way ANOVA with Tukey test.

RESULTS: SEM showed *S. aureus* adhering to all standard metal osteosynthesis surfaces, and significantly less to the hydrophilic coatings, PLL-*g*-PEG and hyaluronic acid (Fig. 1). Fluorescence microscopy confirmed the SEM results (Fig. 2). Electropolishing the TAN surface also had a significant effect on *S. aureus* adhesion compared to the standard TAN surface. Coatings impregnated with an antiseptic also decreased bacterial adhesion (Fig 2), but the antiseptic was found to be cytotoxic to host cells.

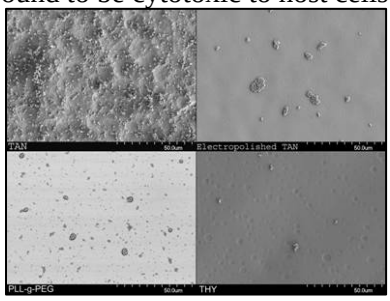
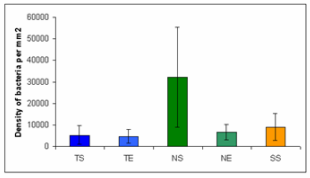
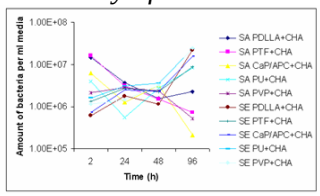


Figure 1: SEM images of S. aureus on different surfaces.

Figure 2: Graphs showing the density of S. aureus adhering to standard surfaces(left), and S. aureus (SA) and *S. epidermidis (SE) on different coated surfaces impregnated with antiseptic (right).*





DISCUSSION & CONCLUSIONS: *S. aureus* adhered to all standard biomaterials, although electropolishing TAN had a significant effect in decreasing adhesion compared to standard TAN. Hyaluronic acid & PLL-*g*-PEG coated titanium surfaces also significantly decreased the density of *S. aureus* & *S. epidermidis*, and hence have potential use as coatings for orthopaedic implants. The polymeric coatings impregnated with antiseptic studied have potential to be used as drug-delivery systems in association with implantable biomaterials, however using an antiseptic is not recommended due to host cell cytotoxicity.

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Bioactive implant coating for local drug delivery

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Demographic data reveal that due to the increasing aging of the population, complications with the musculoskeletal system will increase in the next years.
One major problem in orthopedic and trauma surgery are the delayed healing or non-unions of long bone fractures. The exogenous application of growth factors can stimulate the bone healing to reduce this complication. Beside the choice of the optimal growth factor the application system is important.
Therefore, we developed a new bioactive coating method for implants, which is based on a biodegradable poly(D,L-lactide) (PDLLA, coating thickness: 10 µm) [1]. This coating allows the incorporation of growth factors and the controlled release of substances during the healing process without the need of further devices. The effect of different growth factors such as IGF-I and TGF-ß1 locally released from coated intramedullary implants on fracture healing was investigated in a rat fracture model. Radiologically an enhanced healing was detectable due to the growth factor application (Fig. 1). The biomechanical and histomorphometrical results demonstrate a significant stimulation of the fracture healing due to the locally applied growth factors compared to control at days 28 and 42. At the latest investigated time point, 84 days after fracture, no differences were measurable in the biomechanical stability and the callus composition between the groups. The callus was consistently in the late phase of remodeling without any cartilage left. These results demonstrate, that the local growth factor application enhances the healing in the early phase without alteration of the normal healing process.

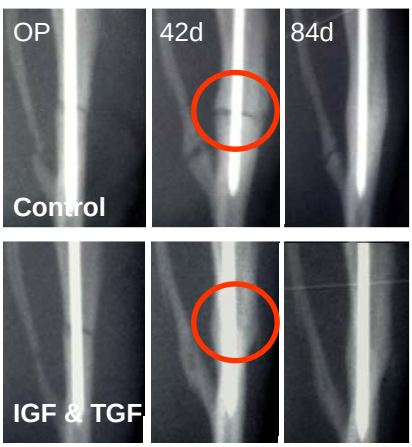

Figure 1 X-rays of fracture healing with and without growth factors Day 42: Better consolidation in the animals treated with IGF & TGF [2]

Besides delayed healing are implant related infections a feared complication in orthopedic and trauma surgery with tremendous consequences for the patient. To reduce this risk, administration of perioperative antibiotic prophylaxis is a routine procedure in orthopedic surgery. To optimize the prophylaxis the above mentioned local delivery system based on a polymer implant coating was used for antibiotics application.
In an animal experiment the efficacy of local prophylaxis was investigated. At surgery the medullary cavities of the rat tibiae were contaminated with *Staph. aureus* and titanium Kirschner wires were implanted. For local antibiotic therapy the implants were coated with PDLLA + gentamicin. All animals treated without local application of the antibiotic developed osteomyelitis (Fig. 2) and all cultures of implants were tested positive on *Staph. aureus* 42 days after surgery. The local application of gentamicin delivered from the PDLLA coating reduced significantly the signs of osteomyelitis in all animals and three of ten implants remained sterile in the microbiological analysis.

Figure 2 X-rays of implant related infection in a rat model 42 days after surgery. Local gentamicin application prevents the infection [3].

Bioactive coating of mechanical well established implants could on the one hand stabilize the fracture and on the other hand serve as a local drug delivery system. The use of gentamicin coated tibial nails is approved in Europe and Canada and the first patients have been treated.

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Design of Biomimetic Microspheres for Biosensing in Packed Microcolumns

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INTRODUCTION: This talk will present recent developments at the University of New Mexico in the analysis of biomolecular recognition in microfluidic systems. The method involves real-time detection of soluble molecules binding to receptor-bearing microspheres, sequestered in affinity column-format inside a microfluidic channel. The packed microcolumn format is (1) well suited for enhancing reaction times of analyte with immobilized receptors, (2) compatible with electro-osmotic pumping, and (3) allows detection of multiple analytes. Identification and quantitation of analytes occurs via direct fluorescence measurements or fluorescence resonance energy transfer (FRET). Several immunoassays have been developed that can potentially detect sub-femtomole quantities of antibody with high signal-to-noise ratio and a large dynamic range spanning nearly four orders of magnitude in analyte concentration in microliter to submicroliter volumes of analyte fluid.

METHODS: Surface modified microbeads (e.g., either silica beads or commercial streptavidin coated beads) are sequestered into microfluidic channels formed by soft lithography or fused silica capillaries to form a packed microcolumns. Sample injection into the columns and subsequent analyte reactions are detected by fluorescence spectroscopy.

RESULTS: Figure 1 shows examples of the packed microcolumns. In this Figure data obtained from a microcolumn containing three distinct sensor regions are presented. A number of different types of biomolecular interactions can be investigated in this type of column. For example, selective binding of antigens to surface immobilized antibodies can be detected directly by monitoring FRET between dyes conjugated to the respective immunological reagents.

Kinetic and equilibrium constants for the reaction of receptor-ligand pairs can be obtained through modelling of kinetic responses of the affinity microcolumn and are consistent with those obtained by flow cytometry. Because of the correlation between kinetic and equilibrium data obtained for the microcolumns, quantitative analysis can be done prior to the steady state endpoint of the recognition reaction.

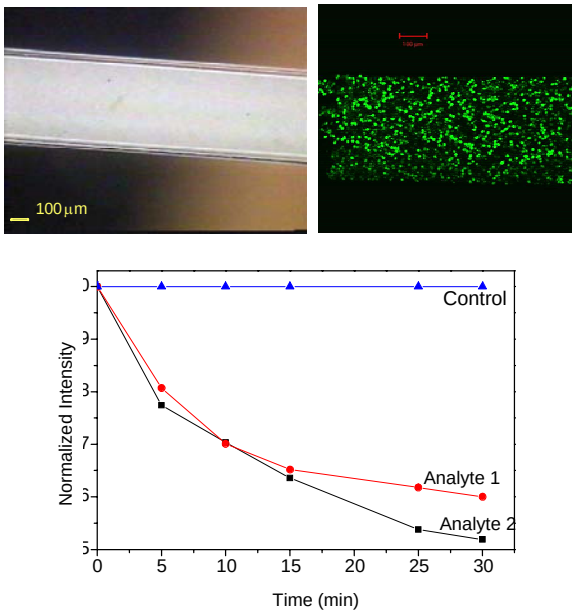

Fig. 1: Top: Optical and fluorescence micrographs of packed microcolumns. Bottom: Typical response of multianalyte microcolumn to injection of equal amounts of two analytes.

DISCUSSION & CONCLUSIONS: This method has the promise of combining the utility of affinity chromatography, with the advantage of direct, quantitative, and real-time analysis and the cost-effectiveness of microanalytical devices. The approach has the potential to be generalized for high sensitivity, high selectivity, rapid detection of a host of bioaffinity assay methods and analyte types.

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Relationships between Surface Properties and Protein Adsorption

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INTRODUCTION: The factors that confer nonfouling character to a surface have been the subject of much discussion. We investigate protein interactions with surfaces using physico-chemical approaches based on classical colloid and surface chemistry, with concepts such as DLVO theory. We are trying to understand protein adsorption or repellence in terms of interfacial forces and how those forces relate to surface chemistry, chain packing density, and other properties. We present key results and how they relate to interfacial interpretations of protein resistance.

METHODS: Si wafer and Teflon FEP substrates were coated with plasma polymer layers from either heptylamine or propanal, to create a functionalized interfacial bonding layer onto which various PEG molecules were covalently linked by cloud-point grafting as in [1]. As a comparative, charged hydrogel layer, polyacrylic acid was also grafted onto an amine plasma layer. Some PEGs were methoxy terminated; others possessed functional end groups that were then modified further. Antibacterial furanone compounds were immobilized onto both types of hydrogels as described in [2]. Characterisation of the coated surfaces was by XPS, ToF-SIMS, and AFM interaction force measurements with a silica probe attached to the cantilever. Protein adsorption experiments were performed with solutions of albumin, fibrinogen or lysozyme; XPS and ToF-SIMS were used to probe for adsorbed proteins.

RESULTS AND DISCUSSION: XPS analysis of the PEG modified surfaces showed that high density PEG coatings had been produced. After immersion in protein solutions, no XPS N 1s signals were observed. By ToFSIMS, however, small contributions from immonium ions derived from amino acids were observed. Their intensities (relative to PEO signals) differed between grafts of 5 kDa, 20 kDa and 40 kDa methoxy-PEGs, with the lowest MW giving the best protein resistance. Both for this and dialdehyde-PEG, the residual protein contributions are extremely low and perhaps caused not by the intrinsic properties of the coatings, but, instead, by unavoidable coating defects induced eg by dust particles. The higher MW PEGs may pack less closely. AFM interaction force measurements against a silica sphere showed a net repulsive force at all separations for closely packed PEG coatings. Using the MWC model [3], the observed steric forces were compared with theoretical predictions, and this enabled determination of the average distance between PEO grafting sites. For dense grafts, this distance was substantially less than twice the Flory radius, confirming the presence of stretched PEG brushes.

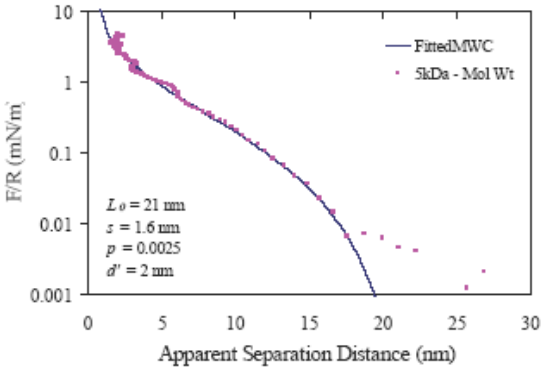

Fig 1: Experimental data (dots) and MWC scaling theory fit (line) for a 5kDa mPEG graft coating.

Functionalized PEGs enable the covalent immobilization of other entities such as oligopeptides and charged small molecules. Of interest is how such entities on top of the PE layer modulate interfacial forces and properties, and protein interactions [4]. PEG grafts have also been compared with other grafted polymer hydrogel layers. Of particular relevance is the fact that polyacrylamide graft coatings analogously showed extremely low protein adsorption [5], which tends to suggest that the interpretation of non-fouling in terms of hydrogen bond acceptance but no hydrogen bond donors [6] needs revision. What is the practical significance of non-fouling coatings? We have observed that furanones immobilized onto various hydrogel spacers inhibit bacterial colonization even when the construct was not protein repellent.

ACKNOWLEDGEMENTS: Support by the Australian Research Council via Discovery Project DP0452838 and the SRC for Particle and Material Interfaces, and Ciba Vision.

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Electrodeposition of Poly(Ethylene Glycol) to Metals

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INTRODUCTION: Poly(ethylene glycol: PEG) is a biofuctional molecule on which adsorption of proteins is inhibited. Therefore, immobilization of PEG to metal surface is an important event to bio-functionalize the metal surface, for inhibition of protein adsorption, blood compatibility, lubrication in bllod vessel, and antibacterial property. In this study, PEG modified both terminals or one terminal with amine bases was immobilized onto titanium surface using electrodeposition and the mode of mobilization was characterized.

METHODS: Both terminals of PEG were modified with NH_2 to form positive electric charge in aqueous solutions. Also, PEG charged only one terminal was prepared. Molecular weights of both PEGs were about 1000. Commercially pure titanium disk with grade 2 was metallographically polished and ultrasonically rinsed in acetone and deionized water.

Titanium was charged as cathode and anode was platinum on electrodeposition. PEG was dissolved to 0.3-mol l-1NaCl solution as a concentration of 2mass%. The pH of solution was 11. The resultant solution was used as an electrolyte for electrodeposition at ambient.

The thickness of immobilized PEG layer was determined with ellipsometory. The immobilization mode to titanium surface and chemical bonding were determined using X-ray photoelectron spectroscopy (XPS).

In order to confirm the prevention of plasma protein adsorption to titanium surface with immobilization of PEG, albumin was adsorbed by immersion in albumin-containing solution and observed with a fluorescence microscope.

DISCUSSION & CONCLUSIONS:

Figure 1 shows the change in thickness of PEG immobilized layer determined using ellipsometry. These thicknesses are measured in air, so the real thickness in solutions is larger than these values. The thickness of the immobilized layer increased with the increase of charged potential. Also, the thickness was larger than that in immersed specimen.

Changes in the ratios, [C-O, C-N]/[C-C, CH_2], in C 1s peak as charged potential are shown in Fig.2. Photoelectron signals in XPS abruptly decays to depth direction. Therefore, C-N bond located at inside of the immobilized PEG layer. In other words, N exists at the interface between PEG and titanium. This phenomenon is also confirmed with result from N 1 s peak. In addition, strong bonding, -N-OH was formed between amine and titanium oxide by electrodeposition while ionic bonding by immersion. Therefore, PEGs are immobilized as shown in Fig. 3.

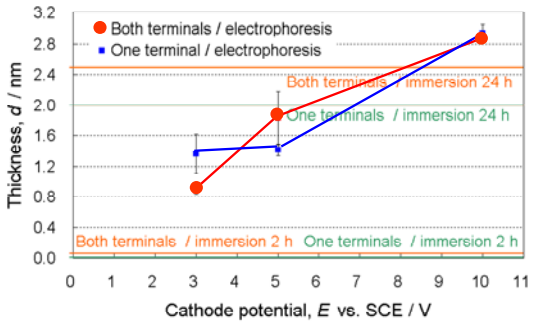

Figure 1: Change in thickness of PEG immobilized layer.

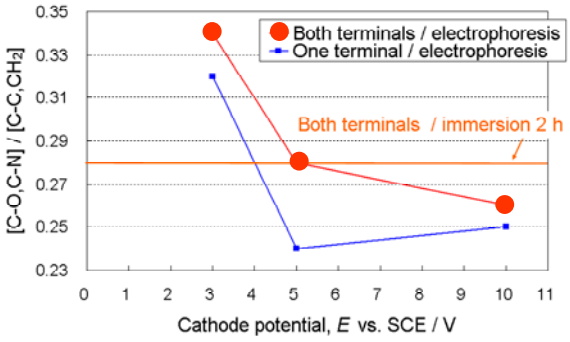

Figure 2: Change in the ratio, [C-O, C-N]/[C-C, CH_2], in C 1s peak as charged potential.

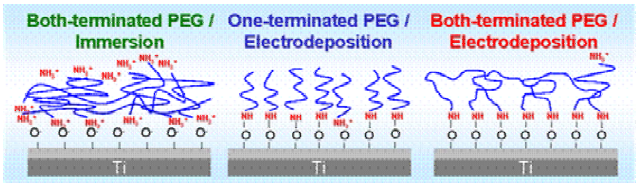

Figure 3: Schematic model of immobilized mode of PEG to titanium surface.

CONCLUSIONS: Control of Immobilization mode of PEG modified with NH_2 to titanium surface by electrodeposition is feasible. This technique could be applied to all metallic materials.

HYBRID OPTICAL AND SCANNING PROBE MICROSCOPIES FOR VISUALIZATION OF BIOMOLECULES ON SURFACES

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ABSTRACT: Effective visualization and quantification of dynamic biological processes involving (multiple) molecular interactions is a key challenge of molecular and cellular imaging. Successfully addressing this challenge requires a multifaceted approach including probe development and bioconjugation strategies, quantitative multi-parameter microscopies, and the judicious combination of optical spectroscopy techniques with optical microscopy or scanning probe techniques. In essence, what is required is microspectroscopy with high spatial, temporal, and spectroscopic resolution.

Fibrillar aggregation of proteins is a self-assembly process of significance to biological function, to specific industrial applications, and increasingly, to disease. Thus, the polymerization of actin is essential to biological function, the fibrillization of specific food proteins provides texture to food, while aggregating proteins forming amyloid are implicated in the pathogenesis of many human diseases. Aggregation is often a consequence of partial or full denaturation of proteins, and provides insights into the mysteries of protein folding and misfolding.

To better address the chemical biology and biophysics of protein aggregation we have developed several hybrid microscopies combining different imaging modes to provide additional insights into biomolecular interactions.

Self-assembly of the human alpha-synuclein protein resulting in protein aggregates of diverse morphology is a feature of Parkinson's Disease and other neurodegenerative disorders known as synucleinopathies. This aggregation process is representative of the interconversion of an unfolded protein into nanostructures with typical amyloid features. The morphologies of the nanostructures formed and the kinetics of the aggregation are modulated, among other factors, by solution conditions, mutations in the protein, and the effect of the support surfaces. We have applied a wide repertoire of biophysical techniques to continuously monitor and visualize at the molecular level the self-assembly of wild-type alpha-synuclein and various mutants. Using a combination of hybrid optical and scanning probe techniques and ensemble and single molecule fluorescence spectroscopy we are seeking to gain direct insight into different modes of alpha-synuclein self-assembly and to identify key factors modulating the aggregation process, contributing further to our understanding of the molecular biophysical bases of disease-related conformational changes of proteins.

The technologies developed for these experiments have broader applications to the study of biological molecules on surfaces.

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Investigation of Bio-Molecular Interfaces Using Photoemission Spectroscopy In Combination With In-Situ Deposition Techniques

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INTRODUCTION: The presented research is motivated by the increasing incorporation of bio-materials in electronic and sensor devices. As bio-materials are used in concert with other materials to form device structures, their interfaces to these materials become a matter of interest. Chemical and electronic properties of contacts crucially define the properties of such structures. Photoemission spectroscopy (PES) has traditionally been a method of choice to explore interfaces of a great variety of materials. However, since in such experiments, the interface of interest needs to be prepared in vacuum to avoid interference with ambient contaminants, bio-materials interfaces have been difficult to investigate in the past. Since bio-molecules usually cannot be evaporated in vacuum due to their thermal fragility, interfaces can in many cases only be fabricated from solution (spin coating, dipping, etc…), which cannot be done in vacuum. The presented experiments address this challenge through the integration of an electrospray based thin film deposition system [1] into a commercially available photoemission spectroscopy system outfitted with a directly attached glove box. Using this set-up, the electronic structure of ribonucleic acid (RNA) homopolymer [2] and L-cysteine [3] interfaces with graphite and gold were investigated.

METHODS: Details about the experimental methods can be found in Ref.[1]. Briefly, RNA and L-cysteine were deposited in several steps using electrospray or dipping in inert atmosphere. In between deposition steps PE-spectra were measured resulting in series of spectra detailing the development of the electronic and chemical structure at the interface. Evaluation of these sequences allowed drawing the orbital line-up at the contacts, and gave insight into the chemical interaction at the interface.

RESULTS: As an example, Fig.1 shows ultraviolet photoemission spectroscopy (UPS) spectra of the highest molecular orbitals (HOMO) of polyguanosine (poly rG) and polyuridine (poly rU) representative for purine and pyrimidine spectra. Poly rG shows a shoulder at the low binding energy side of the spectrum, which is not present in the poly rU spectrum. This indicates a smaller ionization energy of poly rG compared to poly rU.

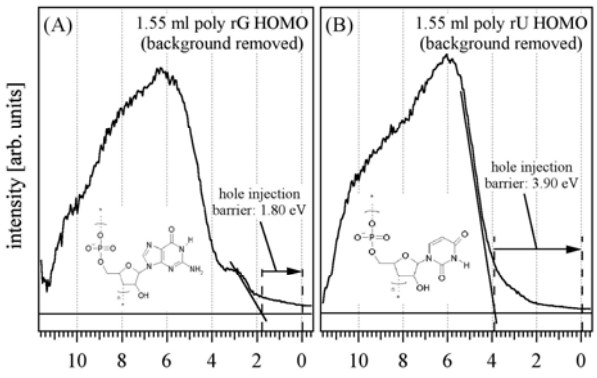

Fig.1: HOMO spectra of polyguanosine (poly rG) and polyuridine (poly rU). A smaller hole injection barrier is evident for poly rG.

DISCUSSION & CONCLUSIONS: Our results demonstrate the application of electrospray as an in-situ deposition technique for the preparation of largely contamination free bio-molecular thin films for surface scientific investigations. Electrospray enables clean repeat deposition of thin films directly from solution without breaking the vacuum. Our results on RNA homopolymers indicate significant differences in ionization energies, where poly rG has the smallest and poly rU the largest. This directly influences the injection barriers relative to HOPG and Au, indicating that charge transfer crucially depends on the particular nucleotide in contact with an electrode. Our experiments on the L-cysteine/Au interface indicate the formation of an interface state caused by the chemical interaction between thiol group and Au surface. This state could potentially act as a "stepping stone" for charge transfer between proteins and Au.

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The Cellular Nano-Machinery Involved in Surface Recognition and Adhesion

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Cell adhesion to the extracellular matrix is a complex process, regulated at multiple levels. Cells can differentially sense, and respond to chemical signals, induced by soluble molecules or by the "insoluble environment", consisting of the extracellular matrix and neighboring cells. In addition, cells can sense a variety of physical signals, including external or internal forces, surface rigidity, topography and fine texture. In this presentation, I will address the role, and possible mechanisms underlying cellular responses to mechanical perturbations of cell adhesion in the nN range, and changes in adhesive ligand density in tens of nm range. I will address possible mechanisms involved in the effect of such environmental features on focal adhesion formation, dynamics and fate. Focusing on different cell types, including fibroblasts, endothelial cells and osteoclasts, different mechano-sensitive processes will be highlighted. The nature of the putative "mechanosensor" in cell adhesions will be discussed, as well as the mechanisms whereby it triggers signaling events within living cells. Particular attention will be devoted to signaling cascades involving phosphorylation and de-phosphorylation of a variety of target molecules. The mechanisms whereby such environmental information is acquired and interpreted will be discussed

Identifying and mimicking features of unfavourable topography and investigating cellular reactions

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INTRODUCTION: Orthopaedic implant manufacturers offer a considerable range of metals. The finish varies from electropolishing of stainless steel (SS) to micro-rough titanium (TS) and Ti-6Al-7Nb (NS). In the context of soft tissue, represented *in vitro* by fibroblasts, rough versus smooth on TS and SS did not significantly affect cell adhesion or subsequent growth. However, fibroblast spreading and cell growth were seriously compromised on micro-rough NS[1]. This led to the question; is the contributory cause chemistry or topography or a combination of both? Here we investigate the influence of chemistry and topography by means of surface coating and microfabrication.

METHODS: Material coating was conducted utilising an e-beam evaporator to deposit 50nm of either gold or titanium on NS, TS and SS. Surface characterisation was performed using Atomic Force Microscopy (AFM), Profilometry and Scanning Electron Microscopy (SEM). Qualitative assessment utilised SEM for cell growth and morphology at 24h, 5 and 10 day timepoints and Fluorescence Microscope (FM) for quantitative cell counts. Intracellular components, vinculin, tubulin, actin and DNA, were fluorescently labelled and imaged using the FM at 48h. A silicon die with inverted micropyramids was produced by photolithography and wet etching methods. Replicas were produced by embossing in polycaprolactone and evaporated with 50nm titanium.

RESULTS & DISCUSSION: Surface coating of NS produced a homogenous layer of either gold or titanium. Cell growth demonstrated no statistically significant differences per sample type at 24h, 5 or 10 days – cell growth was typically depressed on all sample types of NS. Cell morphology and cytoskeletal staining, examined using SEM and FM, demonstrated no differences between uncoated NS and its coated counterparts. To safeguard against coating compatibility issues, SS and TS were also coated and cell growth was demonstrated to be normal for these surfaces in comparison with the uncoated versions – confluent monolayers were observed at 10 days on all. These results indicate that for NS, the surface topography and not the underlying chemistry was the primary cause of inhibited cell growth. This finding was confirmed with coated SS and TS surface models.

Numerically the roughness average of NS (0.77μm) was similar to TS (0.90μm), however when examined utilising SEM and AFM, the surfaces were markedly different with NS displaying a rough microspiked topography. AFM analysis demonstrated that these microspikes had surprisingly uniform dimensions and demonstrated with FM to interfere with cellular processes of adhesion and microtubule formation. This cellular impairment made the microspikes a primary suspect in suppressing cell growth.

A metallic topography inspired by the dimensions and general morphology of the spikes was microfabricated. The topography produced was a uniformly spaced pyramid topography. Cells on these topographies were demonstrated to display low cell growth, low spreading, and their adhesion sites and microtubule networks were visibly influenced by the presence of the pyramids (Fig 1).

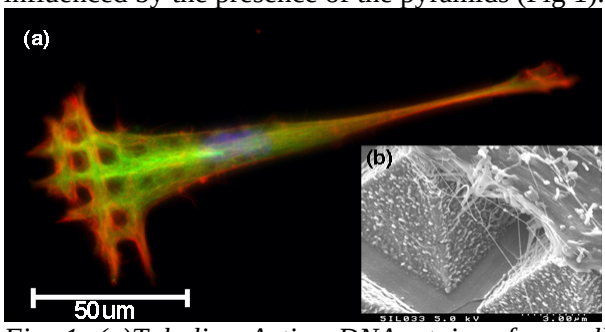

Fig 1 (a)Tubulin, Actin, DNA stain of a cell cultured on the pyramid microtopography. (b) SEM of cell elevated on the pyramid topography.

CONCLUSION: In mimicking such dimensions and eliciting similar cellular reactions, the importance of roughness morphology is illustrated. A reciprocal trend could be developed, as metal topographies could provide inspiration for fabricated microtopographies that in turn could be utilised to produce optimal metallic implant topographies, and not only with regard to fibroblasts.

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Engineering Interfaces for Controlled Cellular Activities

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INTRODUCTION: Cell adhesion to adsorbed extracellular matrix (ECM) proteins and adhesive sequences engineered on synthetic surfaces plays critical roles in biomaterial, tissue engineering, and biotechnological applications [1]. Cell adhesion to these adhesive motifs is primarily mediated by integrin receptors [2]. In addition to anchoring cells, integrin binding activates signaling pathways regulating cell survival, proliferation, and differentiation. While tethering short adhesive peptides derived from ECM ligands (e.g., RGD for fibronectin) promotes cell adhesion and function in several cell systems, these biomimetic strategies are limited by reduced biological activity compared to the native ligand, lack of specificity among integrins, and inability to bind non-RGD integrins. We have engineered biointerfaces that mimic the secondary and tertiary protein structure of fibronectin and type I collagen. These surfaces convey integrin binding specificity, focal adhesion assembly and signaling as well as bone and muscle cell adhesion, proliferation, and differentiation.

METHODS: Self-assembled monolayers of ω-functionalized alkanethiols on gold and microcontact printing were used to engineer surfaces with well-defined chemical properties. Bioadhesive ligands that mimic the primary and secondary protein structure for fibronectin and type I collagen were tethered onto protein-adsorption-resistant supports (19:1 tri(ethylene glycol)-terminated/COOH-hexa(ethylene glycol)-terminated alkanethiols) to target specific integrin adhesion receptors. Cell adhesion was analyzed in terms of integrin binding, focal adhesion assembly and signaling, and adhesion strength using biochemical and functional assays. Cell differentiation (gene and protein expression, mineralization) was assessed via real-time RT-PCR, immunostaining, and histochemical staining.

RESULTS: A recombinant fragment of fibronectin spanning the 7th-10th type III repeats of fibronectin and encompassing the PHSRN and RGD motifs was tethered to non-fouling supports to specifically bind alpha5beta1 integrin and trigger focal adhesion assembly and signaling. Binding of this receptor is critical to osteoblast proliferation, differentiation, and matrix mineralization. To target alpha2beta1 integrin, a triple-helical collagen-mimetic peptide incorporating the GFOGER motif was tethered to model non-adhesive supports. These biomimetic surfaces supported alpha2beta1 integrin-mediated adhesion and focal adhesion assembly and directed osteoblast specific-gene expression and matrix mineralization to higher levels than conventional culture supports. Second-generation interfaces have been developed to display controlled fibronectin-/collagen-mimetic ligand mixed densities to independently target alpha5beta1 and alpha2beta1 integrins. These mixed ligand surfaces synergistically modulate cell adhesive activities. Finally, these approaches have been combined with micropatterning techniques to generate biointerfaces that control cell-substrate adhesive area and integrin binding.

DISCUSSION & CONCLUSIONS: By focusing on bioadhesive ligands that recapitulate the secondary and tertiary structure of ECM proteins, we have engineered surfaces that direct integrin binding and signalling to elicit specific cellular responses. These biomolecular engineering strategies provide a basis for the rational design of robust biointerfaces that tailor adhesive interactions and elicit specific cellular responses for the development of bioactive implant surfaces, scaffolds for enhanced tissue reconstruction, and growth supports for enhanced cellular activities.

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ACKNOWLEDGEMENTS: Funding provided by the National Institute of Health (R01 GM-065918, R01 EB-004496), National Science Foundation (CAREER BES-0093226, EEC-9731643), Arthritis and Whitaker Foundations.

Use of adhesion molecule-coated surfaces to decipher cell contact formation and associated intracellular responses

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How cells sense their environment during embryonic tissues formation and adjust their response in terms of shape, proliferation, differentiation and survival remains one of the more puzzling questions in biology, with high physiopathological implication for tissues homeostasis. In the seventies, cytoskeleton linked cell surface glycoproteins (cadherins and integrins) have been discovered that mediate specific cell-cell and cell-matrix adhesion and transduce mechano-chemical signal from cells in contact.

Cadherins are of special interest for the mechano-transduction at cell-cell contact as they act as both their own ligand and receptor and establish a direct link between adjacent cells. Widely expressed and dynamically regulated during development, they control the aggregation and segregation of embryonic cells, the formation of intercellular junctions and cell differentiation. In complex with their intracellular partners (catenins and signaling proteins), they transduce signals leading to cytoskeleton remodeling and more global intracellular signaling.

However, one of the major limitations for cadherin studies, hampering the elucidation of their mode of action, has been the absence of a method to activate these receptors in a controlled manner. In this context, we developed an approach based on the use of immobilized recombinant cad-Fc ligands[1] to activate cadherins precisely, enabling both the study of the dynamics and molecular regulation of cell contact formation[2,3] and demonstrating the usefulness of biomimetic surfaces to progress in the characterization of the cellular response[4,5].

Using such biomimetic system (Ncad-Fc coated-beads) to specifically activate N-cadherin, we previously described in myogenic C2 cells a fast adhesion-triggered and Rac1-dependent anchoring of N-cadherin to the actin cytoskeleton enabling the transduction of forces generated by the actin tread milling. Recent observations by videomicroscopy in neuronal cells expressing Ncad-GFP (N-cadherin tagged to the Green Fluorescent Protein) indicate that N-cadherin anchoring to the cytoskeleton is followed by a further recruitment of N-cadherin molecules.

We showed previously that cell spreading on Ncad-Fc-coated surfaces induces a strong co-recruitment of cadherin, catenins and actin filaments into focal adhesion-like structures (cadherin adhesions), that we analysed here by RICM (Reflection Interference Contrast Microscopy) and TIRFM (Total Internal Reflection Fluorescence Microscopy). Cadherin adhesions were easily detected by both methods confirming their close apposition to the substratum and their relative stability over time. We followed their formation in real time using GFP-tagged molecules. Tiny clusters of cadherin appear at the tip of growing lamellipodia and then fuse to form cadherin adhesion at the rear of lamellipodia. In some cells, nascent structures were reset and reoriented in relation with global lamellipodial dynamics. Experiments performed with various N-cadherin mutants deleted of their cytoplasm tail confirm that N-cadherin recruitment in cadherin adhesion requires their association to catenins and actin. FRAP (Florescence Recovery After Photobleaching) experiments further show that a population of N-cadherin molecules in cadherin adhesion was diffusive while another was submitted to a slow turnover indicative of a regime exchange limited by the formation/dissociation of N-cadherin adhesive bounds. Interestingly, the association of N-cadherin tail to catenins further regulates this exchange regime.

More stringent control over the adhesion ligand presentation, density, topology, rigidity and mobility is now necessary for further elucidation of the physical regulation of cadherin functions in cell-cell adhesion and associated responses.

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Carbohydrate functionalized surfaces for Glycomic applications

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INTRODUCTION: Glycomics became a field of growing interest in the recent years due to the important role of carbohydrates in many important biological processes like cell-cell and cell-pathogen recognition.[1] Because of missing analytical tools and the difficulties to synthesize or purely isolate complex carbohydrates, there is until now little known about the exact role of carbohydrates in these processes. Therefore, the goal of our work is to contribute to the development of systems that allow a high throughput screening of specific carbohydrate interactions.

METHODS: We have newly developed a method to graft mono- and oligomannosides to the polycationic copolymer poly(L-lysine)-graft-poly(ethylene glycol) (PLL-[g]-PEG), which adsorbs spontaneously on negative charged oxide surfaces(Nb_2O_5, TiO_2). With our system it is possible to control the density and distribution of the mannosides on the polymer backbone by changing on one hand the proportion between sugar terminating and non-functionalized PEG chains or on the other hand the ratio of PEG chains per Lysine unit. We use the label free bio-sensing method Optical Lightmode Waveguide Spectroscopy (OWLS) to study the specific adsorption of the well-known D-mannose specific lectin Concanavalin A (Con A) on our functionalized surfaces. Furthermore the interactions of mannose functionalized surfaces with the mannose specific strain K12 of the bacteria Escherichia coli (E. coli) are tested.

Patterns of the carbohydrate functionalized polymers with a non-fouling background are formed with the photolithographic patterning method MAPL (Molecular Assembly Patterning by Lift-off).[2]

RESULTS: The OWLS results show that the carbohydrate functionalized PLL-[g]-PEG are resistant against non-specific serum adsorption while the amount of adsorbed Con A depends on the mannose surface density. As we can determine the mass of adsorbed polymer qualitatively with a sensitivity around 2 ng/cm^2, we are able to define binding constants for the interaction strength between the lectin and our system as well as IC_{50} values with the inhibitor α-methyl-mannose.

The absence of non-specific interaction and the mannose surface density dependence can be also seen for the adhesion of the E. coli strain K12 via the mannose specific lectin FimH on the functionalized surfaces.

The specific interaction with the biological targets Con A and E. coli can be clearly shown with the patterns of carbohydrate functionalized PLL-[g]-PEGs formed by the MAPL technique as shown in Fig. 1.

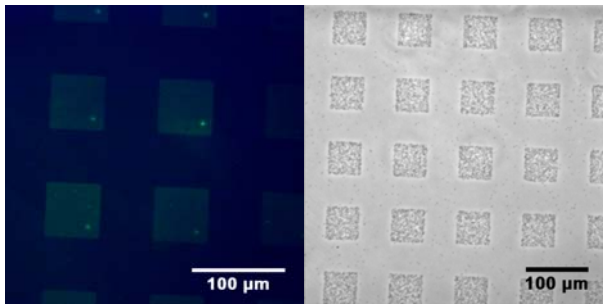

Fig. 1: FITC labelled Con A (left) and E. coli (right) adsorbed on patterns of Mannose functionalized PLL-[g]-PEG formed by the MAPL technique.

DISCUSSION & CONCLUSIONS: Mannose functionalized PLL-[g]-PEG is a simple method to immobilize carbohydrates in a controlled manner on surfaces for the detection of specific carbohydrate interactions. With the well known model systems Con A and E. coli we proof that mannosides immobilized on surfaces are still available for specific interactions while the non-specific interaction is prevented by the ethylenglycol moieties in the polymer. With the possibility to extend this approach to complex oligosaccharides it is a versatile tool for the study of the role of carbohydrates in biological systems.

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Self-Assembling of Particle Monolayers by Spin-Coating

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INTRODUCTION: Monodispers particles dried from suspension form domains of highly ordered hexagonal arrays.[1] The self-assembly of such particle monolayers can be achieved for example by controlled drying[2] and spin-coating.[1] The presented work is a study on self-assembling micron-sized particle monolayers by spin-coating. The motivation for engagement with this matter has the final goal of creating biologically relevant patterns. The use of ordered particle monolayer arrays as etching masks is considered to be a fast and cheap alternative to classic photolithographic patterning techniques which allows for the creation of nanopatterned features with separations in the micron-range. Especially biologically oriented research has a high sample consumption (e.g. cell studies) therefore cheap patterning techniques are highly relevant.

