Investigation of Tissue Engineering Scaffolds for Potential Medical Applications

Ph.D. Thesis

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List of the publications:

- **Providing the basis of the thesis:**

1. Excimer Laser-produced Biodegradable Photopolymer Scaffolds Do Not Induce Immune Rejection In Vivo

Balazs Farkas*, **Adam Zsedenyi*,** Edina Gyukity-Sebestyen, Ilaria Romano, Katalin Nagy, Alberto Diaspro, Fernando Brandi, Krisztina Buzas and Szabolcs Beke *JLMN-Journal of Laser Micro/Nanoengineering Vol. 10, No. 1, 2015* **IF:0.66** (2015) **Q2**

*Equally contribution

2. Gold nanoparticle-filled biodegradable photopolymer scaffolds induced muscle remodeling: in vitro and in vivo findings

Adam Zsedenyi, Balazs Farkas, Gaser N. Abdelrasoul, Ilaria Romano, Edina Gyukity-Sebestyen, Katalin Nagy, Maria Harmati, Gabriella Dobra, Sandor Kormondi, Gabor Decsi, Istvan Balazs Nemeth, Alberto Diaspro, Fernando Brandi, Szabolcs Beke, Krisztina Buzas

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- **Further publications:**

3. Role of epigenetics in EBV regulation and pathogenesis

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I. Introduction

Regenerative medicine seeks to create functional tissues to recover lost tissue or organ functions. Such loss can stem from various conditions that hamper the patient's quality of life, including disease and aging, but congenital diseases, where the function is not lost, but originally missing, also belong here. The field of regenerative medicine is evolving rapidly, and bears the promise of new, more individualized therapies with fewer side effects and complications. The main pillars of regenerative medicine are tissue engineering, biomaterials and stem cell therapy

The first pillar, tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences for the development of biological substitutes that restore, maintain, or improve tissue function. A TE process involves i) well-designed and functional microenvironments called scaffolds ii) cell culture, and iii) highly skilled implantation surgery. These presuppose the cooperation of several fields of science, such as physics, materials science, chemistry, biology, genetics and the medical sciences, especially immunology. This shows that TE is not a homogenous field of science, but a highly interdisciplinary one, where all disciplines have their well-defined role.

In the early days of regenerative medicine, the application of living tissue was the primary means of recovering the damaged area. This is grafting, a technique still used in the clinical practice. Grafts, however, come with a lot of difficulties for the patients. The development of biomaterials opened up the way toward TE, offering an alternative to graft-based tissue regeneration.

The first, 1976 Consensus Conference of the European Society for Biomaterials defined biomaterial as 'a nonviable material used in a medical device, intended to interact with biological systems'. In contrast, the current definition goes: 'a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body'. The two definitions demonstrate how biomaterials have moved from merely interacting with the body to influencing biological processes. Biomaterials can be classified by their substance.

Metals: The use of metals as biomaterials has been reported since as early as the late 18th century when iron, gold, silver and platinum were used to fabricate wires and pins to fix bone fractures. It is also known that gold was used in ancient Egypt in dentistry.

Ceramics as biomaterials are used as filling materials or covering for dental implants. Ceramics have poor fracture toughness, which limits their capability of being used in situations where load bearing is crucial. A wide range of ceramics is used in orthopedic surgery (to fill bone defects), and they are also used for the covering of implant surfaces so as to enhance osseointegration.

Composite biomaterials: Composites are biomaterials which have distinct phases separated on a larger-than-atomic scale, and whose characteristic properties (such as their elastic modulus) significantly differ from those of a homogenous material. In medical applications, they are most often: (1) dental filling composites, (2) reinforced methyl methacrylate bone cement and ultra-high-molecular-weight polyethylene, and (3) orthopedic implants with porous surfaces.

Polymeric biomaterials: Synthetic polymeric materials are widely used in medicine, dentistry and the pharmaceutical industry. The main advantages of these biomaterials over metal or ceramic materials include malleability, good secondary processability, reasonable cost, and availability with customized mechanical and physical properties. The desirable properties of any polymeric biomaterial are similar to those of other biomaterials: biocompatibility, resilience to sterilization, adequate mechanical and physical properties, and manufacturability.

