IAEA
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REPORT

FOURTH RESEARCH COORDINATION MEETING OF THE

IAEA COORDINATE RESEARCH PROJECT /F23030-E31007/

ON

"INSTRUCTIVE SURFACES AND SCAFFOLDS FOR TISSUE ENGINEERING USING RADIATION TECHNOLOGY"

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1. INTRODUCTION

Tissue Engineering brings together cells, materials and suitable biochemical and physico-chemical factors to grow tissues and organs. The primary application for tissue engineering is in medicine for repairing and replacing tissues and organs. However other potential applications include: cosmetic enhancement/alteration; living devices to treat diseases (examples: kidney, liver, Parkinson's disease); food production; living biosensors; implantable, living drug delivery systems; tissues for production of biological molecules; "organs on a chip" and *in vitro* model tissues for drug testing and studies of human disease and pathologies; and basic studies on tissue formation and development.

Tissue engineering is poised to revolutionize medicine by shifting the focus of treatment from addressing the symptoms, roots and causes of diseases to repair and regeneration. Regenerative medicine involving cell therapy is an emerging field that seeks to combine the knowledge and expertise of diverse disciplines towards the aim of restoring impaired tissue/organ functions in the body. This paradigm shift will have huge impact in both developed and developing countries.

Radiation technologies play a role in facilitating and speeding up the development of tissue engineering by addressing some of its challenges and opportunities. These include preparation/optimization of instructive scaffolds, surface grafting, inhibition of cell growth in stem cell feeder layers and sterilization.

Human tissue is an important resource for medical treatment. For example, it is used in burns treatment, reconstructive surgery, cancer care, and heart tissue replacement. Tissue banking exists primarily because of the generosity and goodwill of tissue donors and their families. Many countries face a shortage of donor tissues/organs due to several reasons including religious beliefs, lack of donor registration programs etc. Shortage of donor tissue limits the successful application of tissue reconstruction. Tissue engineering is a promising, relatively new area focused on the development of new tissue created either from "cultured" cells (including stem cells and/or by synthetically produced biomaterials (including the use of nanotechnology). Tissue engineering, whether or not combined with traditional tissue banking techniques will improve the outcome of medical treatment and decrease the need for (sterilized) donor material in the future.

New generations of synthetic biomaterials are being developed at a rapid pace for use as three-dimensional extracellular microenvironments to mimic the regulatory characteristics of natural extracellular matrices (ECMs) and ECM-bound growth factors, both for therapeutic applications and basic biological studies. Recent advances include nanofibrillar networks formed by self-assembly of small building blocks, artificial ECM networks from protein polymers or peptide-conjugated synthetic polymers that present bioactive ligands and respond to cell-secreted signals to enable proteolytic remodeling. These materials have already found application in maintaining functional stem cells and in differentiating stem cells into neurons, repairing bone and inducing angiogenesis.

A few methods can be used to generate instructive matrices to be employed in tissue engineering. Among them, the application of radiation technology for formation and modification of surfaces and matrices has remarkable advantages such as: initiation of low temperature reactions, absence of harmful initiators, high penetration through the bulk materials and curing of different types polymeric materials (by polymerization, grafting and crosslinking). Additionally, radiation synthesized surfaces and scaffolds might simultaneously be modified and sterilized. Radiation sterilization is a well-established technology, it is a reliable and effective process used industrially for nearly 60 years. Medical devices, raw materials for pharmaceuticals, biomaterials, tissue allografts, and cosmetics among other products are routinely sterilized by ionizing radiation.

This International Atomic Energy Agency (IAEA) Coordinated Research project (CRP) provides a forum for knowledge and technology transfer among participating institutions and facilitates the formation of a network between diverse disciplines, as well as promotes the early involvement of developing IAEA member states (MS), thus enhancing their level of competence. It was formulated based on the recommendations made by a Consultants' meetings organized jointly by the ARBR (NAHU) and RPRT (NAPC) sections (held on 23-25 August 2010; and on 12-16 March 2012) and aims to support MS in developing and testing instructive scaffolds and surfaces using radiation technology to create tissue grafts and help decrease the need for human donors. This is a collaborative CRP between NAPC and NAHU. NAPC will implement the part related to the development and testing of the instructive surfaces and scaffolds, while NAHU will implement the biomedical application part related to the intended end-uses. The CRP will build on the knowledge and expertise acquired under the successfully completed CRP on "Nanoscale Radiation Engineering for Biomedical Applications" (NAPC) as well as the on-going CRP on "Safety and Optimization of Radiation Sterilization in Tissue Banking: Studies on Functional Properties of Irradiated Tissue Grafts" (NAHU).

2. CRP OVERALL OBJECTIVES

The goal of the CRP is to engineer instructive scaffolds and surfaces using radiation technology to create tissues from autologous and allogeneic human somatic cells to provide tissue grafts and decrease the need for human donors.

2.1. Specific Objectives

- To investigate and optimize the preparation of instructive scaffolds and surfaces
- To investigate the use of radiation sterilization of the new instructive scaffolds and decellularized matrices
- To study cell-cell-scaffold-matrix-ECM interaction

- To study the possibilities and effectiveness of combining biological and non-biological materials on regeneration/repair
- To bring together researchers from different research directions with end-users in place

2.2. Expected Research Outputs

- Preparation methods for instructive scaffolds and surfaces
- Guidelines on the use of radiation sterilization of the new instructive scaffolds and decellularized matrices
- Data on cell-cell-scaffold-matrix-ECM interaction
- Data on the possibilities and effectiveness of combining biological and non-biological materials on regeneration/repair
- A network of researchers from different research directions with end-users in place

The first Research Coordination Meeting (RCM) was held in Vienna, on 14-18 July 2014 and was attended by 14 participants and one observer. The participants presented the ongoing research in their laboratories/institutions, reviewed the work plan, and identified potential areas for collaboration. The CRP established a SharePoint collaborative workspace at IAEA Nucleus (https://nucleus.iaea.org/sites/arbr/CRP_F23030-E31007/) for direct access to all materials, and activities under the CRP.

