Monitoring tissue implants by field-cycling MRI of quadrupolar-peak contrast agents (acronym: QP-MRI)

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ABSTRACT

In this project, we develop innovative sensors to monitor non-invasively the status of tissue implants by Fast Field Cycling imaging. These sensors enhance image contrast thanks to the ^{14}N of imidazole groups of histidine, which are conjugated to the PLGA polymeric chains forming the scaffold structural matrix. These completely new polymeric sensors are pH-sensitive, biocompatible and biodegradable; they differ completely from current clinical contrast agents (which contain potentially toxic paramagnetic metals) and can report continuously on scaffold degradation and cell proliferation, non-invasively.

Keywords: Biomaterial for tissue engineering; Fast Field Cycling Magnetic Resonance Imaging (FFC-MRI); Imaging Probes.

1. INTRODUCTION

- The aim of the project is the development of an innovative biocompatible, biodegradable and trackable material to be used for the preparation of tissue implants for regenerative medicine applications. The peculiarity of this material is that it contains an intrinsic sensor able to report on the status of the implant, providing insights into its biodegradation and on the fate of implanted cells. Tissue scaffolds act as a temporary substitute for extracellular matrices, providing an initial mechanical support for transplanted cells until the regenerated tissue can stabilise the initial structure. To date there are no methods that can monitor the scaffold status *in vivo* and report on its degradation and on the functionality of the transplanted cells. The biomaterial consists of PLGA (Poly-lactic and glycolic acid) already approved for human use, forming the solid matrix of the scaffold. The sensing part of the biomaterial consists of histidine oligomers (oligo-His) able to generate a relaxation enhancement at a specific frequency well detectable by Fast Field-Cycling Magnetic Resonance Imaging (FFC-MRI). They represent a completely new class of metal-free MRI contrast agents that display remarkable relaxation effects on tissue water protons.
- The breakthrough character of the technology developed in this project is the possibility to monitor the scaffold after its implantation in the target tissue. Most existing degradation assessments are carried out exclusively *in vitro* before the scaffold implantation and under conditions quite different from the physiological state. In comparison, the method we propose can report cell viability without the need to extract cells from the matrix, thanks to its pH dependence.
- PLGA scaffolds conjugated with oligo-His showed a relaxation enhancement (also called Quadrupolar Peak, QP) at 1.39 MHz where the proton nuclear magnetic resonance (NMR) frequency and the ^{14}N nuclear quadrupole resonance frequency coincide. This QP falls at a frequency well distinguishable from the QPs generated by endogenous proteins. The relaxation enhancement is pH dependent in the physiological range 6.5-7.5, thus reporting on scaffold degradation which is often caused by a slight pH decrease. The ability of this new class of contrast agents to generate contrast in an FFC-MRI image acquired at 1.39 MHz was assessed. A good biocompatibility was observed after surgical implantation of oligo-His-PLGA scaffolds in the murine back. A scaffold colonisation with endogenous fibroblasts was observed and proportional changes in the intensity of the relaxation peak were observed. After histological validation the obtained results demonstrated the intrinsic responsiveness of the new material was well using FFC-NMR/MRI.

2. STATE OF THE ART

One of the most important challenges in biomedicine is represented by regenerative medicine, i.e. the possibility to repair and/or replace tissues and organs that have been damaged by disease, trauma or congenital defects.[1] Thus there is an increasing interest in the development of innovative biomaterials that act as a temporary support for tissue regeneration. The administration of stem cells and progenitors has been shown to be the most powerful strategy for regeneration of tissues. However, routine use in clinical applications has been hampered due to rapid scaffold degradation and poor long-term survival of therapeutic cells. In particular, it is not well understood whether graft failure is the consequence of cell death and when this occurs. On this basis, a non-invasive imaging method that can probe implant status and cell viability would accelerate human translation. Most of the existing methods involve the use of probes added into the implant that are detected only *in vitro* before implantation in the patient, therefore in conditions that are very different from physiological ones. In addition, the only way to monitor artificial tissue over time is to resort to the surgical removal of a tissue sample to analyze it with common techniques based on fluorescent dyes. MRI is a powerful high resolution technique capable of providing extremely detailed morphological images. By using Gd-based contrast agents (GBCAs) or highly sensitive iron oxide particles, it is possible to mark various types of cells or implants making them monitorable via MRI. The disadvantage, however, lies in the cytotoxicity of the metals used and the persistence of the contrast in the image even after implant degradation and cell death. For these reasons, probe developers are urgently challenged to find alternative metal-free solutions, in particular that could allow for long-term *in vivo* monitoring applications.