Biodegradable polymeric biomaterials: These materials are broken down spontaneously via enzymatic and non-enzymatic mechanisms. Therefore, they are considered to be environmentally friendly, among their other favorable characteristics. During the past decade, we have witnessed a dramatic increase of interest in these materials. There are two main reasons for this: First, by definition, they do not elicit permanent chronic foreign-body reactions. Second, some of them have recently been found to be promising in the field of tissue engineering, especially for the fabrication of scaffolds. To be suitable for such purposes, any biomaterial must meet a number of criteria. These are:

(i) Biocompatibility is essential. It is only when a material is biocompatible that the cells can migrate onto and inside the scaffold and function normally. After implantation, a biocompatible foreign body must not elicit any immune reaction so that no rejection occurs.

(ii) Biodegradability lies at the heart of the concept of implanted scaffolds. Over time, cells must be able to completely replace the temporarily implanted scaffold. The by-products of degradation should be biologically inert and able to exit the body in natural ways.

(iii) Mechanical properties: the scaffold should have mechanical properties consistent with the receptive site and it must also be resilient enough to endure surgical handling during the implantation. The challenge here is that these scaffolds must have sufficient mechanical integrity to function from the time of implantation through the completion of the healing process.

(iv) Scaffold architecture is not less important. An interconnected pore structure and high porosity are basic requirements so that cellular penetration and adequate diffusion of nutrients to the cells are ensured.

Regarding the manufacturing process, it should be rapid, reliable, and inexpensive. Another key factor is determining how a product will be delivered and made available. Clinicians typically prefer off-the shelf availability without the requirement for extra surgical procedures in order to harvest cells prior to a number of weeks of *in vitro* culture before implantation.

The third pillar of regenerative medicine is stem cell therapy. Stem cells have the ability to renew themselves and differentiate into cells of any tissue (pluripotency). Mesenchymal stem cells harvested from the bone marrow (bone marrow mesenchymal stromal/stem cells; BMSCs) are the best-known and -characterized type of adult stem cells. However, the use of BMSCs has serious disadvantages. These include a low cell yield from the aspirates, the painful harvesting procedure, and the potential complications.

Adipose tissue-derived stromal/stem cells (ASCs) were introduced in 2001. These cells, harvested from fat after liposuction, have the plastic-adherent character and also the ability to differentiate into several cell types (including osteoblasts and myocytes, among others).

In addition, the liposuction procedure is simple, easy and repeatable. The cell yield is higher as compared to bone marrow aspiration, and the procedure is less stressful for the donor. Both BMSCs and ASCs are plastic-adherent under standard culture conditions, with a fibroblastic, spindle-shaped appearance. A further similarity is that both cell types form colonies in culture. However, it was found that ASCs are superior in terms of their proliferative capacity and lower senescence, and they can also be kept *in vitro* for extended periods with a stable proliferation rate. In addition, the osteogenic potential and proliferation of BMSCs seems to decline with age. This decline is less marked in the case of ASCs. Therefore, it was suggested that ASCs would be more suitable for TE purposes than BMSCs. Since then, the osteogenic potential of ASCs have been proven both *in vitro* and *in vivo*.

II. Objectives

The main objective was to establish an innovative application in the field of regenerative medicine. We aimed to investigate a novel, scalable, rapid prototyping (RP) procedure relying on stereo-lithography (STL) and UV photocurable biopolymers *in vitro* and *in vivo.*

We had the following aims:

1. To investigate the suitability of biopolymer scaffolds for cell culturing.
2. To accomplish *in vivo* transfer of ‘scaffold-cell’ system.
3. To investigate biodegrability and biocompatibility of the ‘scaffold-cell’ system.
4. To optimize the scaffold composition for stem cells.
5. To test the tissue growing in the presence of gold nanoparticles (AuNP) added to Poly(propylene-fumarate):diethylene fumarate (PPF:DEF) scaffold.
6. To perform in vivo transfer of ‘stem-cells-on-scaffolds’.

III. Materials and Methods

Biodegradable polymer scaffolds

The scaffolds were bulid with Mask Projection Excimer laser StereoLithography (MPExSL) system. The method relies on a layer-by-layer buildup process where layers are fabricated by image projection, using pulsed excimer laser radiation. The scaffolds are made of resin and photoinitiators

PPF is a versatile biodegradable and photocurable biomaterial, a linear polyester with an unsaturated backbone that allows the crosslinking, and cytocompatible degradation products based on propylene glycol and fumaric acid. To have an appropriate viscosity for STL purposes, the purified PPF resin had to be diluted. Diethyl fumarate (DEF) was applied as diluent to reduce the resin viscosity as needed for proper resin recast. Irgacure 819 or Bis(2,4,6-trimethylbenzoyl)-phenylphosphineoxide „BaPO" is used with the PPF:DEF due to its favorable spectrum in the deep UV, as well as being ethanol-soluble, thus compatible with the resin mixing process.

