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“The effects of biomaterial components on bone cells”

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Abbreviations

BMP	Bone morphogenetic protein
CO_2	Carbon dioxide
DBM	Demineralized bone matrix
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ESEM	Environmental scanning electron microscopy
FCS	Fetal calf serum
HA	Hydroxyapatite
IC 2959	Irgacure 2959
IC 369	Irgacure 369
IGF	Insulin like growth factor
M-CSF	Macrophage colony stimulating factor
PBS	Phosphate buffered saline
PCL	Polycaprolactone
PEGDA	Poly(ethylene glycol) diacrylate
PEGDMA	Poly(ethylene glycol) dimethacrylate
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
RANKL	Receptor activator of nuclear factor κB ligand
SEM	Scanning electron microscope
TGF-β	Transforming growth factor β
TRAP	Tartrate-resistant acid phosphatase
α-MEM	α-modified minimal essential medium

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Aim of the thesis

Engineering synthetic bone grafts is an important field of research with the aim of developing biomaterial scaffolds that mimic natural bone tissue and potentially are even superior to auto-, allo- or xenografts.

If a novel synthetic bone substitute has to be designed, there are several things to be considered, starting with material composition, in vitro tests, up to in vivo performance. Aside from chemical and technical demands regarding structure and mechanical stability, cytotoxicity and cell behavior are important aspects to examine.

In this work, single components, that are possible constituents of scaffolds, were tested with four different cell types to assess cell viability and inhibition of cell growth. This is an important first step in the development process, since unreacted substances can be present in the product after the manufacturing process or be released during the degradation process into the surrounding tissue potentially causing harm.

1 Introduction

1.1 Structure of bone tissue

Bone is a connective tissue, composed of minerals, proteins, lipids, polysaccharides and various cell types. The complex structure provides mechanical stability as well as tensile strength. For biomaterial design, it is important to understand the function and interactions of the different components, in order to mimic the composition and properties of natural bone.

Collagen fibrils, representing the main type of protein in the organic matrix, form a flexible network contributing to tensile strength, whereas non-collagenous proteins can serve as nucleation sites for biomineralization or promote cell adhesion.

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is the idealized formula of inorganic units. In vivo building blocks are not only composed of calcium, phosphate and hydroxyl groups. Magnesium, sodium and carbonate are incorporated as well. The crystals embedded in the collagen framework guarantee rigidity and mechanical support. Minerals can also be released from the reservoir in bone if blood levels of calcium or phosphate are low.

There are two types of bone, differing in macro-structural tissue organization: spongy (i.e. cancellous or trabecular) bone with higher porosity and vascularization, and cortical (i.e. lamellar ore dense) bone. The proportion of either one in the particular type of bone varies depending on anatomical requirements (Muruguan et al., 2010).

1.2 Cellular components of bone tissue

Bone is not a static and stiff tissue. It is subject to constant repair of micro-fractures and remodeling, which is important to beware it from becoming brittle and fragile. Osteoclasts are large, multinucleated cells derived from hematopoietic stem cells. Their differentiation and function is mediated through several cytokines and influenced by various factors, such as blood calcium level or tiny mechanical defects. Osteoblasts incorporated into the tissue during bone formation develop into so called

osteocytes when spatially isolated. They are connected with each other, as well as with osteoblasts and bone lining cells, via filipodial extensions and induce increased bone remodeling if micro-cracks (small fractures) occur in their vicinity. Like osteoblasts they are able to produce RANKL, a cytokine crucial for the development of mature osteoclasts. Various chemoattractants for example released by osteoblasts or lining cells, stimulate osteoclasts to migrate to the remodeling site. Osteoclasts attach to the surface via integrins interacting with matrix proteins, and generate a sealing zone, separating the resorptive site from the surrounding area. They secrete HCl and several enzymes, such as cathepsin K or TRAP, to dissolve respectively inorganic minerals and organic matrix. Osteoclasts also play an important role in recruiting osteoblasts (derived from the mesenchymale linage) by secretion of cytokines or direct cell-cell contact. For example, Ephrin B2, located on the surface of osteoclasts, interacts with its receptor EphB4 on osteoblasts, promoting their differentiation and bone formation. Osteoblasts also deposit osteoinductive growth factors, such as BMPs, IFGs or TGF-β during matrix formation, which are released during bone resorption by osteoclasts. The so called Howship lacuna has to be filled with new tissue. Osteoblasts produce various proteins forming the organic matrix (i.e. osteoid), finally followed by mineral deposition (exact mechanism still unclear). At the end of the remodeling process osteoblasts either undergo apoptosis or become osteocytes or lining cells. Osteocytes do not only act as mechano-sensors as described above, but also produce sclerostin, which inhibits osteoblast proliferation and function and induces osteoclast formation and activity. Lining cells cover the bone surface and maybe also contribute to the regulation of bone remodeling.

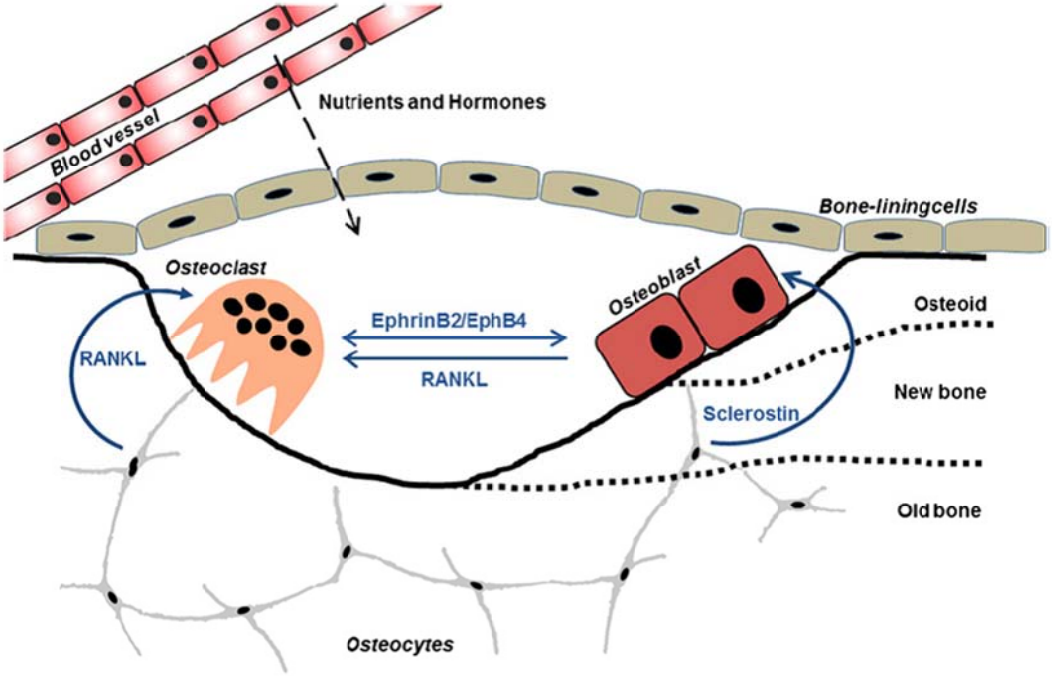

Figure 1: Overview of the bone remodeling process (Kular et al., 2012)

Other cellular components present in bone tissue are vascular endothelial cells, forming capillaries to guarantee sufficient blood supply, as well as resident macrophages (osteomacs) (Kular et al., 2012).

1.3 Bone grafts

The healing of bone defects is a complex process. They can either regenerate spontaneously within a few weeks or may require the help of naturally-derived or synthetic materials if they exceed a certain size (Muruguan et al., 2010). This size is dependent on the particular type of bone, for example 3 cm for the femur and tibia or 5 cm for the forearm (Calori et al., 2011). These so called critical size defects will not completely heal without surgical intervention, thus the development of bone grafts and biomaterials is an important field in tissue engineering. Statistics show, that the market has been growing over the past years and it is predicted to continue increasing (Muruguan et al., 2010).

1.3.1 Ideal properties of bone grafts

Bone grafts are used to bridge the gap of the defect and also to provide mechanical and structural support. They should be readily available, osteoconductive, osteoinductive and osteogenic. Osteoconductivity implies that the three-dimensional structure of the material allows bone cells to migrate into it and fulfill their physiological function, also that it enables new vascularization and thus guides the overall regeneration process. Osteoinductivity means that the graft is able to induce or stimulate bone formation, like promoting differentiation of osteoblast precursors through the presence of growth factors. An osteogenic material contains living bone cells.

Another desirable property is bioresorbability, particularly in correlation with new bone formation and the remodeling process, in order to allow the graft to be replaced by newly formed tissue. Of course materials and degradation products must be biocompatible and should not cause rejection. In addition to biological suitability, economic aspects, such as cost-effectiveness, should also be considered (Calori et al., 2011).

1.3.2 Types of bone grafts

1.3.2.1 Autograft

The transplantation of autologous bone tissue is currently considered the gold standard among the different materials in current use. The patient's own bone is harvested from another skeletal segment, for example the iliac crest, and transplanted to the lesion. A newer method is to take osteoblastic stem cells from the patient's bone marrow and transfer them to the defective site as a composite in combination with scaffolding biomaterials. Advantages of autografts are their osteoconductive, osteoinductive and osteogenic properties. Also they are totally biocompatible and will be replaced by new bone (Muruguan et al., 2010). Disadvantages lie in an increased risk of surgical complications, such as infection, and morbidity at the donor site (Calori et al., 2011). Depending on defect shape and size it can be difficult to obtain the

necessary amount and the required shape of autologous tissue (Muruguan et al., 2010).

1.3.2.2 Allograft

Allogenic bone tissue is obtained from cadaver or other individuals of the same species. It is osteoconductive due to the equivalent structure, but loses its osteoinductive or osteogenic properties during the cleaning and sterilizing process. Compared to autografts, the use of allografts avoids complications due to the harvesting process of the patient's bone. On the other hand it increases the risk of disease transmission and immune response to the allogenic tissue. Therefore it is used only as a second choice, for example to treat large defects when autografting is not possible (Muruguan et al., 2010).

A special type of allograft is demineralized bone matrix (DBM). It consists of the remaining organic matrix after the mineral component of the allogenic bone is removed by acid extraction. The main constituent is type I collagen, in addition to other non-collagenous proteins, including osteoinductive growth factors, like BMPs. Due to its origin from biological material, the content and composition of growth factors vary between samples and are not standardized, leading to great differences in biological activity between the available DBM-products (Nicoll, 2011).

1.3.2.3 Xenograft

In xenografting tissue is transplanted between two different species. Donor material is readily available, but disadvantages are the same as for allografts, and are even worse in terms of antigenicity (Muruguan et al., 2010).

1.3.2.4 Alloplastic or synthetic grafts

Due to the disadvantages of naturally-derived bone grafts, developing adequate synthetic substances (i.e. biomaterials) to fabricate scaffolds that bridge the lesion, is of great interest (Muruguan et al., 2010). The additional surgical risks and increased patient morbidity when using autografts can be avoided as well as disease

transmission. Ideally the properties of synthetic bone substitutes can be adjusted to the requirements of treating different types of defects and even be superior to the use of natural bone transplants.

There are a wide variety of substances and production methods, which will be described in more detail in the next chapter.

1.4 Biomaterials

Scaffolds are engineered as synthetic grafts with the ambition to mimic the properties of natural bone and to achieve osteoconductivity, osteoinductivity, osteogenicity as well as bioresorbability while evading disadvantages of auto-, allo- and xenografting.

Important features to consider are biocompatibility, the shape, size and connectivity of pores, as well as surface properties and degradation profiles. Cells should be able to attach to and migrate into the scaffold to produce new bone, while the biomaterial is gradually degraded. Also the three-dimensional structure should allow vascularization and sufficient fluid transport to deliver nutrients and eliminate metabolic waste (Nicoll, 2011). When in contact with biological fluids or cell culture medium, proteins are adsorbed to the material surface. Quantity and composition of this layer depends on chemical and topological properties of the scaffold, which together influences the cell attachment. Cells generally adhere more efficiently to rough surfaces. Altering the hydrophobicity also contributes to biomaterial-cell interactions (Luo et al., 2007).

Requirements regarding mechanical stability depend on the intended implantation site. For example, weight-bearing bones such as the femur have a much greater demand in terms of mechanical strength compared to the calvaria. To date, many synthetic bone grafts have been adequate to replace trabecular bone, but lack sufficient strength as substitutes for cortical tissue (Nicoll, 2011).

To enhance the function of biomaterials, it is possible to achieve osteoinductivity or osteogenicity by including growth factors or living bone cells respectively into appropriate formulations.

1.4.1 Calcium phosphates

Since the main component in the inorganic part of bone is a highly substituted apatite, it is common to use minerals such as synthetic hydroxyapatite (HA) with a structure of ($Ca_{10}(PO_4)_6(OH)_2$) or β-tricalcium phosphate as grafting materials. Due to the similarity to the natural tissue they are osteoconductive and biocompatible. Degradation time can be controlled through crystallinitiy: the smaller the particles and crystals are, the faster they are resorbed (Nicoll, 2011).

Depending on the structural properties and the manufacturing method, calcium phosphates can be divided into ceramics and cements (Calori et al., 2011).

1.4.1.1 Ceramics

When fabricating ceramics, the mineral powder is sintered, producing a porous, solid scaffold. The sintering temperature influences the degree of porosity and surface properties, hence dissolution and cell mediated degradation (Calori et al., 2011) as well as cell attachment and function.

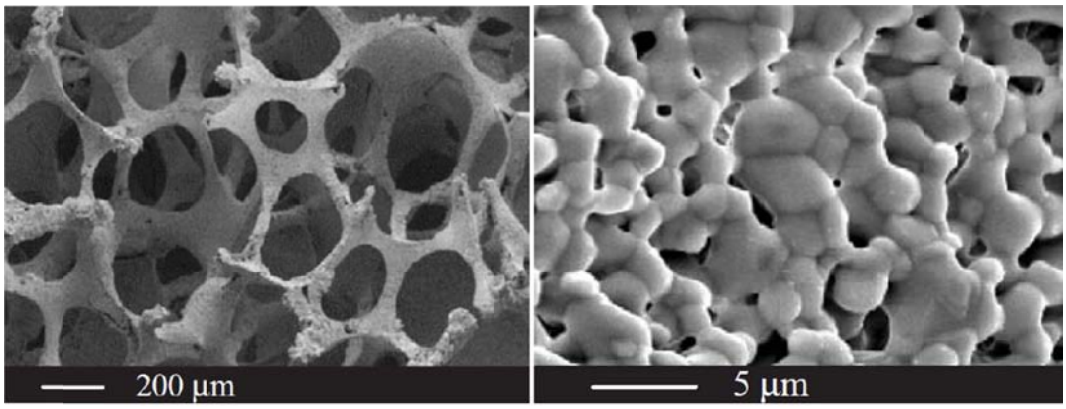

Figure 2: Scanning electron microscope (SEM) images of the porous structure of a sintered hydroxyapatite/β-tricalcium phosphate ceramic scaffold (Ramay and Zhang, 2004)

1.4.1.2 Cements

Cements are more solid compared to ceramics. The calcium phosphates are mixed with water to form a paste, which is applied directly to the defect site, where it hardens to form a crystalline structure, but with limited porosity. Bioresorption occurs mainly through resorption by osteoclasts (Calori et al., 2011).