The second RCM was held on 26-30 October 2015, in Lisbon, Portugal. The participants reported the progress of their work since the first RCM, and agreed on the work plan for the next 18 months. The challenges encountered were also discussed, and closer collaboration proposed. The participants have also prepared a draft article to be sent for publication in Tissue Engineering Journal. The short summary of the work progress as reported by the participants was prepared.

The third RCM was held on 2-5 May 2017 at IAEA Headquarters, VIC, Vienna, Austria. The participants reported the progress of their work since the 2nd RCM and agreed on the work plan for the next 18 months. The challenges and collaboration were discussed. A new a draft article was prepared by participant for publication in Tissue Engineering Journal.

The final, 4th RCM was held on 8-12 April 2019 at IPEN-CNEN/SP, São Paulo, Brazil. The participants reported the progress of their work since the 3rd RCM and agreed to produce an IAEA TecDoc on results of the CRP. Since the long review paper for the Tissue Engineering Journal was rejected, it was decided, as suggested by reviewers, to submit a short *Letter to the Editor*, based

on the overview of the long manuscript. The rest of the "long paper" will be submitted to another journal, i.e. Radiation Physics and Chemistry.

3. PROGRESS OF THE WORK IN INDIVIDUAL INSTITUTIONS

3.1. PARTICIPATING MEMBER STATES

Argentina, Bangladesh, Brazil, Chyna, Egypt, Malaysia, Mexico, Poland, Portugal, Russia, Slovakia, Turkey, United Kingdom, Uruguay

ARGENTINA

In Argentina, human tissue banking activities started in 1948, with the first bone graft from a cadaveric donor. Tissue banks and their regulation were evolving since then, and at the present, there are 26 tissue banks: 8 eye banks, 1 skin bank, 13 bone banks, 2 heart valves and 2 amnion banks. Between them, 5 tissue banks are using ionizing radiation as final sterilization.

All tissue banking activities are depending on tissue donors. The donor rate is still low in Argentina, and the tissue demanding is still not fulfilled. That is why tissue engineering has become a necessary discipline to be investigated.

Our project is conducted to obtain two products for bones replacement: 1) a HDPE/HA permanent prostheses improved by irradiation, with similar mechanical properties than cortical bone; and 2) a resorbable PLA/HA 3D printed scaffold seeded with mesenchymal stem cell, to conduct real bone.

For the permanent prostheses different percentage of HA (0%, 10%, 30 % and 50 %) and different irradiation doses (0, 20, 40 and 100 kGy) and conditions (vacuum and air) were studied to establish the better combination to have tissue bone properties. All the combinations with HA were not cytotoxic and bioactive. For the resorbable scaffold PLA printable filament with different HA % were extruded (3%, 5% and 10%), therefore only de 3% was used to print 3D samples. The PLA/3%HA scaffolds were not cytotoxic and allowed VERO cells adherence. The accelerated hydrolytic degradation test showed not degradation in 90 days, so we have to test different irradiation doses to improve the degradation rate.

In conclusion, we could say the HDPE/HA it seems to be a good biomaterial to bone replacement and the filament of PLA with a load of 3% of HA obtained by extrusion would seem to be an appropriate biomaterial to print 3D scaffolds to be used as bone replacement and in this way get a faster recovery of the patient.

BANGLADESH

In Bangladesh, a large number of people are suffering from different types of bone diseases due to road accident, adulterate food, life style, environmental pollution etc. According to the National Institute of Traumatology and Orthopaedic Rehabilitation (NITOR) report in 2017, a total of 1,76,750 patients were attended and 24,633 patients were admitted in this hospital only. In this way, the numbers of physically inactive people are enormously increasing in Bangladesh. To rehabilitate bone defective people, Bangladesh Atomic Energy Commission (BAEC) owns the facilities for doing research and development work on human tissues and biomaterials. Institute of Tissue Banking and Biomaterial Research (ITBBR) of BAEC has the only tissue bank in Bangladesh, which is devoted to relieving human sufferings throughout the country by reconstructive surgery and providing high quality bone allograft for clinical applications to be used against bone defects. Due to the prevalence of accidents, bone disorder, bone tumor etc., bone tissue grafts are badly needed in Bangladesh and the number of patients requiring graft materials are increasing alarmingly in recent years. To fulfil the current demand, it is necessary to find out an alternative source. In this regard, ITBBR conducts basic and applied research in the field of tissue engineering and regenerative medicine aiming to develop allografts substitutes (scaffolds) and functional biomolecules for the treatment of bone diseases.

Under this coordinated research programme (CRP), hydroxyapatite (HA) was extracted from an available biological waste material such as- bovine and human cortical bone by thermal decomposition process and characterized using several analytical tools. XRD data confirmed that HA was the only crystalline phase when sintered at 950°C. The effects of gamma radiation on HA was studied and found that the HA is stable at 25 kGy gamma radiation. At the same time, we also extracted collagen from another natural bio-waste (rabbit skin) and characterized its properties through FTIR, SDS-PAGE and HPLC analysis. After that, different types of scaffolds were prepared following diverse fabrication method and cross-linked with physical and chemical cross-

linkers. Later, scaffolds were characterized for biodegradability, in vivo biocompatibility along with physical, chemical, and morphological properties. Finally, we implanted our selected scaffolds into the non-load bearing area (maxillofacial region) and load bearing area (long bone) and evaluated their restoration capability.