METHODS: 2 µm polystyrene particles were dispersed in a solution of methanol and surfactant Triton® X-100.[1] A standard spin-coater was used to coat the dispersion onto glass and titanium dioxide substrates. The influence on the monolayer formation was investigated as a function of particle and surfactant concentrations of the coating fluid and of the spinning speed (100 to 6000 rpm). The particle concentrations were varied between 1:3 to 1:100 (v/v). The surfactant concentrations were varied between 1:30 and 1:400 (v/v).

RESULTS: The sizes of the monolayer areas and of the perfectly ordered domains therein were highly affected by the spinning speed and composition of the coating fluid. By adjusting these two parameters monolayers where successfully produced. The main problem interrupting particle self-assembly was the insufficient adhesion between the suspension and the substrate materials. We were able to solve this problem by adding the appropriate surfactant concentration for different spinning speeds. The influence of the surfactant as a bonding agent to support monolayer formation was investigated and showed substantial differences between the different concentrations. In the end large area monolayers formation was achieved (Figure 1) and will be presented in the poster.

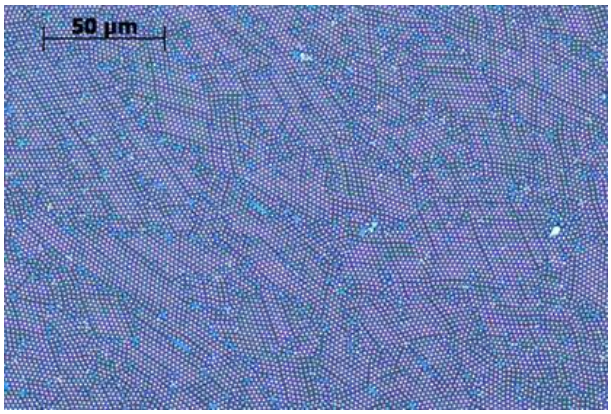

Fig. 1: Light microscopy image of hexagonal ordered domains in a polystyrene particle monolayer on a glass substrate. Such ordered monolayers where achieved with the following spin-coating settings: particle concentration, 1:100; surfactant concentration, 1:200; spinning speed, 200 rpm

DISCUSSION & CONCLUSIONS: We showed that monolayer formation by particle spin-coating is possible even there were some reproducibility problems. The interactions between the suspension and the substrate material and the influence of the surfactant as a bonding agent were substantial and need to be further investigated. The presented particle domains and monolayer areas are already large enough to serve as etching masks which will allow for the creation of biologically relevant patterns.

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ACKNOWLEDGEMENTS: This work, as part of the European Science Foundation EUROCORES Programme "Self-Organized Nanostructures" (SONS, NanoSMAP), was supported by funds from the Swiss National Science Foundation (SNF) and the EC Sixth Framework Programme.

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PEGylated bacteriophage enhances circulation time: towards discovery of tissue targeting peptides

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INTRODUCTION: Combinatorial chemistry approaches for identifying cell and tissue-specific peptides have wide applicability in numerous contexts, ranging from fundamental to clinical applications. Recent in vivo uses of phage-displayed peptide libraries have shown promising results (in both mouse and human models) to identify peptide epitopes capable of homing to specific understood, but has nevertheless allowed remarkably selective targeting of therapeutic agents to tissues, and in several cases, tumors.

On bacteriophage, displayed peptides and even proteins are presented in a manner very similar to their native state. In addition, phage are easily amplified in vitro by infection of E. coli. All of these strengths, however, have been considerably limited by the rapid clearance of phage particles by the reticuloendothelial system (RES). Extensive studies have now shown that poly(ethylene glycol) (PEG) attachment to surfaces and drug delivery vehicles can reduce protein deposition in vivo, and thereby help avoid the RES – prolonging circulation times. By combining the ability of PEG to extend in vivo lifetimes with the ability of peptide-on-phage libraries to identify novel targeting epitopes, we intend to open new possibilities in targeting and therapy. Two main strategies have been employed: (i) non-specific attachment to primary amines using PEG vinyl sulfone and (ii) specific modification to cysteines using PEG acrylates.

METHODS: Standard microbiological techniques were used in the growth and maintenance of E. coli TG1 cells (Stratagene). Wild-type phage was M13KO7 (Amersham), containing a kanamycin resistance gene for screening and propagation.

PEG-VS was synthesized from PEG monomethyl ether and divinyl sulfone with a final molecular weight of 5 kDa. A PEG N-hydroxy-succinimidyl ester, mPEG-SPA, was purchased from Nektar (Hunstville, AL) and was also 5 kDa. Reactions were carried out a room temperature.

Quantitative PCR (qPCR) was performed using a Bio-Rad iCycler, using SYBR Green fluorophore.

RESULTS: In vitro tests quantify any adverse effects of PEG attachment on bacteriophage infectivity and therefore establish a common basis for establishing the biodistribution. Preliminary results show a modest decrease in phage infectivity upon random PEGylation, suggestive of modification of the minor coat protein necessary for infection. Using a chromophoric and/or fluorescamine assay, one can estimate the degree of PEGylation of the bacteriophage to reach values approaching the Flory regime for ideal polymers, providing further evidence of a "Stealth" character. To decouple PEG attachment from adverse effects on infectivity, solvent-accessible single cysteines are introduced via site-directed mutagenesis of the phage major coat protein. Use of PEG-acrylate will then allow for selective attachment to the bacteriophage major coat proteins while preserving the infectivity of the minor coat proteins. This modular approach sets the stage for in vivo studies of peptide-on-phage libraries to identify novel peptide homing epitopes.

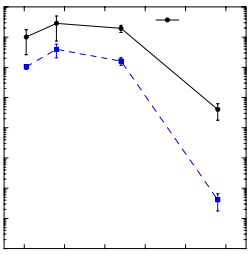

Fig. 1: Amount of phage in blood as determined by real-time PCR.

DISCUSSION & CONCLUSIONS: Initial in vivo studies verify the ability of PEG to dramatically prolong bacteriophage circulation time in mice compared to wild-type phage (Figure 1). Further work aims to establish the optimal PEG surface coverage and chain lengths. Circulation time and biodistribution are determined via phage amplification of the tissue homogenate in E. coli, thus eliminating the need for any fluorescent or radio-labeling. Independently, real-time PCR quantifies the amount of viral DNA recovered, enabling a distinction between surface-associated and cell-uptaken or otherwise inactivated phage.

ACKNOWLEDGEMENTS: We kindly thank H. Schmoekel for assistance with animal experiments.

Optimizing Etching Parameters to Produce Ordered Nanochemical Patterns

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INTRODUCTION: Nanopatterns for bioapplications provide novel tools to address biological problems. For example, protein nanoarrays not only enable molecular level statistics of binding events but also offer an increased sensitivity compared to microarrays. Particles arranged into 2D ordered structures can serve as a template for the fabrication of well-defined nanostructures. Certain novel applications, such as single molecule fluorescence studies, require nano-sized features in geometrically ordered patterns with a separation between the features in the low micrometer range in order to be able to detect individual nanostructures by optical microscopy.

METHODS: To achieve such patterns we have self-assembled micron-sized latex particles by controlled drying from aqueous suspensions on silicon wafers (sputter-)coated with 46 nm SiO_2 (intermediate layer) and 11 nm TiO_2 (top layer). The latex particle patterns were then etched by reactive ion etching (RIE) to homogeneously reduce the size of the latex. The etched latex particle patterns served as a mask to create a metal oxide contrast into the underlying substrate by RIE. This technique produces TiO_2 pillars in a SiO_2 background. Size and morphology of the latex features created after RIE as well as the etch rates of SiO_2 and TiO_2 strongly depend on the parameters, such as gas composition, forward power and chamber pressure used during the RIE. Optimizing the etching parameters was the topic of this work.

RESULTS: We successfully performed above mentioned two-step RIE process to produce a well defined metal oxide contrast with nano-sized TiO_2 features in a SiO_2 background.

Etching of the latex spheres was performed with 50 sccm N_2 and 50 sccm O_2 (100 W, 100 mtorr, 12 min) leading to shrinkage of the spheres from 1.9 µm to 750 nm as shown in Figure 1.

After the latex particle mask was etched to the wanted size, the metal oxide substrates were etched through this mask. Best selectivity for the metal oxides was achieved with 95 sccm SF_6 and 5 sccm O_2 (80 W, 100 mtorr, 2 min). With these settings we successfully achieved metal oxide contrast dot structures. The diameters of the TiO_2 pillars in the SiO_2 background were in the range of 200 nm, separated by the distance of the initial particle diameters (1.9 µm).

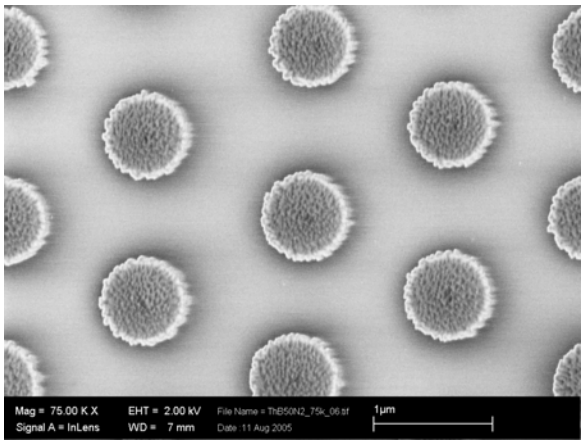

Fig. 1: Scanning electron microscopy image of latex spheres after RIE (50 sccm N_2 and 50 sccm O_2, 100 W, 100 mtorr, 12 min)

DISCUSSION & CONCLUSIONS: We were able to define RIE parameters which allow for the creation of TiO_2 nanopillars in a SiO_2 background with latex particles as etching mask.

The selective molecular-assembly patterning (SMAP)[1] technique can transfer the metal oxide contrast into a biochemical contrast, by two simple dip-and-rinse steps. In short; first alkane phosphates self-assembled monolayers (SAMs) are created on the TiO_2 pillars. In a second step the SiO_2 background is passivated towards unspecific protein adsorption with poly(L-lysine)-*graft*-poly(ethylene glycol) (PLL-*g*-PEG). SMAP patterns are then used for specific protein adsorption on the protein adhesive alkane phosphate SAM nanofeatures while the background is protein resistant.

REFERENCES: [1]R. Michel et al. (2002) *Langmuir* **18**:3281-3287.

ACKNOWLEDGEMENTS: This work, as part of the European Science Foundation EUROCORES Programme "Self-Organized Nanostructures" (SONS, NanoSMAP), was supported by funds from the Swiss National Science Foundation (SNF) and the EC Sixth Framework Programme.

Many thanks to Christoph Huwiler for his help with the SEM and to Otte Homan from the Micro/Nanofabrication Lab (FIRST) at ETH-Hoenggerberg, Zurich.

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Probing the non-fouling behaviour of PEG and sulfonated PEG surfaces: An electrostatic interaction superimposed on the steric repulsion

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INTRODUCTION: The development of non-fouling surfaces has been a significant theme in biomaterial research. Surface modification using ethylene oxide chemistries has proved successful in a number of studies, where both chain density and chain length are important. Further functionalisation with sulfonate groups has more recently been considered to provide greater blood compatibility [1] via the "negative cilia" concept, in which surface grafted chains would repel negatively charged serum proteins by a combination of entropic and electrostatic forces.

METHODS: FEP substrates were coated with an amine plasma polymer layer, followed by cloud-point grafting of dialdehyde PEG molecules [2]. Further modification of the PEG layer was undertaken with sulfonisation of the end groups using $NaHSO_3$.

Characterisation of the surfaces was undertaken with XPS and atomic force microscopy direct interaction force measurements with a silica probe attached to the cantilever. Protein adsorption experiments were performed with solutions of albumin, fibrinogen or lysozyme; adsorbed proteins were detected using XPS (nitrogen signal).

RESULTS: XPS characterisation of the PEG modified surface demonstrated that a high quality of PEG coating was achieved. The sulfonate-modified PEG surface was characterised by a sulphur signal in the XPS spectra.

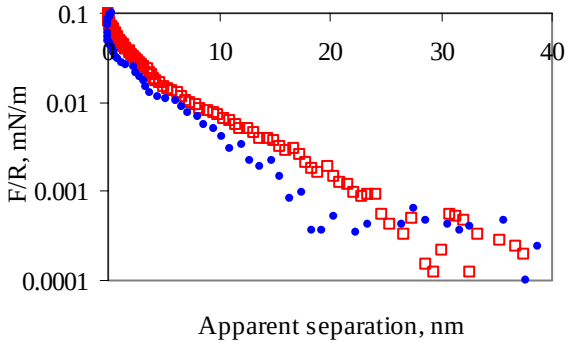

Fig. 1: Interaction forces measured between a silica sphere and the cloud-point grafted PEG surface in PBS (•) and 0.01 PBS(□) .

A repulsive interaction was observed when measuring the interaction forces between a silica sphere and the PEG-cloud-point grafted surface (*Fig. 1)*, indicating that the surface provides a steric barrier to protein adsorption. A similar repulsion was observed on the sulfonate-modified PEG surface, however, the strength of the repulsive interaction energy changed with ionic strength (*Fig.2*). Therefore an electrostatic interaction is responsible in addition to the steric repulsion.

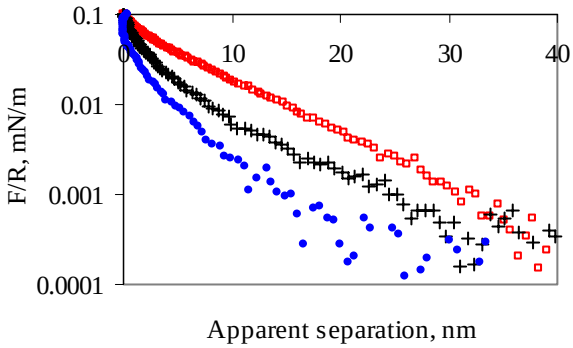

Fig. 2: Interaction forces measured between a silica sphere and the sulfonated PEG surface in PBS (•), 0.1 PBS (+) and 0.01 PBS(□).

Protein adsorption onto the modified surfaces was decreased compared the bare FEP substrate, however was greater on the sulfonated surface, and dependent on the protein charge.

DISCUSSION & CONCLUSIONS: Non-fouling properties of the cloud-point grafted PEG surfaces result from a steric repulsion between the protein molecule and the surface. Addition of sulfonate groups to the PEG chain, provides an electrostatic repulsive interaction superimposed on the steric repulsion.

REFERENCES: [1] Y.H. Kim, D.K. Han, et al. (2003) Biomaterials **24**: 2213-23. [2] P. Kingshott, H. Thissen, H.J. Griesser (2002) *Biomaterials* **23**:2043-56.

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The art of fine surface tuning: electrochemical micropatterning of biomedical surfaces

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INTRODUCTION: Interaction between living matter and an implant surface is a vastly studied subject. From an osseointegration point of view the influence of the surface topography is largely documented [1,2]. Alternatively drug-eluting implants is a booming research field (especially for cardiovascular stents). From a material science point of view, recent advances in electrochemical micromachining methods [3] offer new surface modifications possibilities, which could be of interest for bio-surface applications. The objective of the present paper is to present two illustrative examples. The first one deals with titanium dental implants and the second with stainless steel stents.

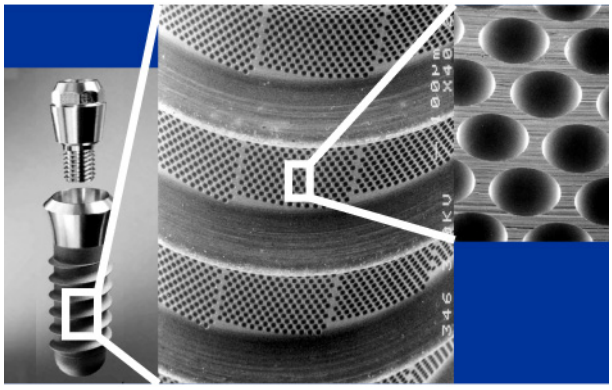

Fig. 1 set of pictures showing the precise micro-structuring of a titanium dental implant (27000 cavities, 50 µm in diameter).

METHOD: After thorough cleaning, the devices are coated with a protective film. This protection is realized through an anodic oxidation process for the titanium implant. On other types of metals as in the stainless stent example, part protection is obtained by the electrodeposition and heat curing of a polymeric resin. The protective film is then locally exposed using an excimer laser (UV). A video imaging setup and motorized stages piloted by specific software allows for the precise positioning of the laser irradiation sites. Laser irradiation locally modifies the oxide film insulating properties. Alternatively clean ablation of the resin is obtained. In both cases the results of this lithographic process is a protected part with well-defined unprotected areas. The pieces are then dipped into an electropolishing electrolyte and connected to a power supply. While applying optimized voltage and hydrodynamic conditions, the unprotected areas are attacked. Under electropolishing conditions (mass transport), dissolution results in perfectly hemispherical, smooth cavities. Precise dimensioning of the features is obtained by controlling the electric charge flown through the system. After dissolution, the titanium oxide film remains on the surface of the dental implant while the polymeric film is removed with a stripper from stainless steel stents.

RESULTS & DISCUSSION: In both cases precise positioning and smooth surface finish is obtained (see Fig. 1 and 2). The laser operation time ranged from 30 s for the dental implant to 5 min for the stent, this time can be reduced by further optimization of the process parameters. Electrochemical dissolution times ranged from 5 to 20 min depending on the cavities diameter.

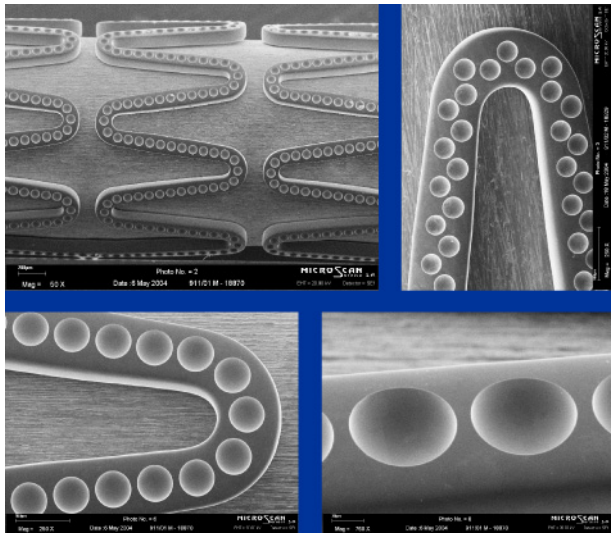

Fig. 2 SEM images of microstructured stainless steel stents showing precise positioning of cavities, geometry versatility (2920/5644 cavities 70/35 µm in diameter) and smooth surface finish.

CONCLUSIONS: The use of modern electrochemical methods allows precise micro-patterning of biomedical devices. The technique presented herein offers new possibilities in the optimization of implant surface topographies. Alternatively surface micropatterning represents an elegant and robust vehicle in drug eluting implants applications.

REFERENCES: [1] C. Larsson et al., *Biomaterials*, **15**, 1062 (1994). [2] A. Curtis and C. Wilkinson, *Trends Biotechnol*., **19**(3), 97 (2001). [3] P.-F. Chauvy et al., *Applied Surface Science*, **208**, 165 (2003).

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Surface modification of poly(ethylene-co-vinyl alcohol) membranes by molecular imprinting technique for biomedical application

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INTRODUCTION: Molecular Imprinting Technology allows to prepare polymeric materials with selectivity towards specific molecules through polymerization or phase inversion in presence of template [1]. This work is focused on searching innovative biomedical uses of these materials as extracorporeal purification systems or recognition-able implantable devices. An other interesting application of this research is the preparation of materials exhibiting molecular recognition behaviour towards biological compounds, able to promote cell adhesion, for application in tissue engineering [2-3]. Recent efforts of research in biomedical field are focused towards the imprinting of compounds such as peptides, aminoacids, phospholipids and proteins. This work regards the imprinting of phosphatidylcholine (PC) in a membrane of ethylen-vinyl alcohol (EVAl).

METHODS: The membranes were prepared by the phase inversion technique. A solution of EVAl in dimethyl sulfoxide (DMSO) was added with a solution of PC in tetrahydrofuran (THF) (PC/total membrane weight =0,11). The resulting solution was spread uniformly on a glass plate using a knife machine and then immersed in a first inversion bath (50/50 DMSO/water) at room temperature. After 1 hr the formed membrane was transferred in a second bath (water) for 15 hr. Finally, the membranes were dried in a ventilated oven at 30°C. The morphological, chemical and physical characterization was carried out by Scanning Electron Microscopy (SEM), Thermo Gravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC). Total reflection Fourier transform infrared (FT-IR) spectra were carried out by means of a Perkin Elmer Spectrum One FT-IR Spectrometer, equipped with a Perkin Elmer Universal ATR Sampling Accessory and a Perkin Elmer Spectrum Spotlight FT-IR Imaging System.

The template (PC) was removed from the membrane using a permeability apparatus in which the solvent (isopropyl alcohol) passes through the membrane, placed into the permeability cell, under a pressure gradient. Biomimetic behaviour of the membranes for the recognition of PC and cellular adhesion tests were performed.

RESULTS: SEM micrographs of the EVAL-PC membrane showed a uniform porosity with size of 1÷2 μm. DSC data evidenced an increase on EVAl cristallinity due to the presence of PC in the membrane. The analysis by spectrophotometer UV-visible of isopropyl alcohol permeated through the membrane for 5 hr showed that the 80% of PC was removed. These data were confirmed from TGA which shown the same events of weight loss in the membrane where PC has been removed compared to an EVAl not imprinted membrane.

The spectrum spotlight FT-IR images, as well as the correlation map of the spectrum, showed the presence of PC uniformly dispersed on the surface before the template removal (dark area in fig.1) and the reduction of PC amount, with a consequent lower correlation, after release in isopropyl alcohol.

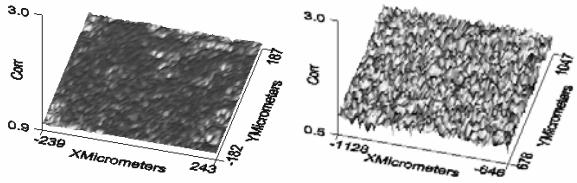

Fig. 1: FT-IR map of an imprinted membrane before (left) and after (right) PC release.

Preliminary tests about the cellular adhesion seem to be promising: cellular adhesion was good for the membrane which had released the template, while any cell was present on not imprinted EVAl membrane and on imprinted sample before PC extraction.

DISCUSSION & CONCLUSIONS: Molecular imprinting of the EVAl membrane with PC has shown an interesting modification of matrix surface. This result suggests the applications of this membrane in tissue engineering and also for the selective recognition of biological compounds.

REFERENCES: [1]L. Ye, P.A.G. Cormack and K. Mosbach (2001) *Anal. Chim. Acta* **435**(1): 187-196. [2]M. Ulbrict (2004) *Journal of Chromatography B* **804**: 113-125. [3]M. Ramamoorthy, M. Ulbricht (2003) *Journal of Membrane Science* **217**: 207-214.

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ELECTRONIC DESORPTION OF TRIBLOCK COPOLYMER POLY (PROPYLENE SULFIDE-*BL*-ETHYLENE GLYCOL) (PPS-PEG) FROM INDIUM TIN OXIDE (ITO)

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INTRODUCTION: Protein-resistant triblock copolymer, poly(propylene sulfide-*bl*-ethylene glycol) (PPS-PEG) has been previously reported to chemisorb onto gold surfaces [1] with high oxidation stability [2]. In this work, we show the adsorption of PPS-PEG onto a transparent and electrically conductive substrate, indium tin oxide (ITO). In addition, we demonstrate the possibility of controlled desorption of PPS-PEG by applying an electrical stimulus.

METHODS: We have used three complementary surface characterization techniques: variable angle scanning ellipsometry (VASE), x-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectroscopy (ToF-SIMS) to analyze the adsorption and electro-desorption of triblock copolymer, PPS-PEG from an ITO surface.

RESULTS: All three methods confirmed the formation of PPS-PEG adlayer on the ITO surfaces.
Based on our experimental XPS and ToF-SIMS results and former publications [3], we postulate that the chemisorption of the PPS-PEG on ITO involves direct sulfide-indium interactions.
When an ascending anodic electrical stimulus was applied to the surface of the modified samples, a gradual and steady polymer removal was observed. At 2000 mV (reference to Ag electrode), a complete removal of the polymer from the polarized ITO surface was observed. Despite subjecting the surface modified samples to an external electrical field, no oxidation effect were observed hence indicating the excellent oxidation stability of PPS-PEG on ITO surfaces. This work is focused towards creating dynamic surface modifications by means of an electrical stimulus.

DISCUSSION & CONCLUSIONS: All the three surface characterizing techniques clearly showed consistent steady and gradual loss of PPS-PEG on the polarized ITO surface with ascending electrical potential. No oxidized species from the polymer were detected despite an electrical polarization up to 2000 mV. The specific formation of PPS-PEG on ITO using an external electrical potential offers high oxidation stability as well as design flexibility for bioanalytical and biodiagnostic devices.

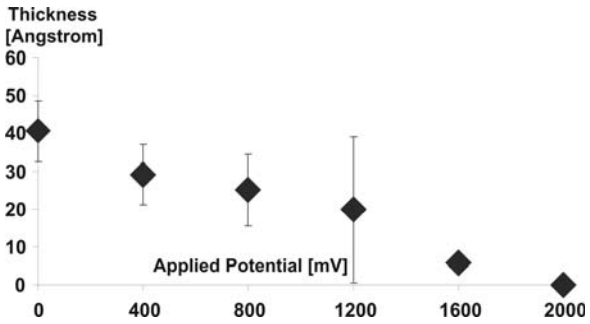

Fig. 1: A thickness-voltage plot obtained from the ellipsometry measurements depicts the amount of PPS-PEG on an electrically polarized ITO surface. At 0 mV, the ITO surface which was exposed to PPS-PEG has a thickness of 40 Å. As the electrical field is further increased, there is a steady reduction in the adlayer thickness until it reached 2000 mV whereby the thickness of the PPS-PEG on the ITO reaches zero. This suggests that the polymer has completely desorbed from the ITO surface.

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ACKNOWLEDGEMENTS: Kind financial support from the Swiss Federal Laboratories for Materials Testing and Research (EMPA) is much appreciated. Special thanks to Marc Bachofner for conducting the feasibility experiments.

A PEG-Based Co-Polymer for Protein-Resistant Surfaces Studied by Ellipsometry and Quartz Crystal Microbalance

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INTRODUCTION: Poly(ethylene glycol) (PEG) has been known as a biomaterial for several decades and is used in many biomedical applications [1]. When grafted onto a surface at a sufficiently high density, PEG chains have the ability to prevent the adsorption of proteins, i.e. to render the surface protein resistant [2]. A possible way to reach high PEG densities is to graft the chains covalently onto a polymeric backbone, as for example poly(L-lysine) (PLL). This graft co-polymer, denoted PLL-*g*-PEG, adsorbs electrostatically on negatively charged surfaces (such as many metal oxide surfaces) from aqueous solution, due to the presence of protonated amine groups. A main advantage of this polymeric system is the possibility to tailor the architecture by varying the molecular weights of both components (PLL and PEG), as well as the grafting ratio (defined as the number of lysine units in the backbone per PEG chain). Pasche et al. [3] showed that the protein-resistance capability of the coating strongly depends on the polymer architecture, and that a high ethylene glycol density is required to withstand protein adsorption. Variable angle spectroscopic ellipsometry (VASE) is a highly sensitive technique that allows the determination of layer thicknesses of adsorbed polymers in the dry state. The quartz crystal microbalance with dissipation monitoring (QCM-D) on the other hand, gives information about *in situ* layer thicknesses as well as about the water content of the layers (when calibrated with an optical technique).

METHODS: PLL-*g*-PEG with different PEG molecular weights (1, 2 and 5 kDa) and grafting ratios (2, 3.5, 6.5, 15 and 30) were adsorbed on cleaned Nb_2O_5. HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 10 mM, pH = 7.4) was used as a solvent. Ellipsometry measurements were carried out in the dry state, while QCM-D allows real-time monitoring of the adsorption process.

RESULTS: The dry layer thicknesses correlate linerarly with adsorbed dry mass (data not shown). Figure 1 shows the direct comparison of dry and wet layer thicknesses. While dry layers have thicknesses between 1-3 nm, the wet thicknesses are much larger (2-12 nm). These differences are attributed to coupled water in the layer that also gets measured by QCM-D.

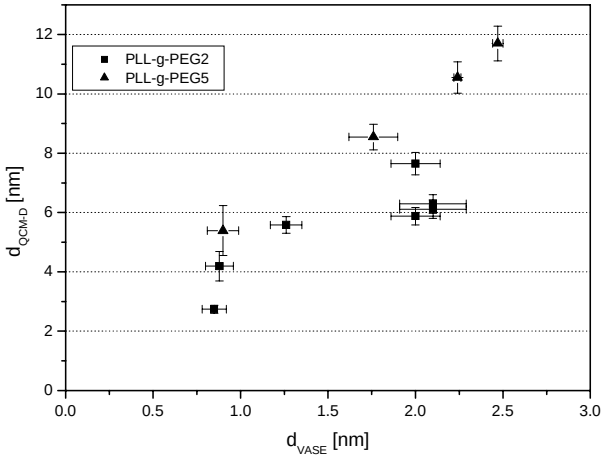

Fig. 1: Layer thickness comparison between dry (ellipsometry) and wet layers (QCM-D). Note the large differences due to hydration of the layers.

For surfaces with densely grafted PEG chains, the number of water molecules per EG monomer unit are lower (~8) than for surfaces with sparse grafting (~25).

DISCUSSION & CONCLUSIONS: The combination of optical and acoustic techniques allows the quantitative determination of the water content in adsorbed polymeric layers. The number of water molecules per EG unit determined for densely grafted PEG chains corresponds to structured water around PEG chains. These layers also gave the best results regarding protein resistance [3], showing the importance of structured water acting as a steric-entropic-osmotic barrier to approaching proteins.

REFERENCES: [1] Harris, J.M., Poly(ethylene glycol) Chemistry, Biotechnical and Biomedical Applications, (1992), New York and London: Plenum Press; [2] Jeon, S.I., et al., J Coll Interf Sci, (1991), 142, 149-158; [3] Pasche, S., et al., Langmuir, (2003), 19, 9216-9225.

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Fundamental DNA-surface analysis to understand microarray assay limitations

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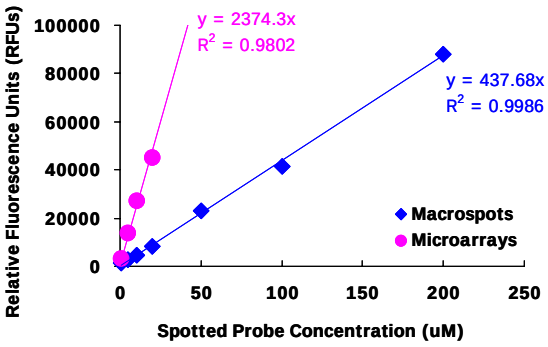
INTRODUCTION: We have characterized amine-derivatized, single-stranded DNA (5'-NH_2-$(CH_2)_6$ CTG AAC GGT AGC ATC TTG AC-3', abbreviated NH_2-Oligo1) attached to amine-reactive commercial microarray slides using X-ray photoelectron spectroscopy, fluorescence imaging and ^{32}P-radioassays. Immobilization efficiencies of NH_2-Oligo1 under microarray format can be reproduced at macroscopic levels for surface analysis using high salt and increased DNA concentrations with good reliability and reproducibility. Hybridization efficiency with complementary DNA (Oligo2) was studied on these surfaces. The macroscopic model provides a platform for study of DNA surface chemistry using highly sensitive, quantitative surface analytical techniques.

METHODS: Two brands of commercial amine-reactive microarray polymer slides were purchased from vendors. (CodeLink™, Amersham, NJ, and OptArray™, Denver, CO). DNA oligonucleotides were purchased from TriLink (San Diego, CA). DNA microarrays were printed using a SpotBot™ microarray printer (TeleChem, Sunnyvale, CA) and hybridized following commercial protocols. For macroscopic immobilization, NH_2-Oligo1 at concentrations ranging from 0 to 200μM in 1M sodium phosphate buffer were applied to commercial slides at 100% humidity. Post-coupling treatment and hybridization were conducted identically to microarray formats. In ^{32}P-radioassay experiments, oligonucleotide probes were labeled with α-^{32}P-ddATP (Amersham Biosciences, Piscataway, NJ) in the presence of terminal transferase[1] (Roche, Indianapolis, IN). Surface reaction procedures were the same as non-radioactive experiments. XPS spectra were obtained using a PHI5800 instrument (Al anode operated at pass energy 58.70eV, 35° take off angle). Fluorescence imaging was performed using a ScanArray Express™ fluorescence scanner at 90% laser power and 45% PMT, with 10μm or 50μm resolution. Phosphor imaging was performed with a Storage phosphor imager™ at 200μm resolution.

RESULTS: We studied the NH_2-Oligo1 immobilization on commercial amine-reactive microarray slides both in microarray and macroscopic format. Ionic strength of immobilization buffer was observed to play a critical role in DNA probe immobilization efficiency. Rapid solvent evaporation in microarray printing procedures result in significantly increased local ionic strength as well as higher DNA concentration. By increasing bulk immobilization buffer salt concentration and DNA concentration, we were able to obtain the same level of immobilization efficiency macroscopically, i.e. spots with diameters of millimeter dimensions. (Figure) Immobilization efficiencies of macro-spots were compared with microarrays using fluorescence imaging. Surface properties of such macro-spots with DNA modified commercial slides were analyzed using XPS and ^{32}P-radioassay. Densities of DNA Oligo1-immobilized surface were found to be at the 10^{12}-10^{13} molecules/cm^2 level and exhibited high hybridization efficiency (70%~100%).[2]



CONCLUSIONS: We have modeled microarray amine-DNA immobilization chemistry and efficiencies at a macroscopic level using high salt buffer and high DNA concentrations. Macro-spots obtained this way provide a good analytical platform for surface analysis of DNA immobilization and hybridization using quantitative surface techniques including XPS and radioisotope labeling.

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Surface immobilization of antibodies and nucleic acid arrays on waveguide optical sensors

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INTRODUCTION: All bioanalytical assays using surface-capture of target analytes suffer from non-ideal sensitivity and selectivity. We have recently focused on microarray formats on optical waveguide surfaces to improve assay performance. Thiol-terminated DNA probe oligonucleotides exhibited substantially higher surface printing immobilization and target hybridization efficiencies than non-thiolated DNA probe oligonucleotides: strong fluorescence signals from target DNA hybridization supported successful DNA oligonucleotide probe microarray fabrication and specific capture bioactivity. Analogously printed arrays of thiolated streptavidin and non-thiolated streptavidin did not exhibit noticeable differences in either surface immobilization or analyte capture assay signals.

METHODS: Sputtered silicon nitride optical waveguide surfaces were silanized and modified with a hetero-bifunctional crosslinker[1] to facilitate thiol-reactive immobilization of contact-printed DNA probe oligonucleotides, streptavidin and murine anti-human interleukin-1β capture agents in microarray formats. X-ray photoelectron spectroscopy (XPS) was used to characterize each reaction sequence on the native silicon oxynitride surface.

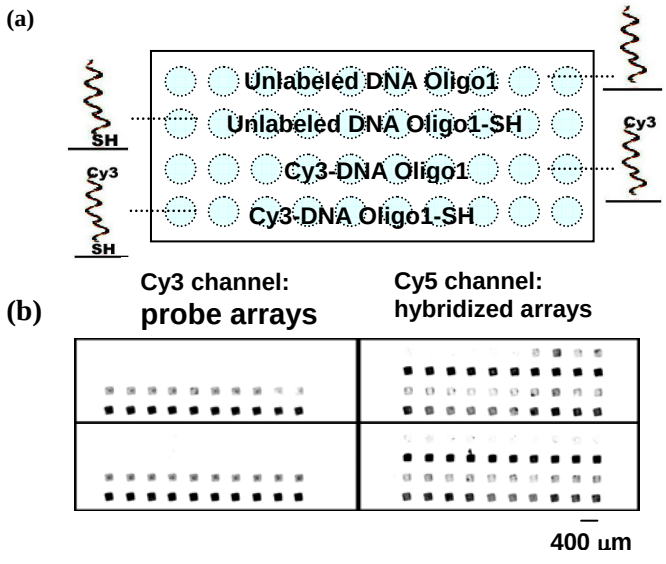

Fig.1. Microarray DNA print and target hybridization fluorescent signals on maleimide-activated surfaces: (a) microarray experimental lay-out; (b) actual fluorescence scanned images (upper/lower = 2 identical arrays) for printed DNA probes formatted as in (a), and hybridized with Cy5-DNAoligo2 targets Relative allocation and amount of resources in research.

RESULTS:

DISCUSSION & CONCLUSIONS: Probe DNA oligonucleotides bearing terminal thiol end groups exhibited significantly improved printing efficiency over non-thiolated analogous oligonucleotides using thiol-maleimide surface coupling. This led to improved hybridization performance in surface-capture assays with complementary DNA target solutions. However, significant target hybridization from non-specific binding of printed non-thiolated oligonucleotide probes was also observed. Printed streptavidin and anti-human IL-1β capture proteins showed little difference in surface retention between covalent and non-covalent attachment modes, demonstrating the fundamental differences between DNA oligomer and protein printing influences on array bioactivity, and the importance of producing surface chemistries that might exploit these differences to improve protein-based microarray assays.