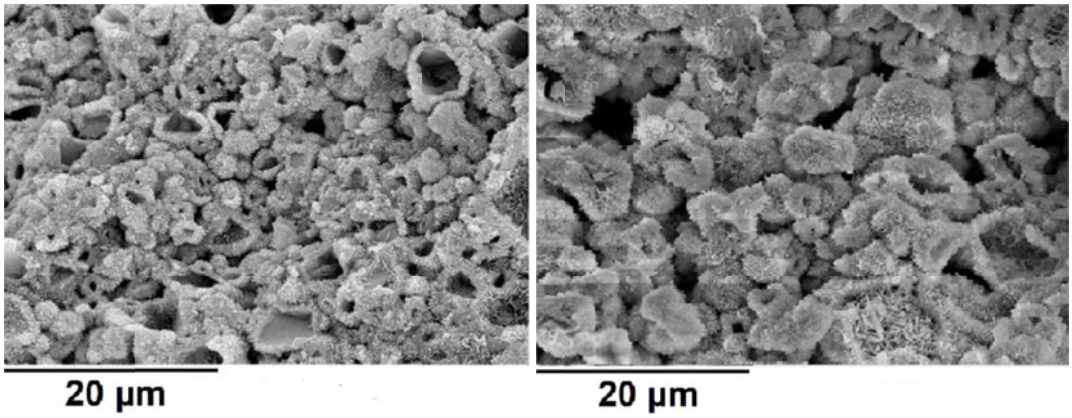

Figure 3: SEM images of two α-tricalcium phosphate cements differing in particle size (Espanol et al., 2009)

1.4.2 Bioactive glasses

Bioactive glasses are osteoconductive, biodegradable, solid materials that allow formation of hydroxyapatite on their surface upon contact with biological fluids (Calori et al., 2011). Depending on the processing method various microstructures can be generated (Fu et al., 2011).

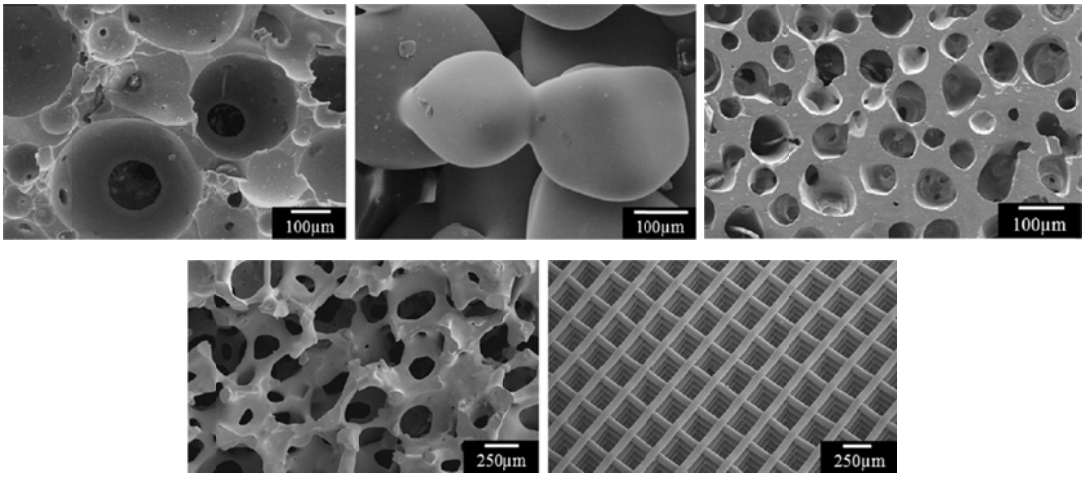

Figure 4: SEM images of microstructures of bioactive glasses generated by different processing methods (Fu et al., 2011)

1.4.3 Metals

Stainless steel or titanium are examples of metals which are biocompatible and exhibit adequate mechanical properties to support defects in weight-bearing sites, however there is only limited use because they are stiff and not biodegradable (Lichte et al., 2011).

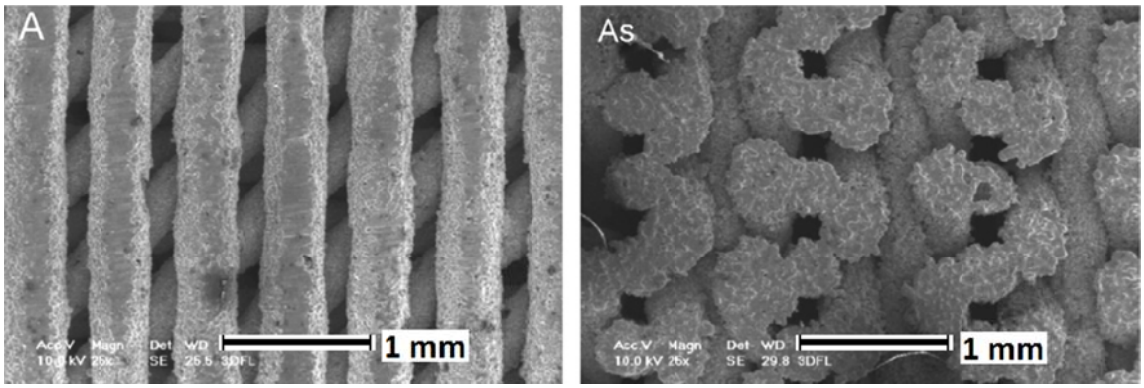

Figure 5: Environmental scanning electron microscopy (ESEM) images of a titanium alloy scaffold (A: top view, As: side view) produced by 3D fiber deposition, which allows fabrication of controlled porosity features (Li et al., 2007)

1.4.4 Natural materials

The most common natural polymer is collagen. Considering the structure of bone tissue, an established approach in developing synthetic grafts is to mimic the bone composition by fabricating collagen scaffolds and including hydroxyapatite crystals. These materials are osteoconductive, biocompatible and biodegradable but show poor mechanical properties and increase the risk of immunogenicity. Also other peptides are able to form nanofiber networks. These can exhibit special features like promoting stem cell differentiation, mineralization or cell adhesion (Nicoll, 2011). Peptide sequences can also be incorporated into synthetic polymer networks, for example to produce materials selectively degradable by special enzymes, like cathepsin K-specific disintegration (Hsu et al., 2011). The fabrication of biomimetic materials is also possible based on DNA structures, which can be alone or in combination with synthetic polymers.

Some other examples of natural substances used in bone regenerative medicine are silk or chitosan (Nicoll, 2011).

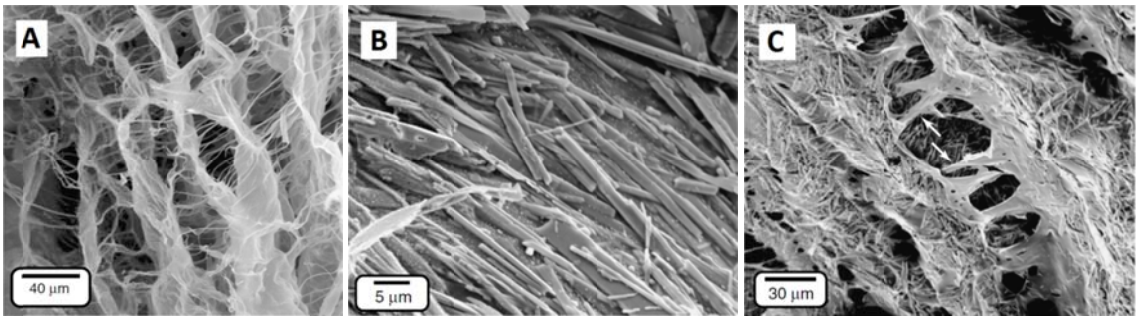

Figure 6: SEM images of freeze-fractured transverse cross-sections of a freeze-dried collagen scaffold without HA (A), HA whiskers (B) and a collagen scaffold reinforced with HA whiskers (1:2 collagen:HA) (Kane and Roeder, 2012)

1.4.5 Synthetic polymers

Commonly used polymers for scaffold design include poly(lactic acid) (PLA), poly(glycolic acid) (PGA), polycaprolactone (PCL), poly(ethylene glycol) diacrylate (PEGDA) and poly(ethylene glycol) dimethacrylate (PEGDMA). The networks can be

assembled out of the corresponding monomers into various three-dimensional forms with distinct porosity and surface characteristics by using different techniques (Nicoll, 2011). A combination with other types of biomaterials allows modulating and improving the properties of the scaffolds.

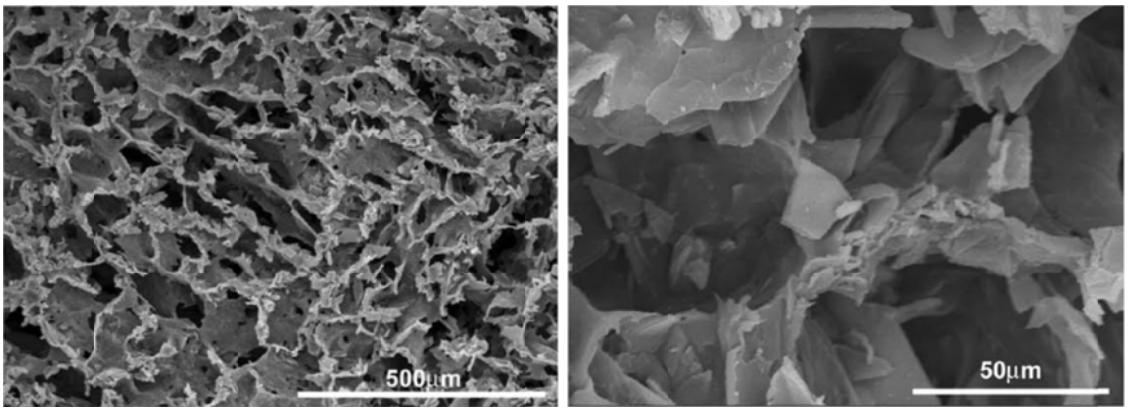

Figure 7: SEM images of a PLA/HA scaffold (Wei and Ma, 2004)

Degradation of synthetic polymers occurs through hydrolysis, enzymatic reactions or can be cell-mediated, depending on composition and structure of the scaffold. Preferably it should be synchronized with the remodelling process, otherwise the scaffold will continue losing mechanical stability if matrix production and mineralization is lagging behind (Grieshaber et al., 2011). Porous, hydrophilic materials are disintegrated throughout the whole structure, whereas in hydrophobic polymers the reaction is limited to the surface (Lichte et al., 2011). Released products can cause immunological reactions by activating macrophages (Lichte et al., 2011) or altering the tissue-pH during acidic degradation processes (Nicoll, 2011).

1.4.6 Hydrogels

Hydrogels are three-dimensional, hydrophilic or amphiphilic polymer networks. Their structural characteristics (Grieshaber et al., 2011) and high water content mimic properties of the natural extracellular matrix (ECM) (Mironi-Harpaz et al., 2012). This provides an appropriate environment for cellular activity and fluid transport.

Components can be natural or synthetic materials or a combination of both (Park, 2011).

Since cell behaviour also depends on mechanical features of their surroundings, there are several strategies to overcome the structural weakness many hydrogels exhibit. One method is to design “double network gels”, composed of different kinds of polymers differing in the degree of crosslinking. This combination of elastic and rigid networks features flexibility and high water binding capacity on the one hand, on the other hand mechanical strength. Also in this context, the ratio of hydrophobicity to hydrophilicity plays an important role.

There are many chemical and physical crosslinking methods (Grieshaber et al., 2011), which in combination with the different materials and technical manufacturing procedures has generated a wide variety of resulting products.

1.5 Two-Photon Polymerization

The two-photon polymerization is a new approach to fabricate arbitrary three-dimensional structures out of synthetic polymers, directly translated from computer designed models (Raimondi et al., 2012).

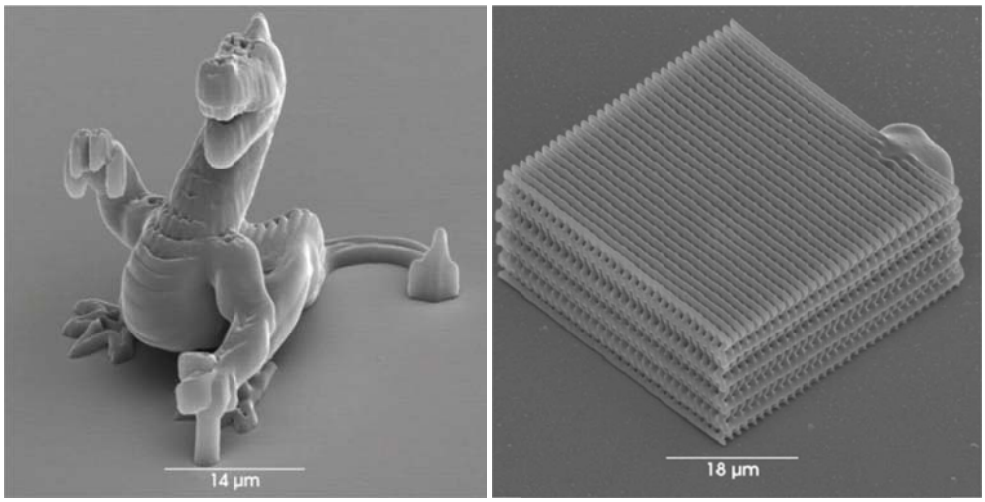

Figure 8: SEM images of structures fabricated by two photon polymerization of Ormocers (Ostendorf and Chichkov, 2006)

Advantages compared to other methods like stereolitography, selective laser sintering or fused deposition modeling, include higher resolution in generating microstructure and porosity, single-step fabrication of the scaffold instead of layer-by-layer preparation and the possibility to polymerize the material in presence of living cells or tissue (Ovsianikov et al., 2011).

Ultrashort (femtosecond) laser pulses are focused into a volume of a photosensitive material (further referred to as photoresist), which leads to two-photon absorption and free-radical reactions. The laser focus is moved through the material until the desired structure is translated. Further development, for instance a washing step, leads to the final three-dimensional structure.

There are two types of materials used in two-photon polymerization: positive- and negative-tone photoresists. In the former, exposure to the femtosecond laser pulses leads to chain scission of a polymer, creating shorter units that can be dissolved and washed away. Negative-tone photoresists can be divided into solid and liquid subgroups. Solid ones are epoxy-based cationic materials. Organically modified ceramics, also called Ormocers (inorganic/organic hybrid polymers) and acrylate-based photoresists (for example PEGDA and PEGDMA) are liquid (Ostendorf and Chichkov, 2006).

When mixed with a photoinitiator, the acrylates can be used to form a hydrogel (Mironi-Harpaz et al., 2012), which can serve as scaffold component in tissue engineering.

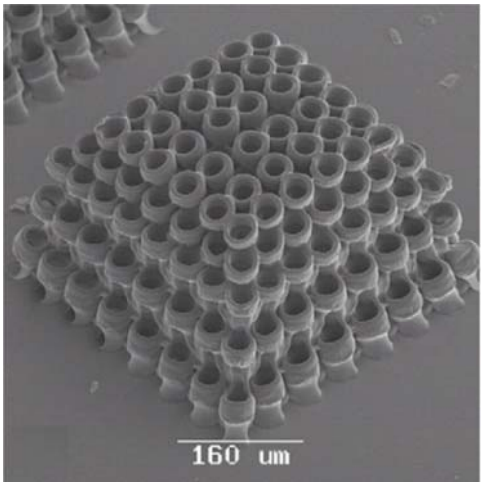

Figure 9: PEGDA (M_n of 742 g/mol) scaffold fabricated by two photon polymerization (Ovsianikov et al., 2011)

The liquid material is placed between two glass cover slips with spacers defining the thickness of the resist. The laser beam, typically generated by a Ti:sapphire system with 800 nanometers wavelength and 100 femtoseconds pulse duration, is focused, for example using a microscope objective (Raimondi et al., 2012). For moving the laser focus through the material, a computer-controlled, highly accurate positioning system is required (Ostendorf and Chichkov, 2006). The ultrashort laser pulses render the exposed volume solid and insoluble to the solvent used in the following developing step, for example ethanol. With an appropriate fabrication setup this method can beat the diffraction limit, which means that the achieved resolution is smaller than the wavelength of light (Raimondi et al., 2012).

Through combinations of different materials, this technique allows the fabrication of scaffolds with defined properties for various applications. Feature sizes can range from hundreds of micrometers down to 100 nanometers, which allows exact determination of structural qualities such as pore shape, size, distribution and connectivity, all important parameters for the behavior of cells.

1.5.1 Mechanism of free radical crosslinking

A photoinitiator, for example Irgacure 2959, is added to a liquid monomer, like PEGDA. Upon laser irradiation, two-photon absorption by the photoinitiator molecule causes

formation of a starter radical. The following chain reaction induces formation of monomer radicals which react with each other to finally form a crosslinked network (Doraiswamy et al., 2006).