From the prepared scaffolds, two scaffolds (hydroxyapatite-collagen-chitosan and hydroxyapatite-chitosan-alginate-polyamide) were fabricated using thermally induced phase separation technique and cross-linked with chemical (GTA) cross-linker and exposed to different doses of irradiation (IR) and de-hydrothermal (DHT) as a physical cross-linker. Another homogenous, biocompatible, inter-connective porous scaffold (HA bare scaffold soaked into collagen-chitosan solution) matrix was developed using the replica method. Designed HA matrix was soaked into collagen-chitosan solution for better cell attachment. In addition, a new type of bone cement (AM+BC) was prepared by using mono-calcium phosphate monohydrate and tri-calcium phosphate with the active solution of amniotic membrane in presence of citric acid. All prepared scaffolds composite were investigated in terms of biocompatibility alongside physical, chemical, and morphological studies. The developed composite scaffolds were found to be porous with 3D interconnected fiber microstructure. In this study, growth factor were also incorporated with the HCC matrix and demonstrated that the introduction of the growth factor did not lead to significant changes in visual and structural properties of the scaffolds. In vitro studies in presence of mesenchymal stem cells revealed that scaffolds were non-cytotoxic and compatible for cell attachment, growth and mineralization. In vivo grafting of scaffold in surgically created rabbit non-load bearing (mandible) and load bearing (long bone) defects model demonstrated the efficacy of scaffolds. For the in-vivo scaffold implantation study, we did not observe any adverse reactions or post-operative complications, such as abnormal bleeding or infection at surgical sites. After 2h of post operation, the rabbits looked alike normal in regard to food eating and movement. Furthermore, we did not observe any sign of inflammation, such as swelling appeared to be minimal, and the grafted materials were confirmed to be intact within the defects. Histological observations indicated the regeneration of new bone and restoration of bone defect at the site of injury. As an implanted biomaterial for bone regeneration, the scaffold composites presented good biocompatibility and osteoconductive properties and induced bone ingrowth into the implant. The substituted materials were also in a creeping replacement mode.

BRAZIL

This project aimed the development and characterization of polymeric scaffolds including PVA/Chitosan membranes, chitosan scaffolds without the use of organic solvents, PVA/Gelatin scaffolds crosslinked by radiation and collagen/chitosan/elastin scaffolds. Radiation was applied for scaffold synthesis in terms of crosslinking the matrices, for the sterilization of the scaffolds and in some cases combining simultaneous crosslinking and sterilization. The developed materials were characterized using physical-chemical assays, biocompatibility tests, also including immunohistochemistry, histology and nondestructive testing such as optical coherence tomography. The isolation and differentiation of mesenchymal stem cells from human skin and/or aesthetic liposuction surgery, biological functional testing including cell adhesion and proliferation were also approached. With such goals, the group sought to contribute and advance the discussion towards obtaining an ideal scaffold for the differentiation of mesenchymal stem cells and furthermore, the understanding of the membrane interaction with cells and mesenchymal stem cells, also addressing the obtention, isolation and differentiation of the mesenchymal stem cells onto scaffolds.

The dissemination of the knowledge and the outcomes of the research was performed via the publication of 4 research manuscripts, 6 oral presentations in scientific conferences. Future activities involve the differentiation of the mesenchymal stem cells present in the scaffold into skin cells, namely fibroblasts, which is under development, and publication of related data in scientific journals and in an IAEA Technical document.

This work is being performed in collaboration with distinct institutes in Brazil IPEN/CNEN-SP, UFRGS, UNIFESP. International Collaboration involves Lodz University of Technology (Poland), Hacettepe University (Turkey) and Universidad de la República (Uruguay).

CHINA

Report from 2017

Collagen-based scaffolds have been dominating the current dermal substitute field, while somewhere restricted by their rapid degradation, less of elasticity and readily to contraction. In the past years, we elavorated on radiation crosslinking of collagen hydrogels using γ-ray. Preliminary

biological properties of the hydrogels were investigated. The comprehensive results suggested that the collagen hydrogels prepared by radiation crosslinking preserved the triple helical conformation, possessed improved thermal stability, mechanical properties and excellent biocompatibility. However, the hydrogels and scaffolds showed excessive rigidity, extensive contraction and relatively slow angiogenesis process, which restricted their utilization as permanent graft. Glycosaminoglycans (GAGs), such as dextran, have gained much attention to be added into collagen for tailoring the properties as well as mimicking the native composition of human skin. Functionally modified dextran has been proved to be capable of enhance angiogenesis, promote neovascularization and even the regeneration of skin appendages.

In this project, the collagen/dextran aqueous complexes were crosslinked by utilizing γ-irradiation, followed by preparing the lyophilized scaffolds. Collagen/dextran scaffolds with the uniform appearance and moderate flexibility has been prepared. Scaffolds with tunable pore size, water absorption, mechanical strength as well as controllable degradation rate could be fabricated by tailoring the protein/polysaccharide ratio related to the crosslinking density. Dextran performed well in improving the surface topography and properties, taking hydrophilicity as an example. In vitro characterization of collagen/dextran scaffolds such as morphology, microstructure, stiffness, degradation has been investigated to evaluate the effects of dextran and crosslinking on the physicochemical properties. The incorporation of dextran was also considered beneficial for stabilizing the collagen system when experiencing the disturbances from the surrounding environment. In vitro cell culture assay and animal experiments revealed that the collagen/dextran scaffolds possess excellent cytocompatibility. The animal assay revealed that the dextran addition significantly facilitated the scaffold-tissue integration and matrix remodeling, promising in repairing dermal defects and inducing tissue regeneration.

In the future years, pilot plant scale equipment for collagen extraction from bovine tendon and for the praparation of collagen/dextran will be established. Clinical trial for the application of the scaffold will be carried on.

EGYPT

Egypt applied the electrospinning technique for preparation of scaffolds intended to be used for tissue engineering. Successful results were obtained when scaffolds were prepared from

polycarbo- lactone (PCL), where fibres were fabricated at the nano and micro structure providing a porous matrix for the growth of Cells. Unsuccessful results were obtained, when fabricating chitosan and polylactic acid (PLA) by the electrospinning technique.

Radiation Technology in this work was applied in sterilization of scaffolds, biostimulation of cells grown on the scaffolds and for testing applicability of fabricated PCL and ionizing radiation sterilized at 25 KGy scaffolds in the treatment wounds induced in irradiated animals.

In vitro studies showed successful growth of amnion and human mesenchymal cells on such scaffolds exhibiting cytocompatibility that might allow for their use as bioscaffolds for tissue engineering. Sterilization by Gamma rays at 25 KGy was effective and did not affect the integrity of electrospun scaffolds.

Exposure to 2 J/cm2 He:Ne laser and 0.25 Gy enhanced cell proliferation and can be used for biostimulating cell growth on such scaffolds.

Animal studies conducted on 7 Gy irradiated and non-irradiated male Albino rats, showed that fiber size and scaffold porosity affected the rate of ulcer healing.