REFERENCES: [1] A. Rezania,, R. Johnson, A.R. Lefkow, K.E. Healy, (1999) *Langmuir* **15**: 6931-6939.

ACKNOWLEDGEMENTS: The authors acknowledge support from NIH EB000726 and the National Science Foundation's Research Experience for Undergraduates (REU) program. Technical guidance from S. S. Saavedra (Univ. of Arizona) and G. Harbers (CSU) are both gratefully acknowledged. Further assistance for fluorescence scanning from Dr. W. H. Hanneman (CSU), silicon nitride sputter coating by M. Stephens (CSU), L. Rea and H. Cross (Thermo Electron Corp., Boulder, CO), and reproducible spotting protocols from Dr. C. Greef (Accelr8, Denver, CO) are all gratefully acknowledged.

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WAVEGUIDE EXCITATION FLUORESCENCE MICROSCOPY: A NEW TOOL FOR SENSING THE BIOINTERFACE

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INTRODUCTION: The ability to investigate interactions between a biological system and a synthetic surface is of critical importance to our fundamental understanding of biomaterials and their many applications in biosensors, medical implants, and tissue engineering. For this reason, (bio)sensing platforms capable of sensing (bio)molecular interactions have become an invaluable tool for discerning which events occur, when they occur, as well as the kinetics and affinity of the interactions at a given biointerface. We have recently developed a general sensing platform that combines the power of ultra-sensitive biosensing with the simultaneous ability for fluorescence microscopy imaging of bio-interfaces in their natural aqueous environment[1].

METHODS: The technique utilizes the evanescent field from a planar optical waveguide to excite fluorescence in the near interface region and is thus refered to as the Waveguide Excitation Fluorescence Microscope (WExFM). The final prototype of the WExFM is intended to be an add-on for a standard inverted fluorescence microscope (FM), thus ensuring ease of adaptability.

The technique is centered around a thin film planar optical waveguide coating on a glass substrate. Incoupling of laser light into the waveguide occurs via an optical grating and will only occur at a specific angle of incidence that is dependent on the wavelength, the grating properties, and the refractive index of the surface overlayers. Thus, as biomolecules adhere to the surface a change in the incoupling angle will occur and this can be converted into a change in mass and thickness.

Furthermore, once the light is traveling along the length of the waveguide, the evanescent field penetrating out from the waveguide into the overlying environment can be used to excite fluorescence in fluorescent molecules. Thus, by placing the waveguide onto a microscope it is possible to image while sensing mass adsorption all in real time and in-situ (i.e. in a liquid environment for example).

RESULTS & DISCUSSION: Studies of the streptavidin-biotin binding event have already demonstrated a sub-picomolar sensitivity for the WExFM technique. In the Fig. 1. a comparison of normal fluorescene and WExFM imaging is shown for a 10ng/mL solution of Alexa 633 labeled streptavidin in contact with a 60x60µm biotinylated surface pattern. The WExFM image (Fig. 1b) illustrates the surface sensitivity of the technique whereby the background is measured to be essentially zero, as expected.

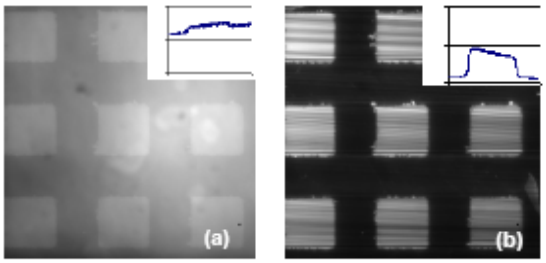

Fig. 1: a) normal fluorescence and b) WExFM image of fluorescently labelled streptavidin binding to biotin immobilised in a 60x60 µm pattern and backfilled with a protein resistant PEG graft co-polymer.

In addition to biomolecular interactions, preliminary cell-surface studies have shown the improved signal to noise of the WExFM technique for the study of focal adhesions when compared to epi- and confocal fluorescence microscopy. In addition, some interesting insights into the processes occurring between lipidic vesicles and synthetic surfaces and subsequent bilayer formation have been obtained using this technique.

CONCLUSIONS: The WExFM provides a new and unique method for the dynamic, in-situ study of the biointerface. Advantages of the technique include high target sensitivity for fluorescence detection (<20 pM already demonstrated), high surface specificity (ca. 200 nm perpendicular to the waveguide), large area analysis with submicron resolution, 'built-in' calibration of fluorescent light gain, and the capability to perform multicolour imaging in-situ and in real time.

REFERENCES: [1] HM Grandin (In Press, 2005) *Biosensors & Bioelectronics*, and references therein.

ACKNOWLEDGEMENTS: Thanks to S. Pasche, L. Bonderer and, B. Staedler for help and the European Commission (FP6-NMP-2002), ETH Zurich, and the National Science and Engineering Research Council of Canada for funding.

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Nano-Colloidal Arrays on Chemically Patterned Surfaces for Biosensing Applications

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INTRODUCTION: Nano–structured functional materials are currently being developed in the still–emerging field of nanotechnology due to promising new or improved optical or electronical properties and its application potential in nanobiotechnological applications. In this work, we report the fabrication of colloidal micro arrays by selectively binding functionalized nano-sized colloidal silica particles on a chemical micro pattern. This approach offers the potential to enhance the sensitivity of current 2D biosensing arrays by decreasing non–specific interactions with bio-molecules while increasing the surface area at distinct spots due to the nano-colloid arrays.

METHODS: SiO_2 suspensions with particles sizes of 41 ± 5 nm and 73 ± 6 nm have been used after characterization. Suspensions were buffered at pH = 7.4 with 150 mM NaCl. Particle suspensions were mixed with the polymer solution to coat the particle surface. A centrifugation step was added to remove unadsorbed polymer. The polymer used was poly(L-lysine)-graft-poly(ethylene glycol) (PLL–*g*–PEG), which consists of a positively charged PLL backbone and PEG chains which are grafted to the backbone and is well known for its protein repellant properties.[1] The substrate patterning technique was developed in our lab and is based on a photoresist lift-off process (Molecular Assembly by Lift-Off (MAPL)[2] (Fig. 1).

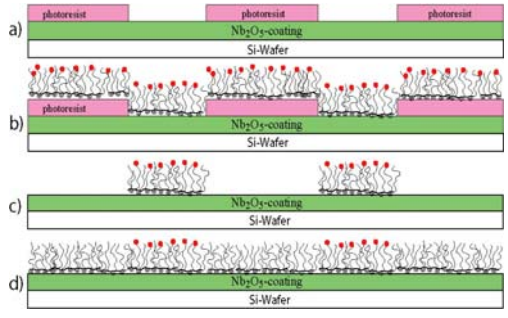

Fig. 1: Overview of the molecular assembly patter-ning by lift–off technique: a) photoresist pattern b) adsorption of biotinylated PLL–g–PEG polymer c) lift–off process to remove photoresist 5. backfilling of the bare niobia withL PLL–g–PEG. For more information on this processes see Ref. [2]

RESULTS & DISCUSSIONS: With the molecular assembly patterning by lift–off technique (Fig. 1), it is possible to create biotinylated surface patches in a non-interactive background. Biotin functionalized particles can then be linked onto the active spots via a streptavidin molecule. The background of these patterns has to be rendered non–interactive in order to avoid unspecific adsorption of colloidal particles to the background (Fig. 2).

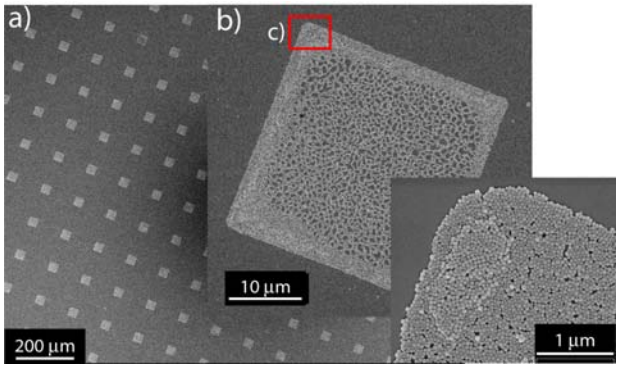

Fig. 1: SEM micrographs showing colloidal arrays obtained by adsorption of PLL–g–PEG–biotin coated 73 nm silica colloids on a MAPL chip. The biotinylated silica colloids were specifically bound to the biotinylated regions of the substrate (see Fig.1). The pattern shows good fidelity (a and b) over a large area. Edge regions of the pattern (c) are sharply confined, but more then a monolayer coverage is often observed as well as depletion effects in center region of the sample (b).

CONCLUSIONS: Self–assembly of functiona-lized colloidal nanoparticles onto chemically patterned substrates was achieved by tailoring the interactions between the functionalized colloid and the surface pattern. The streptavidin-biotin binding system was used to couple the colloidal particles to the surface pattern. Such arrays potentially increase the surface area at given spots without loosing the necessary resistance to non–specific adsorption of biomolecules. Such a system could be used to increase the sensitivity of existing biosensing techniques in the future.

REFERENCES: [1]S. Pasche et al. (2003) *Langmuir* **19**(22):9216–9225 [2] D. Falconnet et al. (2004) *Advanced Functional Materials* **14**(8):749–756.

ACKNOWLEDGEMENTS: Swiss funding program on nanotechnology, Top Nano 21 (Project 5971.2), EPF Lausanne and ETH Zurich.

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Static Secondary Ion Mass Spectrometry Investigation of the Resistance of Polyacrylamide Graft Coatings to Adsorption of Lysozyme

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INTRODUCTION: Characterization of the adsorption of proteins onto biomedical devices is a key issue in bio-interface science. Preventing the non-specific adsorption of proteins is one approach towards controlling interfacial interactions. Much work has focused on PEO for protein-resistant coatings. In this study we investigated polyacrylamide (PAAm) graft coatings and their interaction with lysozyme. As XPS was unable to detect any evidence of protein adsorption onto PAAm graft coatings, we utilized the higher sensitivity of time-of-flight secondary ion mass spectrometry (ToF-SIMS) to probe for any low amounts of adsorbed proteins.

METHODS: Polyacrylamide graft coatings were produced by surface radical polymerization following attachment of isocyanatoethyl methacrylate onto an amine functionalised surface, produced by plasma polymerization of n-heptylamine (HA) [1]. The PAAm grafted surface was contacted with lysozyme solution (100 μg/L in Phosphate Buffer Saline) at 37 ^{o}C for 2 hrs, followed by thorough washing with PBS and de-ionised water. The PAAm graft coating before and after lysozyme contact, as well as lysozyme adsorbed onto Si wafer as reference, were characterised by 10 positive and 1 negative static SIMS spectra. The large quantities of data were processed and interpreted with the aid of Principal Component Analysis [2].

RESULTS AND DISCUSSION: The interaction between PAAm and lysozyme was evaluated by detailed analysis of 3 groups of positive fragments: $[C_mH_n]^+$, $[C_mH_nN]^+$ and $[C_mH_nNO]^+$. The results revealed that a combination of the nitrogen- and nitrogen-oxygen-containing positive ions is best suited for the comparisons. The score plots derived from these fragments are shown in Figure 1. PC1, capturing more than 90 % of the original data variance, clearly separates lysozyme from PAAm and PAAm/Lys. In contrast, PAAm surfaces before and after lysozyme adsorption are not discriminated by PC1. PC2, capturing around 7% of the original data variance, shows some difference between these samples, but also partial overlap.

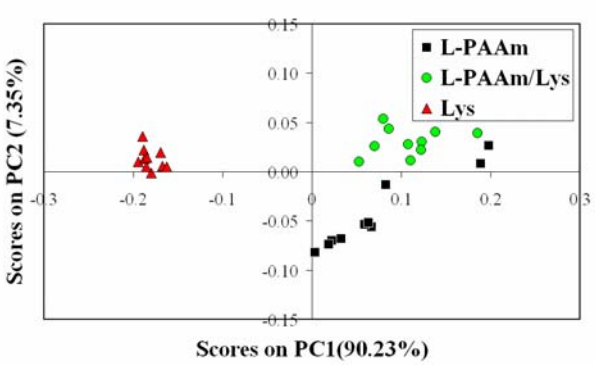

Figure 1:Score plots for L-PAAm, its lysozyme modification and lysozyme derived from combined nitrogen and nitrogen-oxygen based fragments.

The correlation between PC1 and some of the original variables (peaks) is illustrated in Figure 2.

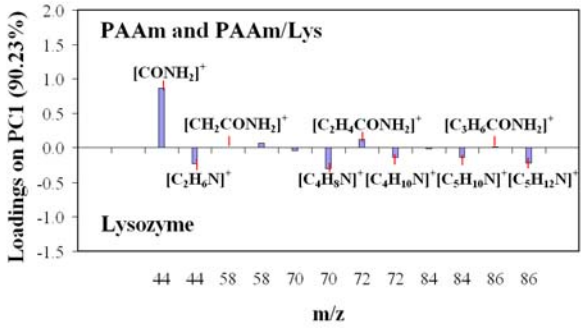

Figure 2: Loadings of individual variables on PC1

$[CONH_2]^+$, $[CH_2CONH_2]^+$, $[C_2H_4CONH_2]^+$ and $[C_3H_6CONH_2]^+$ ions contribute positively to PC1. They are assignable to polyacrylamide fragments. In contrast, most of the $[C_mH_nN]^+$ ions contribute negatively to PC1. They correspond to immonium ions derived from amino acids. The negative ion spectrum shows only a trace amount of sulfur on PAAm/Lys.

CONCLUSIONS: ToF-SIMS can detect some adsorption of lysozyme onto PAAm graft coatings, but the amount is low. The PAAm fragmentation pattern correlates with the linear polyacrylamide structure.

REFERENCES: [1] H.J. Griesser (1989) *Vacuum* **39**: 485. [2] M.S. Wagner, D.J. Graham, B.D. Ratner, D.G. Castner (2004) *Surf Sc* **570**:78-97.

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Amine Functionalisation of Polypyrrole for Bioaplications

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INTRODUCTION: Polypyrrole (PPy) has high conductivity, good environmental stability and excellent biocompatibility in either cell culture or in-vivo applications [1]. To improve the biointeractions of this polymer is necessary to introduce reactive groups in the normally inert surface [2]. In this work PPy was functionalised in controlled areas with -NH_2 groups by the grafting of Allylamine using UV radical activation. To evaluate the activity of the functionalised films, it was test their capacity to improve protein adsorption using fluorescent bovine serum albumin (BSA).

METHODS: PPy deposition on porous silicon was done by galvanostatic oxidation (26A /m^2 for 300s) from an aqueous electrolyte solution of Pyrrole (0.05M) and Lithium Perchlorate (0.05M).

The PPy films covered by a patterned metallic mask (150µm circular mesh) were immersed in a 5% aqueous solution of Allylamine and expose to UV light (intensity = 15 $\times 10^{-4} W/m^2$; λ : 280-450nm) during 20minutes. Further characterization of the functionalised surfaces was done by TOF-SIMS and XPS.

To test the bioactivity, the functionalised films were immerse in a 45µg/ml BSA solution in PBS (20mM pH=7.5) during 20 minutes and after the samples were analysed with a fluorescent microscope.

RESULTS: In the ToF-SIMS image (Figure 1) is presented the $C_3H_4NH_2^+$ ion image of the surface after Allylamine grafting. It is possible to see that this Allylamine characteristic ion is concentrated in the areas were the surface was exposed to UV. XPS studies showed that the grafted areas had 2% of carbon with -NH_2 function. Figure 2 presents a fluorescent microscope image of the BSA adsorption in the PPy surface after the introduction of amine groups. The image shows that the BSA adsorption was concentrated in the grafted areas.

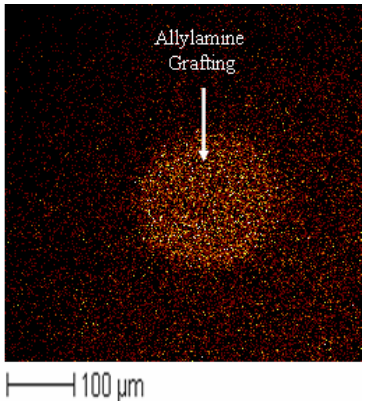

Fig. 1: Tof-SiMS image of $C_3H_4NH_2^+$ ion distribution of controlled Allylamine grafting.

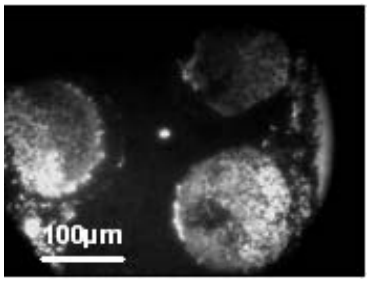

Fig. 2: BSA adsorption in PPy surface after controlled Allylamine grafting.

DISCUSSION & CONCLUSIONS: The studies show that Allylamine grafting using UV light as a radical initiator is a quick and technically simple way of introducing a controlled amount of -NH_2 functional groups onto the surface of PPy. An additional benefit of this method is the ability of directly pattern the functionality. Detailed surface analysis studies have determined the introduced active -NH_2 levels to be sufficient to enhance preferential protein adsorption. The results show that this method is potentially attractive for the fabrication of PPy based electrodes to use in electrochemical biosensors.

REFERENCES: [1] X. Cui, J. Wiler, M. Dzaman, R.A. Altschuler and D.C. Martin, (2003) *Biomat.*, **24** 777-787. [2] L. Cen, K.G.Neoh and E.T. Kang, (2003) *Biosens. Bioelectron.*, **18** 363-374.

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COVALENT ATTACHMENT OF FLUORESCENT LABELS TO PLASMA-MODIFIED SURFACES

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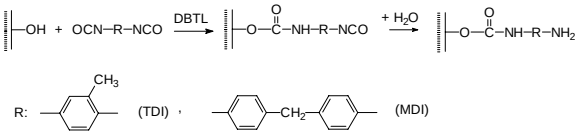
INTRODUCTION: The chemical composition of surfaces plays an important role in many areas like biocompatibility of materials, biosensors, permselectivity of membranes, heterogeneous catalysis, and s.o.. For the adjustment of surface properties, the plasma modification, particularly the plasma functionalization and plasma polymerization with monomers, carrying functional groups, are useful techniques to introduce accurately defined functionalities. Such a modification of the surface provides the opportunity to carry out chemical reactions at surface-bounded functions to covalently attach groups with special features to produce tailored surfaces for different applications.

RESULTS & DISCUSSION: Two ways of surface modifications of PP were used to generate functionalized surfaces. The first one was an oxygen plasma treatment of polypropylene followed by a wet-chemical reduction[1] to transform the different O-containing groups into hydroxyl groups. The number of OH groups was adjusted to 10-14OH/100 C atoms.
The second way was the deposition of plasma polymerized allylamine layers on PP. The number of the so generated primary amino groups amounted up to 18 NH_2 groups/100 C atoms.
The hydroxyl groups of modified PP (PP-OH) were reacted with diisocyanates (TDI, MDI) to get NCO-terminated PP, followed by the reaction with water as given in scheme 1:



Scheme 1

The NCO-modified PP was reacted with NH_2-functionalized fluorophors like dansyl hydrazine, dansyl cadaverine, rhodamine 110 and amino fluoresceine, the NH_2-functionalized PP was reacted with dansyl chloride and fluorescein-isothiocyanate. Fluorescence spectra of PP modified by TDI-linked dansyl hydrazine (DNS-H) are shown in Fig.1:

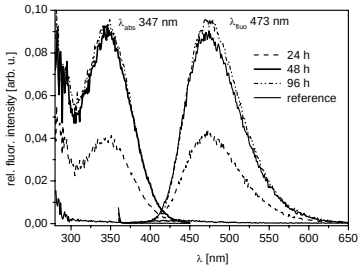


Fig. 1: Fluorescence excitation and emission spectra (uncorrected, excitation wavelength 350 nm;) of DNS-H, bounded to PP-OH, treated various times with TDI

The reaction of plasma polymerized allylamine layers on PP with FITC also resulted in fluorophor-modified surfaces. The fluorescence intensity as well as the S content of the surfaces (S was taken as XPS-tag) showed a linear dependence on the plasma power input.

CONCLUSIONS:
The plasma chemical modification of polymers is suitable for the covalent attachment of special surface functionalities e.g. spacer, sensor or bio molecules as exemplarily demonstrated for surface-bound fluorescent labels. In spite of problems connected with the measurement of the fluorophor tagged foils, described for example by Henneuse-Boxus[3], a correlation between fluorescence intensity and reaction time of the diisocyanate with PP-OH was found.

REFERENCES: [1]J. F. Friedrich, G. Kühn, R. Mix, W. E. S. Unger, (2004) *Polymer Processing and Plasmas* **1**, 28-50. [2] R. Mix, K. Hoffmann, U. Resch-Genger, R. Decker, J. F. Friedrich, *in press.* [3]C. Henneuse-Boxus , A. De Ro, P. Bertrand, J. Marchand-Brynaert, (2000) *Polymer* **41**, 2339-2348

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Tailoring the surface of polyurethanes by plasma immersion ion implantation of nitrogen for controlling calcification

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INTRODUCTION: The flexible structure of polymers has enabled them to be useful in a wide variety of applications due to the possibility to tailor their properties to suit desired applications. Recently, there has been an increasing interest in utilizing polymers as matrices for calcium phosphate-based composites. On the other side, polyurethanes used for urea catheters, heart valves, artificial vessels present a case where calcification and biodegradation should be avoided. The modification of polymer surfaces by plasma immersion ion implantation for reducing the calcium phosphate formation is well known and has a long time effect.

METHODS: In this work, polyurethane films (PU2103 and PU2363 commercial Pellethane materials for medical applications, and newly synthesized PPG-TDI polyurethane with application as urea catheters) are modified by plasma immersion ion implantation with various doses of nitrogen ions ($5x10^{14}$ – $2x10^{16}$ $cm^{-}2$, 20 keV). Polyethylenes modified by the same ion doses are prepared as controls.

The ability of the modified polymer films to induce calcium phosphate formation from a solution resembling the human blood plasma (simulated body fluid) and the effect of the ion doses on the calcification are examined by FTIR, Raman spectroscopy, light microscopy, SEM and EDX. The analysis of the modified polyurethane films is carried out by light microscopy, XPS, FTIR and contact angle measurements.

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Supported Cell Membrane Sheets for Functional Imaging of GPCRs

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INTRODUCTION:

G protein-coupled receptors (GPCRs) are the largest family of membrane receptors and a major target for therapeutic compounds. They transduce external signals (light, hormones, odorants, drugs) inthe activation of intracellular heterotrimeric G-proteins. After activation, G proteins dissociate and induce the production of second messengers by interacting with enzymes or ion channels.

Many unresolved questions remain about the way GPCRs function. For instance, these membrane proteins are able to rapidly and sensitively transduce extracellular stimuli although the physiological concentrations of the proteins involved are usually low. Elucidating these complex processes will require the development of in-vitro systems allowing the selective investigation of particular aspects of the signaling cascade.

We present here the use of cell membrane sheets suitable for *in-vitro* functional fluorescence studies, prepared by direct detachment from cell membranes using poly-L-lysine coated glass slides (Fig 1).

METHODS:

Cell membrane sheets were prepared from HEK-293 cells expressing various proteins of interest. A glass coverslip coated with PLL was pressed to the apical parts of the cells. After several minutes of contact, the coverslip was removed, ripping off from the cells large regions of the apical native plasma membrane.

RESULTS: Cell membrane sheets suitable for *in-vitro* functional fluorescence studies were prepared by direct detachment from cell membranes using poly-L-lysine coated glass slides. The resulting transferred planar membranes conserved the composition as well as most properties of the original plasma membrane, in particular both leaflets remained fluid allowing the investigation of diffusion properties of different cellular membrane components. Measurements on membrane sheets offer several advantages as compared to living cells. First, access to the intracellular leaflet is obtained, in particular to the intracellular part of membrane proteins and to cytoplasmic, membrane-associated proteins, opening the possibility to label them and to modulate their properties with non membrane permeable compounds. Second, the cytosolic autofluorescence of the cells is absent allowing ultra-sensitive measurements to be performed down to the single-molecule level. Third, the complexity of cellular processes occurring at the plasma membrane can be reduced allowing the sequential investigation of selected events from complex biochemical networks. These advantages were used to perform ligand-binding studies on a representative GPCR.

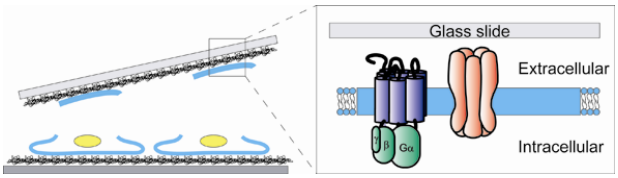

Fig. 1: Formation of cell membrane sheets containing membrane proteins.

DISCUSSION & CONCLUSIONS:

Our results show that supported membrane sheets might find a broad application as ideal *in-vitro* system for the elucidation of complex signaling pathways. We applied for instance this methodology in recent experiments onthe localization and diffusion of the G proteins investigated using single-molecule microscopy (SMM).

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Single Cell Polarization Analysis in Micro-3D Cell Culture Devices

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INTRODUCTION: The merge of micro-fabrication and cell biology allows to address so far experimentally irresolvable questions. It has been shown that the physical and chemical microenvironment of a cell plays a crucial role in controlling its function. Especially the function of epithelial cells as selective barriers between compartments demands asymmetric enrichment of lipids and proteins to specific regions of cells, which is thought to be dependent on contacts to neighboring cells. We are investigating which aspect of the polarized organization of epithelial cells can develop cell autonomously, i.e. independent on cell-cell interactions. To this end, we have developed a set of tools which enables the culturing of single cells in an array format and controlling the three dimensional shape of each individual cell.

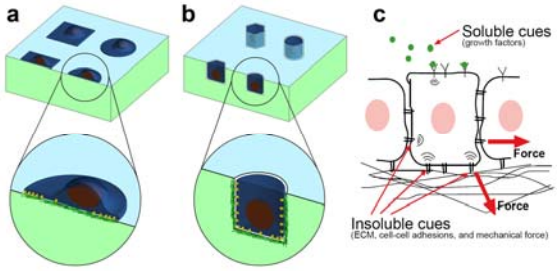

Fig.1: Scheme of the concept A) conventional 2D patterning of cells and B) micro-3D culturing of single cells. The surface of the microwells exhibits cell binding properties, while the plateau surface inhibits adsorption of proteins and attachment of cells. C) Schematic representation of cells in culture[1].

METHODS: The micro-3-D cell culturing combines 2-dimensional chemical patterning with topographical microstructuring presenting to the cells a local 3-D host structure. By the use of microfabricated Si molds and replication techniques, we have created polystyrene chips that exhibit defined microwells of various shapes. By inverted microcontact printing of a *graft*-co-polymer, poly(L-lysine)-*g*-poly(ethylene glycol), which inhibits adsorption of proteins, the plateau surface between the wells could be rendered resistant to cell adhesion, while the surface inside the wells exhibited specific functions for cell attachment[1].

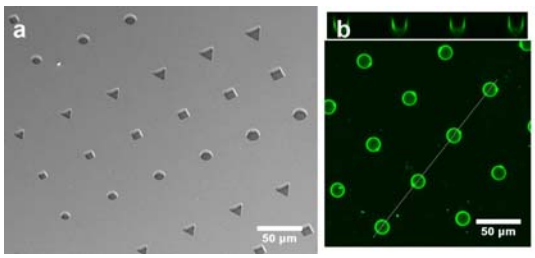

Fig. 2: Microstructured wells: a) SEM micrograph of hot-embossed structures in polystyrene b) CLSM fluorescent image after adsorption of fibronectin Alexa488 on plasma oxidized polystyrene surface, top surface stamped with a PLL-g-PEG loaded hydrogel.

RESULTS: We demonstrated that single epithelial cells can be cultured inside these microwells, remain viable and their three dimensional shape can be controlled[2]. Furthermore, we have created arrays of microwells in other materials such as PDMS and hydrogels, which additionally allow the tailoring of the mechanical properties of the surrounding material to mimic an *in-vivo* microenvironment.

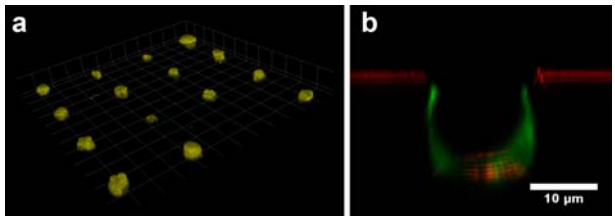

Fig. 3:CLSM 3-dimensional reconstruction of cells a) MDCK cells grown in 15 µm microwells (yellow: YFP-plasmamembrane) b) CLSM z-cross section of a single MDCK cell (transfected with GFP-actin) in a 15 µm circular well (green: actin, red: laser reflection).

DISCUSSION & CONCLUSIONS: We believe that these model surfaces are valuable tools to identify the molecular mechanisms leading to the plasma membrane polarization in epithelial cells.

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Innovative Method for the Production of Similar TiO_2-coated Epoxy Replicas Used in Cell Culture Assays

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INTRODUCTION: Surface topography has been shown to be one of the important surface characteristics affecting cell response[1]. In the past, diverse fabrication methods such as micro machining, plasma spraying, particle blasting and/or acid etching have been applied to fabricate stochastically rough micro- and nano-topographies[2]. However, there is a substantial need for cost-effective methods to produce large numbers of samples with identical surface topographies, without the need for specialized surface treatment such as blasting. Our concept of sample production is based on an epoxy replica technique using dental impression material[3]. The aim of this study was the investigation of surface roughness over several generations of epoxy replicas using standard surface characterization techniques.

METHODS: Masters of a rough (SLA; sand-blasted, large-grit, acid-etched; Institut Straumann AG) CP Ti disc were produced using dental impression material (vinyl polysiloxane). These samples served as negative replicas to cast epoxy resin. Cured epoxy substrates were coated with a 60 nanometer (nm) thick film of titanium oxide using reactive magnetron sputtering. Vinyl polysiloxane masters were cleaned and reused for the fabrication of further generations of epoxy replicas (up to a total of eight casts). Surface topography was characterized with White Light Confocal Microscopy. In the Scanning Electron Microscope (SEM), the same surface area was controlled over several generations. The chemical composition of the sputter-coated titanium oxide film was investigated with X-Ray Photoelectron Spectroscopy.

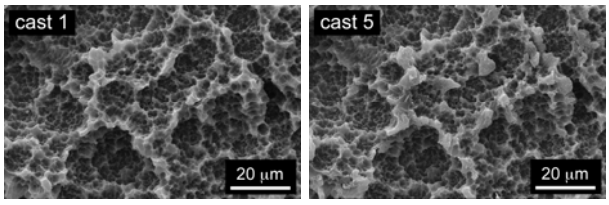

Fig. 1: Scanning electron micrographs of two different TiO_2-coated epoxy replicas made from SLA CP Ti disc. Left panel: first cast; right panel: fifth cast using the same vinyl polysiloxane master.

RESULTS: Roughness values R_a and R_t (measured with optical prolifometry) were the same within experimental uncertainty over all eight generations and in comparison to the original SLA CP Ti disc (Fig. 2).

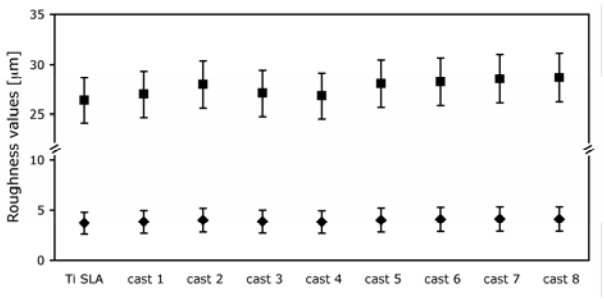

Fig. 2: Roughness values R_a (◆) and R_t (■) measured with White Light Confocal Microscopy (770 x 798 µm^2) of a CP Ti SLA disc and its eight generation replicas.

SEM investigations showed comparable topographies over all the eight consecutive casts. Only very rarely, some additional features were detected due to material transfer between casts. (Fig. 1, right panel). The chemical composition of the titanium oxide film showed pure TiO_2 (data not shown).

DISCUSSION & CONCLUSIONS: The epoxy replica technique is a simple and powerful tool to produce series of samples with essentially identical surface topographies. Such sample sets have the advantage of improving reproducibility and comparability in standard cell culture experiments. It is possible to use the same vinyl polysiloxane negative for the production of at least eight generations of epoxy replicas although the SLA surface used in this study is a highly complex 3-D topography with undercut features. The surface chemistry of epoxy substrates sputter-coated with TiO_2 is comparable to the native oxide film on the original SLA CP Ti surface.

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Fabrication of Functional Nano-structured Surfaces for Protein Based Sensor

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Patterning of surface with active and non-active spots at sub-micron level is one of the main issues for the development of protein and cell based sensors for drug screening application. The vision is that nano-patterned surfaces with high chemical contrast may allow the triggering of specific interactions exclusively and therefore improve drastically the signal to noise ratio of the bio-analytical devices. Many studies have been undertaken to develop reliable method of chemical nanopatterning and to study the effect of the nano-structured materials on the protein adsorption or on the cell adhesion. Several approaches have been used successfully but several issues still to be addressed. On one hand, the excellent results obtained by using a bottom-up approach (molecular assembly, auto-nano-fabrication) are now far to be scaled to high-throughput systems. On the other hand, top-down approach is often too expensive and time consuming (e.g. electron beam lithography) otherwise is giving very promising results. In this work, we present a novel method combining well-established techniques of material processing with low-cost and fast fabrication steps, such as plasma deposition and etching techniques using nanospheres masking of polymeric materials. Several type of nanostructures (ranging from 70 to 250 nm) such nano-domes, nano-wells and nano-spots consisting of materials with chemical and/or biological functionalities suitable for biosensor applications are produced in our laboratory. In this work examples of this research activity will be illustrated. In particular the fabrication process and characterization of Poly Acrylic Acid nanodomes (Fig.1) and nano-wells will be described and discussed. The selective biological response the nano-patterned surface is demonstrated with protein assays i.e. BSA is selectively bound to the functionalised nanostructures, whereas no protein adhesion is detected in the surrounding anti fouling matrix (Fig.2).

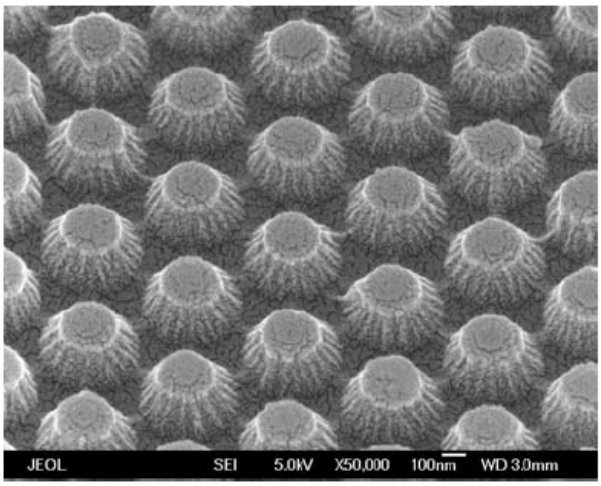

Figure 1. SEM picture of the functionalized nano-domes

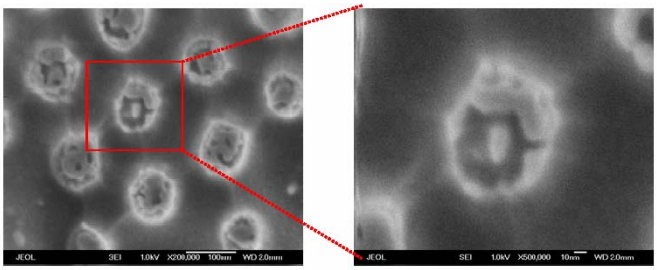

Figure 2. BSA clusters selectively bound on the top of the functional domes

Investigation of the surface properties of SU-8 for biological Applications

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INTRODUCTION: Micrometer structures for fluidic systems are often manufactured in polymers like SU-8 or PDMS. Bioactivity is strongly influenced by the surface properties. Thus, controlling the surface free energy (SE) and the wetting behaviour of the system is crucial [1]. To render polymeric surfaces hydrophilic an O_2 plasma treatment is commonly employed [2]. While previous reports [3,4] focus on the effect of various gases on several polymers such as PMMA and polystyrene we are interested in the O_2 Plasma treated SU-8. Especially the evolution of the SE over time due to ageing effects is important for the calculation of the usability in bio-MEMS applications. To elucidate this processes we carried out contact angle goniometry over time. Topographic imaging by AFM allowed the quantification of changes of the surface roughness induced by the plasma process.

METHODS: Samples were prepared by a conventional spin coating process of SU-8 on 4" Si wafer according to the fabrication instructions of the manufacturer (microchem), including a hardbake process. O_2 Plasma activation has been accomplished at 150W and 13,56MHz. Measuring the contact angle with a DSA10 goniometer from Krüss allowed the determination of the SE over several weeks.

The surface topology was imaged by an AFM from Veeco (Dimension IV) in tapping mode.

RESULTS: The AFM measurements on the surfaces showed a drastic increase of the surface roughness by the plasma treatment. Granular nano-aggregates developed with a size depending on the treatment time.

The effect of plasma treatment can be clearly seen by comparing AFM images of a SU-8 surface before and after plasma treatment (figure 1).

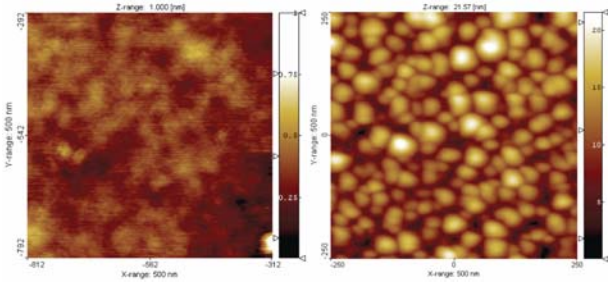

Fig. 1: Topography of SU-8 measured by AFM. (a) before exposure (b) after 120 s O_2 plasma.