3. BREAKTHROUGH CHARACTER OF THE PROJECT

In the search for alternatives to the use of paramagnetic complexes (containing metals such as Gd^{3+}, $Mn^{2+/3+}$, $Fe^{2+/3+}$) we were inspired by the relaxation enhancement brought about by "semi-solid" proteins in biological tissues reported early in the NMR study of immobilized proteins as well in the case of biological tissues.[2,3] In this case the water interaction with quadrupolar ^{14}N atoms present in the protein amidic groups cause a quadrupolar relaxation ($1/T_1$) enhancement (QRE) detectable at specific frequencies. We surmised that other ^{14}N containing functionalities may be the source for proton relaxation enhancements at frequencies different from those reported for the protein amide groups (at 0.6, 2.1 and 2.7 MHz). This approach may lead to a novel class of "frequency-encoding" agents that eventually may prompt the development of MRI scanners operating at magnetic fields very different to the ones (typically 1.5 T or 3.0 T) currently employed for clinical studies.

The aim of the project was to develop a new biomaterial able to self-generate T_1 contrast in an MR image without the addition of paramagnetic metals. The contrast is derived from imidazole groups of oligo-histidine chains causing QRE at 1.39 MHz (far from the protein related QRE), well detectable by an FFC-MRI scanner. Due to its marked dependence on the polymer immobilization, QRE can be exploited for monitoring scaffold water permeation and consequently the scaffold surface or bulk erosion. In addition, the QRE of histidine-containing peptides is pH dependent thus providing at the same time valuable information on cell viability and graft degradation. An increase in poly-His mobility due to the pH decrease associated with cell death may also be detected when the imidazole units are part of a scaffold matrix thus acting as reporters of local physiological and pathological changes. The immobilization of the polymeric chains in a tissue-like state is mandatory for the generation of the QRE and, since pH controls the physical state of the oligo-His solid/liquid status, an estimate of the pH of the microenvironment in which the histidine oligomer is located can be obtained via the changes in the QRE intensity. Accordingly, the conjugation of an oligo-histidine chain to the polymeric biomaterial can allow the physician to assess, in real time, the status of the transplanted scaffold and cells, monitoring the natural biodegradation of the scaffold and cells colonization. Finally, the greatest advantage of the technology developed in this project is the possibility to monitor the implant, *in vivo* and non-invasively, after its implantation in the target tissue.

4. PROJECT RESULTS

Synthesis and purification of oligo-His-PLGA and scaffold preparation. The oligo-His peptide (n=15), synthesized by Solid Phase using the Fmoc strategy, was conjugated to the PLGA-PEG-maleimide derivative, through the coupling between the N-terminal cysteine and the maleimide terminal groups of PEG (Figure 1). Two PLGA with different MW (12 and 46 kDa) were used to prepare oligo-His-PLGA-S and oligo-His-PLGA-L, respectively.

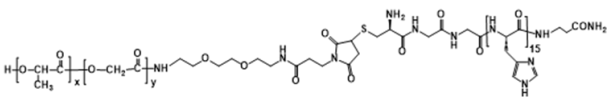

Figure 1. Chemical Structure of Oligo-His-PLGA

Porous oligo-His-PLGA and PLGA-based scaffolds were prepared by the porogen leaching technique.[4] Initially, different polymer formulations were considered in this study. The formulation forming the material with

sufficient consistency and high QRE was obtained using oligo-His-PLGA-L and oligo-His-PLGA-S 75:25 ratio, respectively. The Nuclear Magnetic Relaxation Dispersion (NMRD) profile acquired on this biomaterial showed a characteristic QRE at 1.39 MHz caused by the presence of the imidazole groups.
The QRE was calculated using the following equation

$$QRE = (R_1^{1.39\,MHz} - R_1^{1.68\,MHz})/R_1^{1.68\,MHz} * 100 = \Delta R_1/R_1^{1.68\,MHz} * 100 \quad \text{(eq.1)}$$

The relaxation enhancement (QRE) of oligo-His-PLGA was ca. 40 % corresponding to a $\Delta R_1 \sim 1.1\ s^{-1}$ (Figure 2A). Figure 2B shows the pH dependence of relaxivity (1.39MHz) which results in the ability to monitor changes in the implant status.