It is concluded that Electrospinning is an attractive technique to produce fibrous scaffolds for tissue regeneration and repair since it is composed of nano-to sub-microscale fibres that simulate a native extracellular matrix, provided that the increase in pore size and loosened fibrous structure is very effective in promoting cell infiltration at different depths.

The electron spun scaffolds were effective in vitro even in the absence of stem cells in promoting healing of wounds induced in irradiated rats.

Scaffolds impregnated with Zn were the most superior in promoting wound healing in IR rats.

Sterilization by Gamma rays at 25 kGy was effective and did not affect the effectiveness of electrospun scaffolds.

MALAYSIA

Formulation of Photopolymer Resin Prepared from Acrylated Palm Oil Resin for Tissue Engineering Application.

In this study a new biodegradable photopolymer resin was formulated from synthesized acrylated palm oil resin (APO). The formulation conditions such as polymer concentration, amount of reactive diluent, concentration of photoinnitiator and inhibitor, reaction time were investigated. The fabrication was done using microstereolithography technique, an additive layer process whereby a 3D object is sliced into a series of 2D layers with each of these layers manufactured, one after another, until the 3D part is formed. They are then subjected to swelling ratio and sol fraction to determination the degradability of the structure. Cell adhesion and viability are demonstrated, in order to test whether the fabricated made of acrylated palm oil resin indeed be tolerated by cells in culture. Calibration printing by using 100% Poly(ethylene glycol) dimethacrylate (PEG-DMA) with the presence of 0.5 % photoinitiator has been done. Photoinhibitor was also added into every formulation of resin with different amount as the manipulating variable. The best (optimum) exposure time can be determined by looking at the edge of the printed model. Ratio of APO and selected acrylated polymer is studied to determine the effects on curing time and the optimum formulation. Using CAD (computer-aided design) software, the suitable design of the optimized tissue scaffold is studied. The mechanical and morphological properties are characterized through the studies of tensile, compression, flexural properties as well as porosity and density. Cells LD are culture on the printed scaffold and MTT assay is perform at 1.3, and 5 days to assess cytocompatibility of the resin. Finally, the processability of the resins with MSL technique is demonstrated by constructing multi layer scaffolds with actual measurement that are consistent with the CAD mode. The MSL technique is expected to produce a tissue scaffold with controlled microstructure, tailoring the porosity structure as well as the inclusion phase shape and size; have controlled bioactivity and selected acrylated polymer is studied to determine the effects on curing time and the optimum formulation. Using CAD (computer-aided design) software, the suitable design of the optimized tissue scaffold is studied.

MEXICO

In 2018, IAEA project funds were used to partially cover the participation of Dr. María Cristina VELASQUILLO MARTÍNEZ (INRLGII) in the 5th TERMIS, Sept. 4-7, Kyoto, Japan. ININ acquired some instrumental and partially covered the participation of María Esther Martínez Pardo

in the 5th Congreso International Red Biomat, Mérida, Yuc., México, 24-26 oct. Also, the ININ provided laboratory kits to INRLGII to continue the work. Concerning to amnion supply and according to the agreement between an authorized hospital (Hospital de Gineco-Obstetricia, IMIEM) in Toluca City and the ININ -signed on May 2, 2017-, Mr. Daniel REBOYO-BARRIOS and the IMIEM physician performed the following activities: Selected one suitable donor, obtained written donor consent, carried out the first serological analysis and procured the amnion on December 15, 2017. The second serology results on Jul, 2018 were no reactive, so we can distribute RHA for free to our clients and provide tissue to LB-INR.

Here it was analysed if Radiation Sterilized Pig Skin and Radiation Sterilized Human Amnion, in combination with MSC, were capable to help in wound healing. Both biomaterials has been used since 70s because they are capable to avoid the loss of water and prevent the infections, among others advantages. These biomaterials are also composed by extracellular matrix similar to the human skin and they have great provision and low cost even in low-income countries. The results in this study shows that even when the treatments do not enhance the closure of the wound in comparison with the control, the treatments improve the deposition of collagen in the wound. At day seven post-treatment, RHA and RPS show light deposition of collagen in the skin, Masson staining evidenced this, Herovici and the detection of collagen type I. The addition of MSC to the biomaterials (constructs) show a mild increase in collagen detection, it was also shown by the staining and immunofluorescences, but in all the treatments the deposition of collagen was higher than the control group. For this stage the synthesis of ECM was fast, since at this period there were not reepithelization yet, there were also granulation tissue that denote the period of inflammation, which is very important to induce the cell proliferation. RPS shows an increase of granulation tissue compared with all other treatments but the culture of MSC decrease this exacerbated granulation tissue. These responses could be due to porcine proteins of RPS, even when NU/NU mice were used, B lymphocytes and other immune cells are still present. Previous reports have shown that RPS induces the release of Interleukin-1β, which is capable to promote the keratinocytes proliferation for the reepithelization phase. At the 14 days post-treatment there was more collagen deposition in all conditions, but the increase was considerable when RHA or RPS were culture with MSC. The deposition of ECM is very important to develop new dermis in which the new epidermis will grow.

This research provides evidence that both biomaterials -RHA and RPS- promote the collagen detection during the wound healing process, but the combination of these biomaterials with MSC increase their effect. These constructs have potential to be used in the clinic to treat burn patients, but more studies have to be done to know if the MSC contribute to the wound healing through the release of growth factors.

Conclusion

RHA and RPS promote the collagen deposition in comparison with the control condition without treatment, this collagen deposition was increased when MSC were cultured over the biomaterials.

POLAND

We demonstrate the feasibility of radiation grafting to prepare polymer layers functionalized with short cell promoting peptide ligands and which may be of use as a scaffold in cell sheet engineering. Thermosensitive poly[tri(ethylene glycol) monoethyl ether methacrylate] (P(TEGMA-EE)) layers were synthesized on a polypropylene substrate by post-irradiation grafting. Cell adhesion moiety IKVAV (I-isoleucine, K-lysine, V-valine, A-alanine) peptide modified with methacrylamide functions was introduced to the surface during polymerization process. The amount of IKVAV was easily controlled by changes its concentration in reaction mixture. The changes in the surface composition, morphology, philicity, and thickness that occurred at each step of polypropylene functionalization confirmed that surface modification procedures was successful. The increase of environmental temperature above the cloud point temperature of P(TEGMA-EE) caused the changes of surface philicity. The obtained polymer-peptide surfaces may be applied as a scaffold in cell sheet engineering.