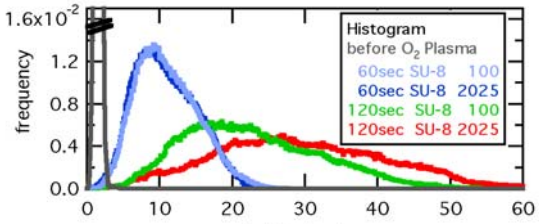

Fig. 2: Height distribution over plasma duration

The surface roughening is illustrated by the analysis of the height histogram (figure 2). Evaluating the maxima in the histogram, a change from 1 nm (before treatment) most frequently populated height to 10 nm (60 s plasma) and 20 nm (120 s plasma) is evident. For 8 min exposure the aggregates grow to 120nm.

The observation of the contact angle over several weeks indicated that after the O_2 plasma activation the hydrophobicity recovered within several days.

The initial SE after plasma activation (80 mN/m) decreased simultaneous to the hydrophobic recovery to 50 mN/m.

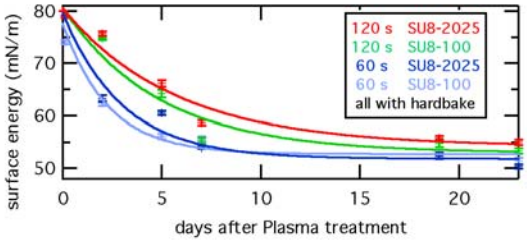

Fig. 3: Surface energy development for 60 s and 120 s plasma duration and different PR solvents.

DISCUSSION & CONCLUSIONS:

O_2 Plasma treated surfaces of SU-8 keep their ultra-hydrophilic properties and high surface energy typically for less than one week. Longer plasma treatment results in a drastically increase of the surface roughness, but the high SE lasts longer. Due to the fact that the bioactivity a.e. of artificial cellular systems depends on the surface energy, constant conditions have to be arranged. For long-term experiments the change in SE has to be considered.

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European Cells and Materials Vol. 10. Suppl. 5, 2005 (page BS23) ISSN 1473-2262

Engineered titanium surfaces for specific interactions with integrin receptors through poly (L-lysine)-g-poly (ethylene glycol) adlayers functionalized with collagen derived mimetic peptide

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Engineering surfaces for specific integrin-ligand interaction and signaling cascades provides a biomolecular strategy for optimizing cellular responses in biomaterials applications. The integrin α2β1 recognizes a specific amino acid binding sequence that is present on type I collagen. Integrin recognition is entirely dependent on the triple-helix conformation of the ligand similar to that of native collagen[1]. This study focuses on engineering α2β1-specific bioadhesive surfaces by immobilizing a triple-helical collagen-mimetic peptide, incorporating the specific binding sequence, onto model nonadhesive substrates. Metal oxide surfaces can be made protein-resistant through spontaneous assembly of poly-(L-lysine)-g-poly-(ethylene glycol) (PLL-g-PEG) grafted co-polymers. This copolymer is used as a basis for developing special surfaces with controlled specific biological properties[2], e.g. through grafting the binding sequence of type I collagen to part of the PEG-chains to induce a direct interaction of the peptide ligands at controlled surface density with cell receptors.

The polymer functionalized surfaces were characterized by Optical Waveguide Lightmode Spectroscopy (OWLS), Ellipsometry and X-ray photoelectron spectroscopy (XPS).

The peptide-modified polymers were adsorbed on TiO2 and preliminary tests with cells were performed. In particular Rat Calvarian Osteoblast and Human Fibroblast were used as substrate: the presence of the collagen-like functionalized polymer seems to induce a preferred cellular adhesion of Osteoblast with respect fibroblast, as compared to the control peptide-functionalized polymer, after 1 day of incubation; nevertheless more detailed experiment have to designed and performed to validate such results.

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European Cells and Materials Vol. 10. Suppl. 5, 2005 (page BS24) ISSN 1473-2262

NTA (nitrilotriacetic acid)-derivatized Poly(L-lysine)-*g*-poly(ethylene glycol): A Novel Polymeric Interface for Binding and Study of 6xHistidine-tagged Proteins

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INTRODUCTION: Interfaces are key elements in the design and fabrication of bioaffinity sensor chips with directed biological response. It has been reported that coating metal oxide surfaces with poly(L-lysine)-*g*-poly(ethylene glycol) (PLL-*g*-PEG) provides an attractive option for producing stable surfaces that are protein-resistant[1]. A novel NTA-functionalized PLL-*g*-PEG is presented that it can assemble on oxide surfaces as a monolayer, allows for the immobilization of proteins through NTA-Ni^{2+}-histidine docking site chemistry.

METHODS: Graft copolymer PLL-*g*-PEG/PEG-NTA was synthesized with a fraction of the PEG chain terminus covalently functionalized with nitrilotriacetic acid (NTA) as a chelating ligand. The polymer was assembled from aqueous solution onto Nb_2O_5 coated optical chips, followed by coordination of Ni^{2+} to the surface-exposed NTA ligand. Subsequently, this sensing platform was used to specifically attach Histidine-tagged proteins, e.g., 6His-GFP(Green Fluorescent Protein) or enzymes, e.g., 6His-β-lactamase. Optical waveguide lightmode spectroscopy (OWLS) was used to monitor quantitatively and *in situ* for each step. Furthermore, the NTA-functionalized polymer was used to produce interactive micropatches in a non-interactive PLL-*g*-PEG background on Nb_2O_5 coated surfaces by a novel approach termed molecular assembly patterning by lift-off (MAPL)[2]. The quality of the 6His-GFP patterns was evaluated by confocal laser scanning microscopy (CLSM).

RESULTS: OWLS studies of 6His-GFP bound to NTA-functionalized polymer modified surfaces proved that the binding of 6xHis-tagged proteins was stable and required the presence of Ni^{2+} attached to the NTA functionalities. The proteins could be fully removed by exposing the surface to imidazole or EDTA. Non-specific adsorptions of 6His-GFP and 6His-β-lactamase were below 2 ng/cm^2. Binding and desorption of 6xHis-tagged proteins was repeated in several cycles demonstrating the excellent regeneration capacity of the novel platform (Fig. 1). Fluorescence microscopy measurement proved that the patterning was successful and that surface-immobilized 6His-GFP was in an active conformation (Fig. 2).

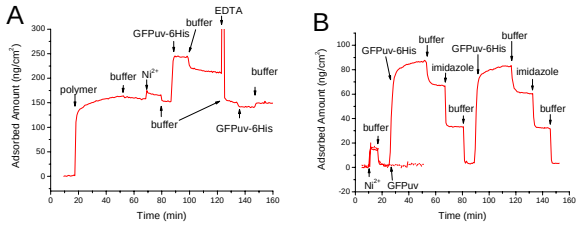

Fig. 1: *Adsorbed mass measured by OWLS of the sequential adsorption of PLL-g-PEG/PEG-NTA, Ni^{2+}, 6His -GFP and 6xHis -β-lactamase. Quantitative regeneration of the surface was achieved by adding either EDTA, which removes Ni^{2+} or imidazole, which removes only the His-tagged protein, but not Ni^{2+}.*

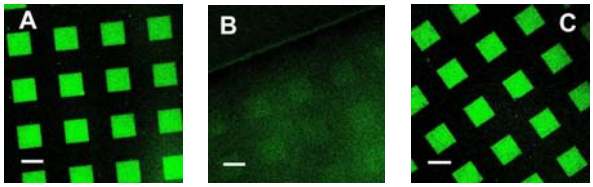

Fig. 2: *Pattern of 6His-GFP on Nb_2O_5 analyzed by CLSM. The scale bar represents 60 µm. (A) 6His-GFP immobilized on MAPL patterned substrate after charging with Ni^{2+}. (B) As A, rinsed with imidazole removing 6His-GFP. (C) As B, reloaded with 6His-GFP.*

DISCUSSION & CONCLUSIONS: We have demonstrated that the novel PLL-g-PEG/PEG-NTA polymeric interface is a promising approach for the binding of 6xHis-tagged proteins in an oriented manner with active conformation. Furthermore, the combination of the MAPL patterning technique with the PLL-*g*-PEG/PEG-NTA system is considered to be a promising technique for the production of functional microarrays in the area of genomics and proteomics.

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FIBRONECTIN ADSORPTION ON MODIFIED POLY(l-LACTIDE) SURFACE AFTER AMMONIA PLASMA TREATMENT

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INTRODUCTION: Gas plasma treatment is extensively used for chemical modification of biomterials[1,2]. In this work, in order to improve the hydrophilicity and surface roughness, ammonia was used as non-polymerizing gase to create reactive sites on the surface of PLLA, such as amine groups. Protein adsorption, depending on the surface property of biomaterials, may influence the biological activities in relation to cell culture *in vitro* and implanted *in vivo*. Therefore, investigation of protein adsorption on the surface of biomaterials is a critical evaluation to elucidate the cell adhesion in vitro and in vivo[3]. Fibronectin, an important extracellular matrix protein[4], was used as a model in this study.

METHODS: PLLA films were fabricated by solution casting method and subsequently their surfaces were treated by ammonia plasma on a Plasma Graft Polymerizer (VTC-FSN-200, Japan, 13.56 MHz) with different powers. The surface chemical composition of the plasma-treated PLLA films was investigated by X-ray photoelectron spectroscopy (XPS). The contact angles of the samples to water were measured to evaluate their hydrophilicity. The surface morphology and roughness of ammonia plasma-treated PLLA films were observed by Atomic force microscopy (AFM). Fibronectin adsorption was studied by ^{125}I-labelled method.

RESULTS: The increased N-functional groups on the treated samples, for example, amino (-NH_2) and imino(-CH=NH), were reflected in the XPS data. Along with the increase of treated power, the hydrophilicity, measured by contact angles, and the roughness, presented with a Ra, were both evidently increased (showed in table 1).

Table 1. Effect of ammonia plasma treatment power on water contact angles and average roughness of PLLA

Sample	θ (°)	Ra(nm)
Before treatment	83.2±1.6	22.32±1.24
50w, 2 min	58.3±2.2	31.35±2.34
100w, 2 min	32.4±1.8	37.50±2.71
150w, 2 min	21.7±0.9	47.26±5.3

The surface morphology of ammonia plasma-treated samples was observed by AFM (showed in Fig 1). It could be seen that the surface morphology depended on the treating power. From the results of fibronectin adsorption test, ammonia plasma-treatment is an effective means to adjust the adsorption kinetics and isothermal adsorption of fibronectin.

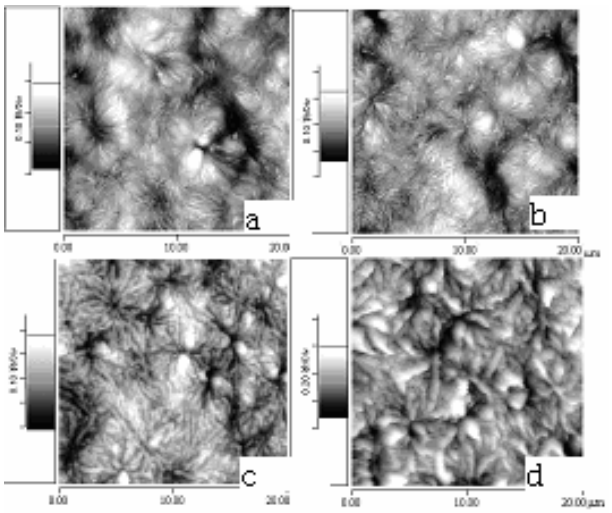

Fig. 1: The morphology of PLLA samples. a: before treatment; b: 50w, 2min; c:100w, 2min; d:150w, 2min.

DISCUSSION & CONCLUSION: The surface properties of ammonia plasma-treated PLLA films were characterized by a series of surface analysis techniques. The hydrophilicity, surface chemistry and surface roughness could all influence the fibronectin adsorption on the surface of treated PLLA films. Furthermore, it could also ultimately affect the biological events of cell, such as adhesion, proliferation and differentiation.[5]

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Engineering Interfaces to Cells using Synthetic Liquid Crystals

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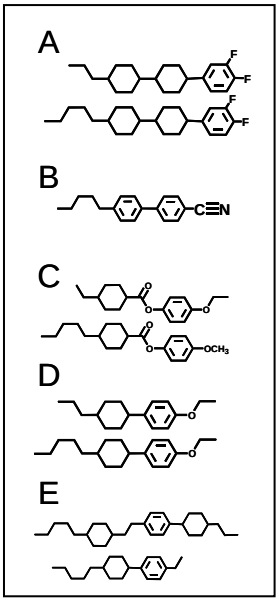
INTRODUCTION: Given the ubiquitous presence of the liquid crystalline state in biological systems and the technological utility of liquid crystals, it is surprising that few examples of the use of liquid crystal technologies involving whole mammalian cell have been reported. One could envisage, for example, the use of liquid crystals to image the expression and organization of receptors either expressed on the surfaces of cells (or secreted into the liquid crystal) in response to biochemical or biophysical cues. Alternatively, liquid crystals might be exploited to deliver chemical or mechanical stimuli to cells and thereby guide their behavior.

To enable the development of technologies that interface cells with liquid crystalline environments, liquid crystals that are not toxic to cells are required. We report here the results of an initial search for liquid crystals that are *not* toxic to mammalian cells.[1] In this work, we carried out experiments using living cells immersed under eight thermotropic liquid crystal mixtures to screen for chemical functionalities in liquid crystals that maintain the viability of cells. Each liquid crystal mixture was comprised of mesogens that contained a unique set of functional groups.

METHODS: TL205, 5CB and E7 were purchased from EM Industries (Merck), NY. Components for the cholesteric series were purchased from Pressure Chemical Company, PA. Components for the "A", "B", "C" and "E" series were purchased from Phentex Corporation, TX.



3T3 fibroblasts were cultured in DMEM (supplemented with 10% fetal bovine serum plus 40 μg/ml gentamycin) and grown in a humidified incubator at 37°C and 5% CO_2. SV-40 HCECs were cultured in supplemented hormonal epithelial medium (SHEM) – a basal medium for epithelial cell growth – with 10% fetal bovine serum (FBS). SHEM is a mixture of DMEM and Ham's F-12 (50/50) plus 0.5% DMSO and 40 μg/ml gentamycin. Both cell lines (3T3 fibroblasts and SV-40 HCEC) were plated at a concentration of 10,000 cells/well on a 96-well plate and allowed to attach and proliferate overnight under each cell lines' corresponding medium. The medium was then removed, 25 μl/well of either liquid crystal, cell culture medium or PBS (pH 7.4) were added to six wells for each solution (total of 120 wells). PBS was used as a control. Saponin (0.1% w/v) was added to one row of cells that had not been exposed to liquid crystal as a control for complete cell death. The cells were incubated at 37°C for 4 or 24 hours under these solutions. The solutions (liquid crystals, cell culture medium and PBS) were then removed. The cells were rinsed three times with PBS. 50 μl of ethidium-homodimer (4 μM) or 50 μl of Calcein-AM (0.6 μM) were added to each well and the plate was incubated for an additional 2 hours. Fluorescence was measured using a Cytofluor 4000TC automated fluorescent plate reader. For ethidium homodimer, the fluorescence was measured using 530 nm (excitiation) and 620 nm (emission). For Calcein-AM, fluorescence was measured using 485 nm (emission) and 530 nm (excitation).

RESULTS: In order to quantify the viability of cells treated with liquid crystals, we carried out a fluorescent assay based on intracellular esterase activity. This viability assay uses the fluorescent precursor calcein acetoxymethylester (CAL-AM), which is permeable to the membrane of cells. The presence of green fluorescence from CAL in cells is evidence of esterase activity as well as an intact membrane that retains the esterase products in the cells, both of which are indicators of a living cell.

By measuring the level of intensity of CAL fluorescence in cells treated with different liquid crystals relative to that measured without treatment with liquid crystal (in culture medium), we quantified the effect of eight liquid crystals on the viability of 3T3 fibroblast and SV-40 HCEC cells (Figure 1)

Inspection of Figure 1 reveals that, in general, the 3T3 fibroblasts and SV-40 HCECs respond in a similar manner to each liquid crystal. In particular, the "E", "A", "B" series of liquid crystals as well as 5CB and E7 are toxic to both 3T3 fibroblasts and SV-40 HCECs. The treatment of cells with these five liquid crystals caused the CAL fluorescence to decrease to less than 30% for 3T3 fibroblasts, and to less than 50% for CV-40 HCECs. In addition, it is evident that the CAL fluorescence reveals the cholesteric series of liquid crystals to be toxic to SV-40 HCECs but not 3T3 fibroblasts. However, treatment of both cell lines with the "C" series and TL205 led to levels of CAL fluorescence that were similar to the CAL fluorescence measured with cells not treated with liquid crystals (both culture medium and pure PBS buffer). We believe these two liquid crystals are not toxic to 3T3 fibroblasts and SV-40 HCECs under the experimental conditions reported in this paper.

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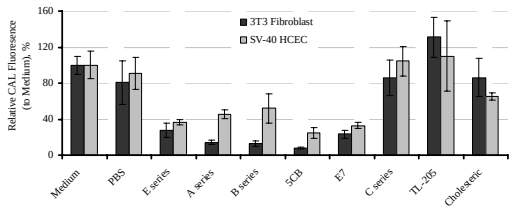

Figure 1: Relative intensities of the fluorescence from CAL-AM added to 3T3 fibroblasts (black bars) and SV-40 HCEC (gray bars) after treatment with each liquid crystal (4 hours, 10,000 cells/well, 6 wells/treatment).

CONCLUSIONS: By investigating the effects of eight liquid crystals with unique sets of functional groups on the viability of two mammalian cell lines immersed in the liquid crystals, we find that the chemical functionality of the liquid crystals correlates closely with the toxic effect (Figure 1). We identified several functional groups that, when incorporated in mesogens, were *not* toxic to cells. Treatment of cells with the liquid crystals containing these functional groups does not affect the post-treatment proliferation of the cells as compared to PBS. The mechanisms by which these functional groups define liquid crystal-cell interactions and thus toxicity are not fully understood and are the subject of ongoing research.

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A Three-Dimensional Hydrogel Matrix for Human Mesenchymal Stem Cell and Urothelial Cell Growth and Differentiation

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INTRODUCTION: There is a critical need for bladder tissue replacements due to malfunction or loss of tissue following bladder diseases or malformations. Numerous studies that have focused on using cell delivery vehicles or acellular scaffolds to improve bladder regeneration have shown the re-establishment of normal bladder urothelium while the success in restoring functional bladder smooth muscle cells (SMC) remains scarce[1]. Both scaffold delivered SMCs as well as host SMCs migrating into an acellular scaffold in vivo have a tendency to switch on a synthetic proliferative phenotype instead of the required quiescent contractile one, eventually resulting in non compliant fibrous scar tissue. We hypothesise that providing the right stimuli, to human mesenchymal stem cells (MSC) will result in their subsequent differentiation into quiescent smooth muscle cells. A biodegradable poly (ethylene glycol) (PEG) hydrogel provides an excellent scaffold for investigating basic biology, as well as the incorporation and delivery of biological signals for tissue regeneration and repair. In this initial study we investigated the optimal gel properties for the growth of MSCs in, and urothelial cells (UCs) on a PEG hydrogel, respectively.

METHODS: Poly (ethylene glycol)-vinyl sulfone (PEG-VS) was synthesised as previously described[2]. The cell adhesion peptide C-RGDSP was reacted with the PEG-VS macromere by Michael Type addition.A matrix metalloproteinase (MMP) sensitive crosslinker (GPQG↓IWGQ) was used to form the gel and to provide degradation sites. To find the optimal gel properties for cell attachment and spreading, the amounts of RGD-SP, PEG and crosslinker were altered. The RGDSP was varied between 100-350µM while keeping the amounts of cross links and percentage of PEG constant. The RGD was then kept constant at 200µM while varying the amount of PEG from 5.5-7.5% (w/v), and the crosslinker from r = 1.0-2.8 where r equals the molar ratio of crosslinker to available PEG, respectively. Human mesenchymal stem cells (Cambrex) were mixed with the gel precursor and polymerised into the gel at a density of 30 000cells per 30µl gel precursor. Primary human urothelial cells were seeded on top of gels, 15000cells per 30µl gel, after 1h of swelling in medium. Cell attachment and spreading was explored by assessment of cell morphology through bright field and fluorescence microscopy. Cells were fluorescently labelled following standard protocols.

RESULTS: The optimal RGD content for both MSCs and UCs was found to range between 200-250µM. At RGD contents below 150µM cells rounded up alone (MSCs) or in aggregates (UCs). Gels with 7.0-7.5% PEG, and an amount of crosslinker ranging between r = 1.4-1.7 were found to be preferential for cell spreading and growth. These optimal gel properties correspond to rather loose, elastic gels. In more cross linked i.e. stiffer gels the cells were unable to degrade the gel in time to make room for attachment and spreading, and in looser, less cross linked gels the cells were less able to attach, and gels difficult to handle.

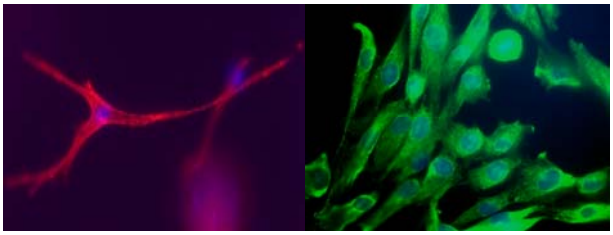

Fig. 1: Mesenchymal stem cells spreading within a three dimensional PEG hydrogel matrix (Phalloidin, DAPI staining)(left). Urothelial cells cultured on top of a PEG hydrogel retain their typical cuboidal shape (pancytokeratin staining AE1/AE3 (FITC), DAPI) (right).

DISCUSSION & CONCLUSIONS: A three dimensional PEG hydrogel scaffold has been modified for the growth of human MSCs and UCs. Current work is focused on investigating gene expressions by qPCR to determine cell phenotypes.

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Hepatocyte apoptosis/anoikis is suppressed by Fas down-regulation on PVLA, a galactose-carrying polymer

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INTRODUCTION: Extracellular matrices (ECMs) have been known to decide cell fate such as differentiation, proliferation, migration, and survival [1]. Especially, it was reported that survival of epithelial cells requires integrin-mediated adhesion to ECM molecules. Integrin interacts with ECM components to activate PI3K/Akt and MEK/ERK signaling pathways and maintains cell viability. When the interaction between integrin and ECM is disrupted, cell is led to apoptosis, named anoikis. In our previous study, a galactose-carrying polystyrene (PSt), poly (*N*-*p*-vinylbenzyl-4-*O*-*β*-**D**-galactopyranosyl-**D**-gluconamide) (PVLA), was developed as a hepatocyte-specific adhesive matrix, and hepatocytes attached to PVLA through asialoglycoprotein receptor (ASGP-R), that could survive. Kim et al. also reported that hepatocyte adhered on PVLA strongly suppressed integrin signaling, because ECM deposition beneath the hepatocytes is inhibited by coated-PVLA molecules [2]. Interestingly, inspite of integrin signaling suppression, hepatocyte can attach to PVLA and survive. It is, thus, suggested that the mechanism of anoikis suppression without integrin signaling might exist.
In this study, we compared the mechanism of anoikis suppression by integrin-mediated adhesion and non-integrin-mediated one.

METHODS: Mouse hepatocytes were cultured on adhesive matrices (PVLA, poly-**L**-lysine (PLL), collagen-1, and fibronectin) and non-adhesive matrices (agarose gel). Hepatocyte death was measured as leaked LDH activity. Akt and ERK phosphorylation levels were determined by Western blot analysis. Fas expression level was examined by RT-PCR method and western blot analysis.

RESULTS and DISCUSSION: FAK autophosphorylation in hepatocytes on PVLA, PLL, and agarose gel was suppressed although that on collagen-1 and fibronectin was strongly detected. This indicates that integrin signal is suppressed in hepatocytes on PVLA and PLL. Hepatocyte anoikis was suppressed on PVLA and PLL without integrin signal although hepatocytes on agarose gel underwent apoptosis. To analyze the suppression mechanism of anoikis, we focused on two survival signalling molecules, ERK and Akt. ERK was phosphorylated in both adherent and non-adherent conditions. In the case of Akt, phosphorylated Akt was detected only on integrin-mediated adhesive matrices (collagen-1 and fibronectin). This result indicates hepatocytes survive on non-integrin-mediated adhesive matrices without involvement of Akt and ERK activation. In the next step, we focused on Fas/Fas ligand system to clarify the mechanism of hepatocyte anoikis. Fas up-regulation was observed in hepatocyte only on agarose gel. To confirm whether Fas/Fas ligand system is involved in hepatocyte anoikis, we used *gld/gld* mice whose Fas ligand has point mutation and loses the ability to induce apoptosis. Anoikis of hepatocytes from *gld/gld* mice was suppressed compared with those from wild type (WT) mice (Figure 1). These results indicate Fas down-regulation without integrin signal rescues hepatocytes from anoikis. Our studies using the artificial matrices that can regulate cell adhesion mechanism will provide us the new insights to regulate anoikis and other cellular behaviour by cell-ECM interaction. Furthermore, these results give us not only information to understand the regulation mechanism of anoikis but also strategical guideline to avoid decline of cell viability in cell transplantation for regenerative medicine and carcinoma metastasis.

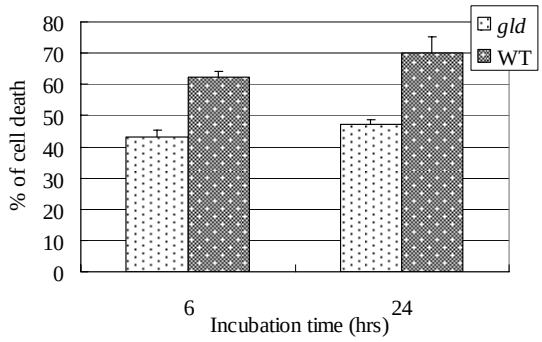

Figure 1: Hepatocytes from gld/gld mice were suppressed anoikis compared with those from wild type mice

REFERENCES: [1] F.G. Giancotti, and E. Ruoslahti, (1999) *Science* **285**: 1028-1032. [2] S.-H. Kim, and T. Akaike, et al (2003) *FEBS Lett.* **553**: 433-439.

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Towards a polymeric patch-on-a-chip design

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INTRODUCTION: Since the patch clamp technique was introduced in 1976, it has become one of the most important tools for the investigation of ion transport mechanisms through cell membranes. A major drawback, however, is that despite recent efforts for automation[1] the technique is still not suited for true high throughput investigations as they are needed in the pharmaceutical industry.

To overcome this drawback we study a new approach which we call polymeric patch-on-a-chip. The general design of this approach is sketched out in Fig. 1

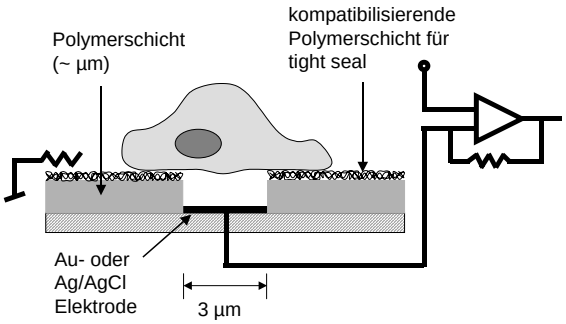

Fig. 1: Patch-on-a-chip design. For details see text.

Here the cell sits on a small hole within a microstructured film and tightly seals the interior of the hole from the medium around the cell. Small electrodes that are placed within the hole and in the medium can then be used to study the ion transport through the cell membrane. Using standard photolithographical techniques this design allows for the generation of many such set-ups on one chip and is therefore in principle well suited for high throughput measurements.[2]

METHODS: The microstructures were generated by using photolithography in a SU8 resist layer. Prior to that the silicon substrate was covered with a structured layer of titanium and gold with a lift off. The modification of the surface of the photoresist was achieved by depositing polymers with photoreactive benzophenone groups which upon UV irradiation for networks that are also chemically linked to the surface. All layers were characterized by XPS and FTIR. The microstructures were characterized by AFM Profiliometer and Light Microscopy.

RESULTS & DISCUSSION: SU-8 technology has been successfully used to generate microstructures with sizes suitable for the approach outlined above. One example is shown in Figure 2. There nine little holes were successfully written into a resist layer. The depth of the holes is of the order of 6 µm and the diameter is 20 µm. Below the resist layer a gold layer which is connected to a big gold spot may later on serve as a connection to the measurement electronics.

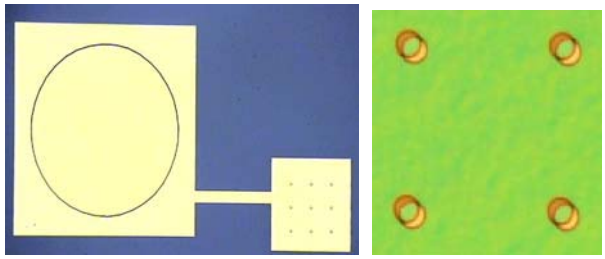

Fig. 2: Test structure for a patch-on-a-chip device.

As SU-8 forms rather hydrophobic surfaces cells cannot be cultured directly on this device. Therefore, we deposited thin coatings of polymers carrying photoreactive groups that upon UV illumination cause the formation of a network. Also bonds to the resist layers are formed and consequently very stable layers are generated that resist delamination even if they were formed from hydrophilic polymers. Using this approach we were able to completely mask the cell hostile properties of the resist. Current investigations concern the deposition of cells such that they come to rest directly above one of the little holes and form a tight seal that separates the inside of the hole from the exterior. Also, other chip designs are studied and optimized in terms of size and spacing.

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Cell morphology, - volume, expression of vinculin and migration are altered as response of human bone marrow cells to topographical characteristics of the culture substrate

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INTRODUCTION: Human bone marrow cells (HBMCs) are a heterogeneous cell population that consists of haematopoietic cells and mesenchymal stem cells. These mesenchymal stem cells (MSCs) are able of self-renewal and can differentiate into various cell types like osteoblasts, chondrocytes and adipocytes. This study investigates the effect of surface topographies on cell architecture and migration of HBMCs using simplified surface models in an *in vitro* culture system.

METHODS: Well-defined inverse Ti topographies were fabricated by electrochemical micromachining through patterned photoresists. Those served as masters for injection molding with polybutyleneterephthalate in which five different surface topographies were imprinted (Fig. 1).

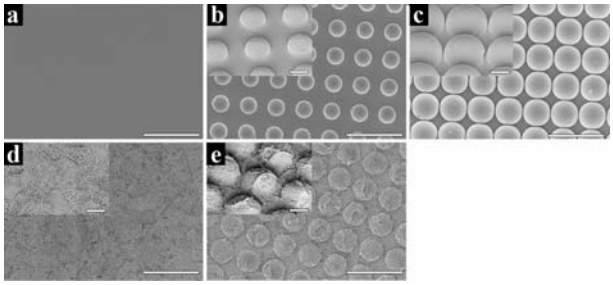

Fig. 1: SEM images of the different surface topographies. (a) smooth, (b) hemispheres of 30 μm and 20 μm spacing (30/20), (c) hemispheres of 50 μm and no spacing (50/00), (d) imprint of etched Ti surface (p/e⁻) and combination of 30/20 with p/e⁻ (30/20e⁻). Big bar: 100 μm, small bar: 20 μm.

The structured polymer substrates were subsequently Ti-coated by physical vapour deposition.

Adult HBMCs were isolated from patients obtaining a total hip prosthesis. After the first passage, the cells were plated out on the different structured culture substrates and grown in α-MEM plus 10% FCS and 1% PSN.

RESULTS: The surface topography influences the morphology and migration of cells. Cells on the imprint of etched surfaces (p/e⁻, 30/20e⁻) were spindle-shaped, showed a condensed morphology with reduced focal contacts (Fig. 2, d, e) and increased migratory activity (Fig. 3). In contrast, actin filaments of cells on the 50/00 structure span from one hemisphere to the neighbouring hemisphere with dense focal contacts on the top of the hemispheres (Fig. 2, c) that caused strong anchorage to the substrate, which was associated with a decreased migratory activity (Fig. 3). Cells on the 30/20 structure were predominately located around the hemispheres (Fig. 2, b) with well-established focal contacts. Interstingly, these cells also displayed high migration abilities (Fig. 3).

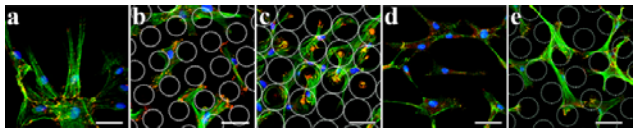

Fig. 2: Adult HMSCs cultured on the smooth (a), 30/20 (b) 50/00 (c), p/e⁻ (d) and 30/20e⁻ (e) surface. Cells were stained for F-actin (green), vinculin (red) and nuclei (blue). Bar: 50 μm.

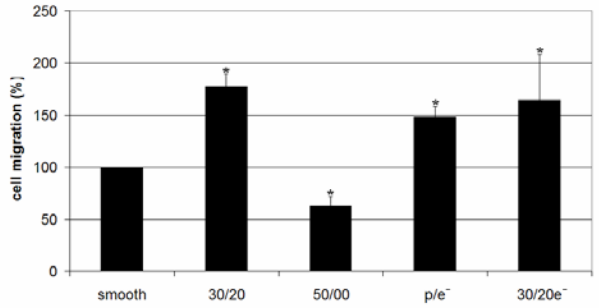

Fig. 3: Cell migration of HBMCs the smooth (a), 30/20 (b) 50/00 (c), p/e⁻ (d) and 30/20e⁻ (e) surface. (*) determines the significance when compared to the reference smooth surface.

DISCUSSION & CONCLUSIONS: Ordered structures in the micrometer range as well as random structures in the submicro- and micrometer range had distinct effects on the morphology, volume and migration. Further work will focus whether by controlling of the cell shape the commitment of HBMCs could be influenced.

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Correlation of Protein Adsorption and Cell Adhesion on Polymeric Surfaces

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INTRODUCTION:

It is widely accepted, that cell growth and viability depend on surface charge, charge density, wettability, hydrophobicity/hydrophilicity and surface-morphology.[1] However, hydrophilicity itself is only a marginal parameter for the attachment of cells to surfaces.[2] It is also known that cells interact with proteins of the extra cellular matrix (ECM) to enhance cell viability.[3] Certain oligopeptide-sequences mimic the ECM- proteins (RGD and others) and ligands (integrins) of the cell membrane and thus improve cell adhesion. [4-8] However, large scale surface modification with oligopeptides such as RGD is impeded by several factors. The most important two are their low bio stability and the high costs when used for surface modification. Synthetic polymers on the other side are stable and readily available with a wide range of properties. Hence, a deeper understanding of the mechanisms that govern the cell-material interactions as a function of physical and chemical characteristics of the polymers is desirable.

In this study we compare selected physical parameters (contact-angle, swelling-degree) of a range of polymers and their tendency to adsorb proteins to their interaction Human Micro Vascular Endothelial Cells (HMVEC).

RESULTS & DISCUSSION:

In this study we used benzophenone containing co- and terpolymers which upon UV-irradiation form surface-attached polymer networks.

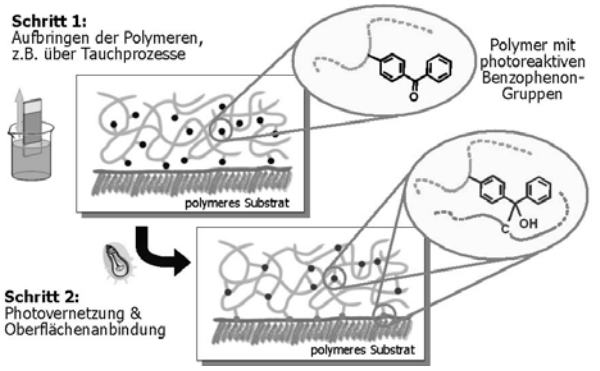

Figure 1: *Illustration of the photo induced cross linking of Benzophenone containing polymers*

During these studies we investigated a wide range of benzophenone containing polymers such as polystyrene, polydimethylacrylamide, poly-2-hydroxymethacrylate, polymethylmethacrylate, polyethyloxazoline and various comb-PEG-polymers.

Protein adsorption and swelling of the polymer layers was investigated by surface-plasmon-spectroscopy and surface wave guide-spectroscopy respectively. As model system for the study of protein adsorption on thin polymer films Fibrinogen was chosen, because it is known to strongly adsorb to a wide range of different surfaces.

Cell culture tests have been conducted using HMVEC on a wide range of different polymer network modified surfaces.

A strict correlation between protein adsorption and cell-growth on uncharged polymer network surfaces was found. Furthermore it could be shown that a protein-repelling, uncharged surface is also not attractive for the attachment of HMVEC.

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Cell Adhesion on Charged Polymer Surfaces

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INTRODUCTION:

Beside other factors, surface charge is an important parameter for cell adhesion.[1] Also, it has been shown that protein adsorption plays a key role in cell-attachment to uncharged polymers.[2]

In this study we aim at a combined investigation of the influence of the type and density of the charges of polyelectrolyte networks on the adhesion of cells on these layers. To do so, we have used a technique that utilizes polymers with photoreactive benzophenone moieties to generate surface attached polyelectrolyte networks and investigated both the adsorption of a model protein (fibrinogen) and of cells (human microvascular endothelial cells, HMVEC) on these samples.

RESULTS & DISCUSSION:

The PEL network layers were generated as described in the literature.[3] In brief, benzophenone layers within polymers are UV activated causing a crosslinking reaction with neighboring chains and also with groups of a polymeric substrates. Accordingly, surface attached network layers are formed that do not delaminate even if placed in solvent that leads to significant swelling of the layers.