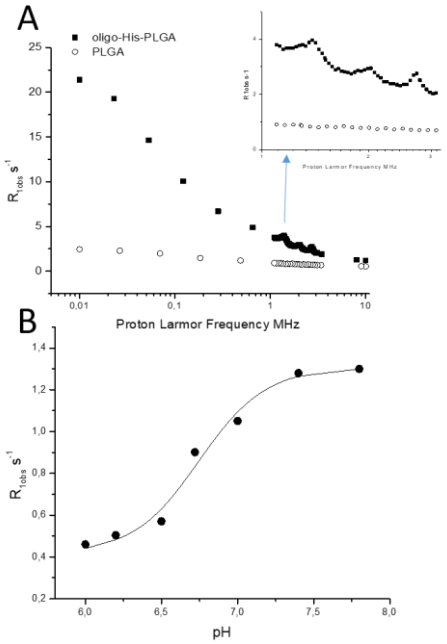

Figure 2. A) NMRD profiles of Oligo-His-PLGA (■) and PLGA scaffolds (○); B) R_{1obs} (s^{-1}) of oligo-His.-PLGA scaffolds measured at 1.39MHz as a function of the pH.

FFC-MRI The contrast-generating ability of oligo-histidine-PLGA scaffold was assessed using the 0.2 T FFC imager built at the University of Aberdeen.[5] Single sample relaxometer measurements of NMRD profiles were obtained from a control PLGA scaffold and two oligo-histidine-PLGA scaffolds at pH of 6.4 and 7.4 respectively. QP contrast images of the scaffold samples submerged in 2% Agarose gel were obtained at 33 mT, by subtracting the approximated baseline of the NMRD profile on a voxel-by-voxel basis. Scaffolds containing polyhistidine showed a distinctive positive image contrast originating from the polyhistidine quadrupolar-induced relaxation differences at 33 mT (Figure 3) The pH 7.4 scaffold yielded 3.8 times higher QP amplitude than the scaffold at pH 6.4.

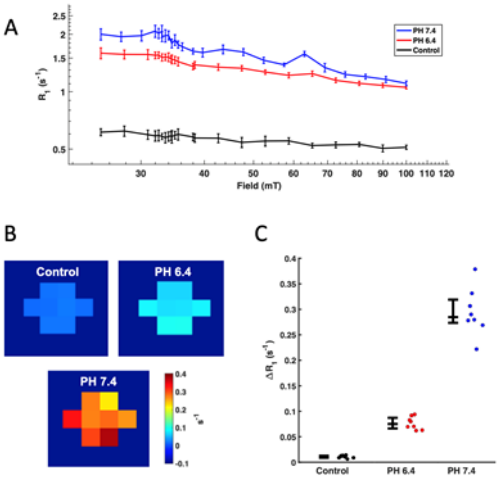

Figure 3. A, single sample relaxometer NMRD profiles where error bars represent the 95% confidence interval. B, QP contrast image measured at 33 mT where the image voxel size is 2.25 × 2.25 × 30 mm^3. C, dot plot of QP magnitude for each scaffold sample. Each dot represents a single image voxel. The median and IQR is indicated for each sample.

Scaffolds implantation. Oligo-His-PLGA and PLGA (control) scaffolds were surgically implanted in the back of Balb/c mice. Mice (n=12) were euthanized 12 and 25 days after surgery, after which FFC-NMR and histological analysis were performed on excised scaffolds. Figure 4 shows that, as expected, QPs are clearly detectable at 1.39, 2.03 and 2.7 MHz only on oligo-His-PLGA scaffolds while PLGA control profiles remain flat. All scaffolds showed a significant increase of the overall relaxation profile and QRE after implantation with respect to pre-implant measurements. (Figure 3A) This is the consequence of the substitution of air by immobilized water into the pores.
Scaffolds analysed 25 days after implant showed reduced QREs with respect 12 days as a consequence of the gradual substitution of scaffold matrix with new generated tissue (Figure 4). This process can be monitored by the QRE quantification using eq.1. Figure 4B shows, 25 days after surgery, a QRE decrease at 1.39 MHz of ca. 18%, probably due to pH change after colonisation of cells. Matrix substitution with the regenerated tissue was further demonstrated by the observation of a less pronounced QRE decrease at 2.03 and 2.7 MHz (ca. 8%) due to the compensation arising from proteins of the new generated tissue. This evidenced once again the potential of the FFC-NMR/MRI technique's ability to image changes in relaxation of materials at different magnetic field strength, thus obtaining unique information not-detectable with clinical scanners.