These modified Petri dishes have been used for cultivation of human being skin tissue composed of patient's own fibroblasts and keratinocytes. Such prepared tissues have been transferred and implanted during patients' treatment in one of Clinics in Poland (Centrum Leczenia Poparzeń w Siemianowicach).

There are additional works devoted to verification and validation of the elaborated procedures for radiation formation of the instructive surfaces. Some new hydrogel scaffolds contain chitosan dissolved in lactic acid as well as composed of methacrylated dextran have been established and tested. A specific action of those scaffolds toward E. coli and S. aureus were demonstrated – inhibition of multiplication of these microorganisms were experimentally tested.

A new, instructive scaffolds for regeneration of broken nerve tissue has been elaborated. It composed of biodegradable tube of poly(methylene carbonate) and poly(lactic acid) filled with carboxymethyl chitosan and sterilized by ionizing radiation. Some tests with animals have shown favorable action such scaffolds toward regeneration of motor functions of animals and minimalization of defective grow of new, nerve tissue.

Numbers of experiments were performed on a new composition of hydrogel scaffolds dedicated for healing of DFU (diabetic food ulcers). The new radiation procedures for preparation of such matrices have been achieved and patented in Polish Patent Office.

The results of above-mentioned investigations and experiments have already been published in form of 7 papers (in the journals with IF over 1), described in 4 patented procedures (Polish Patent Office) as well as presented as 19 contributions to international and national conferences.

PORTUGAL

Aiming to create biodegradable polymeric matrices to be used as skin scaffolds, chitosan based matrices were successfully prepared by gamma irradiation. During the CRP period it was possible to optimize the preparation methodology and refine matrices' composition in order to obtain a very promising acellular 3D scaffolds for skin regeneration.

The matrices were evaluated in terms of preparation methodology, composition, absorbed dose, structural and functional properties and in vitro biocompatibility (cellular viability, morphology and cytochemistry). The best configuration ready for animal experiments was determined as being the one with a 2% content in low molecular chitosan and 5% in poly(vinylpyrrolidone), prepared

by gamma irradiation at 10 kGy (dose rate = 0.5 kGy/h) after 2 freeze-dry cycles. Scaffolds' effectiveness on the healing of wounds and skin regeneration/repair was evaluated by in vivo regeneration experiments in male Wistar rats. The matrix group exhibited a good tissue integration and an accelerated epithelization compared with the control group with no matrix. Histologically, the matrix implant site presented a simpler architectural organization than the normal tissues. Notwithstanding keratinocytes, fibroblasts and new vessels were observed in the region.

In terms of outputs, the work performed under the present project resulted in the publication of a book chapter and a paper (more 2 in preparation), and the dissemination at national and international conferences (7 oral communications and 9 posters). An International Mobility Training of a PhD student from Horia Hulubei National Institute for Physics and Nuclear Engineering (IFIN-HH), Romenia, and a Training for Introduction to Scientific Research (part of the Integrated Master of Chemical and Biochemical Engineering) of a student from the Faculty of Sciences and Technology/New University of Lisbon, included data from the project under the present contract.

SLOVAKIA

The project focused on creation of methods for preparing of different types of scaffolds suitable for skin tissue engineering. The scaffolds are either of biological origin (acellular allodermis, amnion membrane) or biosynthetic (different types of scaffolds based on collagen/hyaluronic acid conjugates). All the scaffolds prepared were sterilized by irradiation. The optimal irradiation dose was tested. The scaffolds prepared were tested for toxicity and suitability for different types of cell cultures. The final products were used clinically in burn medicine and in plastic surgery.

Prepared dermal substitutes from allogenic human, xenogenic porcine skin and amniotic membrane after radiation sterilisation showed disintegration of fine collagen fibres in papillary dermis and of the basement membrane.

No substantial differences in histologically detected fine colagen fibres disintegration after gamma and electrom beam irradiation was detected. Irradiation dose of 25 kGy for sterilisation purpose proved to be suitable in the sence o structural integrity preservaton.

No toxic effects of irradiation or impact on biocompatibility, safety, quality and efficacy of the samples was detected in tissue cultures.

A new, original method for preparation of human acellular allodermis was developed at our institution (CTB) based on combination of enzymatic treatment (trypsin) and repeated hypotonic solution (distilled water) washes. The final product - acellular allodermis was confirmed histologically, immunohistochemically and by molecular biology demonstration of DNA absence. It proved to have no cytotoxicity in different cell types cultivation. This method proved to be effective, easy and non-expensive

The human acellular dermis prepared according to our originally developed method was used clinically in burn treatment as well as in plastic reconstruction surgery. Histologically evaluated biopsy samples showed no adverse host tissue reaction and neovascularisation and ingrowth of host fibroblasts into the acellular dermis implant.

TURKEY

In the development of thermoresponsive cell culture surfaces for non-invasive harvesting of cell sheets, modification of polystyrene cell culture dish surfaces by radiation-induced grafting of poly(N-isopropylacrylamide) has been accepted as the golden standard method. The cell culture dishes with thermoresponsive surfaces enable the control of growth of cells in sheet form and their detachments by simply changing the temperature. PNiPAAm exhibits phase transition in aqueous environment at around 32°C leading to changes in its hydrophobic (>32°C) / hydrophilic (<32°C) properties. The release of cell sheets formed on the hydrophobic surface of PNiPAAm (~37°C) upon transformation into the hydrophilic structure at temperatures below the lower critical solution temperature of PNiPAAm constitutes the rate determining step of the overall process of cell sheet harvesting. This is due slow hydration of hydrophobic PNiPAAm layers.