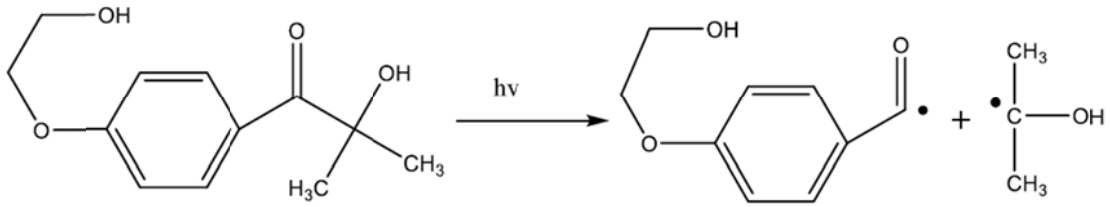

Figure 10: Irradiation leads to radical dissociation of Irgacure 2959 (Mironi-Harpaz et al., 2012)

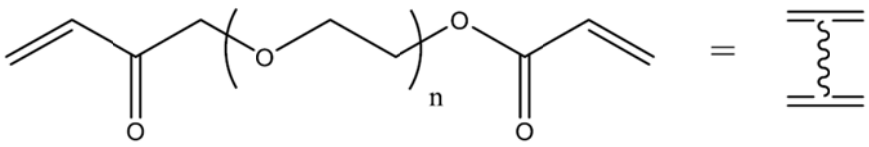

Figure 11: PEGDA (Mironi-Harpaz et al., 2012)

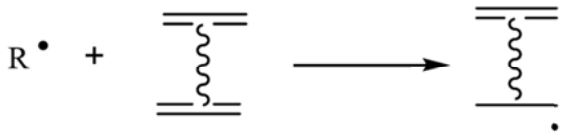

Figure 12: The initiator radical reacts with an acryloyl group of a PEGDA molecule (Mironi-Harpaz et al., 2012)

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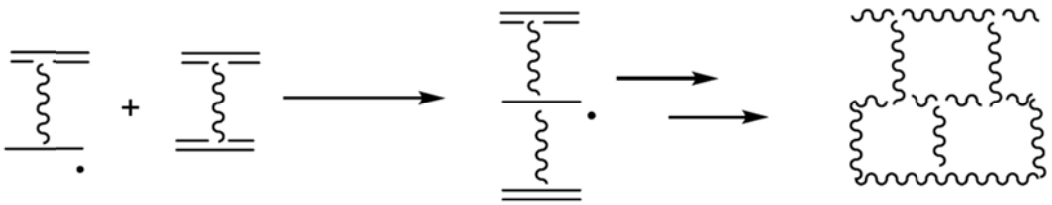

Figure 13: The active center of the monomer radical reacts with other molecules leading finally to hydrogel formation (Mironi-Harpaz et al., 2012)

The double bond of each acryloyl group can react with two other PEGDA molecules, thus the monomers do not only form chains but are crosslinked.

1.6 Primary cell culture versus cell lines

According to R. Ian Freshney, the terms “primary culture” and “cell line” are often used inaccurately, although there are important differences including culture uniformity and life span. Cells directly seeded after isolation from tissue are defined as a “primary culture”, whereas the correct terminology after subculturing is “early passage cell line”, also “secondary” or “tertiary culture” after respectively the first or second split. Passaging of primary cultures containing different cell types finally leads to homogenous cell lines, which can be finite (limited number of population doublings) or continuous (immortalized) (Freshney, 2010).

2 Materials and Methods

2.1 Reagents, Materials and Instruments

Table 1: Reagents

Name	**Supplier**
Acetone	Riedel-de Haen, Germany
$CaCl_2$	Sigma-Aldrich Handels GmbH, Austria
Carbon dioxide	Air Liquide, Austria
CASYton	Roche Diagnostics GmbH, Austria
Collagenase	Sigma-Aldrich Handels GmbH, Austria
Dispase	Sigma-Aldrich Handels GmbH, Austria
DMSO	Sigma-Aldrich Handels GmbH, Austria
EDTA	Sigma-Aldrich Handels GmbH, Austria
Absolute ethanol	Brenntag CEE GmbH, Austria
Fast Red Violet Salt	Sigma-Aldrich Handels GmbH, Austria
FCS	Sigma-Aldrich Handels GmbH, Austria
Formaldehyde 37 %	Carl Roth GmbH + Co. KG, Germany
HCl	MERCK GesmbH, Austria
KCl	MERCK GesmbH, Austria
KH_2PO_4	MERCK GesmbH, Austria
Liquid nitrogen	Vienna University of Technology, Austria
$MgCl_2.6H_2O$	Sigma-Aldrich Handels GmbH, Austria
N,N-Dimethylformamide	Sigma-Aldrich Handels GmbH, Austria
$Na_2HPO_4.2H_2O$	MERCK GesmbH, Austria
NaCl	Sigma-Aldrich Handels GmbH, Austria
NaN_3	Sigma-Aldrich Handels GmbH, Austria
NaOH	MERCK GesmbH, Austria

Naphthol AS-Mx-phosphate	Sigma-Aldrich Handels GmbH, Austria
Penicillin/streptomycin-solution	Biochrom AG, Germany
Sodium acetate	Sigma-Aldrich Handels GmbH, Austria
Sodium tartrate	Sigma-Aldrich Handels GmbH, Austria
Trypsin	Life Technologies, Austria
α-MEM	Life Technologies, Austria
α-MEM + GlutaMAX	Life Technologies, Austria

Table 2: Materials

Name	**Supplier/Company**
24-well plates, tissue culture treated	Costar ®, Corning Incorporated, NY, USA; Falcon, Becton, Dickinson and Company, NJ, USA; Nunc, Thermo Fisher Scientific Inc., MA, USA
48-well plates, tissue culture treated	Nunc, Thermo Fisher Scientific Inc., MA, USA
Centrifuge tube 15 ml	Greiner Bio-One International AG, Austria
Centrifuge tube 50 ml	Greiner Bio-One International AG, Austria
Cryo tubes	Greiner Bio-One International AG, Austria
Eppendorf tube 1.5 ml	Eppendorf AG, Germany
Glass pipets	Brand, Germany
Micropipets	Eppendorf GmbH, Austria
Petri dishes	Iwaki, Japan Sterilin, Bibby Sterilin Ltd., U.K.
Pipet tips	VWR International, Austria Greiner Bio-One International AG, Austria
Plastic sterile pipets	Carl Roth GmbH + Co. KG, Germany

Table 3: Instruments

Name	Company
Autoclave	SX-300E, Tomy Digital Biology, Japan
Camera microscope	D 5100, Nikon, Japan
CASY cell counter	Schärfe Systems, Germany
Centrifuge	Hermle Z 323 K, Bartelt, Austria
Computer	Mac Pro, Apple Computers
Ice machine	AF 103, Scotsman Ice Systems, IL, USA
Incubator	HERAcell® 240, Kendro, NC, USA
Laboratory scale	MC 210 P, Sartorius, Austria
Laminar Airflow work bench	EHRET Labor- und Pharmatechnik, Germany
Magnetic stirrer	Ikamag® RCT, Janke & Kunkel, Germany
Microscopes	Diaphot 300, Nikon, Japan Nikon TMS, Japan
Mr. Frosty Freezing Container	Thermo Fisher Scientific wissenschaftliche Geräte GmbH, Austria
pH-meter	Metrohm, Switzerland
Shaking incubator	GFL-3032, GFL Gesellschaft für Labortechnik mbH, Germany
Vortex	Vortex Genie 2TM, Bender & Hobein AG, Switzerland
Water bath	GFL-1086, GFL Gesellschaft für Labortechnik mbH, Germany

2.2 Experimental set up

To examine cytotoxic effects of monomers and photoinitiators on different cell types, each substance was dissolved in the appropriate medium and tested with osteoblasts, osteoclasts, NIH 3T3 and RAW 264.7 cells. Control and treatment groups were tested in triplets. Every experimental combination of substance and cell type was performed

twice to confirm the results. If results differed, the experiment was repeated a third time.

The methods described below followed the established lab protocols.

2.3 Cell types

2.3.1 Osteoblasts

The cells were obtained from mouse calvaria. To separate other cell types from osteoblasts, cells were seeded, incubated, washed, split 1:4 or 1:5 and then frozen in liquid nitrogen for later use. As defined in the introduction, this is an early passage cell line (Freshney, 2010).

Cells were seeded into 24 well plates, 500 µl per well, at a density of 40,000 cells/cm^2.

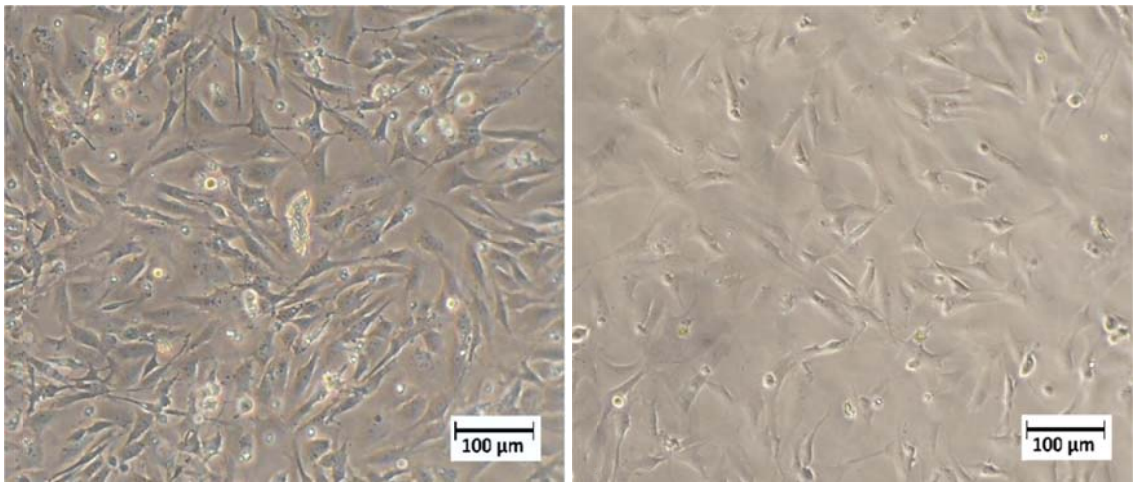

Figure 14: Osteoblasts after 24 hours of incubation in different positions of the well

2.3.2 Osteoclasts

Cells were directly seeded after isolation from rabbit bone, thus it can be considered a primary cell culture, as described in the introduction (Freshney, 2010). The cell suspension was seeded into a 48 well plate. For each substance, repeat experiments were performed, with cells isolated from different individuals.

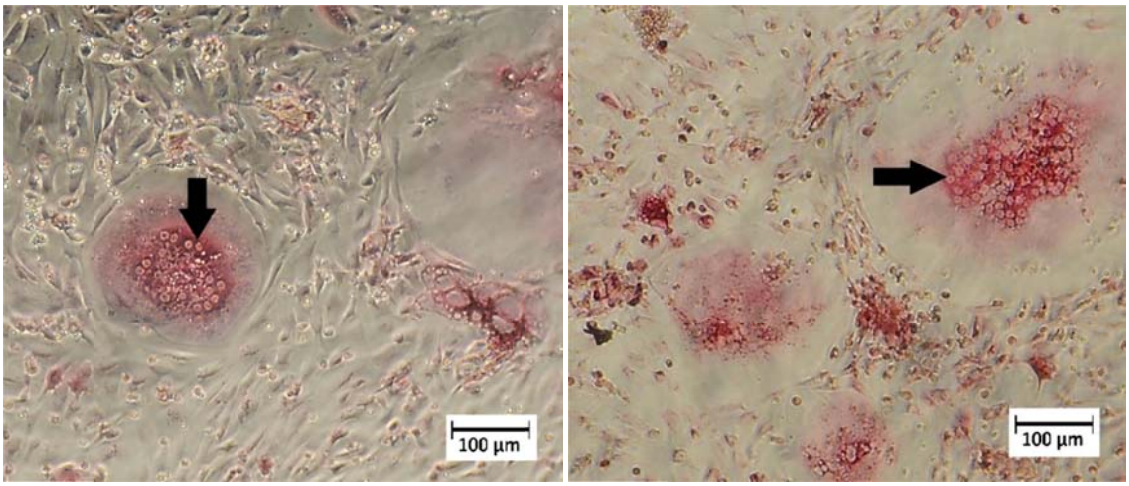

Figure 15: TRAP-stained osteoclasts, arrows indicating nuclei

2.3.3 NIH 3T3

NIH 3T3 is a fibroblast cell line obtained from murine embryo tissue (ATCC® CRL-1658™). Cells were cultivated under the same conditions as osteoblasts (24 well plate, 500 µl per well, 40,000 cells/cm^2).

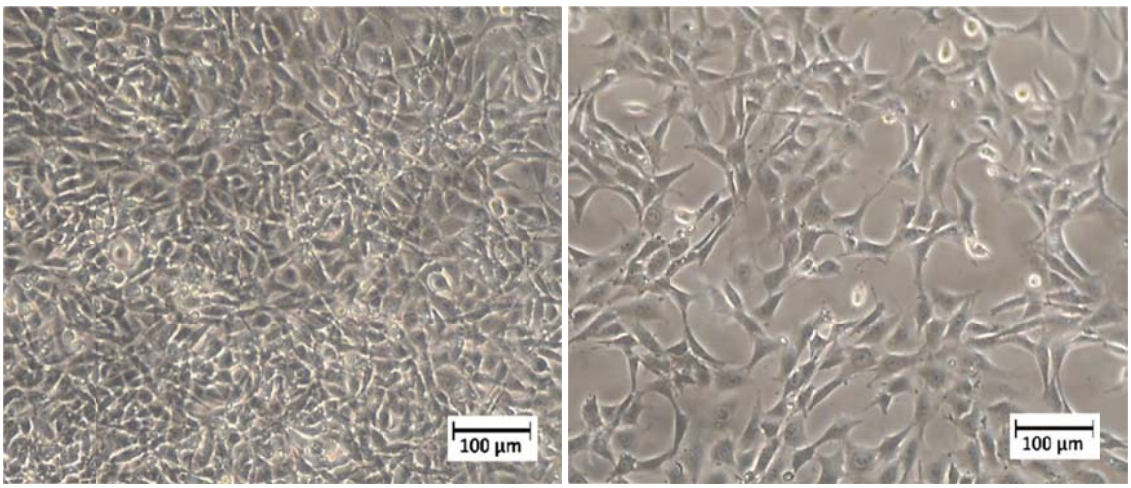

Figure 16: NIH 3T3 after 24 hours incubation in different positions of the well

2.3.4 RAW 264.7

RAW 264.7 is a macrophage cell line (ATCC® TIB-71™) originally established from a tumor induced in a mouse by Abelson murine leukaemia virus (Collin-Osdoby and Osdoby, 2012). In the presence of RANKL and M-CSF, RAW 264.7 cells can fuse and differentiate into mature multinucleated osteoclasts.

Cells were seeded into 24 well plates, 500 µl per well, at a density of 130,000 or 33,000 cells/cm^2.