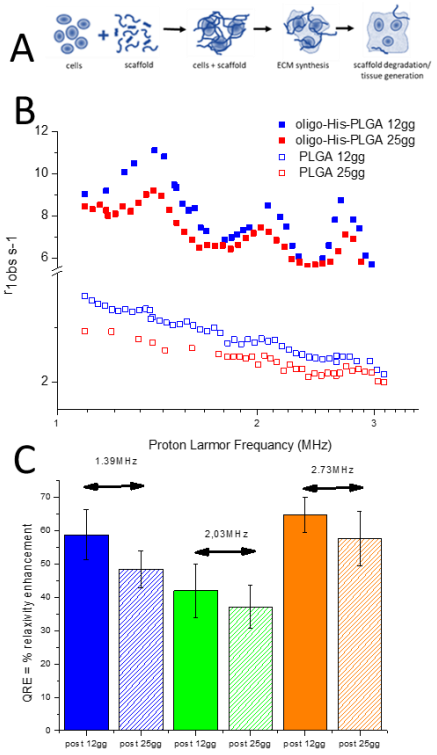

Figure 4. A) Representation of scaffold degradation; B) NMRD profiles acquired on excised scaffolds. C) QRE analysis.

Histological analysis of the explanted scaffolds revealed a progressive colonization by endogenous cells (probably fibroblasts), starting from the periphery and moving toward the centre.(Figure 5A)

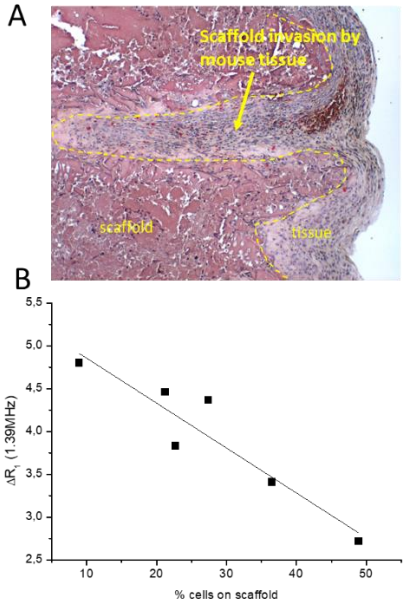

Figure 5. A) H&E staining of tissue implant (12 days); B) DR1 vs % of endogenous cells found in the excited scaffolds.

Hematoxylin and Eosin (H&E) staining showed after 12 days from *in vivo* implant an initial cell invasion of the external scaffold surface. After 25 days, cells colonize the entire scaffold's pores and start to degrade it. The H&E analysis confirmed this result, showing a correlation between the % of the ematoxylin positive area (corresponding to cells invading the scaffold) and days after scaffold implant. The monitoring ability of the biomaterial was demonstrated by the fact that the ΔR_1 decrease at 1.39 MHz linearly correlated (r^2=0.84) with the % of invading cells.(Figure 5B) The lower correlation of ΔR_1 observed at 2.03 and 2.73 MHz (r^2= 0.3 and 0.5, respectively) was due to the contribution of proteins arising from invading cells, thus confirming the scaffold colonization.

5. FUTURE PROJECT VISION

5.1. Technology Scaling

The main phases necessary to reach at least TRL 5 in ATTRACT phase 2 are essentially four:
1) Identification of the leading biomaterial starting from the results obtained in phase 1. Optimization of the synthesis to scale the manufacturing process to a large-scale production. Optimization of scaffold preparation using innovative procedures (i.e. 3D printing, electrospinning)
2) Verification of cell (in particular stem and progenitor cells used in regenerative medicine) engraftment and cell differentiation on biomaterial prepared in step 1).
3) Transplantation of the biomaterial with absorbed progenitor cells on small animals (mice) and monitoring, by *ex vivo* FFC-NMR/MRI, pH changes and cell viability. In a second step, transplantation of the biomaterial on medium dimension animals (rabbits, small pigs) in order to verify biocompatibility and feasibility of the next step: *in vivo* FFC-MR imaging.
4) Acquisition of FFC-MRI images of implanted biomaterial both *ex vivo* and *in vivo* using medium dimension animals. This is a feasibility study of human translation.