Although the preparation of PNiPAAm grafted TCPS dishes is straightforward by electron beam irradiation of monomer solution placed in the PS dish, due to uncontrolled nature of free radical polymerization thickness control of grafted layer has not been achieved so easily. It has been found out that cells do not adhere to modified surfaces with layer thickness of thicker than 25-30 nm. On the other hand, cell detachment from PNiPAAm surface is a very slow process due to diffusion-

controlled hydration of this layer by water. We have attempted to find a solution to control the thickness of grafted layer as well as quick access of water into PNiPAAm layer by using radiation-induced, RAFT-mediated grafting of PNiPAAm from PS cell culture dish surface. RAFT polymerization allows controlling of molecular weight hence chain length of graft layers. RAFT moieties attached to the end of the graft chains allow copolymerization of a second monomer to impart hydrophilicity to the basal plane. The kinetics of RAFT-mediated successive polymerization of acrylamide (hydrophilic monomer) and NiPAAm (thermoresponsive monomer) provides the full control of lengths of chain segments with very narrow molecular weight distributions.

UNITED KINGDOM

Over the term of the project, the following areas of work were carried out in relation to mesenchymal stem cells (MSC), allograft bone, and extracellular vesicles (EVs) within the framework of radiation technologies.

- Clinical application of adipose derived, culture expanded MSC in the treatment of osteoarthritic joints in dogs
- Comparison of canine and human MSC in their phenotypic behaviours
- *In vitro* assessment of paracrine activity of canine and human stem cell derived Conditioned Medium
- Examination of Osteogenesis activity of canine allograft bone (Demineralised Bone Matrix-DBM) on MSC

The clinical use of MSC in dogs demonstrated an anti-inflammatory effect of culture expanded MSC in reducing the pain in the joints which is believed to be responsible by the paracrine activity (cell-cell interaction) of the stem cells being injected. The laboratory experiments showed that both canine and human Conditioned Medium which contains MSC secreted EVs can induce osteogenic, neurotrophic and angiogenic. Both species also shared the same phenotypic behaviors; adipogenesis, osteogenesis and chondrogenesis. In the experiments carried out with canine DBM, the osteogenesis of MSC differentiation into osteoblasts was demonstrated.

The project has produced the results which will now lead to further work on a development of a superior DBM bone allograft loaded with EVs and osteoblasts providing osteoconductive, osteoinductive, osteogenesis and angiogenic properties. This tissue engineering approach to bone allograft should produce superior clinical results that that of the current material.

URUGUAY

Objective:

To develop instructive biological and synthetic scaffolds for tissue engineering, using Human Amniotic Membrane (AM) and Bovine Collagen 1, and fibroblast and human Keratinocytes. Human Stem Cell lines have been used in the development. Radiation technology is used to develop and to sterilize instructive scaffolds.

Progress Results:

1) New pre-clinical laboratory was developed

2) Real networking with Brazil and Mexico is being carried out. The CRP cooperation is promoting us researches

3) Three different kind of AM decellularized (DAM), desepithelialized (DEAM) and with death fetal epithelial layer (FEAM) have been characterized, with Optic Microscopy, Electron Microscopy (scanning and transmission) and Raman RX diffraction. The sterilization was performed according the irradiation AIEA Code of Practice 2012.

4) Physic-chemical modification of Bovine Collagen1(Col 1) were defined, using programmed temperature drop, freeze dry and 15 kGy dose radiations for development and sterilization. Physic characterization and cytotoxicity assay were performed. Mouse Clinical research, with Collagen 1 membrane had been performed and primary observation showed no adverse reaction.

5) 4 Scaffolds, Col1 irradiated, DEAM, FEAM and DAM decontaminated and irradiated were selected as instructive scaffolds, for cell cultures.

7) Human Keratinocyte (HaCaT cell line) and 3T3 NIH mouse fibroblast from UFRGS donation, were expanded and then cultivated over DEAM, FEAM and COL 1.

8)Human Dental Pulp Stem Cells-DPSC- (from other research) were isolated, characterized and expanded. They were grown in the developed scaffolds.

9) Human Mesenchymal Stem Cells (MSC) from Bone Marrow brain dead donors (from another research) were isolated, characterized and expanded. and cultured in the scaffolds.

10) Tissue engineering composite were designed on Millicel® Slides. The Col 1 and 3 treated AM, with its control donor sample, were assembled on slide, the 1cm3/each mini-cell were mounted over scaffolds and then cell was seeding and culture. The viability of cells was analyzed by fluorescent staining (CFSE) immediately and with 2 and 6 days of culture, showing viability on all scaffolds.

11) All experiments were repeated 2 times with the same sample of AM <12 hrs. delivery and the cultures analyzed at 11 and 30 days with Proliferation Rate by division of proliferating cells(+MoAbKi67) between existing cells (+Dapi).

12) The PR for MSC, DPSC and HaCaT was similar on decontaminated and irradiated AM scaffolds and 3T3 even better, verifying the convenience of safety irradiated scaffolds. All the cells grow on COL 1.

Conclusions:

Col 1 irradiated with 15KGy is the better collagen scaffold.

AM treated and irradiated do not loose collagen matrix and is a good instructive scaffold.

Human cells: DPSC, MSC and Keratinocyte grow over the 3 selected scaffolds

12 new safety tissue engineering composites have been developed in vitro, that could be used depending of the clinical needs.

4. COLLABORATION

The information given by the participants during the presentations and discussion has led to the construction of the Table 1 (ANNEX I) describing collaboration summary during the whole duration of the CRP. Additionally, the Table also contains the expertise and or equipment, which were offered by participants to all interested to use when needed. These offers will enhance interaction between participation institutions beyond the end of the CRP.

5. CONCLUSION

Since the formation of the CRP group 5 years ago at the first RCM meeting in 2014, the progress has been made in many areas of the development by the participants as set out in the first meeting (see Annex II for details).

A short communication will be published in an international journal based on the GUIDELINES FOR LOGICAL SEQUENCING OF SCAFFOLD DEVELOPMENT FOR HUMAN USE (TEMPs – TISSUE-ENGINEERED MEDICAL PRODUCTS) prepared by Brazil, Portugal and Argentina.

Since the long review paper for the Tissue Engineering Journal was rejected, it was decided, as suggested by reviewers, to submit a short Letter to the Editor, based on the overview of the long manuscript. The rest of the "long paper" will be submitted to another journal, i.e. Radiation Physics and Chemistry, preparation will be coordinated by EGY.