To study anionic polyelectrolytes we have prepared layers of poly(acrylic acid), poly(*p*-styrene-sulfonic acid sodium salt) (p-SSNa), poly(sulfopropylacrylate potassium salt) (SAK) and of a polymer in which the charged groups (acrylic acid) were diluted with uncharged dimethyl acrylamide (DMAA) residues.

Protein adsorption and swelling of the polymer layers was investigated by surface-plasmon-spectroscopy and surface wave guide-spectroscopy respectively. All of these polymers showed to be protein-repulsive to Fibrinogen as well as to Bovine Serum. In contrast to the expected behavior from studies on non-charged polymers HMVECs seemed to grow well on these surfaces. There, cell adhesion was only observed on surfaces to which proteins adsorbed well. All protein resistant surfaces were also cell repellent. This discrepancy is best illustrated by the behavior of copolymers in which protein- and cell-repellent DMAA residues and cell-attractive styrene sulfonic acid moieties were present. With increasing DMAA and decreasing SSNa content the layers became more and more resistent to the adhesion of HMVECs. This observation is illustrated in Figure 1.

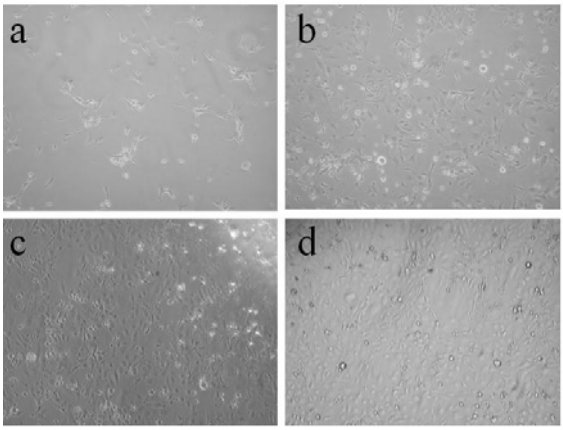

Figure 1: *HMVEC on polyelectrolyte networks composed of DMAA and SSNa with increasing charge density from a) to d). Cell adhesion is largely enhanced when the density of charges is increased.*

On our poster we will present a simple model that explains these findings based on the complexation of Ca-cations from the medium with the anionic groups of the polymers and the extracellular matrixs of the cells. A comparison to the cell adhesion behavior to positively charged polymers will be made.

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FAK-RELATED NON-KINASE (FRNK) DISPLACES FOCAL ADHESION KINASE (FAK) FROM FOCAL ADHESION COMPLEXES IN VASCULAR SMOOTH MUSCLE CELLS

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INTRODUCTION: Myointimal hyperplasia (IH) is the most common cause of late failure after vascular interventions and operations. Vascular smooth muscle cell (SMC) migration from the vessel media to the intima precedes SMC proliferation and matrix deposition. Thus, inhibiting SMC migration may limit the progression of IH. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that contributes to the regulation of cell migration through the promotion of integrin signalling cascades. The C-terminal homologue of FAK, FAK-related non-kinase (FRNK) displaces FAK from focal adhesion complexes in cardiac myocytes, (1) and since it lacks a kinase domain, it is thought to be a negative regulator of the integrin signalling cascade. Here we show that FRNK displaces FAK from focal adhesion complexes in vascular SMCs, and that the FRNK gene can be infected into SMCs that are suspended in a 3-D fibrin glue matrix.

METHODS: FRNK displacement of FAK was shown using canine carotid artery SMCs. SMCs were plated (10^6) on fibronectin-coated chamber slides overnight, then infected with adenovirus (Adv)-GFP or Adv-GFP-FRNK at 100 MOI for 24 hours. The slides were fixed, stained with a rhodamine tagged antibody for the kinase domain of FAK, and visualized under confocal microscopy. Uninfected canine carotid artery SMCs and canine jugular vein endothelial cells were inverted and suspended for 48 hours, and the subsequent cell pellets were entrapped in a fibrin glue matrix and suspended in 24 well plates with growth media. (2) 24 hours after gel suspension, Adv-GFP or Adv-GFP-FRNK was added to the media at 500 MOI, and efficiency of infection was determined at serial time points.

RESULTS: Infection of vascular smooth muscle cells suspended in a 3-dimensional co-culture angiogenesis system was pervasive by 7 days after infection. (Figure 1)

Canine carotid artery smooth muscle cells had 100% infection at 24 hours when plated on chamber slides. FRNK-infected SMCs had FAK displaced from focal adhesion complexes and replaced by FRNK, while GFP-infected cells showed the normal incorporation of FAK into focal adhesion complexes.

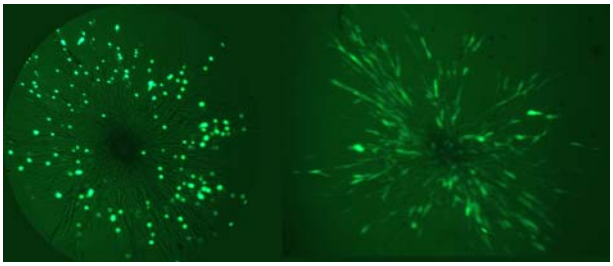

Fig. 1: Successful infection vascular smooth muscle cells in 3-D co-culture with endothelial cells. Adv-GFP-FRNK infected SMCs (left) vs. adv-GFP infected SMCs (right).

DISCUSSION & CONCLUSIONS: FRNK displaces FAK from focal adhesion complexes in vascular smooth muscle cells, and it is readily expressed in SMCs by adenovirus delivery that are grown on/in both 2-D and 3-D culture environments. Substrate specific gene delivery of adv-FRNK may allow down-regulation of FAK-mediated SMC migration, proliferation, and matrix deposition. Cellular activity in 3-D systems is poorly understood, yet these conditions are more similar to the in vivo cellular milieu. This study provides mechanistic and pragmatic proof of concept for the selective manipulation of SMC activity in 3-D matrices.

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ACKNOWLEDGEMENTS: These studies were supported by the NIH [(HPG) HL41272, (AMS) HL34328, (LPB) HL078151, (EMB) HL074594], and the Department of Veteran's Affairs (HPG).

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HUMAN OSTEOGENIC CELLS RESPOND TO ION IRRADIATED POLY-ε-CAPROLACTONE-BASED SURFACE

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INTRODUCTION: A variety of natural and synthetic polymers are under development for filling bone defects. A step to be taken prior to clinical application of engineered materials is the assessment of the osteoconductive potential *in vitro* and *in vivo*. Among compatible polymers suggested for bone engineering poly-ε-caprolactone (PCL), as well as some co-polymers, is receiving considerable attention due to its excellent physical properties, compatibility with tissues, and controlled degradation depending on porosity or co-polymerization.

METHODS: PCL films were deposited on p-doped silicon wafers and some samples were irradiated with low-energy He+ ions. The resulting surfaces were characterized, including surface wettability and roughness by surface free energy measurements and Atomic Force Microscopy, respectively. Human bone marrow was obtained from patients undergoing total hip replacement and isolated by gradient centrifugation. Marrow stromal cells (MSC) were then obtained by adherence on TCPS and expanded in osteoblast-inducing medium. Cells were seeded onto 0.5cm^2 squared PCL samples, with and without irradiation (PCL^{ut} and PCL^{irr}), and analyzed at different time endpoints. Alamar, ALP, collagen, mineralization, as well as fluorescence microscopy and SEM, were employed for detection of bone marrow cell response to bare and irradiated PCL.

RESULTS: At the 1st time endpoint, i.e. 4h, different assays were run to look for early interactions of MSC with the surfaces.

By fluorescence microscopy a higher number of nuclei and an evident spreading of MSC onto PCL^{irr} compared to PCL^{ut} were observed.

Using an image analysis software the views were quantified (table I).

Table I.. Image analysis of MSC (number and spreading) on PCL surfaces at 4 h from seeding.

sample	n° of nuclei	spreading (area fraction)	area (μm^2)	measured area (μm^2)
PCL^{UT}	8 ± 3	~ 0	618.52	6.8 x 10^5
PCL^{IRR}	74 ± 9	0.101	68,481.6	6:8 x 10^5

Cell viability assayed at different time endpoints using Alamar test showed a higher number of MSC on PCL^{irr} (fig. 1).

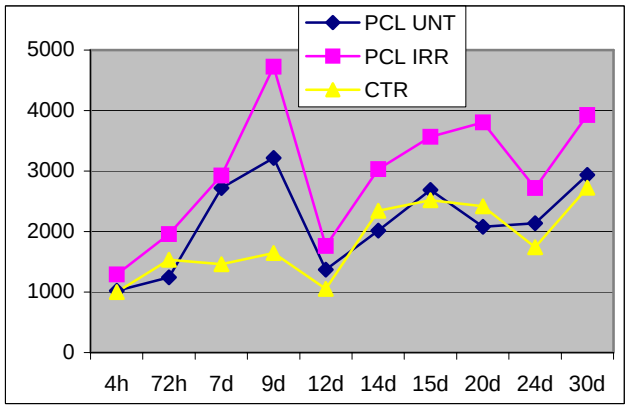

Fig.1 Viability assay of MSC grown on PCL surfaces and TCPS (Alamar blue, RFU units)

At 2 weeks alkaline phosphatase (ALP) was 9.32 mM for MSC on PCL^{irr} compared to 5.69 on PCL^{ut} (control MSC: 14.94). Collagen assay showed that the differentiation of MSC to ECM-forming cells, i.e. osteoblastic cells, was enhanced starting at 2 weeks for PCL^{irr}.

At 4 weeks the MSC were multilayered on PCL surfaces (fig. 2) and start to detach from TCPS.

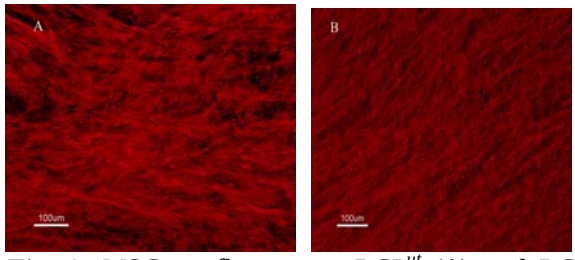

Fig. 2. MSC confluency on PCL^{ut} (A) and PCL^{irr} (B) at 1 month (phalloidin-TRITC)

DISCUSSION & CONCLUSIONS: From the results of biochemical assays, the general trend of cellular activities toward bone deposition is easily recognized for control MSC, while MSC on PCL surfaces show some deviation. Proliferation of MSC on PCL^{irr} is higher than onto PCL^{ut}, and mineral formation also confirms a better behaviour of irradiated PCL surface vs untreated PCL. These *in vitro* results can be correlated to structural features, i.e. wettability and roughness of PCL.

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Quantitative analysis of epithelial cystogenesis revealed serum factors and mechanical restrictions as triggers for lumen initiation.

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INTRODUCTION: Epithelial organs (e.g. lung, kidney) contain a monolayer of polarized cells facing a continuous lumen. The mechanism(s) regulating formation and maintenance of the lumen are currently unclear. Madine-Darby canine kidney (MDCK) cells embedded in collagen-based matrix form self-enclosed monolayers of polarized cells with a central lumen (cysts). Such three-dimensional (3D) cultures are widely used as a model system to study epithelial morphogenesis [1,2].

METHODS: Two stably transfected MDCK strain II cells clones expressing fluorescent protein tags (YMD and GMD cells in the following) were used to prepare 3D cultures as described elsewhere [2]. We characterized individual MDCK cysts' development in 4D by reconstructing the 3D geometry of each cell aggregate from the deconvolved XY-stacks collected at day 1, 4, 7, 10, and 13. From the reconstructed 3D object we obtained a measure of the total aggregate volume and surface. To correlate the mechanical response of collagen gel with lumen formation, we measured the viscoelastic properties of collagen gels using the rheometer Physica MCR 300 (Anton Paar GmbH. Graz, Austria) as described elsewhere [3].

RESULTS: We observed four different stages during cystogenesis (Figure 1): single cell (SC), cell aggregate (CA), cell aggregate with lumen (where more than one layer of cells encloses a single lumen or multiple lumens; CAL), and cyst (a lumen enclosing monolayer). For both cell clones a wide time-range for lumen initiation and cyst formation was observed. Interestingly, all CA to CAL transitions were achieved in a narrow range of total aggregate volume suggesting that either the aggregate volume or a specific cell number in the aggregate trigger lumen initiation. To test the effect of growth factors and mechanical properties of collagen on lumen initiation, we analysed cystogenesis in 3D culture with increased FCS concentration or more rigid collagen preparation.

An increase in matrix rigidity correlates with a slight delay in lumen initiation while a higher percentage of FCS in the medium strongly enhances lumen initiation.

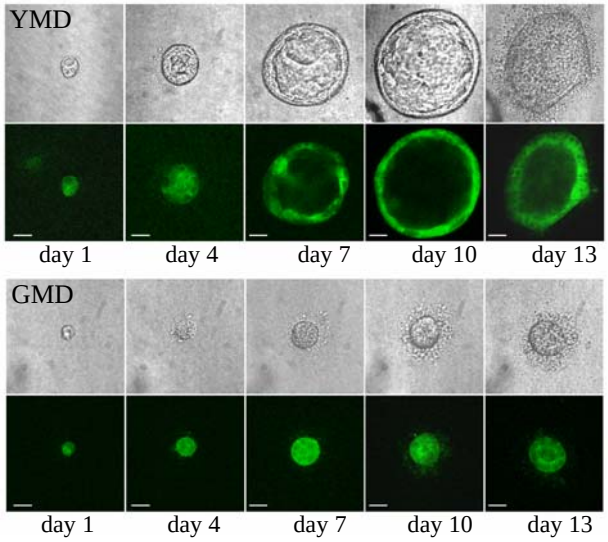

Fig. 1: Middle section of DIC (upper row) and fluorescence (lower row) confocal XY-stacks collected at day 1, 4, 7, 10, and 13 of cystogenesis in representative YMD and GMD evolutions. Bars: 10 µm (YMD at day 1, 4, 7 and GMD), and 20 µm (YMD at day 10, and 13).

DISCUSSION & CONCLUSIONS: Using quantitative analysis of time-lapse confocal microscopy images we derived information about physical conditions under which lumens are initiated. Our data suggest that small cell aggregates initiate lumen in a narrow volume and cell number range that depends on local properties of cell-ECM interactions, nutrients and growth factors [4].

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Neural Progenitor Cell Attachment on IKVAV Functionalized Supported Phospholipid Bilayers

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INTRODUCTION: The ability to control cell behavior on different surfaces is desirable in many biotechnical applications like medical implants and tissue engineering. This control requires knowledge of the cells and how the cells interact with different surfaces. Cell-surface interactions are dependent on cell recognition of ligands, often proteins, peptides or sugars, on a surface, why an appropriate ligand presentation is necessary. One way to present ligands is to immobilize them to a supported lipid bilayer. Bilayers are formed from phospholipid vesicles rupturing on a variety of surfaces, one of which is SiO_2. Bilayers provide a surface inert to cell and protein adhesion, but can be functionalized to expose cell signaling molecules. These molecules can direct different cell functions, for example cell attachment, differentiation or proliferation.

METHODS: All surface modifications were quantified by quarts crystal microbalance with dissipation (QCM-D) and cell attachment were evaluated using immunocytochemistry and fluorescence microscope.

RESULTS: In the present study, maleimido-terminated lipids incorporated in the bilayer, were functionalized with a peptide sequence containing the laminin-derived pentamer IKVAV (CSRARKQAAS**IVKAV**SADR) or a scrambled sequence (CSRARKQAASVKAIVSADR). The IKVAV sequence was found to be specifically recognized by the adult-derived hippocampal progenitor cells (AHP) as proved in comparative studies where cell attachment showed an 11-fold increase to IKVAV functionalized bilayers compared to bilayers functionalized with the scrambled peptide sequence.

Further investigated was cell response to different amounts of IKVAV on the surface, where it was shown that a critical peptide density is required for cell attachment. Surfaces modified subsequently with polyornithine and laminin are standard substrates for AHP cell culture and were used as a reference.

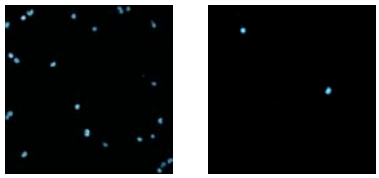

Fig. 1. Cell attachment to a maleimido-bilayer functionalized with IKVAV (left) and VKAIV (right).

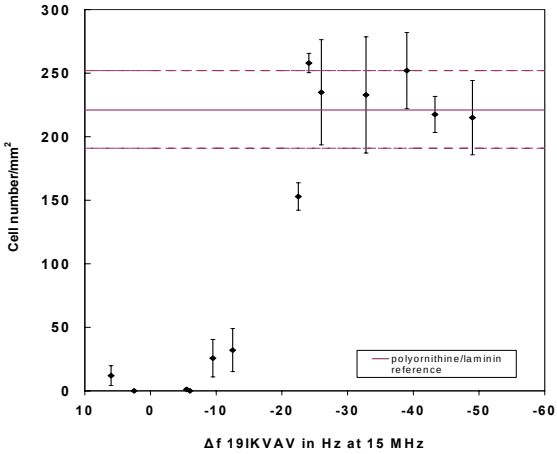

Fig 2. Cell attachment varied with the degree of IKVAV-functionalization and a critical density had to be reached before any cells attach.

DISCUSSION & CONCLUSIONS: Assuming each maleimido-group couples one peptide, the critical IKVAV density for cell attachment is correlated to a maleimido concentration of about 2 mol%. It was also proved that already at a maleimido concentration of 3 mol% cell attachment to IKVAV functionalized maleimido-bilayers was comparable to the control surfaces. The maleimido doped bilayers not presenting any IKVAV showed very low cell attachment, leading us to believe that IKVAV functionalized bilayers are attractive surfaces for stem cell cultivation.

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Selective Surface Modification via Oligoethyleneoxide Derivatives

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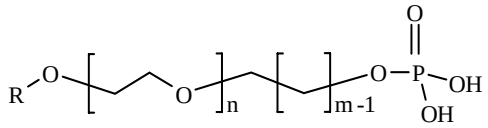
INTRODUCTION: As a flexible method of forming thin and well-defined organic films on a variety of solid surfaces, self-assembly molecules (SAM's) have attracted increased attention in recent years.[1] The use of alkyl phosphorous acids opens a novel route to build molecular thin films on inorganic metal oxide surfaces.[2] Especially, substrate materials showing distinct Brønsted-amphoteric properties, like Ti/TiO_2 or Al/Al_2O_3 seem well-suited for this surface modification procedure. By use of a structured solid surface containing TiO_2 and SiO_2, this kind of molecule will selectively adsorb on TiO_2. When the molecule additionally contains a terminal oligoethylene glycol unit, the adsorbed layers avoid unspecific protein adsorption on the substrate.[3] By further functionalisation of the oligoethylene glycol, specific surface reactions are possible with biomolecules. For these purposes tailor-made ω-functionalised oligo(ethyleneglycol)alkylphos-phates have been synthesised.



R = OCH_3 n = 3, 6 m = 11

R = COOH n = 3 m = 11, 17

Fig. 1: Structure of the ω-functionalised alkane phosphates

METHODS: Specific synthetic pathways have been developed for the synthesis of these novel molecules. The composition and structure were proved by elemental analysis, IR- and NMR spectroscopy.

For the adsorption freshly cleaned substrates (Al/Al_2O_3, Ti/TiO_2) are immersed in a 1 mmol aqueous solution of the molecules. The change of surface properties is determined by contact angle measurements. Orientation of the molecules is studied by angle-dependent XPS. Kinetics of the adsorption process can be followed by SPR.

RESULTS: The synthesis of these ω-functionalised oligo(ethyleneglycol)alkylphosphoric acid esters requires a multi step procedure. The advantage of such kind of molecules is their defined structure and chain length. We synthesised molecules with different chain length and functional groups to investigate their variable behaviour during adsorption and surface reactions. Dynamic contact angle measurements revealed that the surface is homogeneously covered by the monolayers. Compared to the bare substrate contact angle hysteresis strongly decreases indicating smooth surfaces. XPS showed that film quality seriously depended on surface roughness.

DISCUSSION & CONCLUSIONS: Defined bifunctional compounds have been designed and synthesised for surface modification of oxide surfaces to avoid unspecific protein adsorption as well as to improve binding of special proteins. We could prove that monomolecular layers of these compounds were formed on Al_2O_3 and TiO_2 surfaces. The behaviour of these films in the presence of proteins will be tested by means of SPR, XPS or QCM. Because the compounds own defined structures and sizes, variation of these properties will influence the interaction with the proteins.

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INDUCED NEURONAL DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS ON THREE-DIMENSIONAL POLYMER SCAFFOLDS

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INTRODUCTION: Human embryonic stem (hES) cells have the potential to form various cell types. Because neurons have a limited capacity to regenerate, the derivation of neurons from hES cells holds promise to treat neurological pathologies of the central and peripheral nervous system, such as Parkinson's disease, Spinal cord injury, and Glaucoma. However, growth and differentiation of hES cells into complex, viable 3D neural-like tissue is challenging.

It was hypothesized that porous biodegradable polymer scaffolds support the formation of complex 3D tissues during differentiation of hES cells. The scaffold provides physical cues for cell orientation and spreading, and pores provide space for remodelling of tissue structures [1]. The chemical cues to induce and direct neuronal differentiation were hypothesized to be the Neurotrophins [2] and Retinoic Acid [3]. So far, the influence of the Neurotrophins on the differentiation of hES cells on 3D polymer scaffolds has not been described.

METHODS: Cell culture: Human embryonic stem (hES) cells (H9 clone) were grown in an undifferentiated status on mouse embryonic fibroblasts in knockout medium. To induce differentiation, hES cell colonies were dissociated with 1mg/ml collagenase type IV and suspended in differentiation media without lymphocyte inhibitory factor (LIF) and basic Fibroblast growth factor (bFGF). **Scaffold Preparation:** The scaffold consisted of a 50/50 blend of PLGA/ PLLA. The PLGA was selected to degrade quickly (≈3 weeks) to facilitate cellular ingrowth, whereas the PLLA was chosen to provide mechanical stiffness to support 3D structures. The pore size of 250μm-500μm was chosen to facilitate the seeding and ingrowth of cells. The sponges were cut into rectangular pieces of ≈4x4x1mm^3. **hES cell differentiation:** 4- and 9d-old Embryoid bodies (EB) were trypsinized and 0.9x10^6 cells were mixed of a 50% (vol/vol) medium and matrigel. The differentiation medium was supplemented with the Neurotrophins Brain Derived Neurotrophic Factor (BDNF) (20ng/ml), Nerve Growth Factor (NGF) (20ng/ml), Neurotrophin-3 (NT-3) (20ng/ml) each alone and combined with Retinoic Acid (RA) (300ng/ml). For all experiments EBs were grown on the PLLA/PLGA scaffolds for 14d. **Immunohistochemical Staining:** The cell-scaffold constructs were stained with Hematoxylin and Eosin and the primary antibodies Anti-human β_{III}-tubulin (1:500), nestin (1:1000), Cytokeratin-7 (1:25), CD31 (1:20) and SSEA-4.

RESULTS: The results show that neuronal differentiation of hES cells on three-dimensional polymer scaffolds can be directed by Neurotrophins such as BDNF, NGF, NT-3 and RA. The presence of capillary-like networks throughout the scaffolds especially those treated with NGF was shown.

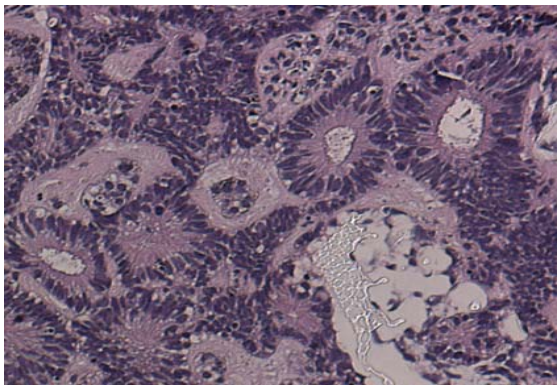

Fig. 1: Neural-like tissue derived from human embryonic stem cells on 3D polymer scaffolds

DISCUSSION & CONCLUSIONS: This approach provides a potential mechanism for creating viable human neural tissue structures for future therapeutic applications in neural pathologies such Parkinson's disease, Spinal cord injury, and Glaucoma.

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TUNABLE PDMS NANOSTRUCTURED SURFACES THROUGH REPLICATION OF NANOSCALE TOPOGRAPHY OF DIBLOCK COPOLYMER MICELLAR THIN FILMS

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INTRODUCTION: This report will focus on creation of nanostructured PDMS surfaces with tunable feature dimensions and periodicities, and the use of these surfaces to study the influence of nanotopography on cell-substrate interactions. The topography on surfaces is known to influence cell-adhesion and growth behaviour.[1] PDMS is attractive as cell-culture substrate owing to its biocompatibility, ease of structuring, flexibility, tunable hardness and transparency. PDMS is recognized as a promising material for making bioactive bandages that assist in wound healing.

METHODS: The nanostructured PDMS surfaces are fabricated by replication of topography offered by poly(styrene-b-2-vinylpyridine),(PS-b-P2VP) micellar thin films on silicon surface. A monolayer of spherical micelles is deposited from o-xylene solution by spin-coating to obtain a quasi-hexagonally ordered array on silicon surface. The tunability of the dimensions of the micelles is achieved by changing the solvent quality, humidity of the environment during film formation, coating conditions and by changing polymer molecular weights. PDMS pre-polymer (SYLGARD 184) is mixed with the curing agent in 10:1 ratio and poured on the micelle coated substrate, cured at 65^{o}C for 3 hours and peeled off. The substrates are then characterized with tapping mode AFM for the topography.

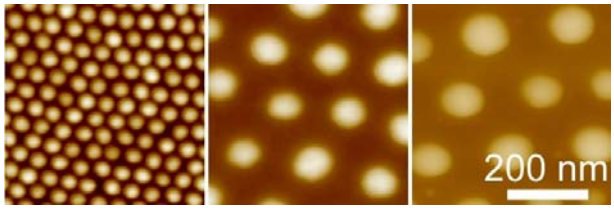

Fig. 1 Tuning micelle dimensions with molecular weight. Molecular weight of the copolymer increases from left to right

RESULTS: The tunability of PS-b-P2VP spherical micelles was achieved in the 30-110 nm range and the periodicity was tuned in the 30-150 nm regimes. Replication of the micellar thin film topography was realized in PDMS forming an array of pits on PDMS surface. The dimension, spacing and depth of the pits were varied by replicating from the appropriate micellar array. Replication of 18nm high micellar topography formed pits that were 7 nm deep in PDMS. Work is in underway to analyse the differences in cell response to the nanostructures.

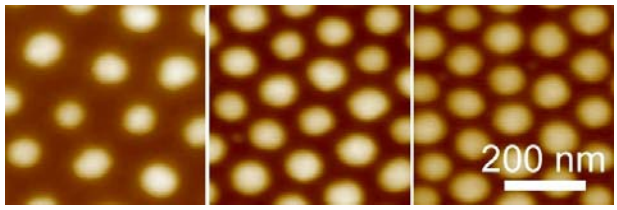

Fig. 2 Tuning micelle periodicity with coating conditions. Left to right the periodicity decreases

DISCUSSION & CONCLUSIONS: We present a continuous tunability of micellar film topography and easy replication of their periodic topography in PDMS. We find a good fidelity of replication in the plane, but poor depth fidelity, apparently due to the surface tension of PDMS. First results with cell culture on nanoscale topographically patterned PDMS substrates are presented.

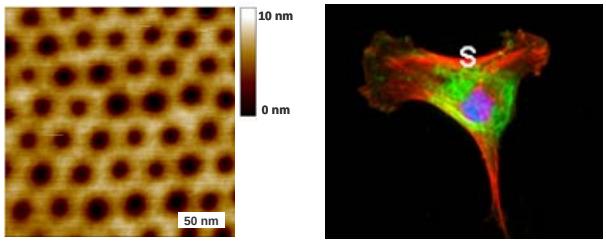

Fig. 3 (left) holes in PDMS obtained by replication of the micellar film topography and (right) an immunofluorescence image of a primary human mesenchymal stem cell grown on a structured PDMS substrate.

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Pattern stability under cell culture conditions – a comparative study of patterning methods based on PLL-*g*-PEG background passivation

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Despite the fast growing number of publications dealing with micro-patterning of cells, little is know about the long term stability of these patterns under cell culture conditions. In the current paper we have investigated the long term stability of cellular patterns created by three different patterning techniques: Selective Molecular Assembly Patterning (SMAP), micro-contact printing (μCP) and Molecular Assembly Patterning by Lift-Off (MAPL). We show that although all three techniques use the same background passivation chemistry – there are considerable differences between their long-term stability under cell culture conditions. Our results suggest that these differences are not cell-dependent but are due to different interactions between the patterned substrate, the passivating molecule and the serum containing cellular medium.

Bacterial adhesion to PLL-*g*-PEG modified surfaces

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INTRODUCTION: Implant associated infections are estimated to cost £7-11 million per year [1], and with the rise in antibiotic resistant bacteria is an important issue [2]. Once adhered many bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *S. epidermidis* form biofilms on the biomaterial surface, which can be difficult to clinically treat since the bacteria are protected from phagocytosis and antibiotics [3], hence the need to prevent initial bacterial adhesion. One approach is to either coat the surface with a PLL-*g*-PEG coating, which is protein resistant [4] and is known to inhibit cell and *S. aureus* adhesion [5-6]; or to coat with an RGD functionalised PLL-*g*-PEG coating, which also minimises protein and *S. aureus* adhesion [5-6] but allows cells to adhere [5]. This study describes the visualisation and quantification of different bacteria to PLL-*g*-PEG (PEG) and PLL-*g*-PEG/RGD (PEG/RGD) coatings on titanium surfaces.

METHODS: To visualize the adhesion of the chosen bacteria (Table 1), the bacteria were cultured on uncoated titanium (Ti), PEG and PEG/RGD coated Ti surfaces for 2h, 4h and 18h at 37°C.

Bacteria	Code
Staphylococcus aureus	SA
Staphylococcus epidermidis	SE
Streptococcus mutans	SM
Pseudomonas aeruginosa	PA

Table 1. List of bacteria used in study.

For SEM study, samples were fixed with 2.5% glutaraldehyde in PIPES buffer for 5 min, post-stained with 1% OsO4 in PIPES for 1h, dehydrated, critical point dried, and coated with Au/Pd, and visualized with an SEM. To quantify the amount of bacteria adhering to the different surfaces, bacteria were cultured as before, then stained with fluorescent redox dye, 5-cyano,2-ditolyl tetrazolium chloride (CTC)5 for 1h, and visualized with a Zeiss Axioplan2 Epifluorescence microscope fitted with an Axiocam camera [6]. The density of adhering live bacteria observed in each image, were counted using KS400 software. Statistical analysis was performed using a one-way ANOVA with Tukey test.

RESULTS: SEM images showed all 4 bacteria strains adhering to the uncoated Ti surfaces, whilst significantly less bacteria were observed on the PEG and PEG/RGD coated surfaces ($p \leq 0.05$) (Fig. 1). This observation was confirmed by the quantification of adherence (Fig. 2).

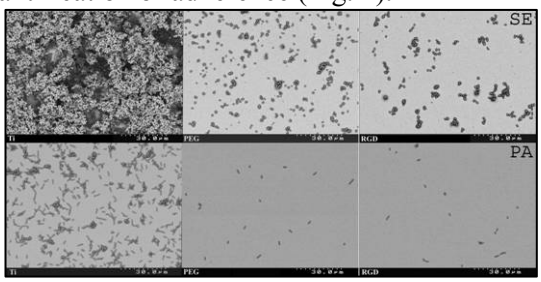
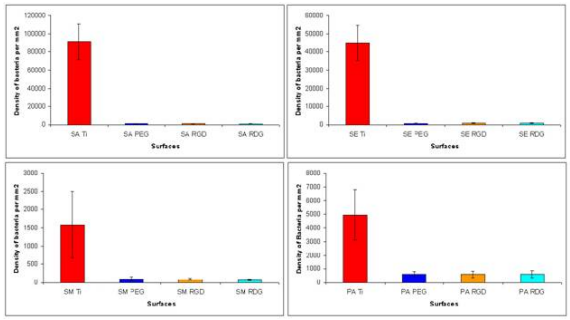


Fig. 1: SEM images of SE andPA on the different surfaces after 18h of culturing.

Fig.2: Graphs showing the effect of PEG and PEG/RGD coatings on SA, SE, SM and PA adhesion.



DISCUSSION & CONCLUSIONS: Coating a Ti surface with PEG or PEG/RGD coating, significantly decreased the adhesion of *S. aureus*, *S. epidermidis*, *Strep mutans*, *and P. aeruginosa* to the surfaces. It was also shown that all four bacteria do not to recognise the eukaryotic cell adhesion moiety RGD.

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DEVELOPMENT AND CHARACTERIZATION OF A NOVEL HIGHLY EXTENDABLE MEMBRANE FOR CELL CULTURE

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INTRODUCTION: Adherent cells tend to change their phenotype in standard culture conditions; this may be partly due to repeated passaging involving enzymes which destroy cell surface proteins. In order to avoid unnecessary passaging, it has been proposed to culture adherent cells on surfaces which can be controllably augmented [1]. By these means it is possible to maintain constant cell density while total cell number increases. However, current commercially available and biocompatible silicone elastomer membranes have limited extendibility of ~50%. In the present study we have developped a highly extendable membrane with a maximum extendibility of ~1000%. We further demonstrate that this novel surface is biocompatible, promotes cell attachment and proliferation and can be provided with a microtopology.

METHODS: The silicone elastomer A-221 (Factor II, Inc., USA) was selected as cell culture substrate for its transparency, nontoxicity, and capacity for elongation near 1000%. A-221 is supplied as a kit with two parts which were mixed in a 1:1 ratio by weight. After mixing and degassing under vacuum for 5 minutes, the mixture was poured onto flat open-faced molds, then cured at 100°C for 48 hours to produce elastomer membranes 0.5 mm thick. The mold with microstructure was fabricated on silicon and photoresist, we employed Bosch process[2] technique (Fig1).

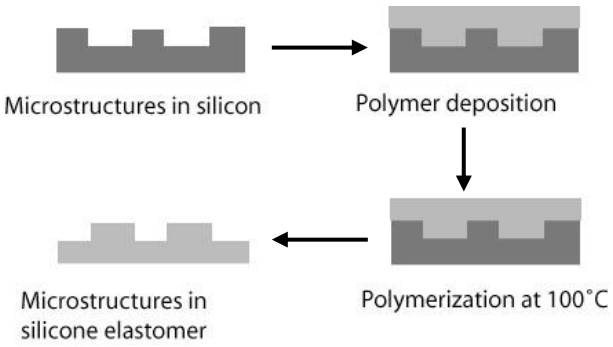

Fig. 1: Principle of membrane microstructuring

Elastomer culture surfaces were then exposured to an oxygen plasma (Tegal) for 45 s at 50 W under 300mTorr. Pilot experiments indicated that the resulting surface chemical activation improved cell attachment if membranes were stored in deionized water after plasma exposure and prior to use in experiments.

Rat lung fibroblasts were seeded onto standard cell culture petri dishes and onto non-structured elastomer membranes at an initial density of 5,200 cells/cm^2. Cell growth was assessed after 1-10 days without passaging and without membrane extension.

RESULTS: Fibroblast morphology on highly extendable transparent elastomer membranes appeared identical to that on standard petri dishes throughout the culture period. Cell attachment and proliferation on non-surface treated elastomer membranes was satisfactory (although apparently somewhat reduced) in comparison to standard cell culture plastic (Fig 2a). With photolithography and etching technology we can develop different structure and design on silicone elastomer surface (Fig 2b).

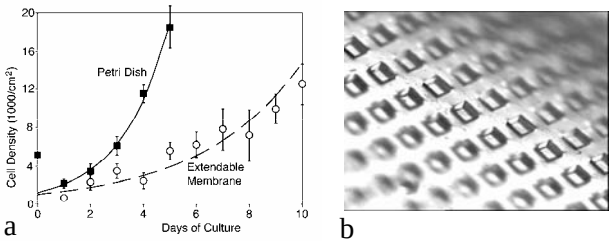

Fig. 2: a) Evolution of cell density on petri dishes versus non-structured extendable membranes (mean ± sem, n=3)
b) Membrane with micro squares of 100µm x 100µm and 50µm depth

DISCUSSION & CONCLUSIONS: With no surface treatment, cell attachment and proliferation on highly extendable elastomer membranes was somewhat lower than on standard cell culture plastic, but nevertheless adequate for future experiments designed to explore effects of substrate stretch. In future we will exploit the combined use of microstructured surfaces topology with elongation to influence cell density and proliferation. Methods for improvement of cell attachment on silicone elastomer surfaces are currently under development.

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Aligned Carbon Nanofiber Materials Direct Neurite Orientation

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INTRODUCTION: Neural materials have experienced reduced effectiveness when implanted for long time periods due to poor interactions between the biomaterial surface and the local neuronal cell populations. The reduced efficacy of these implants is largely due to glial scar tissue formation produced by astrocytes. It is desirable to design implants that retain functionality, but mimic select properties (for instance, dimension) of native tissue in order to reduce chronic implant difficulties such as glial scar tissue formation around implant surfaces. This reduced scar tissue formation would improve the interface between neural biomaterials (for example, electrodes) and neurons. Carbon nanofibers can be formulated to mimic the nanoscale dimensions of macromolecules in the brain (such as proteins) and have unique material properties including high conductivity and high strength to weight ratios. These properties make carbon nanofibers attractive candidates for neural biomaterial applications. Recent results indicated reduced astrocyte functions in vitro [1] and may therefore lessen scar tissue formation in vivo while at the same time enhancing neuronal cell interactions. Further promising neuronal responses could be obtained if neuronal cell orientation (especially neurite and axon extension) could be controlled by carbon nanofiber directionality as it has been in grooved materials [2,3].