There were also nanoparticles integrated to the resin. The particle of choice was gold nanoparticles. AuNPs are ideal platforms for drug/growth factor delivery as well as Deoxyribonucleic acid (DNA) adhesion, and they are also easy to produce

Biological testing

Scaffolds for the *in vivo* and *in vitro* tests were, 5-mm diameter, 100-μm thick with 200 μm-pore diameters, using a BaPO concentration of 1%. Two types of these, PPF:DEF scaffolds with 200 μm pore size were used. The first type of scaffolds did not contain any NPs. The resin included 5.52 μMAu NPs, in the second type of scaffolds.

Three types of cells were tested.K7M2 mouse osteosarcoma cell line cells (provided by Dennis Klinman, NCI-Frederick, MD, USA), RAW 264.7 Cell Line murine, macrophage from blood were obtained from Sigma-91062702 and primary autologous adipose stem cells (ASC). ASCs were isolated from 4 to 6 weeks old Balb/c male mice (Charles River Laboratories International, Inc.).

In the *in vitro* experiments cell-seeded-scaffolds with cells were prepared for scanning electron microscopic (SEM), and confocal microscopic examination.

In the i*n vivo* experiments first K7M2 mouse osteosarcoma cell line was grown on the scaffolds. In case of a successful cell implantation, the presence or absence of selected K7M2 mouse osteosarcoma cell line was followed by histology. Animal care was provided in accordance with the procedures outlined in the protocol authorized by the Institutional and the National Animal Ethics and Experimentation Boards.

We also used ASCs. In case of a successful cell implantation, the presence or absence of selected mouse ASC was followed by histology. All animal experiments were performed in accordance with national (1998. XXVIII; 40/2013) and European (2010/63/EU) animal ethics guidelines. The experimental protocols were approved by the Animal Experimentation and Ethics Committee of the Biological Research Centre of the Hungarian Academy of Sciences and the Hungarian National Animal Experimentation and Ethics Board (clearance number: XVI./ 03521/2011.).

The potential inflammatory reactions of the implanted biomaterial were screened via cytokine and chemokine profiling.

The samples was investigated by fluorescence in situ hybridization (FISH). Thi is a molecular diagnostic technique utilizing labeled DNA probes to detect a gene or specialized sequences.

IV. Results

K7M2 cells were successfully seeded to biopolymer scaffold.

After 14 days of incubation, fluorescent microscopic images were taken of the scaffold, with Tomato red transfected cells .Viable red cells adhered to the margin of pores.

K7M2 seeded scaffold successfully transferred cells *in vivo* and did not induce immun rejection

14 days after implantation, the implantation site, the dorsal skin of the mice were removed and blood samples were collected. A small size nodule was visible in each mosue implanted with K7M2 seeded scaffold, but we could not observe wound, inflammation,

bleeding or ulceration. The experimental animal did not show high temperature, pain, scratching, any symptom or side effect.

The collected blood samples were cytokine and chemokine profiled. No significant difference was detected between the cytokine profiles of the tumor scaffold- implanted group and the control group. We could not find elevated level of proinflammatory cytokines or chemokines.

Upon histological examination of the implanted scaffolds, no signs of inflammation were observed in the dorsal skin, and no signs of rejection were found. In the histological sections of the group implanted with tumor cell seeded scaffolds, viable, anaplastic sarcomatous tumor cells were found. No residual biopolymer piece was found at the transplantation site in the control animals, suggesting the biological degradation of the scaffolds.

AuNP filled scaffolds support stem cell adherence and viability compared to AuNP free material

In vitro, macrophages - a cell type of high adherence - could be seeded on the scaffold surface. The Au-content of the polymer resin had no influence on the adherence of the macrophages.

The adherence of ASCs, however, was apparently affected by the Au content of the scaffold. Adherence to the Au NP hybrid scaffold proved to be much better. Stem cells adhered to the surface of AuNP filled scaffolds. ASCs did not show appropriate proliferation on the surface of the AuNP-free scaffolds, cell aggregates were seen in the middle of pores.