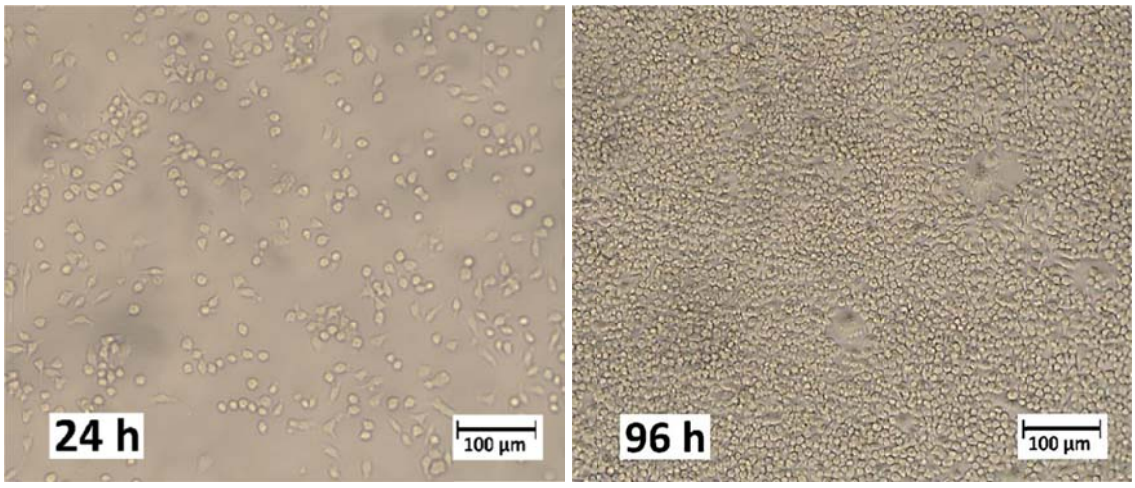

Figure 17: RAW 264.7 are considerably smaller than osteoblasts or NIH 3T3, thus after 24 hours of incubation, cell growth looked less dense compared to osteoblasts or NIH 3T3. When incubated for additional 72 hours (a total of 96 hours), cells had grown to confluency.

2.4 Preparation of media

2.4.1 Heat inactivation of fetal calf serum

Fetal calf serum (FCS) was heated up to 56 °C for 30 minutes in the water bath to denature enzymes and other biological active proteins.

2.4.2 Medium for osteoblasts and NIH 3T3

α-modified minimal essential medium (α-MEM, containing phenol red indicator) was supplemented with 10 % heat inactivated fetal calf serum and 1 % penicillin/streptomycin-solution.

2.4.3 Medium for osteoclasts

α-MEM was supplemented with 5 % heat inactivated fetal calf serum and 1 % penicillin/streptomycin-solution.

2.4.4 Medium for RAW 264.7

α-MEM + GlutaMAX was supplemented with 10 % heat inactivated fetal calf serum and 1 % penicillin/streptomycin-solution.

2.5 Photoinitiators:

2.5.1 Irgacure 2959

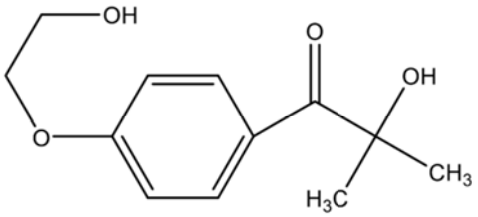

Figure 18: 2-Hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl 1-propanone (Mironi-Harpaz et al., 2012)

Irgacure 2959 was purchased from BASF SE, Germany. It is a white to slightly yellow powder with a solubility in water of 7.6 g/l (Irgacure 2959) and a molecular weight of 224.3 g/mol. The investigated concentrations (3, 1, 0.3 and 0.1 mg/ml) were chosen considering the data regarding acute toxicity in fish (0.147 mg/ml), aquatic invertebrates (0.615 mg/ml) and aquatic plants (> 0.1 mg/ml) published in the BASF material safety data sheet (Irgacure 2959). After a preliminary experiment with

osteoblasts testing 3, 1, 0.1, 0.03 and 0.01 mg/ml, 0.03 and 0.01 mg/ml were eliminated since no reduction in cell number could be observed. Instead a group with 0.3 mg/ml was added.

The substance was dissolved in the appropriate cell culture medium at a concentration of 3 mg/ml and then diluted to the required concentrations.

2.5.2 Irgacure 369

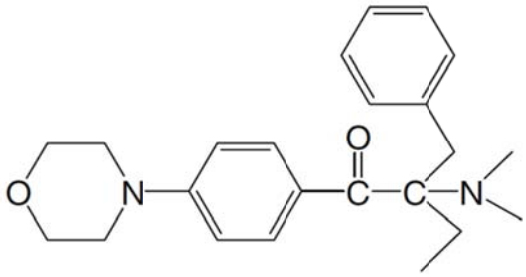

Figure 19: 2-benzyl-2-(dimethylamino)-1-(4-morpholinophenyl)-1-butanone (Shen et al., 2009)

Irgacure 369 was also purchased from BASF SE, Germany. It is a light yellow powder with a water solubility of only 0.0059 g/l (5.9 µg/ml) and a molecular weight of 366.5 g/mol. Reference for choosing the concentrations for the experiments was the ecological information in the BASF material safety data sheet about acute toxicity in fish (0.46 µg/ml), aquatic invertebrates (> 0.8 µg/ml) and aquatic plants (> 2 µg/ml) (Irgacure 369).

In the first experiment with osteoblasts, concentrations of 3, 1, 0.3 and 0.1 µg/ml were tested. A significant reduction of cell number could not be observed in any of the groups, hence for the following experiments concentrations of 6, 3 and 1 µg/ml were examined. As mentioned above, 6 µg/ml represents the solubility limit in water.

Since the substance is not easily soluble in water, it was dissolved in absolute ethanol at a concentration of 6 mg/ml. This was diluted with the appropriate cell culture medium 1:1000 to obtain a concentration of 6 µg/ml, which was further diluted to 3 and 1 µg/ml. The groups were compared to a control containing 1 µl ethanol per ml. Also a control with culture medium alone was included.

2.5.3 Ini1

Ini1 is a benzylidene cyclopentanone dye, received as a gift from IBA Heiligenstadt, Germany. It is a dark red powder, readily soluble in water and shows an intense red colour when dissolved. Since it is still an experimental substance, no other data are published yet.

To identify the cytotoxic range, concentrations of 1000, 100, 10, 1 and 0.1 µg/ml were examined in a preliminary experiment with osteoblasts. Since only 1000 µg/ml showed a significant reduction in cell number, concentrations between 1000 and 30 µg/ml (1 and 0.03 mg/ml) were chosen for the following experiments. The substance was dissolved in the appropriate cell culture medium at a concentration of 1 mg/ml and further diluted to 0.3, 0.1 and 0.03 mg/ml.

2.6 Monomers

2.6.1 PEGDA

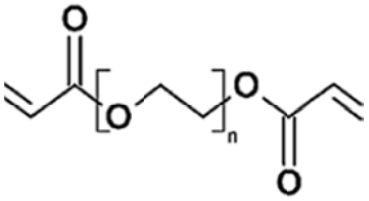

Figure 20: Chemical structure of Poly(ethylene glycol) diacrylate (Tan et al., 2012)

Poly(ethylene glycol) diacrylate was purchased from Sigma-Aldrich Handels GmbH, Austria. It is a viscous, colourless liquid that is soluble in water (Poly(ethylene glycol) diacrylate). The number average molecular weight (M_n) is 575 g/mol. Since there was no data available regarding cytotoxicity or ecological toxicity, several random concentrations were tested in a preliminary experiment with osteoblasts. PEGDA was diluted with α-MEM + 10 % FCS to 100, 10 and 1 µl/ml. PEGDA strongly reduced cell number and altered cell morphology. Thus, for the other experiments lower concentrations were examined: 1, 0.1, 0.03 and 0.01 µl/ml.

2.6.2 PEGDMA:

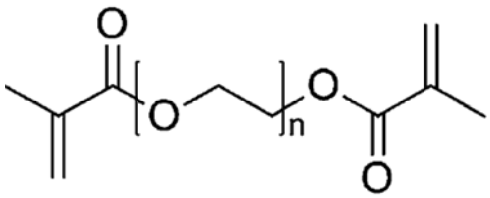

Figure 21: Chemical structure of Poly(ethylene glycol) dimethacrylate (Ito and Meyer, 2012)

Poly(ethylene glycol) dimethacrylate was also purchased from Sigma-Aldrich Handels GmbH, Austria. It is a clear, colourless liquid (Poly(ethylene glycol) dimethacrylate) and the number average molecular weight (M_n) is 330 g/mol. In order to gain comparable results for the two monomers and due to scarce toxicity data also for PEGDMA, examined concentrations were the same as for PEGDA. In a preliminary experiment with osteoblasts, PEGDMA was diluted to concentrations of 100, 10 and 1 µl/ml medium. Since similar results like in the experiment with PEGDA (impaired adherence and reduced cell number compared to control) were observed, PEGDMA was also diluted to 1, 0.1, 0.03 and 0.01 µl/ml of the appropriate cell culture medium for the following experiments.

2.7 Cell culture

Osteoblasts and NIH 3T3 were seeded into 24 well plates (500 µl per well, 40,000 cells/cm^2). After seeding, cells were incubated at 37 °C with 5 % CO_2 for 24 hours. Depending on the cell type and the amount seeded, they were 50 to 80 % confluent by then.

To prepare the treatments, monomers and photoinitiators were dissolved in α-MEM + 10 % FCS and diluted to the desired concentrations. After aspirating the old medium, 500 µl of treatment were added to each well. For the control groups a medium change without any further treatment was performed. The plates were incubated for 72 hours, then trypsinized and counted with the CASY cell counter.

In the initial experiment with RAW 264.7, 130,000 cells/cm^2 were seeded, according to lab protocols of previous experiments using RAW 264.7 cells. Apart from the seeding density, the experimental set up was supposed to be the same as for osteoblasts and NIH 3T3. After incubation of 24 hours, cells were already confluent and the previously pink α-MEM + GlutaMAX + 10 % FCS looked yellowish. Metabolizing cells had produced an acidic environment, thus the colour of the phenol red indicator had changed. This implied that a medium change was necessary.

Respectively treatment or fresh medium were added the same way as in the other experiments mentioned above, but at this density cells were metabolizing very fast. When checking the cells 24 hours later medium was yellowish again. To avoid more medium changes within the 72 hours incubation time and to keep experimental conditions as equal as possible to the other experiments, 1000 µl of fresh medium or treatment were added to each well respectively. This was sufficient, no pH changes could be observed until cells were trypsinized and counted.

For the following experiment, the seeding density of RAW 264.7 cells was reduced to 33,000 cells/cm^2. After incubating for 24 hours, confluency was below 50 %, so they were incubated a further 24 hours. Then old medium was aspirated and 500 µl of respectively fresh medium or treatment were added. Following 72 hours of incubation, cells were trypsinized and counted with CASY cell counter.

Results of these two experiments with RAW 264.7 were similar for all substances except Ini1, so only for this photoinitiator the experiments were repeated again with 130,000 and 33,000 cells/cm^2.

Osteoclasts isolated from rabbits were incubated for 3 hours before adding treatment or changing the medium in the control groups. Afterwards cells were incubated for 48 hours, fixed and TRAP stained.

2.8 Isolation of osteoblasts from mouse calvaria

The method was modified based on the “Isolation and Culture of Bone Cells from Neonatal Mouse Calvaria”, published in the “Bone Research Protocols” 2012 (Bakker and Klein-Nulend, 2012).

Calvaria were isolated from the mice and shaken with a solution of 0.1 % collagenase and 0.2 % dispase in α-MEM at 37 °C. After aspiration, fresh enzyme solution was added to the calvaria and shaken again at 37 °C. The supernatant was collected in a centrifuge tube on ice. This was repeated three times. The combined enzyme solutions were centrifuged and the liquid aspirated. The pellet was resuspended in α-MEM containing 10 % fetal calf serum and 1 % penicillin/streptomycin-solution and the cell suspension was seeded into petri dishes, which were incubated for 24 hours at 37 °C. The medium was changed to remove non adherent cells. After another 24 hours of incubation, the cells were washed with warm PBS without calcium and magnesium. Trypsin-EDTA-solution was added and incubated at 37 °C for some minutes. The enzyme-solutions from the different petri dishes were combined in a centrifuge tube containing α-MEM + 10 % FCS on ice. To collect remaining cells, the petri dishes were washed with medium, which was added to the cell suspension in the tube. After centrifugation at 4 °C and resuspending the pellet, cells were seeded into the new petri dishes at a split rate of 1:4 or 1:5. They were placed in the incubator for 48 hours to grow to confluency.

Afterwards medium was removed and cells were washed once with warm PBS without calcium and magnesium. Cells were trypsinized as described above and centrifuged at 4 °C for 5 minutes. The supernatant was aspirated and the pellet resuspended in medium. To determine the cell number, an aliquot was measured with the CASY cell counter. Depending on the portions the cells should be frozen in, the necessary amount of freezing medium, consisting of α-MEM + 50 % FCS containing 10 % DMSO, was prepared. DMSO was added to avoid the formation of ice crystals which would damage the cells when frozen. Cells were centrifuged again, resuspended in freezing medium and portioned into cryogenic tubes. These were stored overnight at -80 °C in

Mr. Frosty Freezing Containers and the next day transferred to liquid nitrogen for long term storage.

2.8.1 Preparation of solutions

2.8.1.1 0.1 % Collagenase-solution

To prepare 50 ml, 0.05 g of collagenase was weighed wearing a face mask, dissolved in 50 ml of α-MEM without fetal calf serum and sterile filtered.

2.8.1.2 0.2 % Dispase-solution

To prepare 50 ml, 0.1 g of dispase was weighed wearing a face mask, dissolved in 50 ml of α-MEM without fetal calf serum and sterile filtered.

2.8.1.3 Trypsin-EDTA-solution

Trypsin-EDTA solution was prepared as a tenfold concentrate in advance and stored at -20 °C for later use. To prepare 100 ml 0.5 g of trypsin were weighed wearing a face mask and dissolved in demineralized water. The pH was adjusted with NaOH to 7.7. Then 0.155 g of EDTA and 0.9 g of NaCl were added and the pH was adjusted again to 7.4-7.6. The total volume of the solution should be 100 ml. Finally the solution was sterile filtered and frozen in aliquots of 10 ml.
To prepare the trypsin-EDTA-solution for the experiments, the tenfold concentrate was thawed and diluted 1:10 with PBS without calcium and magnesium.

2.8.1.4 PBS without calcium and magnesium

PBS without calcium and magnesium was also prepared as a tenfold concentrate and stored at 4 °C. 2.00 g of KCl, 2.00 g of KH_2PO_4, 80.00 g of NaCl and 27.07 g of $Na_2HPO_4.2H_2O$ were dissolved in 1000 ml of double-distilled water.
For laboratory use this solution was diluted 1:10 with double-distilled water and stored at 4°C.

2.9 Isolation of osteoclasts from rabbits

Tibia and femur were isolated from the legs of 4 to 5 days old rabbits and cut to small pieces in a centrifuge tube containing α-MEM + 5 % FCS. The medium containing cells was transferred to another centrifuge tube with a wide mouth pipet. The remaining bone pieces were resuspended in medium, vortexed and then placed on the bench top to settle. The supernatant was combined with the other fraction (bone pieces were discarded), centrifuged at 4 °C and then aspirated. The pellet was resuspended in α-MEM + 5 % FCS and the cell suspension was seeded into 48 well plates, 300 µl per well, using a special wide mouth pipet tip, in order to prevent damaging the large cells when passing through the opening. Cells were incubated for 3 hours. Afterwards they were washed once with warm medium to remove non adherent cells. Then 300 µl of treatment or fresh medium respectively were added and the plates were placed in the incubator at 37 °C with 5 % CO_2 for 48 hours. Finally cells were fixed and TRAP stained.

2.10 Trypsinization of adherent cells

Cells were washed with warm PBS without calcium and magnesium. Then 200 µl of warm trypsin-EDTA-solution were added. The plate was incubated at 37 °C for about two minutes. When the cells started to lift, the content of each well was flushed through the pipet tip thoroughly several times to completely detach and separate the cells. Then 600 µl of cold cell culture medium containing 10 % FCS were added to each well, to stop the trypsinization reaction. The cell suspension was transferred to a 1.5 ml Eppendorf tube on ice. To collect remaining cells, each well was flushed with another 200 µl of cold medium + 10 % FCS which were added to the tubes, so the total volume was 1 ml. The Eppendorf tubes were slightly vortexed and kept on ice.

According to the lab protocol a centrifugation step should followed. This is necessary to eliminate the trypsin, especially if cells are seeded again. For measuring with the CASY cell counter, the cell suspension has to be stable for only a short time. Also it was diluted with medium containing fetal calf serum, which inhibits damage to the cells

caused by the enzyme. Hence to save time the content of the Eppendorf tube was used directly.

PBS without calcium and magnesium and trypsin-EDTA-solution were prepared as described above.