METHODS: For this purpose, we describe the use of polycarbonate urethane (PCU) and aligned carbon nanofiber composites for neural applications. Specifically, this study aligned 60 nanometer (diameter) carbon fibers in PCU matrices by placing them between parallel electrodes and applying a homogeneous electrical field immediately before the polymer hardened. To determine neuron responses, pheochromocytoma cells (PC-12 cells) were seeded onto the aligned carbon nanofiber substrates coated with laminin and were cultured for 11 days in standard conditions.

RESULTS: In Fig. 1A and 1B, the blue color highlights the aligned carbon nanofibers with the yellow arrow indicating their general orientation in PCU. Most importantly, results of this study showed that the aligned carbon nanofibers guided the general direction of neurite growth from neuronal cells (in green).

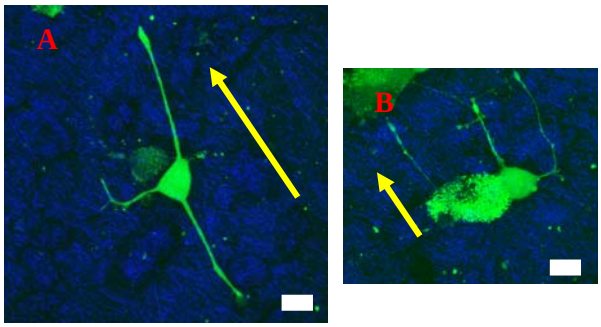

Fig. 1: Neurite alignment in neurons parallel to carbon nanofiber orientation in PCU composites. Panels A and B show confocal images of PC-12 cells (green) on aligned carbon nanofibers (blue) with neurite extension parallel to the general direction of carbon nanofiber alignment in PCU. Scale bars are 20 μm.

DISCUSSION & CONCLUSIONS: These results suggest the possibility of using aligned carbon nanofiber materials to control neurite and axon extension for various central and peripheral nervous system applications including neural networks [4], electrodes, neural tissue engineering bridges [5], and neural probes. Collectively, this series of studies highlights the promise carbon nanofibers have to reduce scar tissue formation at a neural implant interface while at the same time increasing interactions with neurons for guided control over neurite and axon orientation to improve various neural applications.

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ACKNOWLEDGEMENTS: We wish to thank Applied Sciences, Inc./Pyrograf Products, Inc. for the carbon nanofibers. Experiments were supported by an NSF Integrative Graduate Education and Research Training Program Therapeutic and Diagnostic Devices Grant, Grant DGE-97-72770.

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GENE INDUCTION BY CYCLIC SURFACE STRAIN: ROLE OF ACTIN CONTRACTILITY

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INTRODUCTION: When cells are attached to an elastic surface, strain is transmitted to the cells via their extracellular matrix (ECM) adhesion sites. The resulting mechanical stress triggers intracellular signals which change the pattern of gene expression. We have shown previously[1,2] that in chick fibroblasts cyclic tensile strain results in a twofold increase in the mRNA level for the ECM protein, tenascin-C, within six hours. This response is not affected by inhibitors for mitogen activated protein kinase (MAPK) pathways, but strongly attenuated by Y27632, a specific inhibitor for Rho-dependent kinase (ROCK)[2]. Here we explored the function of the actin cytoskeleton and of RhoA/ROCK-controlled contractility in the induction of tenascin-C by cyclic tensile strain.

METHODS: Chick embryo fibroblasts were cultured on fibronectin coated silicone membranes and subjected to equibiaxial cyclic strain (10%, 0.3Hz) by means of a custom made device[2]. Activators (lysophosphatidic acid [LPA], thrombin, colchicin) or inhibitors (Y27632, latrunculin A) of RhoA/ROCK mediated actin contraction were added 30 min prior to the application of cyclic strain for 6 hours. Tenascin-C and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNAs were quantified from Northern blots.

RESULTS: We found that chemical activation of RhoA/ROCK by thrombin[3] (Fig.1) or LPA is sufficient to double the amount of tenascin-C mRNA in resting fibroblasts within 6 hours. When cells were pretreated with these drugs, cyclic strain (6h) caused a super-induction (3.5-fold) of the tenascin-C mRNA level (Fig.1); the additional increase was suppressed by ROCK inhibitor Y27632. Microtubule disruption, which is known to trigger ROCK-dependent actin contraction, also induced tenascin-C mRNA[1]. Conversely, disorganization of the actin cytoskeleton with latrunculin-A completely abolished induction of tenascin-C mRNA by either chemical RhoA/ROCK activators, mechanical stress, or both (Fig.1). Cyclic strain itself increased the amount of active RhoA in fibroblasts after 5 min as measured by a pulldown assay, and within 30 min triggered ROCK-dependent contraction of a collagen gel layer by the cells. Moreover, myosin II activity was shown to be required for tenascin-C induction by mechanical stress.

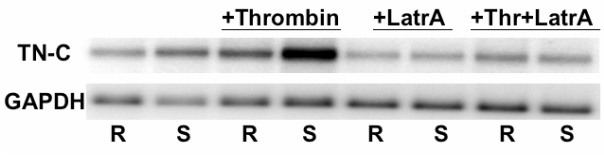

Fig. 1: Northern blot with RNA from chick embryo fibroblasts seeded on a silicone membrane and either left at rest (R) or subjected to cyclic strain (10%, 0.3 Hz) for six hours (S). The blot was hybridized with radioactively labeled chick cDNA probes complementary to tenascin-C (TN-C) mRNA, and to GAPDH mRNA for control. Note that thrombin (Thr, 1U/ml) increased the tenascin-C mRNA in cells at rest, and synergistically enhanced its induction by cyclic strain. Latrunculin-A (LatrA) abolished the cyclic strain-dependent up-regulation of tenascin-C mRNA, as well as its thrombin-mediated super-induction.

DISCUSSION & CONCLUSIONS: From our results, we conclude that RhoA/ROCK-mediated contractility of the actin cytoskeleton has a mechanosensory function in fibroblasts that relates directly to the expression level of the tenascin-C gene. Furthermore, we suggest that prior activation of actin contraction, by either chemical or mechanical signals, renders fibroblasts more sensitive to further external mechanical stress. This principle might be important in connective tissue regeneration and wound healing.

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Polymerized peptide-amphiphile microstructures for cell-based microsystems

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INTRODUCTION: Biomimetic interfaces that allow interactions with biological cells in a specific desired way are forming a core area of research in bioengineered materials[1]. Cell-based microsystems are becoming increasingly important in diagnostics and therapeutics. We have recently described the synthesis of polymerizable peptide-amphiphiles that can be used to form planar as well as 3-D architechtures[2, 3]. The well known "RGD" peptide linked to a polymerized diacetylenic lipid monolayer is shown to promote cell-adhesion in a controlled way. These biologically active structures are stable, and can be organized on conventional solid substrates. We also introduce a design principle for the development of cell-based microarrays by using surface-immobilized polymerized vesicles that expose a bioactive ligand at the outer surface.

METHODS: Peptide synthesis was done on solid-phase using standard F-moc chemistry protocols. Peptide amphiphiles were purified on RP-HPLC and characterized by ESI-MS, NMR and FTIR. Self-assembled Langmuir monolayers were polymerized on the water-air interface and transferred subsequently to a substrate, which is coated with a surface-attached polymer gel. The monolayer was fixed to the polymer by photochemical[4] means [Fig. 1A]. These monolayers were studied with respect to the phase-behaviour and topography using LBK techniques, BAM and AFM, respectively. Vesicles were prepared in water and studied by UV-Vis spectroscopy, FTIR and TEM. Microarrays were prepared by microcontact printing of the polymerized vesicles on a cell-inhibiting hydrogel with a subsequent photochemical immobilization of the polymerised vesicles.

RESULTS: Several parameters have been mapped out with respect to the phase-behaviour and polymerization in order to produce highly stable bioactive surfaces. The surface concentration of the polymerized peptide-amphiphile monolayer was controlled by addition of an analogous polymerizable amphiphile that carried no peptide head-group. The monolayers having various amounts of peptide exposed at the surface were found to influence the adhesion of human microvasculature endothelial cells in a concentration dependent manner [Fig. 1B].

Polymerized vesicles were prepared using the same peptide-amphiphiles mixtures. The polymerized vesicles were printed on a polymer hydrogel that does not promote cell-adhesion, and subsequently, the vesicles are covalently linked to the underlying surface by photochemical means. The microarrays were characterized with respect to the surface-morphology, immobilization efficiency, and the possibility to direct the cell-adhesion in a spatially controlled manner.

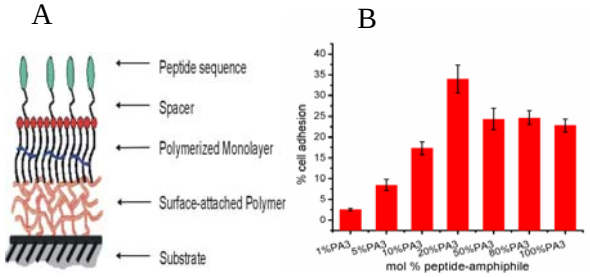

Fig. 1:[A] Scheme of a polymer supported peptide amphiphile monolayer,[B] Amount of adherent cells as a function of the molar concentration of ligand in the peptide-amphiphile monolayers.

DISCUSSION & CONCLUSIONS: 2D and 3D architectures composed of polymerized peptide-amphiphiles have been prepared. Supported polymerized monolayers show enhanced stability and promote the adhesion of microvasculature endothelial cells in a concentration dependent manner. Polymerized vesicles can be used to design microarrays that consist of cell-adhesion promoting areas (i.e. spots) surrounded by a hydrogel layer that does not support the attachment of cells.

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Surfactant-like peptides: Supramolecular assembly and peptide-membrane interaction to engineer novel drug delivery systems

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In the last few years, significant advances have been made in the use of peptides as building blocks to produce biological materials for a wide range of applications [1]. Thus, synthesis and characterization of short peptides that can adopt a stable three-dimensional structure in solution are crucial for the construction of bio-active and bio-inspired *de-novo* polypeptides.

Selected peptides and hybrid peptides (combinations of peptide sequences with organic moieties in a single molecule) self-assemble in a variety of motifs to form pores, channels and tubules. Various physiological functions, such as ion transport through cell membranes, and physical functions such as solubilizing difficult-to-dissolve molecules, are facilitated by the tubes that are formed by molecular assemblies [2]. Peptide nanotubes are constructed by highly convergent non-covalent processes by which cyclic peptides rapidly self-assemble into well-ordered three-dimensional structures when triggered appropriately by a chemical or by the medium. It is well known that the secondary structures are part of a larger system and that their conformational stability depends on non-covalent interactions, both intra- and inter-chain interactions, such as van der Waals', electrostatic and hydrophobic forces as well as hydrogen bonds [3].

Peptide-lipid membrane interactions play a critical role in the regulation of several biological phenomena, including the insertion and folding of membrane proteins, the translocation of polypeptides through membranes and the cytolytic action of antimicrobial peptides. However, a systematic study of the interaction of different peptide assemblies with membranes is still missing [4].

The aim of the present study is the systematic design and synthesis of cationic surfactant-like peptides, the structural characterization of spontaneously formed supramolecular entities, and the evaluation of their interactions with supported bilayers serving as a model for cell membranes. The development of such biologically inspired delivery vehicles should fulfill several criteria that are important for the envisaged application such as biodegradability, cell-membrane transit promotion and lack of cytotoxicity.

To assess the approximate size of the peptide assemblies, dynamic light scattering measurement as well as transmission electron microscopy (TEM) will be used to study the supramolecular assembly of the samples in solution as a function of concentration and pH, complemented by morphological studies of peptide assemblies at surfaces based on atomic force microscopy.

Molecular mechanics calculations are performed to simulate the assembly of selected peptide sequences in aqueous media, and the comparison of such results with those experimentally obtained is also discussed.

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STIMULI-RESPONSIVE NANO-STRUCTURES OF PH- SENSITIVE GRADIENT COPOLYMERS

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A novel type of amphiphilic ionic copolymers comprising hydrophilic poly(acrylic acid)-gradient-poly(acrylic acid)/poly(styrene) blocks has been synthesized by direct Nitroxide- Mediated Polymerization (NMP). The aggregation behaviour of the copolymers in aqueous medium have been studied by small angle neutron scattering (SANS), fluorescence spectroscopy, static light scattering (SLS) in a wide range of pH and salinity and compared to that of the homologous diblock copolymers.

We have demonstrated that the solution properties and aggregation behavior of the gradient copolymers are markedly different from those of the diblock copolymers. In particular, the gradient copolymers are directly soluble in water at room temperature without special experimental procedure. The SANS spectra for the copolymer solution exhibit a correlation peak confirming the formation of micelles with a repulsive corona. Our results indicate, that micelles of the amphiphilic gradient copolymers exhibit strong pHresponsive properties. In contrast to "frozen" aggregates of the homologous diblock copolymers, these micelles are capable to rearrange the aggregation state (aggregation number) and dimensions as a triggered response to variation of the environmental pH or salinity. This property determines potential application of the water-soluble amphiphilic gradient copolymers for design of the nano-scale delivery systems for low molecular weight drugs and biological molecules (proteins, nucleic acids).

Morphological and aggregation behavior of PEG-PPS block copolymers

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INTRODUCTION: Block copolymers are composed of covalently bound hydrophilic and hydrophobic polymer chains. Due to their amphiphilic character when put in a specific solvent they are able to associate and form a wide variety of aggregates[1]. In this study we characterize the relationship between block copolymer composition and kind of aggregate formed in water by cryo TEM[2] technique and by measuring the critical aggregation concentration (cac) of a series of poly(ethylene glycol-*bl*-propylene sulfide) (PEG-PPS) block copolymers. Depending on the block ratios, nanoparticle, spherical micelles, rods, or unilamellar vesicles similar to liposomes can be formed. Our goal is to be able to optimize the design of these materials for use in the diagnosis and treatment of disease.

METHODS: The synthesis of the PEG-PPS di-block copolymer used in this study was performed as shown in the literature.[2] The polymers aggregates were prepared in two ways, first, preparing a concentrated solution of the polymer in THF then diluting few µl of it in water, second, using the standard thin film hydration technique followed by extrusion through a 200nm membrane. The resultant aggregates were then visualized using the cryo-TEM technique.[3] For the cmc measurements we used the pyrene fluorescence technique as described elsewhere[3]. The results are reported respect to the hydrophilic fraction (f_{PEG}) and respect to the hydrophobic block, (nPPS).

RESULTS: Different aggregates are formed by increasing the nPPS of the polymers, showing also lower cac (easier aggregation) for more hydrophobic polymers. This follows the well established pattern already studied for similar block copolymer systems, Pluronics (PEO-PPO).[4] The aggregation is in both cases driven by the hydrophobic effect. Depending on the specific polymer, different or same kind of aggregates could be formed in water, from either of the two ways used to form the aggregates, indirectly from THF or directly from simple hydration. Analysis of a specific triblock copolymer showed that the aggregates formed from THF are thermodynamically stable, while those formed from water are kinetically trapped.[4]

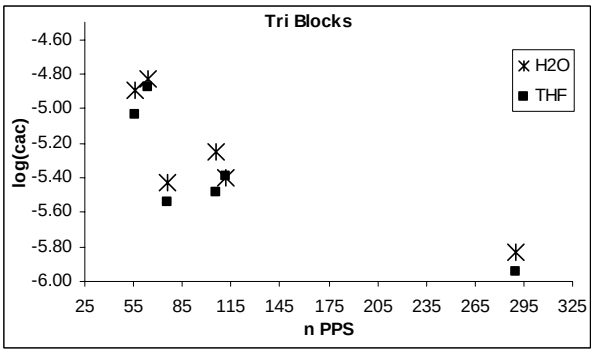

Figure 1 cac of a series of PEG-PPS triblock copolymers calculated by fluorescence technique. An increase in the hydrophobic block makes the cac decrease.

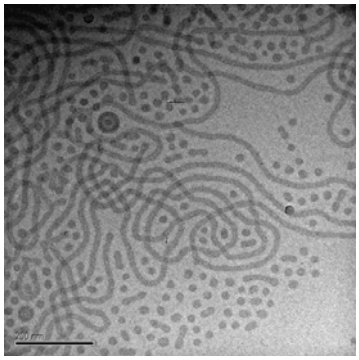

Figure 2 Example of aggregates formed by the E45S76E45 triblock copolymer at 1wt% in water

DISCUSSION & CONCLUSIONS: The study of the relationship between *f*PEG, MW, hydrophobicity and the different aggregates formed in water, are of key importance for the rational design of block copolymers for different applications. One of the examples of particular interest is the vesicular structure, which could be useful in the drug delivery field.

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Supramolecular assembly of amphiphilic peptides: characterization and preliminary studies of peptide- cell interactions

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Surfactant-like peptides (peptide sequences with both hydrophobic and hydrophilic moieties in a single molecule) self-assemble in a variety of motifs to form pores, channels and tubules. Ion transport through cell membranes, and physical functions such as solubilizing difficult-to-dissolve molecules, are facilitated by the tubes that are formed by molecular assemblies [1]. Peptide nanostructures are constructed by highly convergent non-covalent processes, both intra- and inter-chain interactions, such as van der Waals', electrostatic and hydrophobic forces as well as hydrogen bonds [2]. In this study the supramolecular assembly of four different amphiphilic peptides is studied and preliminary results on peptide-cell interactions shown.

Transmission electron microscopy (TEM) is used to study the supramolecular assembly of the samples in solution, complemented by conformational analysis as a function of concentration and pH- achieved by circular dichroism, and morphological studies of peptide assemblies based on atomic force microscopy (AFM).

Peptide- cells interactions are studied by Fluorescence microscopy, while molecular mechanics calculations are performed to simulate the assembly of selected peptide sequences in aqueous media, and the comparison of such results with those experimentally obtained is also discussed.

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Polyelectrolyte Multilayer- Based Gene Delivery Systems

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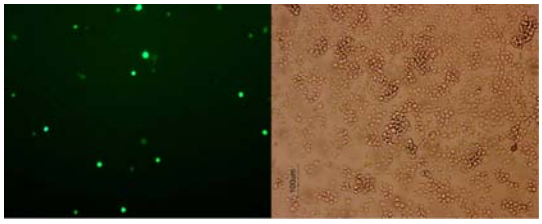
INTRODUCTION: Polyelectrolyte multilayers (PEM) are highly efficient adsorption substrates for charged particles [**1- 3**]. Here, the ability of PEM to deliver positively charged polyplexes has been tested on non adherent monocytic THP-1cells and adherent NIH3T3 fibroblast cells with gene transfer systems based on the adsorption of plasmid DNA/poly(ethylenimine) (PEI) complexes on (poly-L-lysine/ poly-glutamic acid)$_5$ and (chitosan/hyaluronan)$_{10}$ multilayers, (PLL/PGA)$_5$ and (CHI/HA)$_{10}$, respectively.

METHODS: PEM were built by alternated immersion of glass coverslips during 10 min, in presence of 0.15 M NaCl, in polyelectrolyte solutions (300 µL) at the concentration of 1 mg/mL for PLL, PGA and HA and 0.5 mg/mL for CHI at pH=7.4 for PLL/PGA and pH=5 for CHI/HA. After each deposition the coverslips were rinsed three times during 10 min with 0.15 M NaCl. 22 KDa linear PEI and plasmid DNA (pEGFP-C1) were mixed at various nitrogen/ phosphate (N/P) ratios to produce PEI/DNA complexes. Positively charged polyplexes (1µg of DNA in 300µL) were adsorbed on polyanion-ending architectures for 1 hour. Cells were washed with PBS and seeded on architectures at a density of 5.10^5 in complete medium (1mL). The effect of the N/P ratio on transfection efficiency was observed by fluorescence microscopy. The transfection rate, expressed as the ratio of EGFP positive cells and the total number of cells, was analyzed by flow cytometry. Dynamic light scattering as well as electrophoretic mobility measurements were performed on a zetasizer 3000 HS device (Malvern Instruments, UK) to determine the size and charge of the complexes.

RESULTS: The size of the complexes resulting from N/P ratios of 5 to 12 averaged between 897.6 ± 79.8 nm-678.6 ± 78.1 nm with a polydispersity of 0.2 - 0.086, and their zeta potential ranged between +6.8 mV and +16.2 mV, respectively. The most favorable N/P ratio for in vitro transfection, evaluated by fluorescence microscopy, was N/P 12 (Fig.1). Transfection rates of THP-1 and NIH3T3 cells determined with (PEI/pEGFP-C1) complexes from (PLL/PGA)$_5$ and (CHI/HA)$_{10}$ PEM after 24 hours were 1.97 ± 0.63 % and 5.26 ± 0.45 % respectively for the N/P ratio of 12. In order to quantify the amount of pDNA initially bound to the multilayer, and analyze its eventual release with time, we built multilayers functionalized with PEI/ FITC-labeled pDNA complexes and measured the fluorescence resulting from the dissociation of the multilayers in presence of 5M NaCl after different periods of incubation with the transfection medium. The complete dissolution in presence of 5M NaCl was checked by ellipsometry and the amount of fluorescein-labeled pDNA measured by fluorometry. The initial amount of pDNA bound to PLL/PGA and CHI/HA PEM was about 50 and 65 ng (25-30 ng/cm$^{2)}$ respectively. No significant DNA release from PLL/PGA architectures was observed with time over a 6 day-period of incubation with the culture medium, indicating that DNA availability for cells was not mainly through passive release but due to complexes uptake from the surface by cells. As for functionalized CHI/HA PEM, about 50% of the initially bound pDNA was passively released from (CHI/HA)$_{10}$ after 48 h, indicating that the stability of pDNA on CHI/HA PEM was lower than on PLL/PGA PEM.

Fig.1: THP-1 cells transfection after contact for 24h with (PLL/PGA)$_5$ (PEI/pEGFP-C1) architectures (left).



Control photograph showing cell density (right).

DISCUSSION & CONCLUSIONS: Gene transfer from PLL/PGA and CHI/HA architectures might be quite efficient in absence of any additional auxiliary cell transfection agent, suggesting the generality of the system. Depending on the nature of constitutive polyelectrolytes, DNA delivery mode might be different.

Polyplexes-functionalized PEM can act as efficient gene delivery tools from surfaces.

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Biocompatible colloidal glass formation in response to temperature

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INTRODUCTION: We here present a sol-gel transition in response to temperature, observed for concentrated aqueous dispersions of polymeric nanoparticles (NPs). The possibility of encapsulating drugs in the NPs, combined with a demonstrated lack of toxicity allow this novel physical gelling system to be considered for a number of drug delivery applications.

METHODS: Nanoparticles composed of cross-linked PEG and Pluronic® were prepared via inverse emulsion polymerization as previously described(*1*). Their structure enables the encapsulation of hydrophobic drugs, as was shown using doxorubicin, an anti-proliferative agent.

Rheometry was used to investigate the nature of the thermal hardening of NP dispersions. Temperature scans revealed the characteristic gelation temperature whereas frequency and stress sweeps provided insight on the transition mechanism.

Dissolution kinetics of the structured colloid material were compared to Pluronic® physical gels.

Cytotoxicity was assessed by performing the gelation process on top of cultured HeLa cells and comparing their viability and morphology to controls.

RESULTS: Stable, hydrogel NPs with an average diameter of 100nm were used in this study. Pluronic self-assembled structure was preserved during polymerization, providing the colloids with hydrophobic drug loading capabilities and thermally-responsive character.

A gradual hardening of the material was observed with increasing temperature above a concentration threshold (~4% wt.); the cross-over point of elastic and viscous modulus was at 27°C for a 15% dispersion, slightly increasing with lower NP concentrations. The gel-like behavior of the material was reversed at very low frequencies and high shear stresses: a high relaxation time and the presence of yield stress suggest that the solidification is better described as a structural and dynamic arrest of close-packed elastic spheres. At low temperature, hydration of NPs results in a transformation to soft, deformable particles able to flow (Fig. 1).

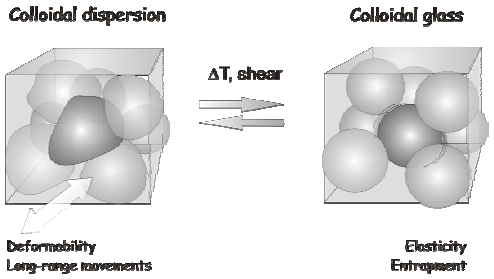

Fig. 1: Schematic representation of thermally-induced colloidal glass formation

Upon exposure to dilution, as would be the case in *in vivo* applications, the close-packed structure dissolves; however kinetics are much slower than Pluronic® physical gels and do not affect the release kinetics of encapsulated drugs.

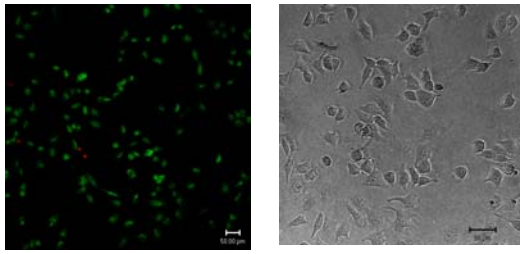

Fig.2. Viability (left; green:live, red:dead) and morphology (right) of HeLa cells under an in situ formed colloidal glass. Results are similar to controls

DISCUSSION & CONCLUSIONS: The physical 'gelation' scheme presented here is based on an intra-particular transition event in response to temperature. The mild and biocompatible character of this material make it an ideal candidate for drug delivery applications, for example through *in situ* forming injectable formulations or as surface coatings of biomedical devices.

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From Virus- to Nonvirus-based Technologies to Improve Endogenous Wound Healing Capacities by Local Gene Therapy

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INTRODUCTION: Deficient angiogenesis is a major clinical incidence and affects wound healing in elderly persons, diabetes- and bed-ridden patients. Our study aims to locally increase blood perfusion and improve the endogenous wound healing capacity with the introduction and the expression of necessary components by both virus- and nonvirus-based gene delivery systems. Although increased angiogenesis was observed in the chicken chorioallantoic membrane (CAM) by a lentiviral-based system [1], we aim to develop a novel nonviral-based gene delivery system [2], since nonviral transfection systems are safer and less immunogenic for the patients.

METHODS: Lentivirus-derived particles expressing vascular endothelial growth factor ($VEGF_{121}$) were produced and applied onto chicken embryo's CAM as described [3]. On embryonic day 12 the CAMs were examined by *in vivo* fluorescence microscopy following intravenous injection of 100µl 2.5% FITC.

RESULTS: To evaluate lentiviral-induced angiogenesis *in vivo*, we transduced the CAMs using $VEGF_{121}$-expressing lentiviral particles. SEAP-expressing particles were used as a control.

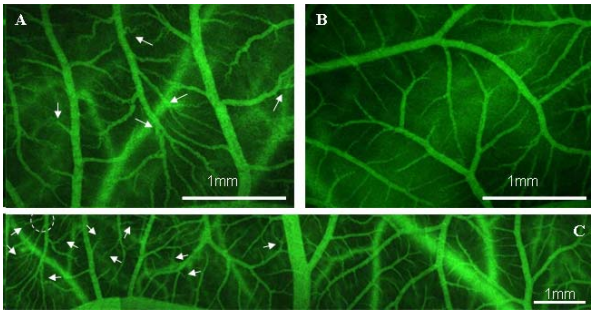


Fig. 1: In vivo angiogenic response in CAMs after transduction of $VEGF_{121}$- (A and C) and control SEAP-expressing lentiviral particles (B).

Microscopic analysis and morphometric analysis of $VEGF_{121}$-mediated neovascularization and vessel morphology showed a correlation between use of $VEGF_{121}$-expressing lentiviral particles and angiogenesis: a general increase in blood vessel number, atypical endpoint patterns (Fig. 2), irregular tortuous vessel shape and multiple vessel branching are present when $VEGF_{121}$ was expressed. $VEGF_{121}$-induced effects were confined to a 4mm radius around the site of application and could not be observed on the same CAM beyond this perimeter (Fig. 1A, C). On the contrary after application of SEAP-expressing particles (Fig. 1B), the blood vessel pattern remain unaltered.

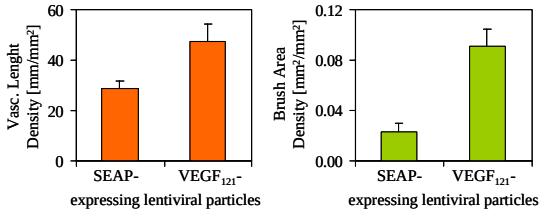
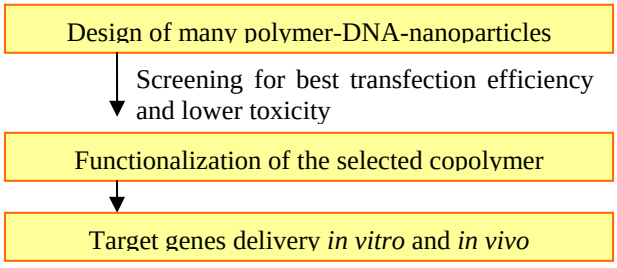


Fig. 2: Morphometric analysis of mean vascular length density and brush-like area density in CAMs transduced with $VEGF_{121}$- or SEAP-expressing lentiviral particles.

DISCUSSION & CONCLUSIONS: Although angiogenetic response was observed with a lentiviral-based gene delivery system, the severe security problems still associated with these vectors drove our study towards the development of nonviral gene transfer vehicles. Our project can be summarized in this schema:



Target genes to be delivered induce stimulation of endogenous wound healing capabilities by the expression of relevant growth- or transcription factors such as $VEGF_{121}$. Local administration of such a DNA delivery system may represent a powerful tool in tissue engineering and therapeutic angiogenesis where such matrices should be analyzed for delayed wound healing, induced by underlying microvascular pathology, as in case of diabetes.

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Enhanced Bone Regeneration by a Novel Bioactive PLGA Membrane

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INTRODUCTION: In guided bone regeneration, a membrane is implanted to create a compartment that can easily be occupied by bone. In order to avoid a second surgery required for its removal, a bioresorbable poly (lactide- co - glycolide) membrane was developed. The intrinsic rigidity of the polymeric material was overcome by using the plasticizer NMP (N-methyl-2-pyrrolidone), which has recently been shown to be bioactive, as it enhances osteoblastic maturation *in vitro* as well as bone regeneration *in vivo* [1].

METHODS: MC3T3-E1 pre-osteoblastic cells seeded onto TCPS plates were tested for early and late maturation responses: ALP (Alkaline phosphatase activity) and Alizarin Red mineralization assay respectively. Also, quantitative real time RT-PCR for specific osteoblastic markers (Osteocalcin-OCN, Bone Sialoprotein-BSP) was assessed after stimulating the cells for 6 days. *In vivo*, rigid membranes without NMP, flexible ones containing NMP, the commonly used Osseoquest membranes, as well as empty defects for control samples (n=6 for each case), were implanted into non-critical size defects on a rabbit calvarial model. 4 weeks after implantation, bone regeneration was analyzed both by qualitative and quantitative methods including X-ray analysis, histology and histomorphometry.

RESULTS: The combination of rhBMP-2 and N-methyl-2-pyrrolidone (NMP) treatment on MC3T3-E1 pre-osteoblastic cells seeded onto TCPS, promotes both their differentiation into mature osteoblasts (ALP activity), the formation of mineral-like nodules (Alizarin red assay), as well as highest expression of mature osteogenic markers (OCN, BSP) via qRT-PCR.

In vivo, the polymeric NMP-containing membranes successfully filled the defect (80%) with newly formed bone, while control samples failed to bridge the bone defect. Both PLGA rigid membranes and Osseoquest filled up to 60% of the initial defect area.

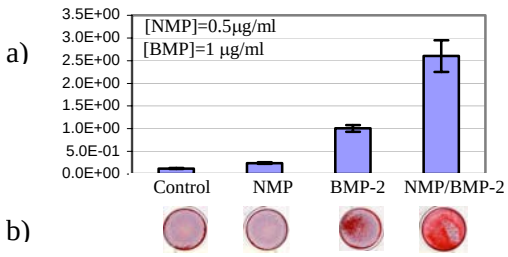

Fig.1. Effect of NMP , rhBMP-2 and NMP/ rhBMP-2 on a) ALP activity after 1 week and b) mineralization by Alizarin Red staining after 4 weeks of MC3T3-E1 cells cultured on TCPS in medium supplemented with ascorbic acid and β-glycerol phosphate.

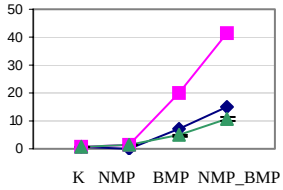

Fig.2. BSP fold increase of the different treatments normalized to control cells.

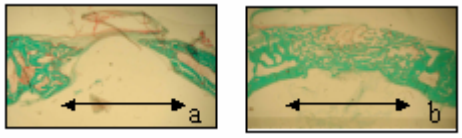

Fig.3. Histological in vivo results from empty defect (a) Vs membrane loaded with NMP (b). Bone stains dark green whereas fibrous tissue light green. Arrow indicates initial 6mm width defect.

DISCUSSION & CONCLUSIONS: Both *in vivo* as well as *in vitro* results demonstrate that the guided bone regeneration membrane and its NMP bioactive principle is based on a synergistical interaction with BMP which, in turn, enhances bone formation. Other approaches including *noggin* (BMP-inhibitor), MALDI and a Biacore membrane confirmed this interaction (results not included).

The significance of this study lays on a possible reduction of the exogenous growth factor addition required for bone regeneration in the case of critical-size defects, and an acceleration of the healing in non-critical defect cases as shown in this study. These findings could be translated into novel treatment strategies for bone regeneration, where NMP improves the biological activity of BMP.

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FUNCTIONALIZED SUPERPARAMAGNETIC IRON OXIDE NANOBEADS FOR SINGLE CELL RNA EXTRACTION

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INTRODUCTION: Only about 1 % of total RNA is mRNA, representing the current gene expression pattern of the cell. Typical RNA extraction is done from 10^7-10^8 cells. The detection of specific mRNA transcripts from a single cell is a technical challenge due to the very low amount of mRNA. This limitation largely increases in the case of cells with a complex architecture such as neurons where the mRNA is transported to the dendrites. The dendrites of the neuron are embedded in a tri-dimensional matrix of other cells making it impossible to extract the intact neuron from the matrix for gene expression analysis.
This poster shows the production of 100 nm superparamagnetic iron oxide beads (SPIOs) and their biofunctionalization for RNA extraction.

METHODS: SPIONs have been synthesized according to Chastellain et al. [1]. The coating and the formation of beads has been done with (3-aminopropyl)triethoxysilane (APS) via an ultrasonic synthesis route. The SPIO beads have been analyzed by means of TEM, FTIR, and PCS. N-sulfosuccinimidyl-6-(4´-azido-2´-nitrophenyl-amino) hexanoate (Sulfo-SANPAH) has been used to immobilize neutravidin onto the surface of these beads.

RESULTS: For the extraction of single cell RNA 10 nm superparamagnetic iron oxide nanoparticles have been synthesized and consequently beads have been formed in an APS silica matrix with a final size of 88 ± 27 nm. The SPION beads show an open fractal structure. These beads have been tagged with neutravidin, capturing biotinylated poly(T). The final product as well as the intermediates have been characterized in terms of size, colloidal stability, morphology, and concentration. The magnetic beads show a fractal structure with high protein immobilization efficiency. 100% sedimentation occurs on a permanent magnet (300 mT) within 20 min for protein modified nanoparticles. Protocols for RNA extraction and RT-PCR have been developed. It is shown that RNA extraction is possible with the developed and investigated 100 nm nanobeads and RT-PCR is not inhibited by these nanoparticles.

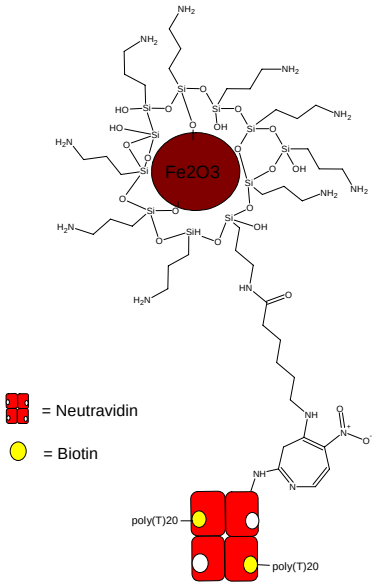

Fig. 1: Scheme of APS modified SPIONs coated with avidin

DISCUSSION & CONCLUSIONS: We show an easy synthesis of SPION beads with sizes smaller than 100 nm. These nano-sized beads show an open fractal structure with a high surface to volume ratio. This surface is ideal for the immobilization of proteins in order to achieve a high protein loading. With the same chemistry as used for the immobilization of neutravidin onto the APS modified SPION beads other proteins can be grafted onto these particles. The beads might be used as drug carriers for magnetically directed drug delivery.

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Degradation Behavior of Chuanxiongqin-derived Polymers

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INTRODUCTION: Chuanxiongqin(tetramethypyrazine,TMPZ) is an active ingredient of a Chinese herbal medicine--Ligusticum chuanxiong Hort, which has been widely used by Chinese physicians for at least 2000 years. TMPZ is reported to have multiple pharmacological modes of actions, including anticoagulant and thrombolytic, protection against vascular endothelial cell injury, antipyretic and antitumor activities[1]. But its use is limited due to the ease of metabolism with half-life of $t_{1/2}$=2.89 h in vivo [1]. Parmacodynamics studies found that TMPZ's medicinal properties attributed to its pyrazine[1]. So we designed two chuanxiongqin-derived polymers, TMPZ/ poly(ethylene glycol)(PEG) conjugate and TMPZ /polyacrylic acid(PAA) conjugate, in which the oxidated side groups of TMPZ were linked by ester bonds to polymers to extend drug moieties' lifetime in blood. In this paper the degradation behavior of the conjugates we synthesized was investigated. Since most Chinese medicine have the problem of fast metabolism *in vivo* and must be frequently administrated for keeping an effective plasma concentration, this work will develop the application field of traditional Chinese medicine.