National and regional funding opportunities for exchange of scientists and expertise were utilized by the participants during the course of the projects.

Collaborators of BRA team received FAPESP grant for chitosan matrix and biological testing.

URU collaborators received grant from CSIC for Dental Pulp Stem Cells and MSCs.

SLK received VEGA 2/0021/15 grant on Molecular mechanisms of cardiovascular damage induced by irradiation; options of targeted prevention.

UK Collaborators received a four year, fully funded BBSRC grant to study canine mesenchymal stem cells.

Peter Myint was a guest editor for the Cell Tissue Bank, 2018, 19(2) and 3 CRP-related papers were published in this special issue.

Radiation induced grafting, crosslinking and sterilization techniques were used extensively by the participants to develop instructive surfaces and scaffolds for regenerative medicine, which gave promising results.

6. RECOMMENDATIONS

Treatment of Diabetic foot ulcers (DFUs) with hydrogel and other composite matrixes were discussed. It was recommended to consider starting the preparation of application for IAEA TC inter-regional or other project on this topic under leadership of Poland. All participants of the CRP expressed their willingness to contribute.

Another project is recommended to produce Regulatory Guidance on development of new products involving scaffolds, cells and composites etc.

All participant institutions agreed on the need of organising training courses/meetings for transferring the knowledge in the following areas:

- the characterisation of materials used for radiation preparation/sterilisation of scaffolds and instructive surfaces;
- biological testing;
- preclinical and clinical tests.

Transfer CRP findings to research and clinical practice is an important task for the future.

As one of outputs of the CRP an IAEA TECDOC will be published.

To identify the best practices from this CRP participants in the various areas of application.

ANNEX I

	ARG	BGD	BRA	CPR	EGP	MAL	MEX	POL	POR	SLK	TUR	UK	URU	USA	
ARG	Biological testing, determination of sterilisation dose for sensitive materials						Knowledge transfer								ARG
BGD				Clinical trials											BGD
BRA			Biological testing and characterisation of materials, irradiation services	Clinical trials and biological testing			Knowledge transfer	Knowledge transfer			Biological functional tests		Membrane characterisation		BRA
CPR		MoU in progress													CPR
EGP					EGY offered gamma irradiation	Know-how transfer									EGP
MAL															MAL
MEX	Corneal tissue		Know-how transfer				Biological testing and irradiation services				Biological functional tests		Knowledge transfer		MEX
POL			Tech transfer, training in good progress, common papers are being prepared					Characterisation of materials				Veterinary trials			POL
POR			Structural characterisation						Gamma-rays irradiation services		Physico-Chemical characterisation of materials				POR

SLK								Knowledge transfer		Preparation of accelular matrix + histology and histochemistry					SLK
TUR			Biological functional tests						Characterisation of materials		Characterisation of materials		Biological functional tests		TUR
UK												Veterinary trials			UK
URU			Membrane characterisation and biological testing	Collagen	Biological tests of NFS		Knowledge transfer				Biological functional tests		LITYC lab for GMP manufacturing human products is avalible		URU
USA															USA
	ARG	BGD	BRA	CPR	EGP	MAL	MEX	POL	POR	SLK	TUR	UK	URU	USA	

Legend: plans | in progress | offer to all partners | Completed | Canceled

ANNEX II – TABLE

Specific research objectives	Countries involved	Assessment of progress
To investigate and optimize the preparation of radiation synthesized three-dimensional scaffolds.	URU, ARG, BRA, CPR, BGD, EGY, TUR, POL, MAL, POR	Collagen (URU); HDPE+HA (ARG); PVA+chitosan, chitosan, PDLLA and collagen mixed (BRA); Collagen and chitosan matrixes (CPR); HCG and HPCA (BGD); PCL+ chitosan (EGY); PNIPAAm and PAAm grafted PS (TUR); PTGMA grafted PP (POL); PEGDA and EPOLA (MAL); chitosan+PVA, chitosan+PVP (POR)
To investigate the use of radiation sterilization of the new instructive scaffolds and decellularized matrices.	URU, MEX, ARG, BRA, CPR, BGD, EGY, POL, POR	Collagen and amnion (URU); amnion and porcine skin (MEX); HDPE+HA, 3D printed PLA+AH (ARG); human skin, amnion, PVA+chitosan, chitosan, PDLLA and collagen mixed (BRA); Collagen and chitosan matrixes (CPR); HCG and HPCA (BGD); PCL and PCL+ chitosan (EGY) PTGMA grafted PP (POL); chitosan+PVA, chitosan+PVP (POR)
Biological testing	URU, MEX, ARG, BRA, POL, POR, EGY	Cytotoxicity (URU, MEX, ARG, BRA, POL, POR); biodegradation (ARG, MEX, URU, BRA), growth and cell death (EGY)

To study cell-cell-scaffold-matrix-EMC (Extra Cellular Matrix interactions).	SLK, POL, USA, MEX, BRA, URU, ARG	Proliferation, attachment, detachment of different types of cells: fibroblasts, epithelial cells (POL); amnion and pig skin with mesenchymal stem cells, pig skin with keratinocytes (MEX); PVA+chitosan, chitosan, PDLLA and collagen mixed matrix with fibroblasts, keratinocyte and MSCs (BRA); treated amnion and collagen I irradiated with keratinocytes, dental pulp stem cells and MSCs brain-dead donors (URU); irradiated 3D printed PLA+AH with MSCs (ARG), fibroblast and epithelial cell culture on acellular allogenic skin preparations (SLK)
To study the effectiveness of combining biological and non-biological material on regeneration/repair.	UK, US, POL, ARG	DBM with carriers (UK), fibroblast layers (POL), HDPE+HA with osteoblasts (ARG)
Bring together researchers and end-users to form a network.	All institutions	Collaboration established, review paper is prepared for submission