2.11 Cell counting with CASY cell counter

100 µl of the cell suspension in the Eppendorf tubes were added to 10 ml of CASYton, a special electrolyte solution designed for this system. CASYton has to be sterile filtered before use to guarantee a particle free measuring environment. The volume of cell suspension depends on the amount of cells per well and the capillary used.
A defined volume of the sample is aspirated through a measuring pore in the capillary and electrical resistance is measured as cells pass through the pore individually. Live cells act as isolators hence resistance increases proportionally to the size of the cell. Cells with damaged membrane are recorded by the size of their nucleus (Roche Innovatis AG, 2002).
The quantity of cells smaller than 8 µm, between 8 and 30 µm and larger than 30 µm diameter was measured.
Counts between 8 and 30 µm represent healthy cells, smaller than 8 µm dead and damaged cells or debris, and larger than 30 µm cell aggregates (Roche Innovatis AG, 2002). According to previous testing this classification is applicable for osteoblasts and NIH 3T3 cells, as well as for RAW 264.7 cells.

2.12 Fixation before TRAP-Staining

Cells were washed once with warm PBS with calcium and magnesium. 300 µl of a 3.7 % formaldehyde solution were added to each well and incubated at room temperature. After 3 minutes it was aspirated and cells were incubated with another 300 µl of 3.7 % formaldehyde solution for 10 minutes. After 3 washing steps with PBS

with calcium and magnesium cells were either immediately TRAP stained or stored in PBS with calcium and magnesium at 4 °C to do the staining later.

2.12.1 Preparation of solutions

2.12.1.1 PBS with calcium and magnesium

PBS with calcium and magnesium was prepared as a tenfold concentrate and stored at 4 °C. 2.00 g of KCl, 2.00 g of KH_2PO_4, 80.00 g of NaCl, 14.34 g of $Na_2HPO_4.2H_2O$, and 1.00 g of $MgCl_2.6H_2O$ were dissolved in 1000 ml of double-distilled water. The pH was adjusted with 5 M HCl to a value of 2 to 3 (optimal pH for dissolving $CaCl_2$) and 1.00 g of $CaCl_2$ was added.
For laboratory use this solution was diluted 1:10 with double-distilled water and pH was adjusted to 7.2 with 5 N NaOH (storage at 4 °C).

2.12.1.2 3.7 % formaldehyde solution

37 % formaldehyde-solution was diluted 1:10 with PBS with calcium and magnesium.

2.13 TRAP-Staining

Tartrate-resistant acid phosphatase (TRAP) is commonly used as a marker to identify osteoclasts. It plays an important role in the bone resorption process, but is also expressed in other cell types (Hayman et al., 2000). Therefore counting criteria were positive TRAP-staining and 3 or more nuclei.

After fixation, PBS was aspirated, 500 µl of TRAP-buffer were adder to each well and the plate was incubated at 37 °C for 30 minutes. After removing the TRAP-buffer, each well was washed with ethanol/acetone-solution and left to dry for 2 minutes at room temperature. The short treatment with ethanol/acetone contributes to the intensity of the staining by increasing the permeability of cells for the dye.

After adding the staining solution (500 µl/well) the plate was placed at 37 °C for 5 to 10 minutes. Following 2 washing steps with double-distilled water, 500 µl of storage solution were added to each well and the plate was kept at 4 °C until counting.

2.13.1 Preparation of solutions:

2.13.1.1 TRAP-buffer

To prepare 50 ml, 0.2722 of sodium acetate were dissolved in double-distilled water. The pH was adjusted to 5 with 1 M HCl, then 0.1151 g of sodium tartrate were added.

2.13.1.2 Ethanol/acetone-solution

96 % ethanol was mixed 1:1 with acetone and kept in a closed glass bottle to avoid evaporation.

2.13.1.3 Staining solution

6 mg of naphthol AS-MX phosphate were dissolved in 600 µl of N,N-dimethylformamide. This solution was used immediately, otherwise it would have become cloudy. 30 mg of fast red violet salt were dissolved in 50 ml of TRAP-buffer and 500 µl of the naphthol-solution were added.

2.13.1.4 Storage solution

0.1 g of NaN_3 was dissolved in 1 ml of double-distilled water. This solution was diluted with PBS with calcium and magnesium 1:500.

2.14 Statistical analysis

Cell numbers generated by CASY or TRAP counting were analysed with Statview statistical software. P-values were calculated by Fisher's protected least significant difference (Fisher PLSD) test and stated for 95 %, 99 % and 99.9% ($p < 0.05$, $p < 0.01$ and $p < 0.001$).

Since cell numbers varied strongly between the experiments, especially with different cell types, values were normalized to percentages. Cell numbers of the 3 control groups were averaged, then every cell number value was divided by this mean. Graphs were created by Prism Software (Graphpad).

3 Results

In each experimental set-up, 3 wells per group were seeded with cells. The examined concentrations were selected based on available toxicity information after performing preliminary experiments, to span ranges between maximal and no inhibition.
The morphology of osteoblasts, NIH 3T3 and RAW 264.7 was assessed in the microscope. Then, cells were quantified using a CASY cell counter. Normalized values for cell counts between a cell size of 8 and 30 µm are shown as percentages of the control group in the graphs below. TRAP positive multinucleated osteoclasts were counted using a microscope. Cell numbers are also presented as percentages in the graphs.

3.1 Irgacure 2959

Examined concentrations were selected after a preliminary experiment using osteoblasts (data not shown) referring to available toxicity data (Irgacure 2959). In this experiment concentrations of 3 and 1 mg/ml caused a reduction of cell number, and cells looked smaller and more compact. At 0.1, 0.03 and 0.01 mg/ml, the quantity and morphology of cells was comparable to the control group without any test compound. In order to describe the cytotoxic properties more precisely, a treatment group (0.3 mg/ml) was added between the inhibiting concentration of 1 mg/ml and the concentration without any effect on osteoblasts, 0.1 mg/ml.

3.1.1 Effect of Irgacure 2959 on osteoblasts and fibroblasts

The Figures 22 and 23 show that Irgacure 2959 reduced the number of osteoblasts and fibroblasts respectively in all treatment groups compared to the control, though just slightly with 0.1 mg/ml. The cell morphology was affected strongly at 3 mg/ml. At 1 mg/ml, cells adhered and stretched, but cell growth looked clearly less dense in the microscope.

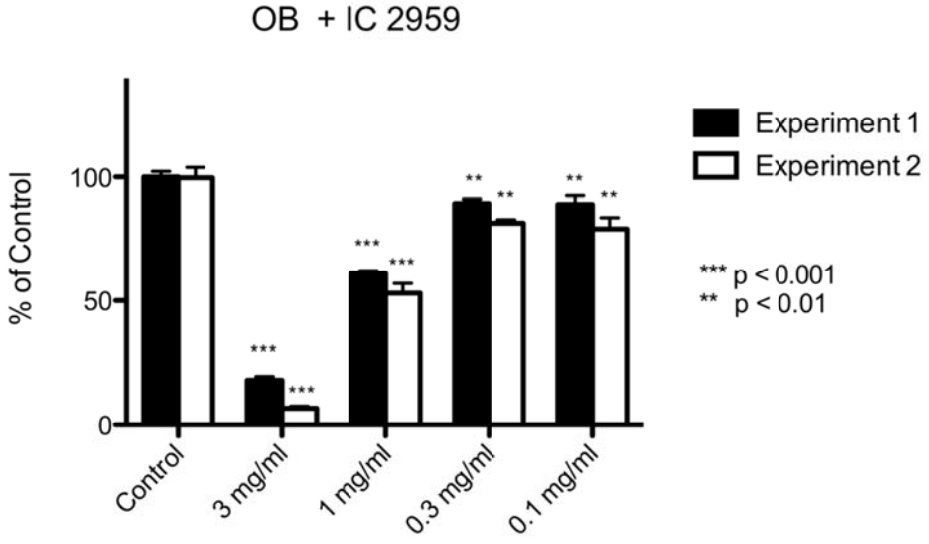

Figure 22: Effect of the photoinitiator Irgacure 2959 on osteoblasts (control, mean cell numbers: experiment 1 = 157,367 cells, experiment 2 = 103,400 cells)

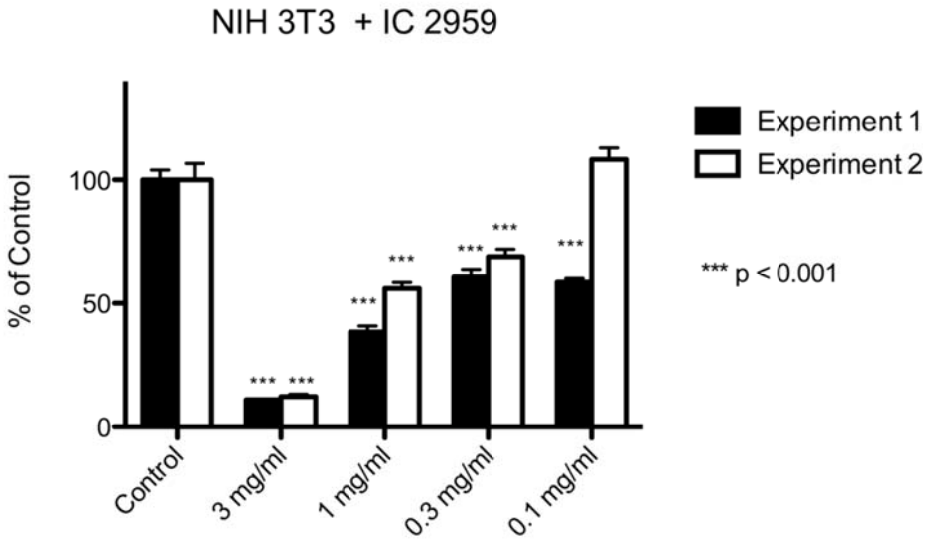

Figure 23: Effect of the photoinitiator Irgacure 2959 on fibroblasts (control, mean cell numbers: experiment 1 = 367,700 cells, experiment 2 = 216,700 cells)

3.1.2 Effect of Irgacure 2959 on osteoclasts and osteoclast precursors

Osteoclasts and RAW 264.7 were affected by all examined concentrations of Irgacure 2959, although impairment was minor at 0.1 mg/ml.

The study of the effect of this photoinitiator on osteoclasts (Figure 24) was repeated twice. In the first experiment, cell numbers were clearly reduced, also at the lowest concentration, while the experiments 2 and 3 showed no or just slight decrease at 0.1 mg/ml. Results were similar for osteoclast precursors (Figure 25). The morphology of the RAW 264.7 cells was altered at 3 and 1 mg/ml.

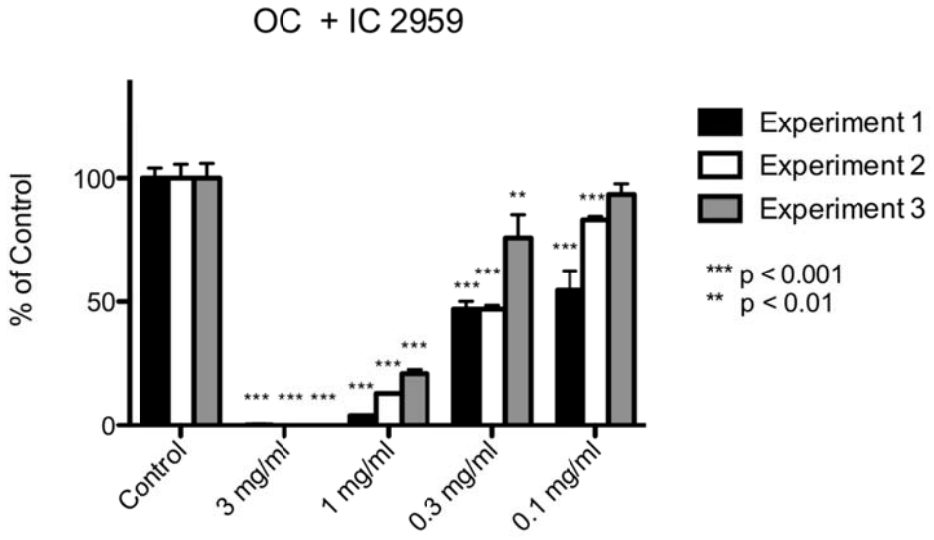

Figure 24: Effect of the photoinitiator Irgacure 2959 on osteoclasts (control, mean cell numbers: experiment 1 = 369 cells, experiment 2 = 135 cells, experiment 3 = 248 cells)

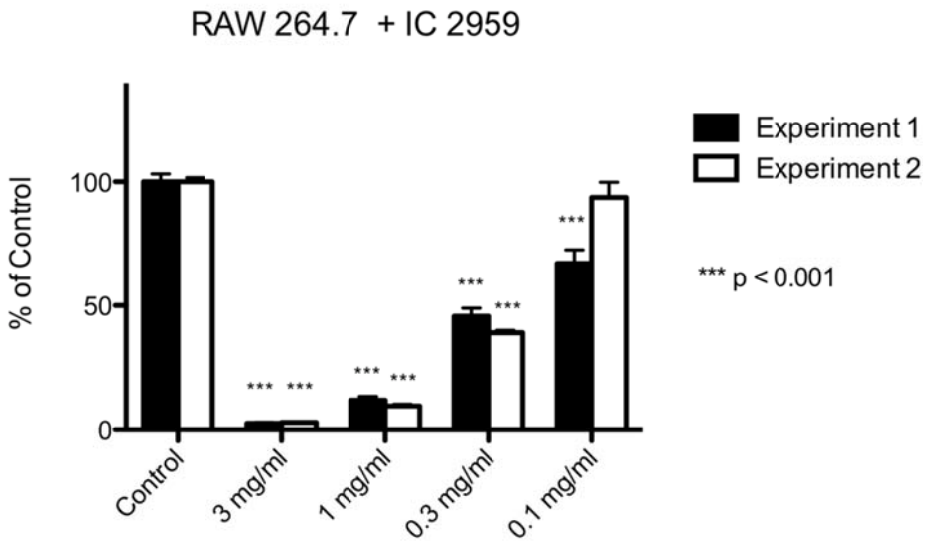

Figure 25: Effect of the photoinitiator Irgacure 2959 on osteoclast precursors (control, mean cell numbers: experiment 1 = 1,059,667 cells, experiment 2 = 716,267 cells)

Table 4: Mean percentages (n = 3) of cell numbers in the groups treated with Irgacure 2959 compared to control

	Osteoblasts		NIH 3T3		Osteoclasts			RAW 264.7	
	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2
Control	100 ± 2.31	100 ± 4.26	100 ± 4.00	100 ± 6.66	100 ± 4.16	100 ± 5.69	100 ± 6.03	100 ± 3.22	100 ± 1.73
3 mg/ml	18 ± 1.53	7 ± 0.88	11 ± 0.00	12 ± 0.88	0 ± 0.33	0 ± 0.00	0 ± 0.00	3 ± 0.33	3 ± 0.00
1 mg/ml	61 ± 1.00	53 ± 4.04	38 ± 2.40	56 ± 2.52	4 ± 0.00	13 ± 0.00	21 ± 1.73	12 ± 1.53	10 ± 0.67
0.3 mg/ml	90 ± 1.76	81 ± 1.45	61 ± 3.18	69 ± 3.06	47 ± 3.06	47 ± 1.53	76 ± 9.45	46 ± 3.28	39 ± 1.00
0.1 mg/ml	89 ± 3.46	79 ± 4.58	59 ± 1.45	109 ± 4.70	55 ± 7.97	83 ± 1.45	93 ± 4.33	67 ± 5.51	93 ± 6.17

3.2 Irgacure 369

Based on toxicity information presented in the BASF material safety data sheet (Irgacure 369), concentrations of 3, 1, 0.3 and 0.1 µg/ml were suggested and examined in a preliminary test with osteoblasts (data not shown). At these concentrations, Irgacure 369 did not cause a reduction of cell number, or a change in cell morphology. Therefore concentrations of 6, 3 and 1 µg/ml were examined in the following experiments.