METHODS: Both metabolism products of TMPZ, 2-cardoxyl-3, 5, 6-trimethylpyrazine and 2,5-dihydroxyl-3,6-dimethylpyrazin , and their polymer conjugates were synthesized as described previously(patented)[2,3]. The products were purified by gel filtration chromatography and characterized by FTIR and UV spectrometer. FTIR(KBr); 1732-1735cm-1 (C=O), 1448-1468cm-1(-N=C-). The maximum values of TMPZ in the conjugates measured from the UV absorbance at 280 nm are 2 and 14 wt% for both TMPZ/PEG($M=10^3$-10^4) and TMPZ/PAA($M=10^3$-10^5), respectively. The hydrolytic degradation of TMPZ/polymer conjugates were performed in phosphate buffer saline of pH 5.8, 7.4 or 9.6 at 37 *in vitro* for 30 to 60 days and the degradation media periodically was analyzed by using HPLC and UV spectrometer.

RESULTS & DISCUSSION: The drug release rate is pH-dependent. Esters bonds of TMPZ/ PEG conjugate are stable to acidic media even after 60 days, but 50% of the total contents of drug moieties were released by one day at pH 9.6 and 4 days at 7.4, respectively. The phenomena can be explained that the TMPZ/PEG conjugate will release acidic drug moiety during the degradation and they may inhibit the hydrolytic reaction. For TMPZ/ PAA conjugate ($M=10^3$-10^5), no hydrolysis observed after 30 days at pH 7.4 or 9.6. It is probably because carboxyl groups on PAA backbone give the conjugate local acidic environment. It is interesting that Chinese physicians usually use TMPZ phosphate or hydrochloride in clinical therapy for enhance drug efficiency, we also found that TMPZ/ PAA conjugate show stronger anticoagulant activity than TMPZ/ PEG conjugate and their parent drug. It suggests that the TMPZ/ PAA conjugate could not only be used as a long-term drug releasing materials and also as a potent medicament.

Table 1. Time for releasing 50%,90%,100% contents of drug moieties from TMPZ/PEG at pH 5.8,7.4 and 9.6.

	pH5.8	pH7.4	pH9.6
50%	-	4d	25h
90%	-	10d	72h
100%	-	35d	124h

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INFLUENCE OF A NANOSTRUCTURED TITANIUM SURFACE ON CULTURED HUMAN OSTEOBLASTIC CELLS

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INTRODUCTION: Surface modifications have been applied to titanium (Ti) in an attempt to accelerate/enhance contact osteogenesis. Chemical deoxidation and controlled reoxidation using H_2SO_4/H_2O_2 results in the formation of a unique surface nanotopography that affects early events in osteogenic cell cultures derived from rat calvaria [1,2]. The aim of this study was to evaluate the effect of the nanostructured Ti surfaces on human osteoblastic (hOB) cells.

METHODS: Machined, commercially-pure grade II Ti discs, 12 mm in diameter and 2 mm thick, were chemically treated with H_2SO_4/H_2O_2 for 2 h [1]. Osteoblastic cells were obtained by enzymatic digestion of human alveolar bone and cultured in standard osteogenic condition until subconfluence. First passage cells were cultured ($2x10^4$ cells/well) on nanostructured and machined Ti discs in 24-well culture plates. For cell attachment assay, adherent cells were enzymatically released at 24 hours and counted. Data were expressed as percentage of initial cell number. For proliferation, cells were cultured for 1 and 7 days, enzymatically released and counted. Data were expressed as doubling time in hours [3]. At day 7, cell viability was evaluated using trypan blue and expressed as percentage of viable cells. At days 1, 3, and 7, cell morphology was evaluated with phalloidin and DAPI; alkaline phosphatase (ALP) labeling was performed using a primary mouse anti-human ALP antibody (B4-78). Data were compared by Mann-Whitney test.

RESULTS: Chemical treatment resulted in a nanopitted surface (compare Fig. 1A and 1D). There were no statistically significant differences between nanostructured and machined Ti in terms of cell attachment (p=0.70), proliferation (p=0.28), and viability (p=0.83) (Table 1) of passaged hOB. At day 1, cells were spread and exhibited an elongated polygonal shape on machined Ti whereas on nanostructured Ti they were mostly stellate-shaped (Fig. 1B and 1E). At day 7 both Ti surfaces exhibited areas of cell multilayering and cells were predominantly elongated. ALP positive cells were observed at all time points with a similar labeling pattern on both Ti surfaces (Fig. 1C and 1F).

Table 1. Cell attachment, proliferation, and viability (Mean±SD, n=3) of hOB cells cultured on nanostructured and machined Ti.

	Nanostructured	Machined
Attachment	34.7±6.4	40.3±10.5
Doubling time	50.4±2.7	48.1±2.1
Viability	92.1±2.8	93.1±1

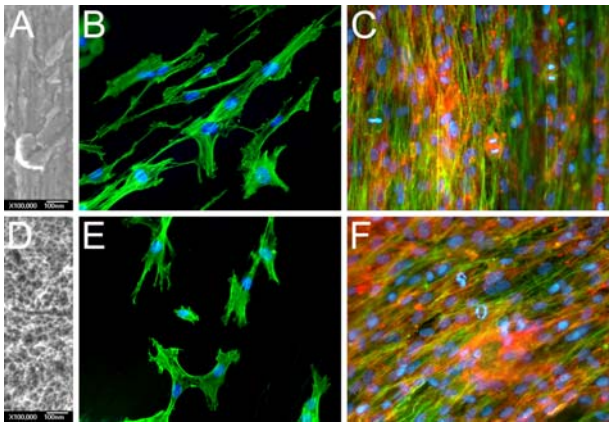

Fig. 1. (A-C) machined, (D-F) nanostructured Ti. (A,D) FE-SEM. (B,E) Cell morphology at day 1; (C,F) ALP labelling (red) at day 7, X40.

DISCUSSION & CONCLUSIONS: Results from these initial studies indicate that nanostructured Ti induces no significant differences in cell attachment, proliferation and viability of passaged hOB when compared to machined Ti. Nanotopography may, therefore, affect other key parameters of in vitro osteogenesis such as the observed influence on cell shape or possibly cell differentiation.

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GUIDED TISSUE REGENERATION: IN VITRO BIOCOMPATIBILITY OF A NOVEL MEMBRANE OF THE COMPOSITE POLY(VINYLIDENE-TRIFLUOROETHYLENE)/BARIUM TITANATE

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INTRODUCTION: Guided tissue regeneration (GTR) is a technique that uses a barrier membrane, which can allow the repopulation of local sites by their own cells. This technique has been applied in clinical dentistry to various cases including dental implant therapy. The most popular membrane used for GTR is an expanded polytetrafluoroethylene (e-PTFE) membrane and some investigators have demonstrated successful bone regeneration by using this material [1-2]. Despite the advantages of this membrane, alternative materials could be employed in GTR. This study aimed at investigating the in vitro biocompatibility of a novel membrane of the composite Poly(vinylidene-trifluoroethytele)/Barium titanate (PVLTrFE).

METHODS: Human alveolar bone fragments (explants) were obtained from healthy donors, under approved research protocols of Committee of Ethics in Research. Osteoblastic cells were obtained from these explants by enzymatic digestion and cultured in α-MEM supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin, 0.3 μg/ml fungizone, 5 μg/ml ascorbic acid, 7 mM β-glycerophosphate, and 10^{-7} M dexamethasone until subconfluence. Cells from first passage were subcultured ($2x10^4$ cells/well) on PVLTrFE and e-PTFE (control) membranes in 24-well culture plates. During all the culture period, cells were maintained at 37ºC, 5% CO_2 and 95% air, and the medium were changed every 3 or 4 days. For attachment evaluation, cells were incubated for 24 hours, enzimatically released and counted. Data were expressed as percentage of adherent cells. For proliferation, cells were cultured for 1 and 10 days, enzimatically released and counted. Data were expressed as doubling time in hours [3]. At 14 days, ALP activity was measured using a commercial kit (Labtest) and expressed as μmol thymolphtalein/h/mg protein. Bone-like nodule formation was stained by alizarin red at 21 days and examined by epiluminescence under a conventional fluorescence microscope. Data were compared by Student t-test.

RESULTS: Data are shown in Table 1. Cell attachment (p=0.001) and ALP activity (p=0.0001) were greater on PVLTrFE membrane. Doubling time was greater on PVLTrFE membrane (p=0.03), which indicates a decreased proliferation rate. Bone-like nodule formation occurred only on PVLTrFE membrane (Fig. 1).

Table 1. Attachment, proliferation, and ALP activity of osteoblastic cells cultured on PVLTrFE and e-PTFE membranes. Data were expressed as mean ± standard deviation (n=5).

	PVLTrFE	e-PTFE
Attachment	62.5±11.4	25.0±8.8
Doubling time	69.2±8.0	55.5±7.8
ALP activity	23.8±3.8	4.32±1.6

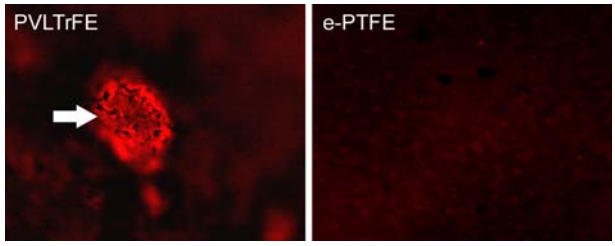

Fig. 1. Photomicrograph of membranes stained by alizarin red. Bone-like nodule formation was observed only in PVLTrFE membrane (arrow). Objective 10X.

DISCUSSION & CONCLUSIONS: The present results showed that both membranes are biocompatible. However, PVLTrFE presented a better in vitro biocompatibility when compared with e-PTFE. Also, PVLTrFE allowed bone-like nodule formation, the final event of in vitro osteogenesis. Therefore, PVLTrFE membrane could be an alternative to be used in GTR.

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Development of Tunable Surface Coatings via Layer-by-Layer Deposition

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INTRODUCTION: A promising approach to the surface engineering of biopolymers is the so-called "blank slate" – the creation of a non-interactive surface which can then be functionalised to provide only the desired interactions when placed in a biological environment. One highly versatile avenue towards this goal is layer-by-layer electrostatic deposition of polyelectrolytes (biologically derived or otherwise). The oppositely charged polysaccharides hyaluronic acid (HA) and chitosan in particular appear to be promising candidates for this technique.

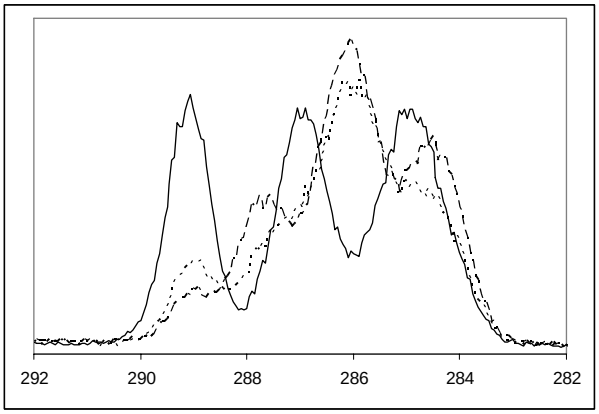
METHODS: Numerous surfaces, including a number of alkanethiol monolayers, aminolysed poly(lactic-*co*-glycolic acid) (PLGA) and tissue culture polystyrene (TCP) were coated layer-by-layer with HA and chitosan in the presence of carbodiimide as a crosslinking agent. Buildup, stability, and protein resistance/binding were followed using a quartz crystal microbalance (QCM). X-ray photoelectron spectroscopy (XPS) was used to follow the build-up on PLGA surfaces. In addition, the *in vitro* cell response to these surfaces were analysed using fluorescence microscopy techniques.

RESULTS: QCM analysis showed very similar build-up behaviour on different surfaces and at 10-fold different polymer concentrations (50 and 500 μg/mL). No change in mass was observed when a multilayer surface was challenged with fibronectin, albumin or collagen IV under physiological conditions. However, collagen IV bound readily in a reduced salt, low-pH buffer, in the presence of carbodiimide. This collagen was further used as a template for the self-assembly of proteins from dilute Matrigel.

When placed in contact with 10% foetal bovine serum in DMEM, mass was lost corresponding closely to the mass of the final HA layer, presumably due to the action of hyaluronidase. However, this loss was prevented by incubating the surface with 10 mM acetate in the presence of carbodiimide – it is hypothesized that the carbodiimide mediated the esterification of the acetate to the HA hydroxyl groups. Interestingly, no mass increase was noted in this case, indicating that the surface retained its non-adhesive properties.

XPS analysis of the carbon 1s spectrum of treated PLGA surfaces showed a progressive change as the number of layers increased, reaching a steady state with no evidence of the characteristic peaks of PLGA after 5 layers.

The *in vitro* response of NIH-3T3 fibroblasts to the treated surfaces strongly reflected the results observed in the QCM. While cells spread and multiplied quickly on untreated and aminolysed PLGA and TCP, cell adhesion was almost entirely removed upon the application of 3 ½ HA/chitosan bilayers, with all observed cells remaining rounded. On collagen IV-coated multilayer surfaces, adhesion, spreading and multiplication were restored to control levels.



.*Figure 1. XPS C1s spectrum of aminolysed PLGA (solid line), a 3 ½ bilayer HA/chi coating (dotted line) and 3 ½ bilayers + collagen IV (dashed line).*

CONCLUSIONS: Layer-by-layer modification provides an extremely facile, versatile method firstly for the production of highly non-interactive surfaces, and the subsequent functionalisation of these surfaces to provide specific interactions with cells. Much of the flexibility inherent in this method remains to be tapped.

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Scanning Force Arthroscope

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INTRODUCTION: In severe knee injuries, the surface of the joint is often affected. Depending on the age of the patient and the severity of the damage, the surgeon try to repair the cartilage or, in the worst cases, the knee is replaced by prosthesis. After a cartilage replacement, it would be highly desirable to have a minimally invasive device that allows post-operative monitoring of the status of the repaired area. A recent study based on *ex-vivo* indentation of normal, diseased and enzymatically altered cartilage shows that the scanning force microscope (SFM) is a very sensitive tool that allows quantitative measurements of the bioechanical properties of cartilage tissue[1]. We design a robust, reliable and quantitative diagnostical tool, the scanning force arthroscope (SFA), for monitoring the long-term clinical outcomes of such surgical procedures for knee cartilage defects.

INSTRUMENT: The SFA (Fig. 1) has a diameter comparable with current arthroscopic operative instruments (roughly 5 mm). The stabilization of the SFA inside the knee-cavity relative to the surface to be inspected is perform by a pneumatic system: Two sets of balloons, similar to those used in angioplasty, wedge the instrument between the bones, ligaments and the fat pad. A standard four segmented piezoelectric tube is used as scanner. The deflection of the cantilever is detected by an integrated piezoresistor. During the insertion, under arthroscopic control, of the instrument inside the knee, the fragile tip needs a special mechanical protection, which is withdrawn once the instrument is in place.

Fig. 1: A complete SFM setup has been inserted into an arthroscopic cannula.

MEASUREMENTS: The stability of the pneumatic stage was checked in a series of tests, for which the knee cavity was modeled by a rubber tube. Load-displacement curves (Fig. 2) have been recorded, using a prototype of SFA in a knee phantom. Recently, this instrument has been inserted in a knee cadaver for testing stabilization in an anatomical environment. Further development and evaluation are in progress.

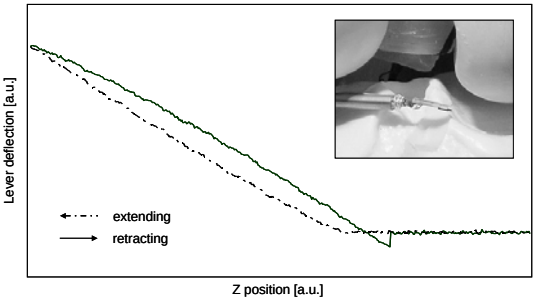

Fig. 2: Load-displacement curves have been recorded in a knee phantom, with the prototype of SFE shown in Fig. 1.

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Anodic Titanium Oxides Grown in Sulphuric and Phosphoric Acid Electrolytes

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INTRODUCTION: Depending on the electrolyte, its concentration and the applied potential, anodic oxide films on Ti can grow mostly dense or porous, amorphous or crystalline [1, 2]. Such films are frequently used for biomedical surface preparation. They are thought to protect the un-noble substrate from corrosion and enhance its biocompatibility, while the thickness dependent interference colors allow for color-coding of implantable devices. In the present study, growth mechanism and oxide morphology upon anodization of Ti in sulfuric and phosphoric acid electrolytes is investigated with non-destructive ac impedance spectroscopy (EIS), transmission electron microscopy (TEM) and Raman analysis. Chemical depth profiling is performed by glow-discharge optical emission spectroscopy (GD-OES), X-ray photoelectron spectroscopy (XPS), and Rutherford backscattering (RBS) combined with elastic recoil detection analysis (ERDA).

METHODS: C.p. Ti discs (grade 2) were used. Anodic oxidation (5 V/s) was performed up to different end potentials in 1 M H_2SO_4 or 1 M H_3PO_4 at 25°C. EIS was done using an Autolab PGSTAT30 with FRA module. Raman spectra were measured in backscattering geometry on a Renishaw Ramascope 2000 using a HeNe-laser. Ellipsometry was performed on a spectroscopic ellipsometer (MOSS model ES 4G). TEM was performed on Philips CM-300 and EM-430 microscopes at 300 keV. GD-OES (Jobin-Yvon 5000 RF) was used for estimation of film thickness and chemical depth profiling. RBS and ERDA spectra were obtained by irradiation with a 2 MeV He-ion beam on a Van de Graaff accelerator.

RESULTS: For a given potential, oxides grown in H_3PO_4 are slightly thinner than in H_2SO_4. Already at 10 V, a small amount of anatase is observed in the Raman signal of both oxide types. For a given potential the crystallization is found more progressed in H_2SO_4 than in H_3PO_4. TEM observations reveal a multilayer structure consisting of a porous central part and a dense part, already observed at 20 V (Fig. 1). Besides the higher amount of crystalline phase in H_2SO_4, a significant difference in the location of first nanocrystallites is observed in both oxide types. While crystallites are present around the central porous part and in the outermost dense part in H_2SO_4 oxides, nanocrystals are exclusively found at the interface metal/oxide in H_3PO_4. The film porosity is confirmed by the appearance of a 2nd capacitance in impedance spectra above 20 V. Based on TEM, a 2-layer model is chosen for data fitting. The ongoing crystallization with increasing potential can be correlated to systematic changes in resistances and capacitances. Impedance data confirm the slower crystallization in H_3PO_4.

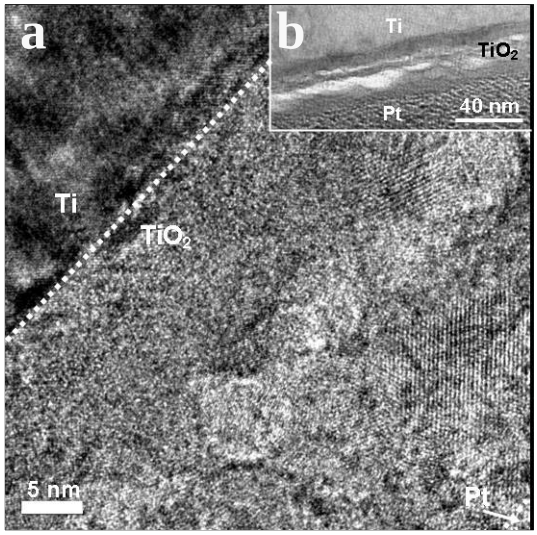

Fig.1 (a) TEM bright field image of a 20 V sulfuric acid oxide. (b) Overview of its layered structure.

XPS on 80 V oxides reveal stoichiometric TiO_2. A S contamination located at the metal/oxide interface (max. 2.5 at-%) and a P peak at the surface, rapidly decreasing to around 5 at-% inside the oxides, are found. An accumulation of H around the interface metal/oxide is seen by GD-OES, the maximum concentration being quantified to 20-30 at-% by RBS/ERDA. Ion beam analysis allows to estimate the film porosity to 34-38%.

DISCUSSION & CONCLUSIONS: Crystallization of anodic oxides on Ti is found to start at potentials as low as 10 V, strongly influencing the fitted capacitance and resistance values obtained from ac impedance data. The presence of a porous layer is observed at potentials as low as 20 V by TEM and EIS. Further crystallization is found to progress slower in H_3PO_4 than in H_2SO_4.

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Influence of the Molecular Charge of PEG-Conjugated Catechol Equivalents on the Formation of Self-Assembled Adlayers

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INTRODUCTION: Highly controlled physicochemical surface properties are key factors in the development and optimization of new biomaterials such as load bearing hard-tissue (i.e. bone) implants or catheters, as well as biosensor surfaces that have regular contact with complex protein mixtures. In recent works, L-3,4-dihydroxyphenylalanine (DOPA), a catechol which is believed to be the key residue in mussel adhesive proteins (MAPs), was attached to monomethoxy-terminated poly(ethylene glycol) (mPEG) for the purpose of rendering surfaces protein resistant [1]. By using an adhesion foot consisting of three repeating DOPA units, Dalsin *et al.* showed that protein adsorption could be reduced up to 99% [1]. However, the fact that DOPA is a non-proteinogenic amino acid which carries a negative charge at physiological pH values and that at the same conditions most metal oxide surfaces are negatively charged as well, electrostatic repulsion effects could impede adsorption processes and decrease the stability of adsorbed molecules with only one or two DOPA groups per PEG chain.

In order to investigate the influence of the charge of the surface binding group on the adsorbed adlayer, its stability and subsequent protein resistance upon serum exposure, we developed new single-side catechol derivatives (neutral, negative and positive charged) and coupled them to PEG chains.

The design of the positively charged compound was inspired by a natural product evolutionary selected by cyanobacteria to bind Fe(III). Indeed, these organisms evolved sophisticated strategies for iron sequestering and the iron chelator anachelin was evolutionary optimized to effectively bind to Fe(III) [2]. We wondered whether this exceptional binding phenomenon could be extended to bind as well to *metal oxide surfaces* [3].

METHODS: The molecules were/will be investigated in terms of adsorption behavior and subsequent resistance against serum adsorption, using variable angle spectroscopic ellipsometry (VASE), optical waveguide lightmode spectroscopy (OWLS) and X-ray photoelectron spectroscopy (XPS). For polymer adsorption standard conditions were defined as follows: 1 mg/ml resp. 0,1 mg/ml of polymer dissolved in cloud-point buffer, adsorption at 50°C for 4 hours. Adsorptions were done on TiO_2 coated silicon wafers for VASE and XPS experiments and on TiO_2 coated waveguides for OWLS measurements.

RESULTS: Our preliminary results demonstrate that the charge of catechol PEG conjugates has a major influence on polymer adsorption and stability. In order to confirm these observations further results, including long term stability study will be presented.

DISCUSSION & CONCLUSIONS: VASE and OWLS experiments revealed that neutral and positive charged mPEG-DOPA derivatives show an enhanced adsorption on TiO_2 and lead to a superior protein resistance compared to negatively charged mPEG-$DOPA_1$ used in a related work by Dalsin et al. [1].

REFERENCES: [1] J.L Dalsin, et al (2005) *Langmuir* **21**: 640-646. [2] Selected examples: K. Gademann, (2005) *ChemBioChem* **6**: 913. [3] K. Gademann, Y. Bethuel, (2004) *Org. Lett.* **6**: 4707. K. Gademann, Y. Bethuel, (2004) *Angew. Chem., Int. Ed.* **43**: 3327.[3] D. Wäckerlin (2005) *Biomimetic Surface Modifications with Differently Charged PEG-conjugated Catechol Derivatives. Diploma Thesis, Dept. of Material, ETH Zürich,* soon available at www.textorgroup.ch

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Differently Charged PEG-Conjugated Catechol Derivatives

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INTRODUCTION: Highly controlled physicochemical surface properties are key factors in the development and optimization of new biomaterials and devices for different fields of applications such as dentistry, surgery and for manufacture of devices that have regular contact with moisture. In recent works L-3,4-dihydroxyphenylalanine (DOPA), a catechol which is believed to be the key molecule in mussel adhesive proteins (MAPs), was attached to monomethoxy-terminated poly(ethylene glycol) (mPEG) for the purpose of rendering surfaces protein resistant. It is being a non-proteinogenic amino acid which carries a negative charge at physiological pH. As at these conditions most metal oxide surfaces are negatively charged as well, electrostatic repulsion effects could impede adsorption processes and decrease the stability of adsorbed molecules. Dalsin et al. [1] observed a multivalency effect by studying peptides containing up to three DOPA residues conjugated to mPEG. We would like to present new single-side derivatives of DOPA connected to PEG and investigate the effect of the molecular charge. Therefore, a neutral, a negatively and a positively charged catecholic derivatives were synthetized and adsorbed on metal oxide surfaces [2].

METHODS: The molecules were investigated in terms of adsorption behavior and subsequent resistance against serum adsorption, using variable angle spectroscopic ellipsometry (VASE), optical waveguide lightmode spectroscopy (OWLS) and X-ray photoelectron spectroscopy (XPS). For polymer adsorption standard conditions were defined as follows: 1 mg/ml resp. 0,1 mg/ml of polymer dissolved in cloud-point buffer, adsorption at 50°C for 4 hours. Adsorptions were done on TiO_2 coated silicon wafers for VASE and XPS experiments and on TiO_2 coated waveguides for OWLS measurements.

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REFERENCES: [1] J.L Dalsin, et al (2005) *Langmuir* **21**: 640-646. [2] D. Wackerlin (2005) *Biomimetic Surface Modifications with Differently Charged PEG-conjugated Catechol Derivatives.*

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Bioactive Coatings for Implants by Electrophoretic Deposition

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INTRODUCTION: In the field of orthopaedics, hydroxyapatite (HA) plasma-spray coatings have been used in order to enhance implants fixation. However, due to high temperatures, the plasma spray process does not allow for a rigorous control of the final chemical and structural coating properties. Moreover, coatings on complex shapes are critical[1]. Electrophoretic deposition (EPD) is an alternative coating technique with the following advantages: Control of the stoichiometry, uniformity on any shape, flexibility in the choice of materials, simplicity and low cost. We aim to develop graded coatings by EPD, consisting of a biocompatible matrix and bioactive elements with different rates of bioresorbability. In the present study, composite coatings of Bioglass® (45S5) and a "bioresorbable phase" were prepared by EPD on Ti6Al4V substrates.

METHODS: As received 45S5 (wt%: 45.0 SiO_2, 24.5 Na_2O, 24.5 CaO, 6.0 P_2O_5, Schott Glas Export GmbH) powder and additional "bioresorbable particles" were used as precursors for the coatings. The powders were characterized by particle size distribution analysis (Beckman Coulter LS 230 instrument) and SEM (Hitachi S-4800). Cathodic EPD was carried out in an electrochemical cell (parallel plate geometry, 50V/cm). Etched Ti6Al4V discs were used as cathode. A mixed suspension of 45S5 and "bioresorbable particles" in isopropanol was used as electrolyte. After EPD, the samples were rinsed, dried and sintered in Ar flow (900°C, 2h). Roughness and thickness of the coatings were measured by white light profilometry (Altisurf). The morphology was investigated by SEM (Hitachi S-3600N). Chemical depth profiling with Glow Discharge Optical Emission Spectroscopy (GD-OES, Jobin-Yvon 5000 RF) was performed. Hardness tests (Zwick ZHU 2.5) were carried out as a preliminary characterization of the coatings mechanical properties.

RESULTS: The measured mean size of the 45S5 particles (not agglomerated) was 4,3 μm. The coatings thickness ranged from 25 to 35 μm, with R_a between 2.5 and 3.5 μm. The sintered coatings showed some cracks. The 45S5 particles were partially melted forming a coherent matrix (Fig. 1).

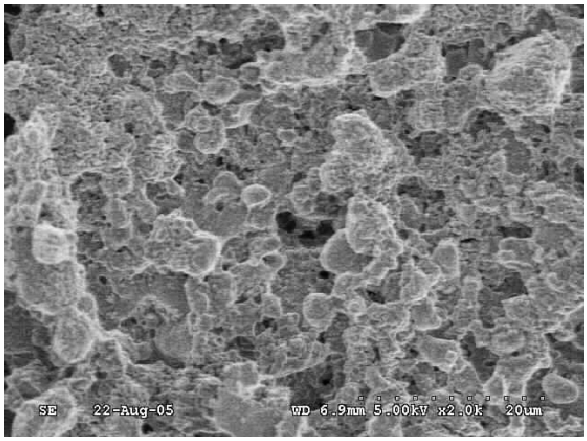

Fig.1: 45S5 coating with bioresorbable phase.

The smaller "bioresorbable particles" seemed preferentially adsorbed on the 45S5 particles. The GD-OES analysis confirmed the chemical composition as expected from the powders nominal chemistry. Hardness tests showed a clear improvement in mechanical properties after sintering.

DISCUSSION & CONCLUSIONS: The bioactive glass developed by Hench (Bioglass®) is known for its bioactivity[2]. Moreover, its glass transition temperature of 560°C[3] allows for sintering at relatively low temperatures, avoiding alteration of the substrate's properties. However, 45S5 particles gain a negative charge in organic suspensions[4] and cannot be deposited cathodically. We found that the addition of the "bioresorbable particles" acts as surface functionalization of the 45S5 particles, charging them positively. This enabled to prepare 45S5 coatings deposited by cathodic EPD. The mechanical properties of these coating could be improved, for example by incorporating of ZrO_2 particles in the 45S5 matrix. The biological behavior of such composite coatings needs further investigation.

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ACKNOWLEDGEMENTS:, H. J. Schindler for the particles size analysis, G. Bürki for SEM, H. P. Feuz for Altisurf, M. Aeberhart for GD-OES, S. Siegmann and M. Hadad for helpful discussions (EMPA).

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Ways to decrease the Adhesion of *Pseudomonas Aeruginosa* Bacteria to the Surfaces of Endotracheal Tubes

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INTRODUCTION: The objective of the research presented here is to develop non-adhesive surface coatings on endotracheal tubes, in order to prevent bacterial growth. Many hospital-acquired pneumonias follow colonisation of the intubation devices by *Pseudomonas aeruginosa*[1]. This could be prevented by making the surface of the tubes non-adhesive to bacteria. Furthermore, such tubes would prevent infection without the use of antibiotics and, thus, discourage the formation of antibiotic resistance in bacteria.

METHODS: The surface of the endotracheal tube is modified using several approaches including dielectric barrier discharge treatment and wet chemical treatment. The hydrophilicity of the surface is, in this way, increased which hinders the adhesion of substances in body fluids that can allow bacteria to anchor. Also, by including silver ions, the tube surface can be made toxic to bacteria (but not to humans), which further decreases bacterial adhesion and growth[2]. The present research develops and examines different surface modifications of medical grade poly(vinyl chloride) tubes using techniques such as X-ray Photoelectron Spectroscopy (XPS), Fourier Transformed Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). These techniques give the possibility to monitor structural changes and obtain chemical information about the surfaces. In a later stage of the project, the altered surfaces will be tested for adhesion of different strains of *P. aeruginosa*. This will enable the biological response to be correlated to the chemical properties of the surfaces, and make it possible to identify the most bacterial resistant surface

RESULTS & DISCUSSION: Both the wet chemical treatment and dielectric barrier discharge treatment increase the roughness of the surface (Fig 1). The wet chemical treatment is more efficient in producing a large uptake of silver ions with resulting silver content of approximately 4-7 at % (obtained from XPS) while the discharge treatment only results in 2-3 at %.

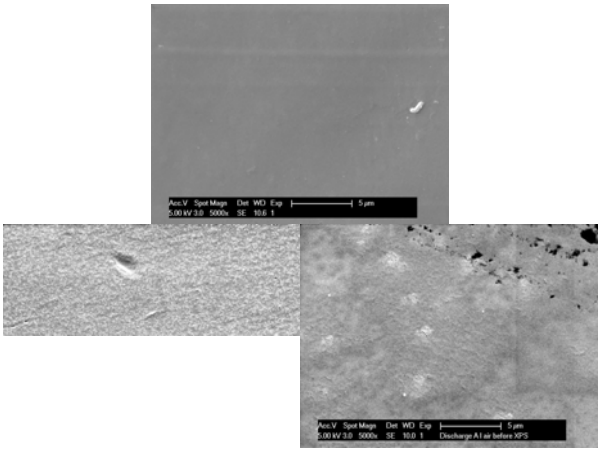

Fig. 1: Effect of wet chemical and dielectric barrier discharge treatments on surface morphology: non treated surface (top) vs. wet chemically treated (bottom left) and discharge treated (bottom right).

XPS data show that silver is present in ionic form. Furthermore, FTIR and XPS data suggest that the interaction between the silver ions and tube surface is through bond formation with chloride and/or oxygen containing groups.

CONCLUSIONS: The wet chemical treatment of the surface gives a higher content of silver ions than the dielectric barrier discharge treatment. Thus, this treatment is hypothesized to be the most efficient one for reduction of bacterial adhesion.

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ACKNOWLEDGEMENTS: This work is supported by the Swedish Research Council. The authors would like to acknowledge R. Houriet for assisting with FTIR instrumentation and expertise.

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Frictional behaviour of silicone structured surfaces simulating tongue roughness

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INTRODUCTION: Food when eaten is squeezed and sheared between the tongue and other oral surfaces. Tribological properties of interacting surfaces in relative motion can be used to explain some physico-chemical phenomena that govern oral perception of food structure [1]. Although several tribology studies were performed between soft/hard surfaces to emulate more realistically the forces applied in the mouth [2-3], the influence of the rough nature of the tongue surface was not addressed. Indeed, the human tongue has a rough surface with a large density of papillae in the sub millimetre range.

The objective of the present study is to assess the influence of surface structure on friction coefficient of a model tribosystem representing the tongue/palate contact under dry conditions and in the presence of oil and aqueous solution having the same viscosity at 36°C.

METHODS: Soft silicone samples with well-defined surface structures, based on hemispherical pillars of different dimensions (Table1 and Figure 1) were fabricated by a moulding technique to simulate tongue roughness. The friction experiments were performed on a reciprocating motion sliding tribometer involving contact between a relatively hard ball representing the palate and one of the soft silicone surfaces simulating the tongue. Tests conditions (0.31 MPa, 10 mm s^{-1} and 36°C) were designed to represent those encountered in the mouth when thin films of food residues coat the oral mucosa surfaces.

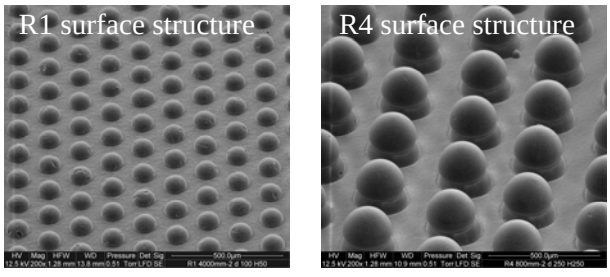

Fig. 1 SEM images of silicone surface structures R1 and R4 with hemispherical pillars.

RESULTS & DISCUSSION:

The frictional behaviour of the investigated tribosystem is strongly affected by the surface structure of the contacting surfaces as pointed out in Figure 2 (where R0 denotes a flat specimen).

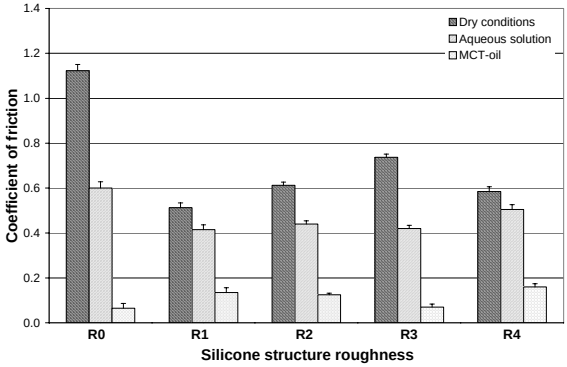

Fig. 2 Effect of the surface structure on the coefficient of friction under dry conditions and with aqueous or oil-based lubricant.

Under dry conditions, the coefficient of friction decreases significantly with increase of hemispherical pillar density. With aqueous solution or MCT-oil between the contact surfaces, these differences are relatively lower. For lubricated surfaces, higher pillars with an optimal high density increase the friction coefficient.

Table 1. Geometric parameters of silicone structured surfaces of different dimensions.

Silicone surfaces	Density mm^{-2}	Diameter μm	Height μm
R1	40	100	50
R2	8	250	125
R3	2.5	250	125
R4	8	250	250

CONCLUSIONS: The use of moulded silicone surfaces appears to be interesting to mimic tongue properties. This novel approach constitutes a promising way to assess the effect of tongue topography on "in-mouth" friction.

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In vitro osteogenesis on chemically modified (ASD-AK) titanium surface

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INTRODUCTION: Titanium (Ti) has been widely used as bone biomaterial in dentistry and medicine. Several modifications of Ti surface were described aiming to improve its biocompatibility. A chemical modification (ASD-AK) consisted of anodic spark discharge using consecutively solutions containing phosphorous ions, calcium ions, and alkali etching was described [1]. ASD-AK produces Ti surfaces containing Ca and P that allows higher cell proliferation rate compared to untreated Ti [2]. However, it remains to be showed whether ASD-AK improves bone response on Ti surface. The aim of this study was to compare in vitro osteogenesis on ASD-AK and on untreated Ti.