ANNEX III

IAEA International Atomic Energy Agency *Atoms for Peace and Development*	**PROGRAMME OF THE 4TH RESEARCH COORDINATION MEETING (RCM) OF THE COORDINATED RESEARCH PROJECT (CRP) F23030-E31007** **INSTRUCTIVE SURFACES AND SCAFFOLDS FOR TISSUE ENGINEERING USING RADIATION TECHNOLOGY** **IPEN-CNEN/SP, SÃO PAULO, BRAZIL, 8-12 APRIL 2019**	
Monday, 8 April 2019		
08:00 – 09:00	Registration at IPEN	
09:00 – 09:30	Dr Wilson P Calvo, Head of IPEN Dr Margarida Hamada, CTR (IPEN)	IPEN Opening remarks
09:30 – 10:00	Aldo Malavasi, vice DDG-NA[1] (IAEA), tbc Bum Soo Han, PO (IAEA)[1]	IAEA Coordinated Research Activities
10:00 – 10:30	Oleg Belyakov, PO (IAEA)[2]	IAEA Opening remarks
10:30 – 10:45	Election of the chairperson of the meeting	
10:45 – 12:00	**Brunch and group photograph**	
12:00 – 18:00	Visit to CNPEM Facilities (CNBIO, LNNANO, and SIRIUS)	
Tuesday, 9 April 2019		
09:00 – 09:30	Maria Carolina Anessi (Argentina)	Regenerating Tissue Using Biomedical Engineering Approaches
~~09:30 – 10:00~~	~~SM Asaduzzaman (Bangladesh)~~	~~Fabrication of Biocompatible Bone Tissue Scaffold~~
10:00 – 10:30	**Coffee break**	
10:30 – 11:00	Gustavo Varca and Monica Beatriz Mathor (Brazil)	Development and Evaluation of Mesenchymal Stem Cell on Scaffolds for Skin Regeneration
11:00 – 11:30	Ling Xu (China)	Gamma Radiation Cross-linked Collagen/Dextran Scaffolds with Improved Mechanical Property and Angiogenesis Capability for Dermal Tissue Engineering
11:30 – 12:00	Soheir Korraa (Egypt)	Fabrication and Modification of Surfaces and Scaffolds for Tissue Engineering Using Radiation Technology
12:00 – 12:30	Questions/Discussion	
12:30 – 14:00	**Lunch break**	
14:00 – 14:30	Marina Talib (Malaysia)	The Development of 3-Dimensional Tissue Scaffolds for Tissue Engineering Application via Microstereolithography Technique
14:30 – 15:00	Maria Esther Martinez Pardo and Roberto Sanchez Sanchez (Mexico)	Development of Biological Skin Substitute Using Radio Sterilized Aminos and Pig Skin Seeded with MSC, Fibroblasts and Keratinocytes

[1] IAEA Deputy Director General, Head of Department of Nuclear Sciences and Applications
[2] Project officer E31007

15:00 – 15:30	Questions/Discussion	
15:30 – 17:00	Finalisation of the review: An IAEA Initiative: Instructive Surfaces and Scaffolds for Tissue Engineering Using Radiation Technology	
Wednesday, 10 April 2019		
09:00 – 09:30	Janusz M Rosiak (Poland)	Radiation Processing to Make Cell-Sheet Releasing Instructive Surfaces and Scaffolds for Regeneration of Tissue
09:30 – 10:00	Maria Helena Casimiro (Portugal)	Biodegradable Polymer Matrices Obtained by Ionizing Radiation for Skin Scaffolds
10:00 – 10:30	**Coffee break**	
~~10:30 – 11:00~~	~~Kirill Golokhvast (Russia)~~	~~Direct Management of the Cell Growth and Differentiation using Artificial Surfaces~~
11:00 – 11:30	Pavel Babal (Slovakia)	Irradiated Scaffolds for Tissue Engineering of Skin
11:30 – 12:00	Olgun Güven (Turkey)	Modification of Cell Culture Dishes by Spin Coating and/or Grafting of Poly (NIPAAm) for Cell Sheet Recovery
12:00 – 12:30	Questions/Discussion	
12:30 – 14:00	**Lunch break**	
14:00 – 14:30	Peter Myint (United Kingdom)	The Use of Combined Autologous Stem Cells and Biologics in Orthopaedics
14:30 – 15:00	Inés Álvarez (Uruguay)	Comparative Studies Between Artificial, Biological and Composites Instructive Scaffolds for Cell Culture to Therapeutical Purposes
15:00 – 15:30	Questions/Discussion	
15:30 – 16:00	Bum Soo Han (IAEA)	Guidelines and format of IAEA publications
16:00 – 17:00	Finalisation of the review: An IAEA Initiative: Instructive Surfaces and Scaffolds for Tissue Engineering Using Radiation Technology	
Thursday, 11 April 2019		
09:00 – 10:30	CRP Report Preparation	
10:30 – 11:00	**Coffee break**	
11:00 – 12:30	CRP Report Preparation	
12:30 – 14:00	**Lunch break**	
14:00 – 15:00	Visit to IPEN facilities	
15:00 – 17:00	Report Preparation	
19:00 – 22.00	**Meeting dinner (self-paid)[3]**	
Friday, 12 April 2019		
09:00 – 09:30	Presentation of the final draft of the CRP report	
09:30 – 10:00	Review and acceptance of the final draft of the report	
10:00 – 10:30	**Coffee break**	
10:30 – 11:00	Review and acceptance of the final draft of the report	
11:00 – 11:30	Final remarks/Closure of the meeting	
11:30 – 13:30	**Lunch and departure**	

[3] Self-paid, tbc

ANNEX V

IAEA International Atomic Energy Agency *Atoms for Peace and Development*	**LIST OF PARTICIPANTS OF THE 4TH RESEARCH COORDINATION MEETING (RCM) OF THE COORDINATED RESEARCH PROJECT (CRP) F23030-E31007** **INSTRUCTIVE SURFACES AND SCAFFOLDS FOR TISSUE ENGINEERING USING RADIATION TECHNOLOGY** **IPEN-CNEN/SP, SÃO PAULO, BRAZIL, 8-12 APRIL 2019**

Lists of participants sorted in alphabetical order acording to the country name

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[4] IAEA Deputy Director General, Head of Department of Nuclear Sciences and Applications
[5] Radiation Chemist, Project officer F23030
[6] Radiation Biologist, Project Officer E31007