Due to insufficient water solubility, Irgacure 369 was first dissolved in absolute ethanol and then diluted to the desired concentrations. Since cells tolerate only a limited amount of alcohol, the dilution was calculated to result in a maximum of 0.1 % ethanol in the highest examined concentration. Therefore treatment groups were compared to a control group with medium containing 0.1 % absolute ethanol.

3.2.1 Effect of Irgacure 369 on osteoblasts and fibroblasts

Osteoblasts showed no statistically relevant decrease in cell number (Figure 26), whereas fibroblasts were affected at all examined concentrations (Figure 27).

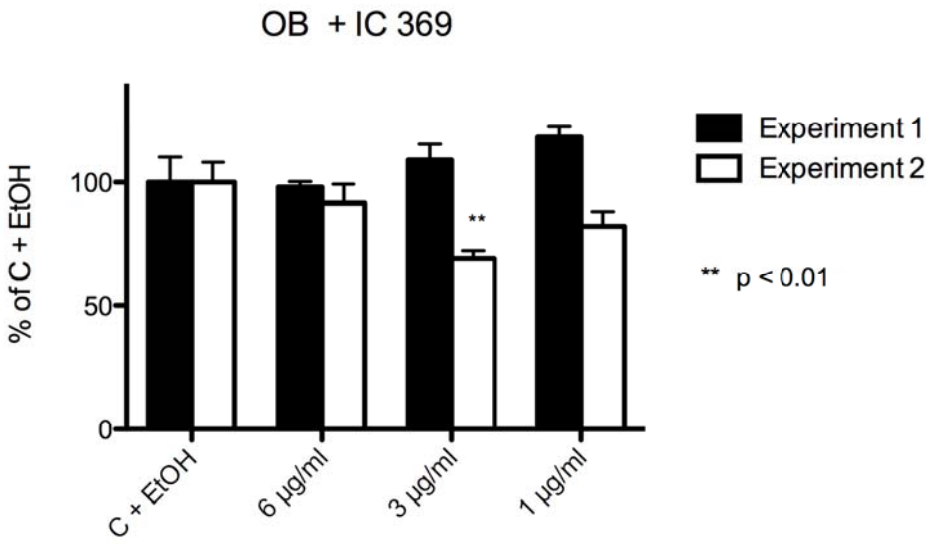

Figure 26: Effect of the photoinitiator Irgacure 369 on osteoblasts (control, mean cell numbers: experiment 1 = 109,200 cells, experiment 2 = 128,833 cells)

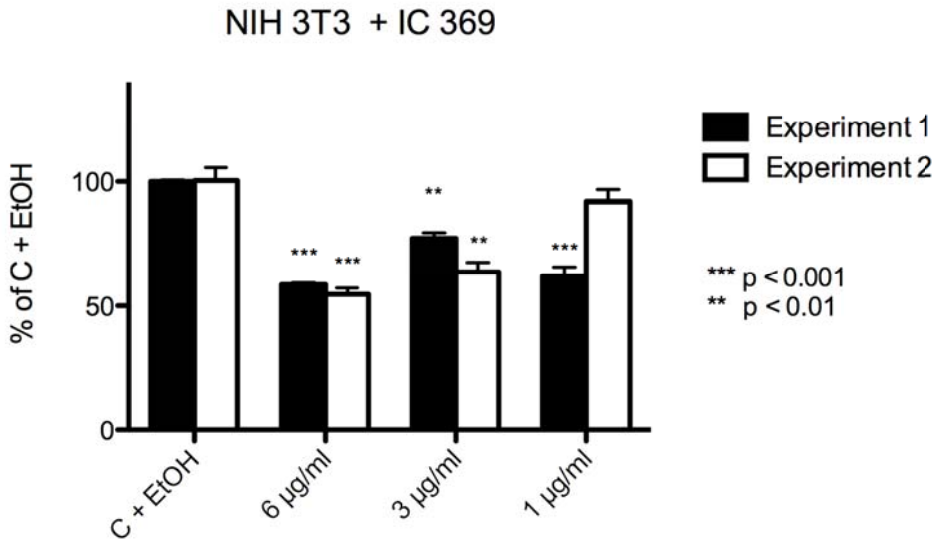

Figure 27: Effect of the photoinitiator Irgacure 369 on fibroblasts (control, mean cell numbers: experiment 1 = 307,933 cells, experiment 2 = 250,367 cells)

3.2.2 Effect of Irgacure 369 on osteoclasts and osteoclast precursors

The study with osteoclasts was repeated twice (Figure 28). Results differed but showed the same trend at 6 and 3 µg/ml, while no inhibition was observed at 1 µg/ml in any of the experiments. Similar results were found for osteoclast precursors (Figure 29).

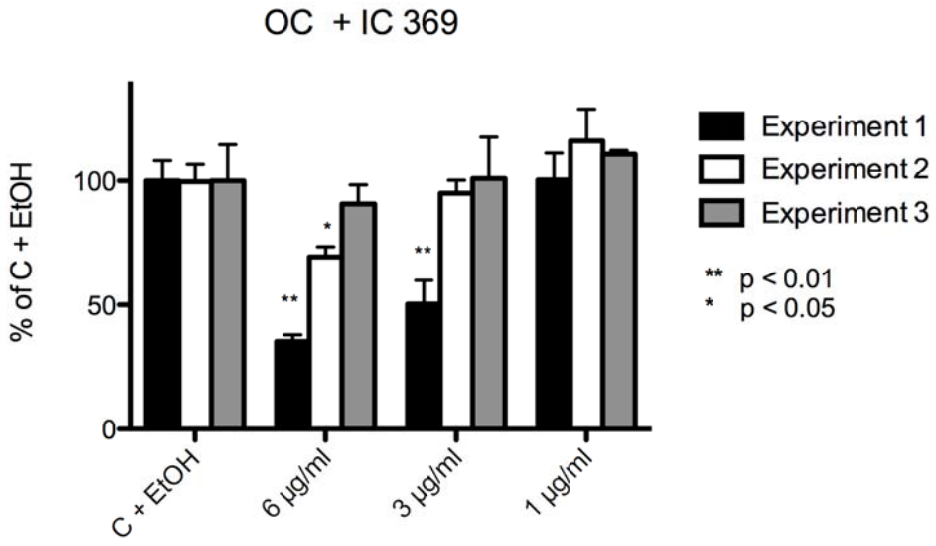

Figure 28: Effect of the photoinitiator Irgacure 369 on osteoclasts (control, mean cell numbers: experiment 1 = 223 cells, experiment 2 = 116 cells, experiment 3 = 206 cells)

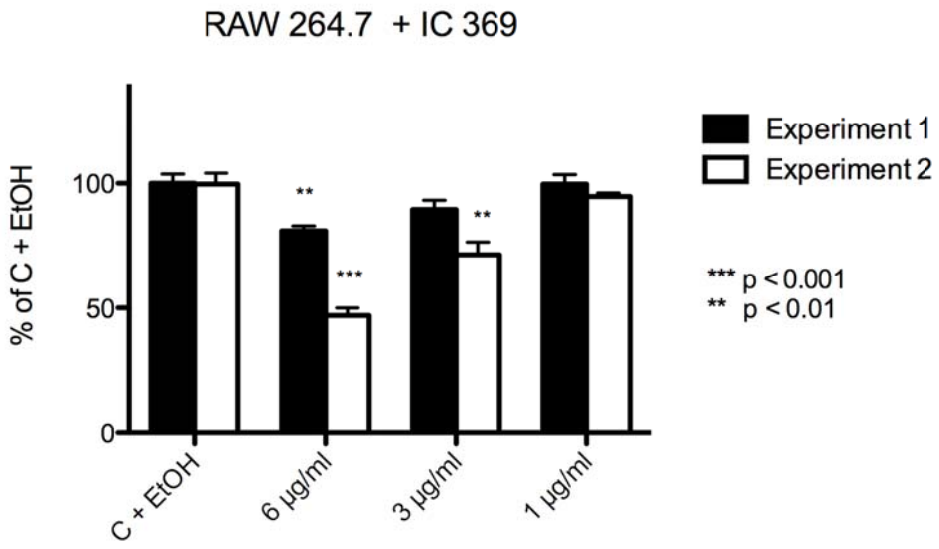

Figure 29: Effect of the photoinitiator Irgacure 369 on osteoclast precursors (control, mean cell numbers: experiment 1 = 1,009,667 cells, experiment 2 = 760,633 cells)

Table 5: Mean percentages (n = 3) of cell numbers in the groups treated with Irgacure 369 compared to control

	Osteoblasts		NIH 3T3		Osteoclasts			RAW 264.7	
	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2
C + EtOH	100 ± 10.21	100 ± 8.15	100 ± 0.58	100 ± 5.33	100 ± 8.15	100 ± 6.89	100 ± 14.50	100 ± 3.79	100 ± 4.41
6 µg/ml	98 ± 2.31	92 ± 7.54	59 ± 0.67	55 ± 2.60	35 ± 2.60	69 ± 4.10	91 ± 7.75	81 ± 2.00	47 ± 3.00
3 µg/ml	109 ± 6.43	69 ± 3.18	77 ± 2.19	64 ± 3.84	50 ± 9.56	95 ± 5.29	101 ± 16.52	90 ± 3.53	72 ± 5.18
1 µg/ml	119 ± 4.18	82 ± 5.90	62 ± 3.61	92 ± 4.73	100 ± 10.75	116 ± 12.86	111 ± 1.45	100 ± 3.84	95 ± 1.45

3.3 Ini1

Due to the recent development of this compound, there was no toxicity data available. In a preliminary experiment concentrations were chosen to span ranges comparable to Irgacure 2959 as well as Irgacure 369: 1000, 100, 10, 1 and 0.1 µg/ml (data not shown in graphs). Only 1000 µg/ml (1 mg/ml) exhibited considerable cell impairment, therefore concentrations of 1, 0.3, 0.1 and 0.03 mg/ml were examined in the following experiments.

3.3.1 Effect of Ini1 on osteoblasts and fibroblasts

Cell number of osteoblasts was significantly reduced ($p < 0.05$ or $p < 0.001$ respectively) at a concentration of 1 mg/ml (Figure 30). Also the cell morphology was altered. For the other treatment groups no impairment was observed. Figure 31 shows that NIH 3T3 were hardly affected by Ini1. Cell morphology in all groups was comparable to the control.

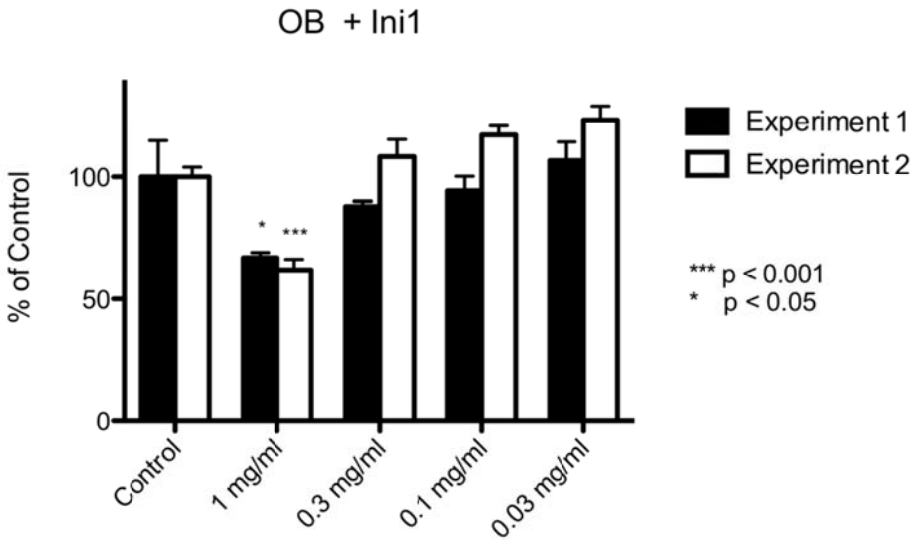

Figure 30: Effect of the photoinitiator Ini1 on osteoblasts (control, mean cell numbers: experiment 1 = 151,100 cells, experiment 2 = 85,633 cells)

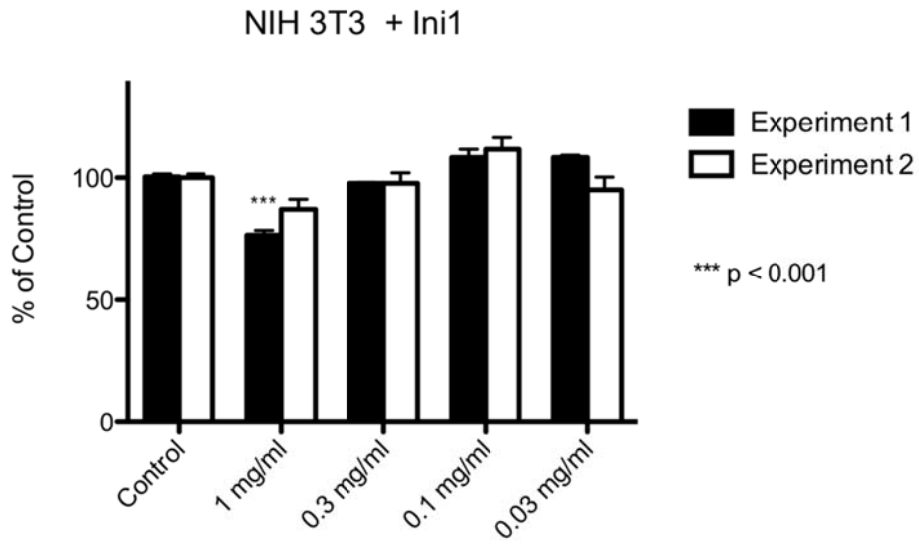

Figure 31: Effect of the photoinitiator Ini1 on fibroblasts (control, mean cell numbers: experiment 1 = 289,633 cells, experiment 2 = 238,967 cells)

3.3.2 Effect of Ini1 on osteoclasts and osteoclast precursors

Osteoclasts exhibited greater sensitivity to the photoinitiator in the first study, but no inhibition could be observed with 0.03 mg/ml in either experiment (Figure 32).

RAW 264.7 cells were hardly affected by the presence of Ini1, not even at the highest examined concentration (Figure 33), and the cell morphology did not differ from the control.

The studies with the osteoclast precursors needed to be repeated three times. In the first examination (data not shown), strong reduction of cell number and alteration of their morphology was observed at 0.1 and 0.03 mg/ml, but not at higher concentrations. These findings were not confirmed by the following experiment (experiment 1 in Figure 33). The two studies differed in seeding densities, which caused differences in the incubation times and the number of medium changes (see 2.7). Considering a possible influence of the experimental set-up on the results, a repeat experiment had to be performed for each seeding density. The findings of experiment 1 were confirmed, independent from the amount of cells seeded.