METHODS: ASD-AK and untreated Ti coupons (10x10x1 mm) were prepared as described elsewhere[1]. Human alveolar bone fragments (explants) were obtained from healthy donors, under approved research protocols of Committee of Ethics in Research. Osteoblastic cells were obtained from these explants by enzymatic digestion and cultured in α-MEM supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin, 0.3 μg/ml fungizone, 5 μg/ml ascorbic acid, 7 mM β-glycerophosphate, and dexamethasone 10^{-7} M until subconfluence. Cells from first passage were subcultured at a concentration of $2x10^4$ cells/well on ASD-AK and untreated Ti in 24-well culture plates. During all the culture period, cells were maintained at 37ºC, 5% CO_2 and 95% air, and the medium were changed every 3 or 4 days. For attachment evaluation, cells were incubated for 24h, enzimatically released and counted. Data were expressed as percentage of seeded number. For proliferation, cells were cultured for 1 and 10 days, enzimatically released and counted. Data were expressed as doubling time in hours, with higher values meaning lower proliferation. At 10 days, cell viability was evaluated using the trypan blue method and expressed as percentage of total cell number. At 14 days, alkaline phosphatase (ALP) activity was measured using a commercial kit (Labtest) and expressed as μmol thymolphtalein/h/mg protein. Bone-like nodule formation was stained by alizarin red at 21 days. Average area of the nodules was measured using an image analyzer and the amount of bone-like formation was expressed as percentage of sample area. Data were compared by Student t-test.

RESULTS: All data are shown in Table 1. For cell attachment (p=0.9), viability (p=0.35), and bone-like nodule formation (p=0.12) there was no statistically significant difference between ASD-AK Ti and untreated Ti. Cell proliferation (p=0.02) and ALP activity (p=0.02) were both affected by treatments, being higher in untreated Ti.

Table 1. Attachment (% of seeded number), doubling time (hours), viability (% total cell number), ALP activity (μmol thymolphtalein/h/mg protein), and bone-like nodule formation(% of sample area) of osteoblastic cells derived from human alveolar bone cultured on ASD-AK and untreated Ti. Data were expressed as mean ± standard deviation (n=5).

	ASD-AK Ti	Untreated Ti
Attachment	18.4±10.85	18.3±13.57
Doubling time	337.4±185.52	96.3±11.38
Viability	100±0	98.5±3.44
ALP activity	16.9±1.62	26.4±5.18
Bone-like nodule formation	4.3±5.52	6.4±1.26

DISCUSSION & CONCLUSIONS: In this study both initial and final events of in vitro osteogenesis were evaluated. Initial events were evaluated by cell attachment and proliferation and final ones by ALP activity and bone-like nodule formation. Cell attachment was not affected by any Ti surfaces but cells cultured on ASD-AK Ti presented a slower proliferation rate that could not be explained by differences in their viability. Also, ALP activity was lower for cells cultured on ASD-AK Ti but it did not affect bone-like nodule formation. It was observed that both Ti surfaces were capable of sustaining in vitro osteogenesis despite some differences between them. These results failed to show that ASD-AK treatment could stimulate in vitro osteogenesis on Ti surface, suggesting that ASD-AK treatment does not affect bone biocompatibility of Ti.

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Osteoblast proliferation on bone biomaterials with different biocompatibility profile

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INTRODUCTION: Several biomaterials have been used as bone substitute including ceramics, metals and polymers. Biomaterials can be classified according to its biocompatibility profile as biotolerated, bioinert and bioactive[1]. This study aimed at evaluating proliferation and viability of osteoblastic cells cultured on biotolerated (polymetylmetacrilate-PMMA), bioinert (titanium-Ti and stainless steel-SS), and bioactive (hidroxyapatite-HA and glass ceramic-45S5) biomaterials.

METHODS: Discs (12mm diameter and 3mm height) were prepared (PMMA, HA, and 45S5) or purchased (Ti and SS). Human bone marrow mesenchymal cells (hBMMC) were obtained from healthy donors, under approved research protocols of Committee of Ethics in Research. hBMMC were cultured in osteogenic medium containing α-MEM supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin, 0.3 μg/ml fungizone, 5 μg/ml ascorbic acid, 7 mM β-glycerophosphate, and dexamethasone 10^{-7} M until subconfluence. Osteoblastic cells from first passage were subcultured at a concentration of $2x10^4$ cells/well on biomaterial discs in 24-well culture plates (n=5). Wells with no discs were used as control. During all the culture period, cells were maintained at 37ºC, 5% CO_2 and 95% air, and the medium were changed every 3 or 4 days. Cells were cultured for 7, 14 and 21 days, enzimatically released and counted. Proliferation was expressed as cell number/well at 7, 14 and 21 days and as doubling time between 7 and 14 days [2]. Cell viability was evaluated using the trypan blue method and expressed as percentage of total cell number. Data were compared by one-way ANOVA and Bonferroni test for multiple comparisons and considered to be statistically significant for $p \leq 0.05$.

RESULTS: Proliferation expressed as cell number/well is showed in Fig. 1. At 7 days, there was difference only between 45S5 and control ($p=0.05$). At 14 days, there were more cells on 45S5 than on any other biomaterial and control ($p<0.0001$). At 21 days, cell number ranking was as follows: control=45S5=SS>Ti=HA=PMMA ($p<0.0001$). Doubling time between 7 and 14 days (Fig. 2) was significantly lower only for cells cultured on 45S5 compared to other biomaterials and control ($p=0.03$). Cell viability was always higher than 90% in any period and there was no difference among any biomaterial.

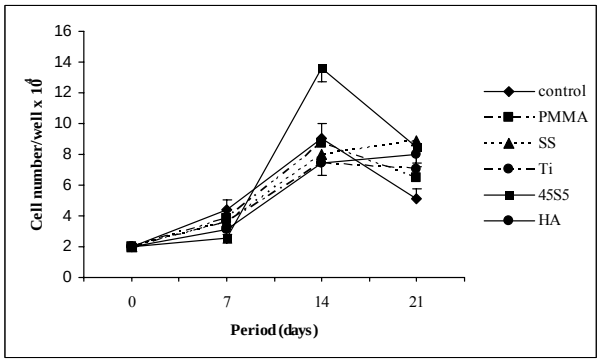

Fig. 1 – Proliferation of osteoblastic cells cultured on biomaterials for 7, 14 and 21 days. Data showed as mean ± SD (n=5).

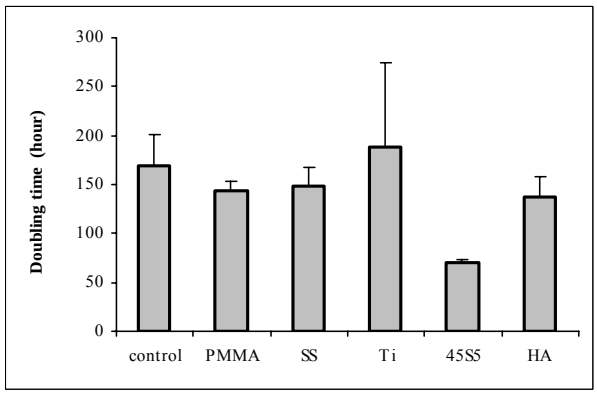

Fig. 2 – Doubling time in hours between 7 and 14 days of osteoblastic cells cultured on biomaterials. Data showed as mean ± SD (n=5).

DISCUSSION & CONCLUSIONS: All tested biomaterials allowed cell proliferation, regardless its biocompatibility profile. For all of them the pattern of proliferation was similar to control that was an increase in cell number from day 0 to day 14 and a decrease thereafter. Among them only 45S5 presented higher proliferation rate as showed by greater cell number at 14 days and lower doubling time. None of them affected cell viability in any period. It remains to be evaluated whether biocompatibility profile affects cell responses related to bone formation.

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Influence of Seeding Concentration of Cells on Efficiency of Occupation of Ca-P 3D Scaffolds with Bone Marrow Stromal Stem Cells and Differentiation in Bone-like Tissue .

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INTRODUCTION: Bone marrow-derived stromal stem cells (BM SSCs) have the capacity for renewal and the potential to differentiate into multiple lineages of mesenchymal tissues (bone, cartilage, fat and muscle). These cells are very attractive cell source in tissue engineering. BM SSCs are capable of forming bone when implanted ectopically in an appropriate scaffold. Two important parameters in scaffold-based tissue engineering include the cell seeding efficiency and the uniform distribution of cells in the matrix. The practical application of BM SSCs in 3D scaffolds is hampered by its low cell seeding efficiency. The aim of this study was to assess the efficiency of seeding concentration of cells on bone formation with BM SSCs in CaP scaffolds.

METHODS: Cells were isolated from BM obtained by puncturing iliac crest under the intravenous anesthesia of patient, expanded and detected by expression of surface antigens by flow cytometer FACS (Epics Elite Coulter). For derivation of human 3D transplants of bone tissue we used CaP scaffolds (BD Biosciences). Cell suspension containing 2.5 x 10^5, 1 x 10^6, 2 x 10^6 cells in 200 ml of media loaded onto scaffolds, respectively, and incubated the tube with gentle agitation (~50 rpm) on an orbital shaker at 37^{o}C for 2-hours. The osteogenic culture media was DMEM–LG supplemented with 10% FBS, 0.2 M ascorbic acid, 1x10^{-7}dexamethasone, 10mM β-glycerophoshate. Cell-scaffold constructions fixed in 4% paraformaldehyde for 2 days, then decalcified for further 10 days in 10% EDTA prior to embedding in paraffin. For histological analysis, 5 mm sections of the implants were prepared and stained with hematoxylin and eosin. Immunohistochemical analysis of samples was detected using Abs for osteopontin, produced by R&D Systems.

RESULTS: BM SSCs were positively stained with Abs for the Ags: CD10, CD13, CD29, CD44, CD49a,b, CD54, CD71, CD73, CD90, CD105, CD166, HLA ABC. The cells were negative for CD34, CD45, CD133, CD31, Flk, CD117, STRO-1, HLA DR, DP and DQ. Comparative analysis of results has shown that at loading cells in concentration 2.5 x 10^5, 1 x 10^6, 2 x 10^6 in scaffolds, 2 x 10^5, 8.5 x 10^5 and 1.26 x 10^6 cells occupied porous sponges, accordingly (Fig.1 a, b, c). Furthermore, only seeding cell concentration 2 x 10^6 allowed cells to occupy scaffolds densely and to differentiate in osteogenic pathway. Culturing of cells in this concentration in scaffolds with osteogenic medium for 21 days led to formation bits of bone-like tissue in vitro. Histochemical analysis showed that cells had morphology of osteoblasts and osteocytes. Immunohistological analysis detected expression of osteopontin (Fig1.d). The cells supported an uniform area of bone formation in scaffolds.

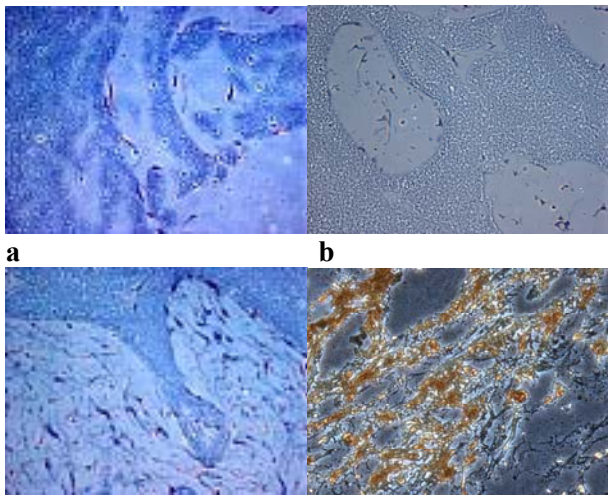

Fig.1:Effect of seeding cell concentration on occupation of Ca-P scaffolds and differentiation BM SSCs in cells of bone tissue after 3 week of culture: a) 2.5 x10^5, b) 1 x 10^6, c) 2 x 10^6 d) expression of osteopontin.

DISCUSSION& CONCLUSIONS: A typical Ca-P scaffold for tissue engineering should consist of inter-connected macroporous structures 200-400 μm to facilitate cell distribution and enhance diffusion rates to and from the center of the scaffold. Therefore, low cell seeding efficiency is a prevalent problem for such 3D scaffolds with similar architecture. Results of our research have shown that seeding cell concentration influences not only settling scaffolds with cells, but also on a differentiation of BM SSCs in osteogenic pathway. So, seeding cell concentration 2 x 10^6 is optimal for engineering of bone tissue in vitro in CaP scaffolds.

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Collagen-Hydroxyapatite scaffolds for Hard Tissue Engineering with a predefined architecture and shape

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INTRODUCTION: The aim of hard tissue engineering is to produce tissue to repair damaged or diseased skeletal bones. A porous matrix (the scaffold) comprising collagen-hydroxyapatite (Col-HA) can be tailored to the shape of any defect using a 3-D printing technology and its internal architecture is controlled, through control of the porosity and internal micro-vasculature during the manufacturing process.

METHODS: The external and internal geometry of a mould is pre-designed (Mechanical AutoCAD 2005) and printed using solid freeform fabrication[1]. Hydroxyapatite powders and bovine collagen (Sigma-Aldrich) were suspended in an acidic aqueous solution at pH=5, poured into a mould and frozen. The freezing temperature (-20°C, -80°C), crosslinking by dehydrothermal treatment (DHT) and solute concentration (1-5wt% collagen) were varied in order to optimise scaffold porosity and its resistance to collagenase degradation. The mould was removed by ethanol, and the water removed by critical point drying[2]. A low voltage scanning electron microscopy (SEM; JSM-840F JEOL) was used to measure porosity through image analysis (Image-J); differential scanning calorimetry (METLER DSC-821e), swell ratios and resistance to collagenase were used to determine the amount of crosslinking. Finally, human osteosarcoma cells (MG63) were suspended in a culture medium and seeded (1×10^5 cells/scaffold) onto Col-HA scaffolds.

RESULTS: Figures 1 illustrates the ability to control the porosity of a Col-HA scaffold by changing the solute concentration or the freezing rate. Pore sizes varied between 10µm to 350µm. Pore size distribution curves revealed a higher percentage of large pores (mean distribution peak shifts from 20µm to 50µm), for scaffolds manufactured with low solute content and lower freezing rates. DHT has not induced any denaturation of the scaffold and has altered the thermal stability, the swell resistance and the degradation resistance to collagenase. These can be correlated to the amount of crosslinking present.
The external and internal shape of the scaffolds is controlled by printing out different moulds of different size and design.

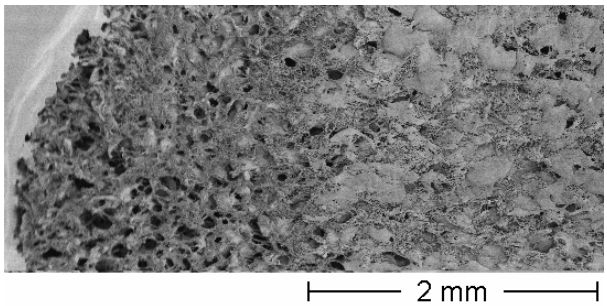

Fig. 1: Varying the pore size across a Col-HA scaffold (left: 1wt% collagen, right:5wt% collagen)

Figure 2 illustrates the viability of osteogenic cells (MG63) on a collagen/HA scaffold surface after 14 days of culture.

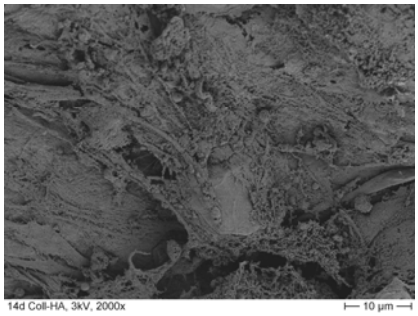

Fig.2: SEM image of cells cultured for 14 days onto a Col/HA scaffold

DISCUSSION & CONCLUSIONS: Choosing biocompatible materials for the moulds and scaffolds will lead to the reduction of implant rejections. The ability to control pore geometry within the scaffold has the potential to optimise mechanical and biological properties. The current issues facing tissue engineering of 3D tissues arise from the passive diffusion limitation of cells, which cause cellular necrosis within the centre of engineered scaffolds. The potential to have an internal vasculature system within a Col-HA scaffold may overcome such problems.

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RECOMBINANT CHIMERIC FIBRINOGEN SYNTHESIS FOR DIRECT INCORPORATION OF PROTEIN THERAPEUTICS

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INTRODUCTION: Fibrin, a polymer comprised of nanoscale fibers formed by the conversion fibrinogen into fibrin monomers by the serine protease thrombin, serves the primary role of hemostasis and an additional function as a provisional structural scaffold for invading cells and an immunomodulator of innate immune cells. The attraction to fibrin-based biomaterials is based on its central role in tissue binding and in the initiation of tissue repair and defence. Using CHO cells stably transfected with expression vectors encoding fibrinogen B-beta and gamma chains and transiently transfecting A-alpha chain truncation mutations we have identified the C-terminal domain of the A-alpha chain as a suitable site for the fusion of interesting molecules, including growth factors. This initial system is capable of generating truncated fibrinogen that is biologically active. We are currently generating chimeric fibrinogen species to study release profiles and functional significance of this novel polymer-delivery system.

METHODS:

1**.** Fibrinogen ELISA:
Capture IgG: goat anti-human fibrinogen (1:1000)
Detection IgG: HRP-conjugated goat anti-human fibrinogen (1:50,000)

2. GPRP purification:
Binding buffer: 0.1M HEPES, 20 mM CaCl
Elution buffer: 1M NaBr, 50 mM NaAc, pH 5.3

3. Fibrinogen Assays: Polymerization rate, Percent clottable protein, Plasmin degradation rate, SDS-PAGE

RESULTS: All truncated fibrinogens are capable of being produced and assembled.

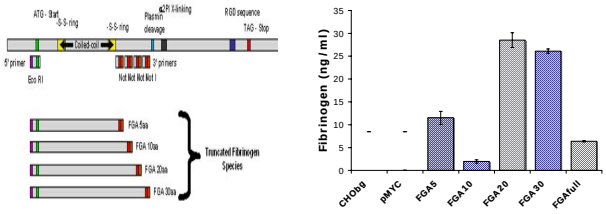

Fig. 1: Transfection of FGA expression vectors into Chinese Hamster Ovary cells stably expressing fibrinogen Bβ and γ chains (CHOBγβ).

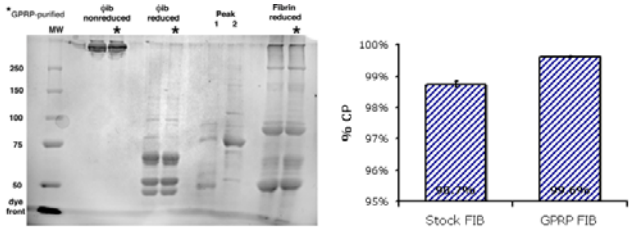

Fig. 2: GPRP-purified fibrinogen retains its native activity.

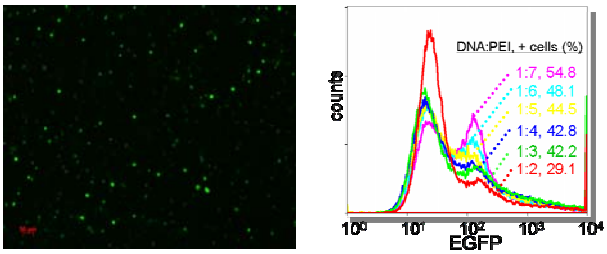

Fig. 3: FACs analysis of CHOβγ cells in suspension transfected with pEGFP

DISCUSSION & CONCLUSIONS: Fibrinogen Aα chain can be truncated to amino acid 184 while maintaining the capacity to be assembled into a secreted, detectable fibrinogen. Current problems exist with current technology for the production of recombinant fibrinogen that require additional development. Pilot studies indicate that anchorage-independent CHO cells may be capable of producing significant quantities of fibrinogen. So, the creation of recombinant chimeric fibrinogens is feasible and provides a means for the direct incorporation, at the genetic level, of protein therapeutics into fibrin matrices.

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CONTROLLED PREPARATION OF BIOMOLECULAR INTERFACES BETWEEN CELLS AND SYNTHETIC MATERIALS

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INTRODUCTION: A contact of synthetic materials with cells and tissues in vivo is preceded nearly always by the adsorption of biological macromolecules, especially proteins, from ambient aqueous media, such as, cell cultivation media in vitro or extracellular body fluids in vivo, onto the material surface. Subsequent effects of the surface on cell adhesion, proliferation, and differentiation are mediated by the adsorbed protein assembly. Thus, the precoating of synthetic scaffolds with adequately stable protein assemblies of specific composition and architecture seems to be a potential way in which their interaction with cells and tissues could be controlled.

METHODS: The growth of smooth muscle cells was studied on polystyrene surfaces coated with various molecular multilayer assemblies composed of extracellular matrix proteins collagen I and IV, laminin, and fibronectin) or coated with fibrin fiber networks and the fibrin networks modified with the extracellular matrix proteins. Multilayer assemblies were prepared by the successive adsorption based on physical interactions, mainly the electrostatic ones, between different macromolecules. The preparation of fibrin networks growing out of the surface comprised the immobilization of thrombin onto a fibrinogen monolayer adsorbed on the surface followed with the activation of fibrinogen solution at the surface by the immobilized thrombin. Some coatings were stabilized with covalent bonds formed by carmodiimide-succinimide method or by Factor XIII in fibrin networks. The formation and stability of the assemblies on solid surfaces was observed in situ by IR reflection spectroscopy and surface plasmon resonance

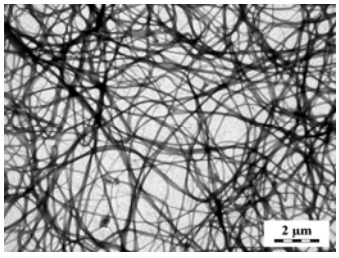

Fig. 1: Fibrin network growing out of the polystyrene surface (Transmission electron microscopy)

RESULTS: Tables I and II show relative numbers, n, of smooth muscle cells attached onto polystyrene surface (PS) coated with some of the tested molecular assemblies 7th day after the seeding (n=100% on uncoated PS).

Table 1 The growth of cells polystyrene surface coated with molecular multilayer

	COI PS	Lm COI PS	COIV H COI PS	Lm COIV H COI PS	COIV Lm COI PS
n %	125	142	155	155	136
std	5.8	5.2	6.1	5.2	3.9

Molecular layers COI (collagen I), Lm (laminin), H (heparin), and COIV (collagen IV) were successively deposited on PS

Table I1. The growth of cells polystyrene surface coated with fibrin networks.

	Fb PS	Fn Fb PS	Lm Fb PS	COI Fb PS
%	139	129	109	125
std	2.8	5.2	2.5	5.1

Fn (fibronectin), Lm, and COI were attached Fb (fibrin network)

DISCUSSION & CONCLUSIONS: The coating of polystyrene with biomolecular assemblies supports the growth of cells on the surface. Analogous coatings can be prepared on surfaces on which a first protein layer can be adsorbed, generally on any materials except of the highly hydrophilic uncharged hydrogels.

ACKNOWLEDGEMENTS: This research was supported by the Grant Agency of the ASCR. (IAA400500507) and by the Ministry of Education, Youth, and Sports CR (1M0021620803).

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COMPARISON OF TISSUE REACTION TO IMPLANTED POLYIMIDE AND SILICON MICROELECTRODE ARRAYS

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INTRODUCTION: This work compares the tissue reaction to implantable polymer-based and silicon-based microelectrode arrays. Although silicon probes have long been used in neural recording, the mechanical mismatch between the stiff, brittle probe and the tissue is thought to be an aggravating factor in the inflammation at the implantation site, and micromotion between the probe and tissue can contribute to shear-induced inflammation [1]. In response to brain injury, astrocytes and microglia proliferate and form a glial scar which encapulates the implant. The use of flexible arrays may reduce this reaction and contribute to long-term viability of future neural prosthetic devices.

METHODS: Silicon-based probes (15 µm thick) were obtained from the University of Michigan CNCT. Polyimide test structures (22 µm thick) of the same shape were microfabricated at the Center of MicroNanoTechnology (CMI) in Lausanne. The structures were patterned using reactive ion etching and were released from the substrate by anodic dissolution of a sacrificial aluminum layer underneath the polyimide [2]. All implantations were performed at the Georgia Institute of Technology. The silicon and polyimide structures were implanted into the brains of anesthetized male Sprague-Dawley rats, one in each hemisphere. One week post surgery, the rats were perfused, the probes were carefully removed, and the brains were sectioned and immunostained with GFAP, ED1, and NeuN antibodies to identify astrocytes, reactive microglia/macrophages, and neuron nuclei, respectively.

RESULTS: Preliminary results show that the polyimide structures induce slightly more inflammation than the silicon probes.

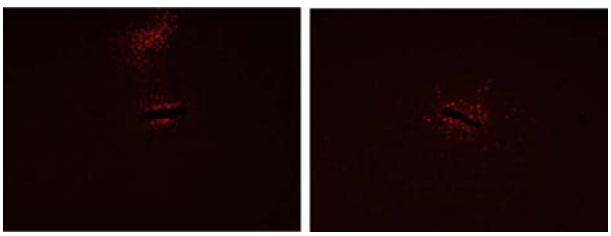

Fig. 1: ED1 staining for reactive microglia and macrophages surrounding the polyimide (left) vs. silicon (right) implant sites.

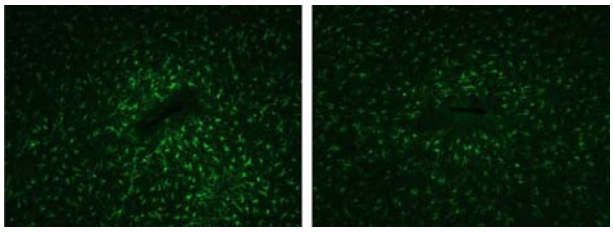

Fig. 2: GFAP staining for reactive astrocytes surrounding the polyimide (left) vs. silicon (right) implant sites.

Tissue surrounding the polyimide structures showed less neurofilament staining than around silicon probes.

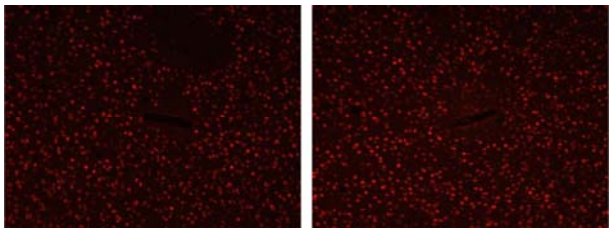

Fig. 3: NeuN staining for neuron nuclei surrounding the polyimide (left) vs. silicon (right) implant sites.

DISCUSSION & CONCLUSIONS: One possible cause for the slightly increased inflammation around the polyimide is that the polyimide structures (22 µm) are thicker than the silicon structures (15 µm), thus causing more injury in the cortex during implantation. Another possibility could be that the polyimide surface activates microglia more than silicon.

Current implantation tests seek to compare implant structures that are the same thickness. In addition, *in vitro* tests will compare the adhesion of microglia to polyimide and silicon and whether one surface is more proinflammatory.

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ACKNOWLEDGEMENTS: This research is supported by the Healthy Aims project IST-2002-1-001837, NIH R01-DC-06849-01 and NIH R01-NS-45072.

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Interaction of functionalized superparamagnetic iron oxide nanoparticles with the brain

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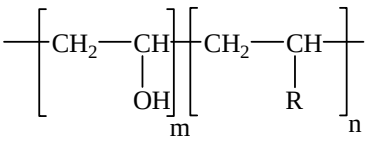
INTRODUCTION. The use of Super Paramagnetic Iron Oxide Nanoparticles (SPIONs) combined with MRI is under clinical evaluation to enhance the detection of vascular leakage associated with human diseases. A major improvement would be to link therapeutic drugs to the SPIONs to achieve targeted drug delivery together with active disease detection, without inducing cell reaction. Our objectives are to develop drug-derivatized SPIONs and to define the characteristics of SPIONS able to achieve cell-specific delivery of therapeutic drugs to defined brain-derived cells, in particular endothelial and mononuclear cells.

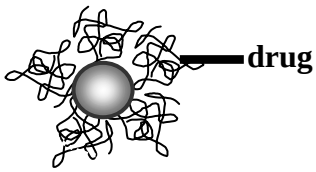
METHODS. Our system consists in an ironoxide core (9-10 nm diameter) coated with various polyvinylalcohol (PVA) polymers, which have been functionalized on the hydroxyl groups with either amino, carboxylate or thiol groups[1] and schema 1:

PVA :



R=-OH, -NH_2, -COOH, -SH

SPIONs:



Schema 1: Functionalization of the PVA coating of the SPIONs and structure of the functionalized SPIONs.

Cellular uptake, cell reaction and cytotoxicity of these various nanoparticles were evaluated using the rat EC219 brain-derived endothelial cells, the murine N9 and N11 microglial cells and differentiated aggregate brain cells in 3-dimensional cultures.

RESULTS. Only aminoPVA-coated SPIONs were taken up by brain cells, but externally added aminoPVA-coated SPIONs did not invade brain cell aggregates deeper than the first cell layer (Figure 1A). None of the SPIONs coated with the various PVAs was cytotoxic or induced the production of the inflammatory mediator nitric oxide (NO) used as a reporter for inflammatory cell reaction. AminoPVA-coated SPIONs did not induce cell activation in cell and in the aggregate cultures. AminoPVA-coated SPIONs derivatized with a fluorescent reporter molecule demonstrated in microglial and endothelial cells that the fluorescence can be found strongly associated with cells. We demonstrated in confocal microscopy, that the fluorescence was localized inside microglial cells (Figure 1B).

A B

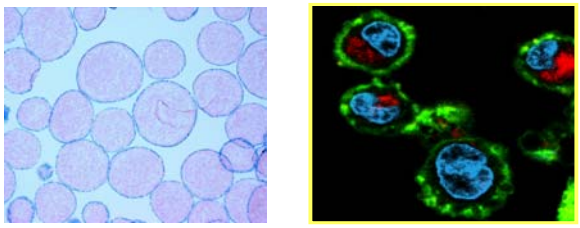


Fig. 1: Localization of functionalized SPIONs in 3-D (A) aggregate cultures of differentiated brain cells or 2-D (B) cultures of brain-derived microglial cells.

CONCLUSIONS. In conclusion, these approaches gave a proof of concept of the feasibility of delivering drugs to defined brain cells. Brain-derived cells take up SPIONs according to an active, saturable mechanism only when exposed to aminoPVA-coated SPIONs, and these SPIONs and their associated coating are internalized by the cells, without inducing inflammatory reaction and without unselectively invading the whole brain.

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Case Report TALUS: Application of Selective Laser Sintering Technology in Orthopaedics

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[3] *Zimmer GmbH, Winterthur, Switzerland.*

INTRODUCTION: Basing on the example of a tumour infested anklebone (*Talus*), an innovative production method for an implant is being illustrated in the present case report.

A malignant tumour at the right talus of a 32 years old, male patient was to be explanted.

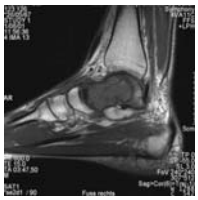

Fig. 1: Tumour affecting entirely the right talus

After the explantation, an external fixation was made to prevent the resulting "cavity" from collapsing. At this stage, the patient had two options:

a) A stiffening of the bone forming the heel (*Calcaneus*) over the navicular bone (*Os naviculare*) and the shin-bone (*Tibia*)

b) Innovative method: substitution of the affected talus with a CoCr alloy talus, obtained from Computer Tomography pictures from the left, healthy talus, mirrored, modelled, cast and implant as right talus

Having thoroughly considered the two variants and referred to Dr Christoph Lampert's advice, Medical Director and Head of Paediatric Orthopaedics at the Hospital of the Canton of St. Gall, the patient decided on new variant b).

METHODS: This new production method consists of a series of diverse single steps: First, CT pictures of the left, healthy talus are being scanned. With the special software called MIMICS (Materialise Belgium) these pictures are processed to allow the "extraction" of the healthy talus from its ankle bone system. This separately sliced talus is then converted into a 3D file, mirrored and scaled with the shrinkage factor of the steel material (CoCr alloy) in which it will be cast. The 3D file is selective laser sintered (SLS), layer by layer, in a wax/polystyrene material. The investment caster wraps the model into several ceramic shells and fires them to obtain a solid mould. During this process, the wax melts away and the polystyrene evaporates, leaving the required space volume inside the ceramic form for the casting of the CoCr metal alloy. Subsequently, the cast is being surface finished, polished, packed, sterilised and implanted.

RESULTS: Today the patient walks painless. As post surgical X-ray pictures show, the patient puts his ankle under load again, and the internal bone structure of the tibia (Spongiosa) grows again. The next steps will be to apply that new methode to more complex shaped bones.

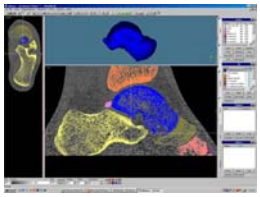

Fig. 2: separated and 3D converted left healthy talus (blue) before mirroring

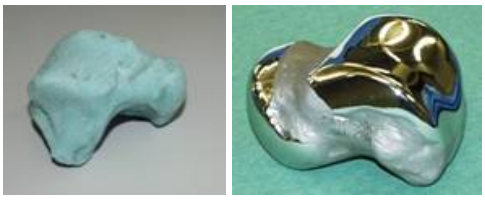

Fig. 3/4: Selective laser sintered, mirrored respectively reversed wax model and CoCr cast of new right talus, polished

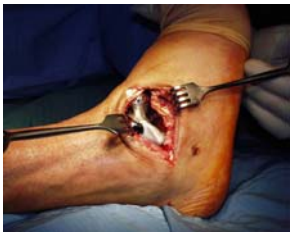

Fig. 5: Implanted right talus

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European Cells and Materials Vol. 10. Suppl. 5, 2005 (page TM3) ISSN 1473-2262

NEW SURFACE TREATMENT TO REDUCE ALUMINA CONTAMINATION OF GRIT-BLASTED IMPLANTS

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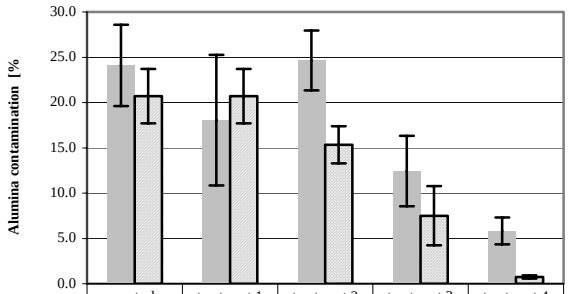
INTRODUCTION: For more than 20 years alumina-blasted surfaces have been successfully used for cementless surgical implants, because of their good biocompatibility and rapid osseointegration. However, it has been shown recently that grit-blasting leads to partial embedding of aluminum oxide particles that can cover 24% to 40% of the surface of commercial hip implants [1-2]. These particles can invade the joint space when dislodged and result in third body wear of the articulating surface [3]. Therefore, there is a need to develop a method which can reduce the hard particles contaminating grit-blasted surfaces without affecting the existing topography. The efficacy of four different surface treatments is presented regarding surface contamination, roughness and topography.

METHODS: Samples were made from three titanium alloys (cpTi, Ti-6Al-4V and Ti-6Al-7Nb). All reference samples were alumina-blasted with 3 or 4 bars depending on the alloy to reach a Ra of 4-6 μm, representing the roughness of commercial implants. After optimisation, four different surface treatments were analysed: #1 chemical; #2 mechanical; #3 chemical & ultrasounds in water; and #4 chemical & mechanical combined.
The "chemical" process loosens the embedded alumina particles. The "mechanical" process removes mechanically the loosened particles.
Two methods based on image analysis were used to measure the surface contamination of alumina. The first analysis named "BSE", uses the chemical contrast obtained by the Back-Scattering Electrons (BSE). The accuracy of the method was controlled by Energy-Dispersive X-ray (EDX) mapping. The second analysis named "Optical", uses a short pickling to enhance the optical contrast under polarized light. The roughness parameters of the surfaces after the different surface treatments were measured with a non-contact optical profilometer.

RESULTS: Alumina contaminations measured with both methods are presented in Figure 1 for Ti-6Al-7Nb alloy. Surface treatments #1 and #2 do not show significant reduction of contamination. However, treatment #3 reduces the contamination by around 48% when measured with BSE and contamination is 76% lower than the control when using treatment #4 (considering the mean values). Measured with the optical method, the reduction of contamination is of the order of 96% for treatment #4. The results for roughness parameters Ra and Rt do not show significant differences between the various surface finishing methods (results not shown). The different treatments show similar trends in contamination, as well as roughness parameters, for cp Ti and Ti-6Al-4V (results not shown).



	control	treatment 1	treatment 2	treatment 3	treatment 4
BSE	24.1	18.1	24.6	12.4	5.8
Optical	20.7	20.7	15.3	7.5	0.7

Fig. 1: Al_2O_3 contamination after 4 surface treatments on grit-blasted Ti-6Al-7Nb substrates measured with 2 methods (control = grit-blasted surface); mean and STDEV from 5 measures.

DISCUSSION & CONCLUSIONS: The BSE method is very sensitive to small particles and takes also into account the particles that are deeply embedded into the substrate material. On the other hand the Optical method takes into account the large particles which are on the outer surface and is therefore more representative for the potential particle shedding. The best results are obtained with the combined surface treatment #4.

· The chemical process combined with the mechanical surface treatment is able to reduce the alumina surface coverage of grit-blasted titanium implants by up to 96% without changing the existing surface micro topography.

· Potential particle shedding of alumina is virtually eliminated.

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