5.2 Project Synergies and Outreach

The consortium will be reinforced by adding: i) a partner expert on tissue engineering in order to optimise the product design and to demonstrate its clinical potential; ii) Physicians expert in tissue transplant to find most challenge medical needs; iii) an industrial partner for the construction and commercialisation of FFC-MRI scanners; iv) a chemical/pharmaceutical company for the large-scale production of the biomaterial. Public dissemination will be focused on the involvement of relevant stakeholder groups as well as participation at meetings and public events relevant for this topic. A plan for the exploitation of project results will be set up also including definition of end users and markets in which project results will be exploited.

5.3 Technology application and demonstration cases

Tissue scaffolds are excellent tools for the regeneration of tissues such as bone, cartilage or cornea, providing initial mechanical support until the regenerated tissue stabilizes. However, successful regeneration depends strongly on their stability. It is crucial to assess their status in vivo after insertion to take early corrective actions. No solution currently exists that allows non-invasive monitoring of the viability of implanted scaffolds. Our contrast agent has the unique ability to monitor the tissue scaffold in vivo, non-invasively and repeatedly after its implantation in the target tissue, paving the way to monitoring strategies. We expect that regular monitoring via our histidine probe will dramatically improve patient outcome by allowing early corrective actions in order to maintain the scaffold's structural integrity. University of Torino is one of the nodes of the European Research Infrastructure Consortium (ERIC) named Euro-BioImaging which provides life scientists with open access to a broad range of technologies and resources in biological and biomedical imaging. Of course, during phase 2 of the ATTRACT project we will exploit the possibility to assess the different imaging technologies provided by EuroBioImaging.

5.4. Technology commercialization

We exploit an innovative contrast mechanism that can generate a completely new class of contrast agents that are non-toxic and have exceptional abilities for monitoring, two essential and urgent needs in the field. This, together with the patent already in place (PCT/IB2020/055017), will enable negotiations to take place to establish commercialisation strategies with contrast agent companies such as Bracco, Guerbet and GE Healthcare, with which UNITO has long-standing relationships. A customer discovery study will also be conducted, approaching physicians expert in tissue transplant, to better target the market and to help design products that meet the needs of a realistic customer base. Research-focused applications will be sought first, to circumvent the long and expensive process of CE marking for contrast agents. The commercialisation strategy of FFC systems is currently being developed at the University of Aberdeen and the exciting applications of this FFC contrast agent augments the already demonstrated ability of this technology for non-invasive applications, which provides stronger rationale for funding.

5.5. Envisioned risks

Risks	Mitigation strategies
Failure in the interaction of stem or other progenitor cells with the biomaterial	Collagen or other specific compounds will be added to the biomaterial; physical methods, as centrifugation, to increase cell adhesion will be also used
Failure in the large-scale manufacturing of biolpolymers	Chemical companies included in the consortium will provide the necessary expertise
Failure in in vivo QRE detection	The biomaterial will be prepared with a higher poly-histidine content

5.6. Liaison with Student Teams and Socio-Economic Study

During ATTRACT Phase 2 we are planning to organize summer schools for MSc students covering the different topics at the basis of the project (regenerative medicine, material for tissue implants, MRI and FFC-MRI). An experienced person will be nominated for the organization of the program that will consist in both theoretical lessons and practical parts. In this way we would like to attract students with different backgrounds (chemistry, Biology, Medicine, Physics) giving the possibility to acquire a basic knowledge of these multi-disciplinary technologies. Moreover during phase 2 we will consider in deep the impact of the innovative technology developed in our project on ecosystem. To this purpose, the use of synthetic protocols based on "green chemistry" will be considered for the large scale production of the biomaterial. In fact, Green Chemistry reduces pollution at its source by minimizing or eliminating the hazards of chemical feedstock, reagents, solvents and products; or encouraging the invention and innovation of new and non-hazardous solvents, materials, and processes.

6. ACKNOWLEDGEMENT

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