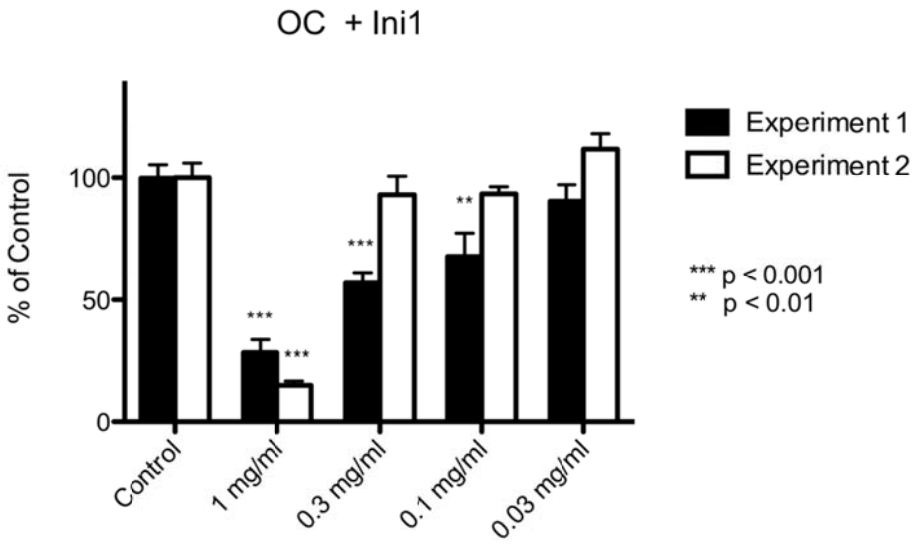

Figure 32: Effect of the photoinitiator Ini1 on osteoclasts (control, mean cell numbers: experiment 1 = 97 cells, experiment 2 = 248 cells)

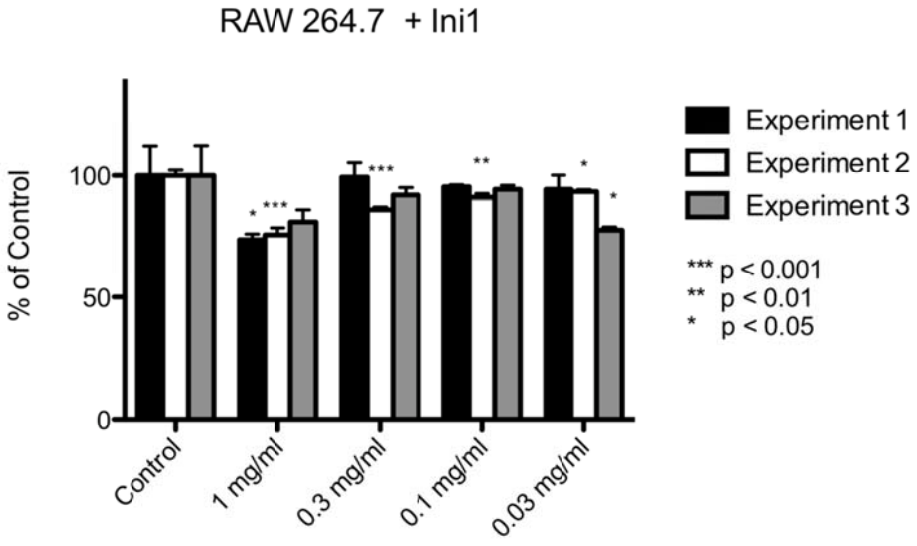

Figure 33: Effect of the photoinitiator Ini1 on osteoclast precursors (control, mean cell numbers: experiment 1 = 777,000 cells, experiment 2 = 1,235,667 cells, experiment 3 = 408,833 cells)

Table 6: Mean percentages (n = 3) of cell numbers in the groups treated with Ini1 compared to control

	Osteoblasts		NIH 3T3		Osteoclasts		RAW 264.7		
	Exp.1	Exp.1	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2	Exp.3
Control	100 ± 15.01	100 ± 1.20	100 ± 1.20	100 ± 1.53	100 ± 5.47	100 ± 6.03	100 ± 12.00	100 ± 2.31	100 ± 12.12
1 mg/ml	67 ± 2.08	77 ± 2.03	77 ± 2.03	87 ± 3.84	28 ± 5.36	15 ± 1.73	74 ± 2.33	76 ± 2.96	81 ± 5.03
0.3 mg/ml	88 ± 2.08	98 ± 0.33	98 ± 0.33	98 ± 4.33	57 ± 4.36	93 ± 7.57	99 ± 6.01	86 ± 1.16	92 ± 3.06
0.1 mg/ml	94 ± 5.90	108 ± 3.33	108 ± 3.33	111 ± 4.84	68 ± 9.54	93 ± 2.96	96 ± 0.88	91 ± 1.53	95 ± 1.67
0.03 mg/ml	106 ± 7.75	108 ± 0.88	108 ± 0.88	95 ± 5.29	90 ± 6.77	112 ± 6.39	94 ± 5.84	93 ± 0.88	78 ± 1.20

3.4 PEGDA

No information about cytotoxicity of pure PEGDA could be found in the literature. Thus, in a pilot experiment with osteoblasts (data not shown) concentrations of 100, 10 and 1 µl/ml were tested. All groups exhibited altered cell morphology, and also strongly reduced cell number and attachment. For the following experiments, concentrations of 1, 0.1, 0.03 and 0.01 µl/ml were chosen.

3.4.1 Effect of PEGDA on osteoblasts and fibroblasts

Figure 34 shows the effect of PEGDA on osteoblasts. Cell number was strongly decreased at all examined concentrations. The results for fibroblasts were similar (Figure 35), except at a concentration of 0.01 µl/ml no statistically significant reduction could be found. The cell morphology of both cell types was affected at 1 and 0.1 µl/ml.

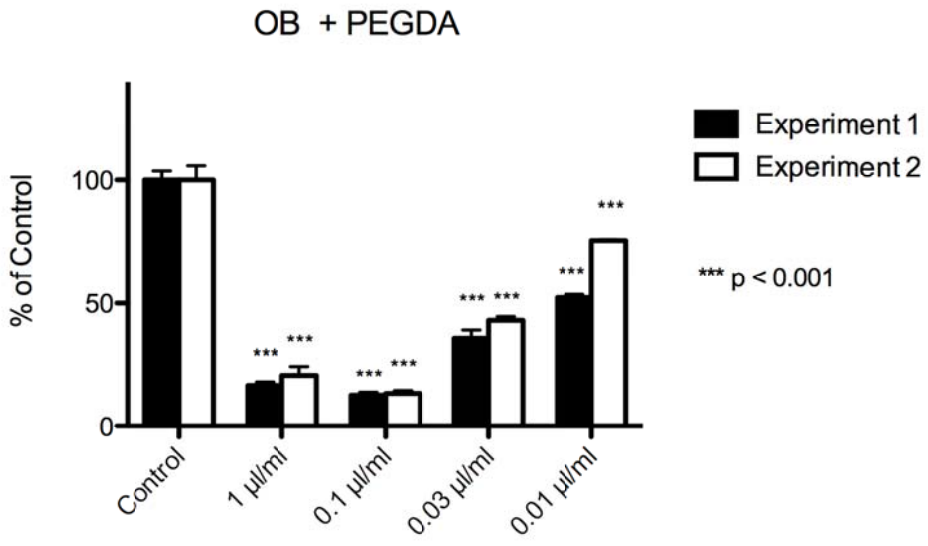

Figure 34: Effect of the monomer PEGDA on osteoblasts (control, mean cell numbers: experiment 1 = 123,200 cells, experiment 2 = 93,533 cells)

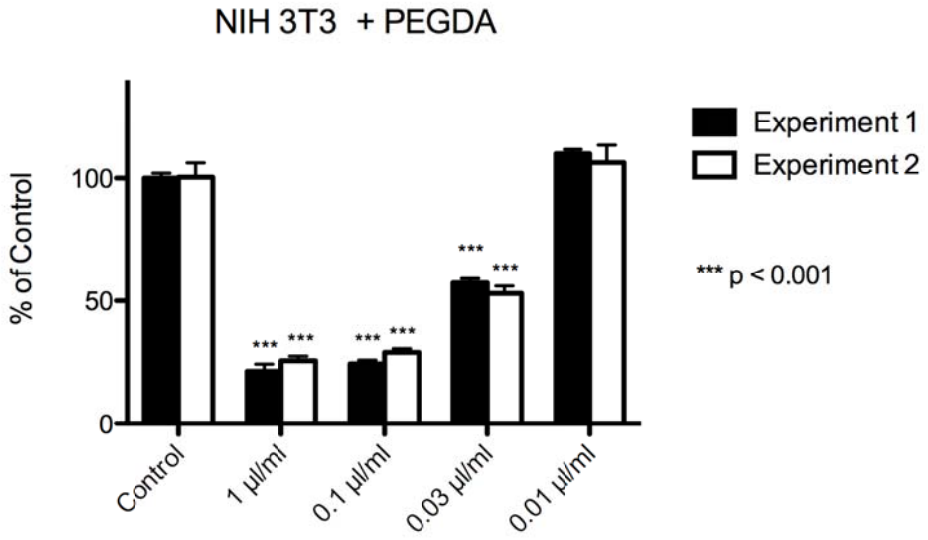

Figure 35: Effect of the monomer PEGDA on fibroblasts (control, mean cell numbers: experiment 1 = 316,400 cells, experiment 2 = 219,500 cells)

3.4.2 Effect of PEGDA on osteoclasts and osteoclast precursors

Cell number of osteoclasts was around zero at 1 and 0.1 µl/ml, but increased at the lower concentrations (Figure 36). Results for the osteoclast precursors were similar (Figure 37). Their cell morphology was clearly altered at 1 and 0.1 µl/ml.

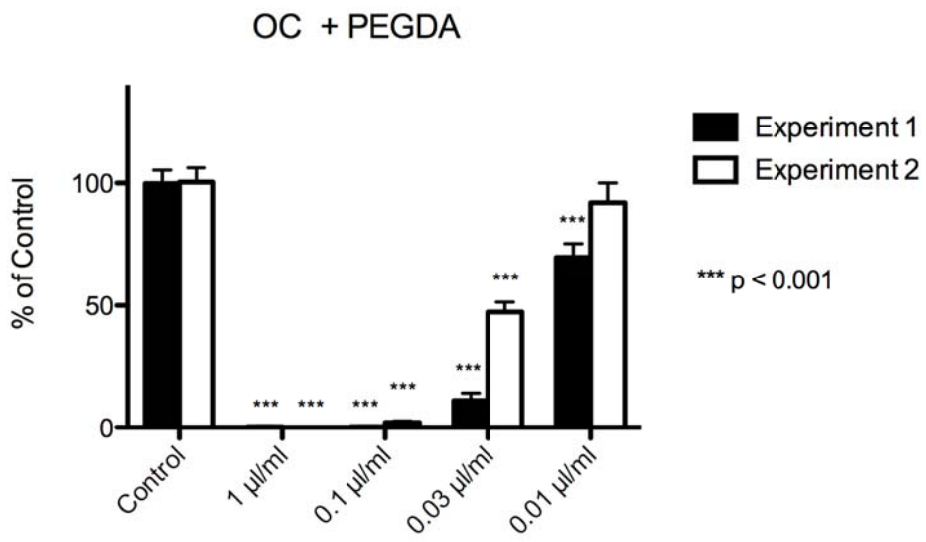

Figure 36: Effect of the monomer PEGDA on osteoclasts (control, mean cell numbers: experiment 1 = 97 cells, experiment 2 = 254 cells)

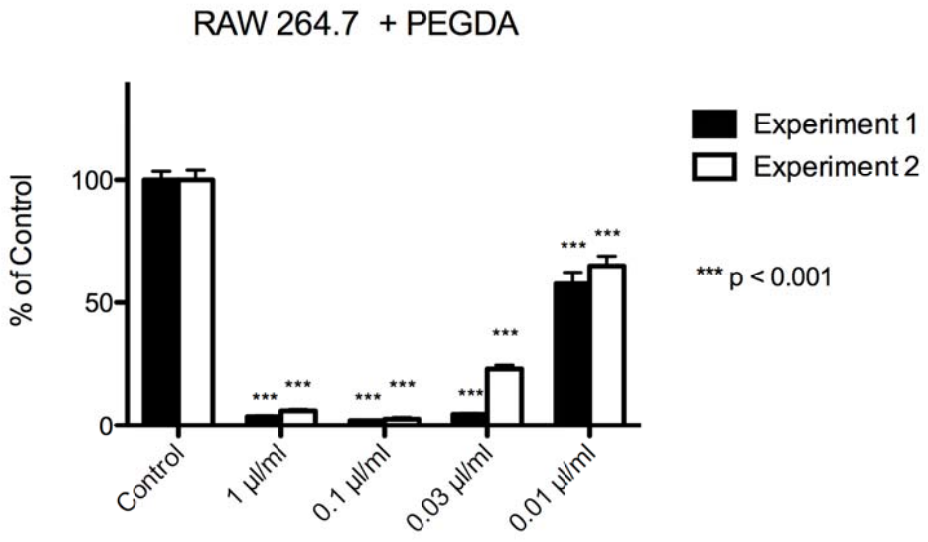

Figure 37: Effect of the monomer PEGDA on osteoclast precursors (control, mean cell numbers: experiment 1 = 1,036,900 cells, experiment 2 = 955,167 cells)

Table 7: Mean percentages (n = 3) of cell numbers in the groups treated with PEGDA compared to control

	Osteoblasts		NIH 3T3		Osteoclasts		RAW 264.7	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Control	100 ± 3.61	100 ± 5.77	100 ± 2.08	100 ± 5.90	100 ± 5.47	100 ± 5.90	100 ± 3.51	100 ± 4.04
1 µl/ml	17 ± 1.33	21 ± 3.67	21 ± 2.91	26 ± 1.86	0 ± 0.33	0 ± 0.00	4 ± 0.33	6 ± 0.58
0.1 µl/ml	13 ± 1.20	13 ± 1.20	24 ± 1.45	29 ± 1.53	0 ± 0.33	2 ± 0.58	2 ± 0.00	3 ± 0.67
0.03 µl/ml	36 ± 3.38	43 ± 1.53	57 ± 1.76	53 ± 3.06	11 ± 3.06	47 ± 4.10	5 ± 0.33	23 ± 1.53
0.01 µl/ml	52 ± 1.33	76 ± 0.33	110 ± 1.73	106 ± 7.13	70 ± 5.61	92 ± 8.08	53 ± 4.70	65 ± 4.04

3.5 PEGDMA

For PEGDMA no reference data regarding cytotoxicity was available. To ensure comparability of the two monomers, all experiments with PEGDA and PEGDMA were performed at the same concentrations. Similarly to PEGDA, PEGDMA showed severe cell impairment at concentrations of 100, 10 and 1 µl/ml.

3.5.1 Effect of PEGDMA on osteoblasts and fibroblasts

At a concentration of 1 µl/ml, cell number of osteoblasts was significantly reduced ($p < 0.001$) and cell morphology differed from the control (Figure 38). At the other concentrations, cell number increased gradually. No inhibitory effect could be observed at 0.01 µl/ml of PEGDMA. The decrease in cell counts for NIH 3T3 was statistically significant ($p < 0.001$) in all groups (Figure 39), whereas cell morphology was only affected with 1 µl/ml.

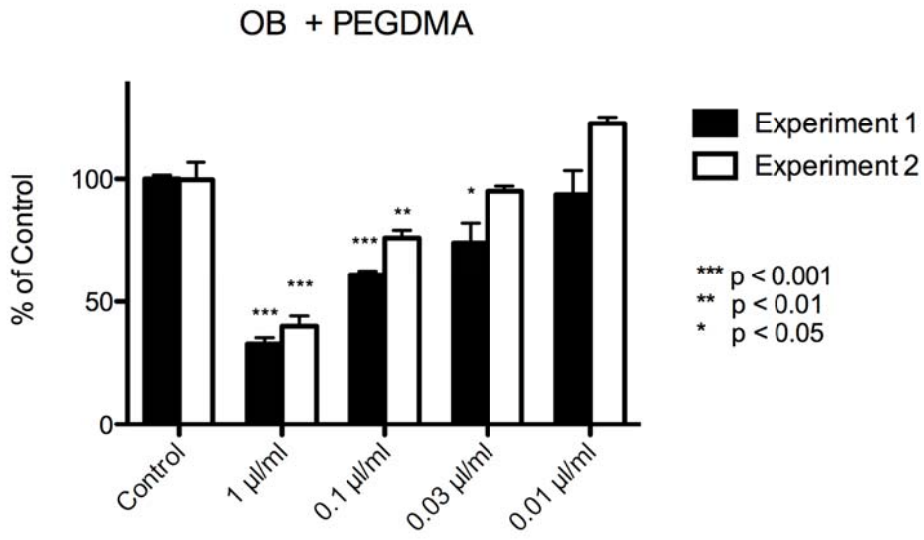

Figure 38: Effect of the monomer PEGDMA on osteoblasts (control, mean cell numbers: experiment 1 = 103,933 cells, experiment 2 = 72,677 cells)

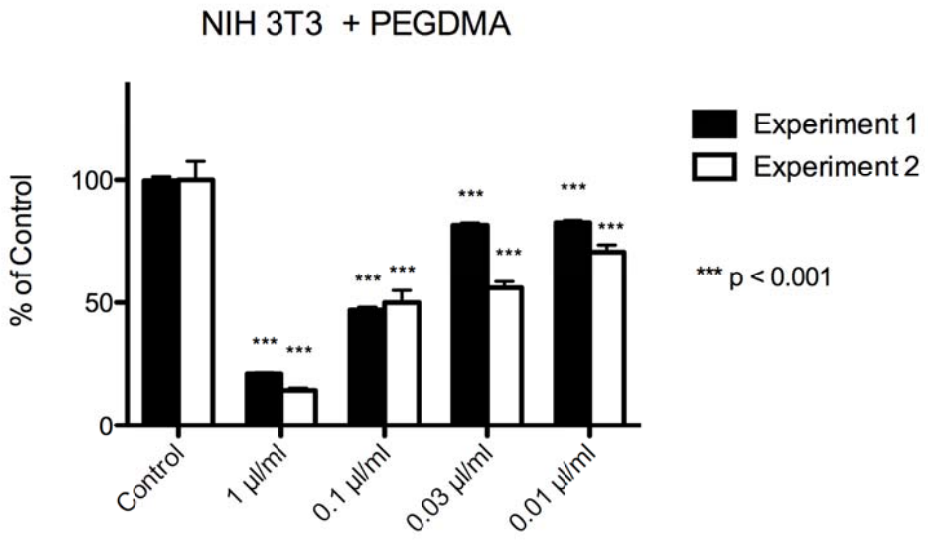

Figure 39: Effect of the monomer PEGDMA on fibroblasts (control, mean cell numbers: experiment 1 = 354,733 cells, experiment 2 = 251,267 cells)

3.5.2 Effect of PEGDMA on osteoclasts and osteoclast precursors

The studies with osteoclasts (Figure 40) showed toxic effects only at a concentration of 1 µl/ml in the first experiment. The osteoclast precursors showed greater sensitivity to PEGDMA (Figure 41). The cell number was greatly reduced at 1 and 0.1 µl/ml, but a smaller reduction was observed at the lower concentrations. Altered cell morphology was observed at 1 µl/ml and 0.1 µl/ml.