Stem cell seeded scaffold induced muscle regeneration in vivo

The excised skin areas showed no visible signs of reactive inflammation, and the cytokine profiling did not indicate inflammation either. Interestingly, we detected not only the implanted ASCs with X-Y chromosome but also cells containing Y and more than one X chromosomes, most likely resulting from regenerative cellular fusion between ASCs and stromal mesenchymal cells. This suggests that the biopolymers applied together with ASCs were able to initiate tissue repair in the appropriate tissue environment.

V. Discussion

The main objective we set was to test a novel, scalable, rapid prototyping (RP) procedure relying on stereo-lithography (STL) and UV photocurable biopolymers *in vitro* and *in vivo*.

The first experiment was built around the fundamental questions of scaffold use. We investigated cell adhesion, biocompatibility and biodegradability. Tissue engineering scaffolds are usually made of polyester. Our scaffold material, PPF:DEF is a relatively new material. However for bone TE applications the photocross-linking of poly(propylene fumarate) (PPF) was investigated to form porous scaffolds. For the formation of cross-linked, degradable polymer networks with tunable material properties cross-linking factors have been examined in combination with PPF, but this precise combination was first described by Kasper and colleagues in 2009 and it needed further investigation.

In our study, we covered PPF:DEF scaffolds with K7M2 mouse osteosarcoma cells, and we were able to transfer this 'scaffold-cell' system into the living tissue, i.e. into recipient mice. No signs of inflammation were detected, either by the cytokine/chemokine profiling or with histology. No scaffold remnants were found in the histological sections, which indicates that the scaffold was biologically degraded. In this sense, we can say that our system met the three fundamental requirements towards scaffolds: adhesion, mechanical strength, and biocompatibility/biodegradability.

Our results showed that the stem cells are sensitive to the composition of the scaffolds. We performed *in vitro* experiments with scaffolds of different concentration of incorporated AuNPs. While the stable but transformed macrophage cell line was not especially sensitive to the Au-content of the polymer, the adherence and distribution of ASCs was definitely better on the Au hybrid polymer. This is important because of the potential value of these cells in regenerative tissue engineering. The incorporated AuNPs helped the stem cells adhere to the scaffolds. The results of the *in vivo* experiment showed that our cell-scaffold complex could be successfully transferred into the living tissue of experimental animals and the stem cells induced muscle regeneration.

Tumor formation is an ever-present danger of stem cell transplantation, but our system proved to be safe in this respect. The use of the scaffold ensures that the cells are kept in place, and the chance of stem cells getting into the bloodstream is significantly reduced. The use of autologous stem cells reduces the risk of rejection, ensures better regeneration, and ASCs are readily available. ASCs are easy to harvest in a safe, minimally invasive way from almost any patient

Our research about PPF:DEF scaffolds are pointing in the future. The results show that this scaffold is an adequate option in regenerative medicine. It needs further research until it gets to the clinical use, but the first steps were made.

VI. Conclusion

The studies described in this thesis were planned to take the PPF:DEF scaffolds closer to the clinical application.

In vitro and *in vivo* experiments were conducted to prove that the scaffolds meet the *sine qua non* requirements of medical use. When it was established, we turned our attention to application with stem cells.

The PPF:DEF scaffold proved to be a competent tissue engineering device, because it was biocompatible, biodegradable, and cells could adhere to it, and it's meeting the expectations when it gets to the application. Furthermore, the adipose stem cells could be transported with the scaffold to experimental animals. We also noticed that AuNPs supplementation of the scaffold enhanced cell adhesion and proliferation.

The future of this research should be continued towards the clinical usage. We did the first steps, and after the basics, it should carry on, because the scaffold is promising. Further experiments need to show, that the scaffolds can be used in bigger size, with more cells, to cover up a bigger surface. It is necessary to work out more precise experiments *in vivo* towards regenerative medicine. A standardized method could facilitate the hard and soft tissue regeneration, renewing thereby restorative, regenerative medicine.

VII. Summary of accomplished objectives:

- PPF:DEF biopolymer scaffold supported cell adhesion and proliferation.
- Cell seeded PPF:DEF biopolymer scaffold successfully transferred living cells into experimental animals.
- PPF:DEF biopolymer scaffold biocompatible and biodegradable tissue engineering devices, without significant immune rejection.
- Incorporated NPs (especially Au NPs) in the scaffold composition can enhance tissue growing.
- Stem cell-seeded- PPF:DEF scaffolds successfully induced tissue regeneration *in vivo*

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