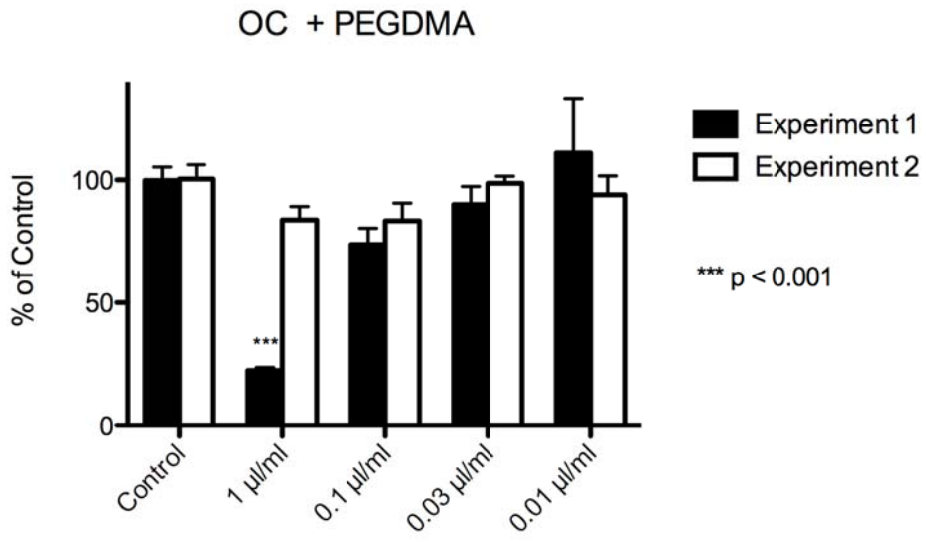

Figure 40: Effect of the monomer PEGDMA on osteoclasts (control, mean cell numbers: experiment 1 = 97 cells, experiment 2 = 254 cells)

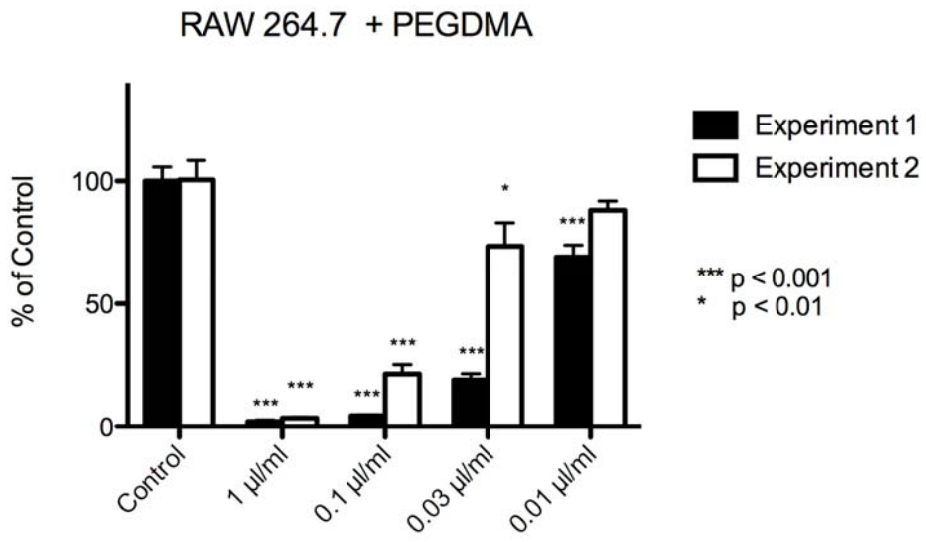

Figure 41: Effect of the monomer PEGDMA on osteoclast precursors (control, mean cell numbers: experiment 1 = 1,045,700 cells, experiment 2 = 697,900 cells)

Table 8: Mean percentages (n = 3) of cell numbers in the groups treated with PEGDMA compared to control

	Osteoblasts		NIH 3T3		Osteoclasts		RAW 264.7	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Control	100 ± 1.53	100 ± 7.13	100 ± 1.67	100 ± 7.57	100 ± 5.47	100 ± 5.90	100 ± 5.77	100 ± 8.01
1 µl/ml	33 ± 2.60	40 ± 4.16	21 ± 0.58	14 ± 0.88	22 ± 1.33	83 ± 5.55	2 ± 0.58	4 ± 0.33
0.1 µl/ml	61 ± 1.53	76 ± 3.22	47 ± 1.16	50 ± 5.00	74 ± 6.64	83 ± 7.31	4 ± 0.33	22 ± 3.84
0.03 µl/ml	74 ± 8.08	95 ± 2.08	81 ± 0.88	56 ± 2.65	90 ± 7.37	99 ± 2.85	19 ± 2.52	74 ± 9.62
0.01 µl/ml	94 ± 9.70	123 ± 2.60	83 ± 0.88	71 ± 2.85	111 ± 22.34	94 ± 7.64	69 ± 4.93	88 ± 3.79

4 Discussion

The development of novel biomaterials to serve as scaffolds in synthetic bone grafting is a complex process with many parameters to consider and assess. Therefore it is important to understand biological and chemical interactions between cells, tissue and materials.

Unreacted photoinitiators or monomers can be present in the fabricated product or released during degradation. Thus, examining cell behavior at different concentrations of all components involved is a reasonable first step in biomaterial design.

Cell numbers measured in the experiments described in this thesis are a result of two different processes. On the one hand, cell viability or function was decreased by the presence of photoinitiators or monomers. On the other hand, the tested substances could interfere with cell proliferation and growth. The morphology of the cells also gives important information about their reaction to the treatments. If cells do not adhere firmly or stretch across the surface, they look smaller and more compact. Also apoptotic or necrotic processes influence cell appearance.

Irgacure 2959 exhibits low cytotoxicity on osteoblasts (Figure 22) and osteoclasts (Figure 24) at the lowest tested concentration (0.1 mg/ml). Results for osteoclasts show a more differentiated inhibition. Cell number clearly increases between 0.3 and 0.1 mg/ml. This might be due to a change in the degree of cytotoxicity around this concentration range. Potential reasons might be deviations in the concentrations applied, or variations due to differences in cell resistance, against any impairment. Also, cell culture is generally subject to variation in several biological aspects, such as cell growth, which can be demonstrated for example when cell numbers of treatment groups exceed 100 % compared to the control. With each NIH 3T3 (Figure 23) and RAW 264.7 (Figure 25), one experiment showed significant reduction in cell numbers at 0.1 mg/ml, whereas the repeat did not. This suggests that 0.1 mg/ml is close to a

non-toxic concentration. With all examined cell types, 3 mg/ml caused severe impairment, but it is questionable if these amounts of substance will be released in vivo. At this concentration, Irgacure 2959 is not easily water soluble. Also, molecules are constantly eliminated from the potential implant site. Therefore, it is not only important to know the cytotoxicity of a substance in an isolated situation, but also for example, in case of photoinitiators and monomers, how much of it remains unreacted in the product after fabrication and what proportion can be washed out before it will be used.

Irgacure 369 has no cytotoxic effects on osteoblasts (Figure 26). Since significant reduction occurred only at 3 µg/ml, and not at the higher concentration of 6 µg/ml, this decrease could be due to variations independent from impairment by the photoinitiator, as described above. With osteoclasts (Figure 28), Irgacure 369 induced a less severe reduction, but the same deviations in the increase of cell number could be observed as with Irgacure 2959 (Figure 24). The experiments were performed in parallel for both substances, using osteoclasts isolated from the same rabbits. With both photoinitiators, the strongest reduction of cell numbers occurred in the first experiment, lowest in experiment 3, which indicates that differences are based on varying sensitivity of cells obtained from different individuals.
In the first experiment with Ini1 tested with RAW 264.7 cells (Figure 33), a strong impairment of cell growth and alteration of morphology were observed for 0.1 and 0.03 mg/ml, whereas groups at 0.3 and 1 mg/ml remained unaffected. Repeat experiments neither showed a reduction of cell number, nor changes of cell morphology at the lower concentrations used. Reasons for this surprizing outcome of the first experiments could not be explained sufficiently.

In summary, a comparison of the results of the 3 photoinitiators shows that Ini1 has the lowest cytotoxic effect on cells under investigation.
A. Ovsianikov et al. stated that the influence of photoinitiators on the biocompatibility of fabricated scaffolds does not only depend on the cytotoxicity of the pure substance.

They showed that, as expected, increasing concentrations of photoinitiator in the freshly prepared scaffold correlated with stronger cytotoxic effects, which were in contrast reduced if samples were aged for 6 days in distilled water. This suggests a potential benefit of high water solubility of the photoinitiator. Residual unreacted molecules can be easily washed out of a porous scaffold during a pre-incubation period, and will not gradually be released when the material is degraded in vivo (Ovsianikov et al., 2011). These findings indicate that Ini1 is the most favourable of the 3 examined photoinitiators, not only due to minor cytotoxicity compared to the others, but also because of its high water solubility.

Osteoblasts were less affected by PEGDMA (Figure 38) than by PEGDA (Figure 34). Also the experiments with osteoclasts (Figure 36 and 40) displayed clearly less toxic effects caused by the methacrylate (Figure 40). Only in experiment 1 cell number was drastically reduced at 1 µl/ml, but rose to a level comparable to the control in the following group. With NIH 3T3, cell numbers increased in about the same range, but did not attain 100 % with PEGDMA (Figure 39). Here the methacrylate seems to cause stronger inhibitory effects, whereas RAW 264.7 cells again proved to be more resistant against PEGDMA (Figure 41) than PEGDA (Figure 37).

To assess the biocompatibility of scaffolds, it is not only important to evaluate the cytotoxicity of possible residual monomers. Also degradation products should be determined in structure, chemical properties, such as pH, and concentration of scaffold components released over time.

A. Ovsianikov et al. also studied features of biomaterials produced by two-photon polymerization using two PEGDA-materials differing in molecular weight. Polymerized products from PEGDA with a number average molecular weight (M_n) of about 302 g/mol (which is similar to the PEGDA used for the experiments in this thesis) resulted in more rigid structures without relevant swelling in PBS, whereas the other (M_n of about 742 g/mol) absorbed significant amounts of water. Depending on the intended application, combinations of PEGDA-materials with different average

molecular weights of the monomer could be advantageous to modulate mechanical properties.

Also they found, that due to the hydrophilic character of the material (hence formation of hydrogen bonds with the surrounding fluid), protein adhesion and cell attachment are impaired. They suggest adding of adhesive ligands to overcome this problem (Ovsianikov et al., 2011).

After deciding on the final material composition, based on the knowledge about the various features of the single constituents, the next steps in the development process are assessing cell functions like attachment to the surface, mineralization by osteoblasts or osteoclastic resorption in direct contact with the two- or three-dimensional structures. In addition degradation products and immune response must be examined. Aside from biological aspects, also mechanical features have to be adapted and tested, followed by extensive in vivo studies.

Engineering synthetic bone substitutes, from choosing appropriate biomaterials to clinical testing, is a long way, requiring detailed research involving various disciplines, but necessary to satisfy the needs of different medical applications and cover the increasing demand.

5 Abstract

Biomaterial scaffolds are used in bone grafting as alternatives to naturally derived tissue. A possible way to fabricate arbitrary three-dimensional structures is the two photon polymerization of photosensitive materials consisting of monomers and photoinitiators. In this work, the three photoinitiators Irgacure 2959, Irgacure 369 and Ini1 as well as the two monomers poly(ethylene glycol) diacrylate (PEGDA) and poly(ethylene glycol) dimethacrylate (PEGDMA) were tested with different cell types to assess their cytotoxicity. The substances were dissolved in the appropriate cell culture medium and incubated with the cells for 72 hours. To assess the impairment of cell viability and growth by different concentrations of the monomers or photoinitiators, cell number of osteoblasts, NIH 3T3 and RAW 264.7 was determined with a CASY cell counter at the end of the experiments, while osteoclasts were TRAP-stained and counted using a microscope.

The experimental photoinitiator Ini1 appeared to be the most favourable compared to Irgacure 2959 and Irgacure 369. It exhibited highest water solubility and lowest cytotoxicity.

PEGDMA generally showed lower cytotoxicity than PEGDA, but it is important to also determine structure and release profile of degradation products to predict biocompatibility of a biomaterial scaffold in vivo.

6 Zusammenfassung

Biomaterialien können als Alternative zur Transplantation von menschlichem oder tierischem Knochengewebe in der Therapie von diversen Knochendefekten vielseitig eingesetzt werden.

Die 2-Photonen-Polymerisation bietet eine Möglichkeit zur innovativen Produktion von beliebigen dreidimensionalen Strukturen aus photosensitiven Materialien, bestehend aus Monomeren und Photoinitiatoren. In dieser Arbeit wurde der Einfluss von drei ausgewählten Photoinitiatoren (Irgacure 2959, Irgacure 369 und Ini1) sowie der zwei Monomere Polyethylenglycoldiacrylat (PEGDA) und Polyethylenglycoldimethacrylat (PEGDMA) auf verschiedene Zelltypen getestet, um deren Zytotoxizität zu ermitteln. Die Substanzen wurden im entsprechenden Zellkulturmedium gelöst und für 72 h mit den Zellen inkubiert. Um die Beeinträchtigung von Wachstum und Viabiliät der Zellen durch verschiedene Konzentrationen der Photoinitiatoren oder Monomere zu ermitteln, wurde danach die Zellzahl von Osteoblasten, NIH 3T3 und RAW 264.7 Zellen mittels CASY Zellzählgerät bestimmt, wohingegen Osteoklasten TRAP-gefärbt und im Mikroskop gezählt wurden.

Im Vergleich mit Irgacure 2959 und Irgacure 369 weist der experimentelle Photoinitiator Ini1 die höchste Wasserlöslichkeit gepaart mit niedriger Zytotoxizität auf und erscheint daher am geeignetsten.

PEGDMA erwies sich als weniger zytotoxisch im Vergleich mit PEGDA. Allerdings ist es unumgänglich, Struktur und Freisetzungscharakteristik von Abbauprodukten der Biomaterialien zu untersuchen, um Vorhersagen bezüglich der Biokompatibilität in vivo treffen zu können.

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8 Figures

9 Tables

10 Curriculum Vitae

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