Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Converging volumetric bioprinting and electrospinning to develop a patient-specific hierarchical scaffold for bone regeneration.

B.1.2 Abstract

Bone injuries affect million people every year and they dramatically decrease the quality of life of the patients. Recently, novel biofabrication techniques for instance extrusion-based and inkjet technologies and light-based 3D printing have provided a potential platform for the treatment of bone defects. Although these techniques have been explored, they present significant operational

limitations, such as lack of biocompatibility or materials unaffordability. In this way, the purpose of this study is to enhance the regeneration of critical-size bone defects by converging electrospinning and volumetric bioprinting.

The approach consists in fabricating a silk fibroin electrospun scaffold that will be subsequently mineralized with apatite crystals. The scaffold will then be combined with volumetric bioprinting using the PEGDA resin including MC3T3-E1 cells. On the one hand, mechanical analysis will be carried out to evaluate the resemblance of the nanofibers to the collagen fibers. Additionally, the rheological properties of the bioresin will be assessed and the resistance of the whole scaffold to physiological load will be evaluated. On the other hand, biological assays will be carried out to assess the viability, metabolic activity and differentiation ability of the cells. For that, these cells will be used including MC3T3-E1 cells.

The electrospun scaffold is expected to resemble the nanohierarchical structure of the native bone tissue, while volumetric bioprinting aims to improve the biological behaviour of the scaffold.

In general, combining electrospinning and volumetric bioprinting will mimic the native bone tissue and, thus, it will provide a favourable environment for the regeneration of the host tissue.

B.1.3 Layman's summary

Injuries to the bone have a serious impact on the life of the patients as they reduce their ability to move and can cause severe pain. Bone injuries that cannot heal spontaneously when surgeons perform a stabilization of the area are known as critical-size bone defects. Many treatments have been explored to heal critical-size defects, such as the transplantation of a piece of bone from one part of the body to another one to fill the defect. However, current medical practices have several disadvantages and their availability is limited.

To overcome these drawbacks, scientists have tried to use advanced technologies, like 3D printing, to improve the healing of bone. Several techniques have been exploited to create structures that resemble the bone tissue, by controlling the deposition and the shaping of specific materials, that interact with the human tissue and enhance the healing process. Among the employed techniques are extrusion-based, inkjet technologies and light-based 3D printing. They allow to model the structures layer-by-layer and therefore, they present some notable limitations in terms of speed production and surface resolution quality.

This project combines two different techniques that can model specific materials to create structures similar to the bone tissue, to enhance the healing of bone injuries. The structure of the bone in the native tissue is made of a mineral component, the apatite, and a fiber component, the collagen. Thus, the electrospinning is used and it consists on the deposition of specific materials in the form of fibres with a diameter similar to those of the collagen fibrils. For this technique, silk fibroin, that is a natural

substance produced by many insects, will be used. It interacts well with the human body and cells as well as being able to resist mechanical force similarly to the native collagen fibers. The silk fibers will be covered with a mineral to resemble the mineralized part of the bone. The presence of cells that build bone, called osteoblasts, in the structure can further improve the healing process and can recreate an environment biologically similar to that of the native tissue. MC3T3-E1 cells under specific conditions can become osteoblasts, thus they will be used for volumetric bioprinting. This is a technique that in few seconds allows to shape a material able to absorb a lot of water, a hydrogel, on top of the silk fibers. This technique avoids the layer-by-layer approach, dramatically increasing the surface resolution of the structure. The used hydrogel will be poly (ethylene glycol) diacrylate (PEGDA), that will reduce the inflammation in the host tissue and will create a favourable environment for the cells.

Once all the scaffold is assembled, it will be tested to assess if it is able to resist under physiological forces and the cells will be observed over a month to check if they survive and if they are able to become bone cells. The implant will be eventually tested in mice to check if it can improve the healing of bone defects in a living tissue.

B.1.4 Keywords

Bone regeneration, volumetric bioprinting, electrospinning, patient-specific

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic (what)

Over 150 million bone fractures are recorded every year and today's rapidly ageing global population is leading to an increase in people affected by bone defects [1, 2]. Those unable to heal spontaneously after skeletal stabilization alone are regarded as critical-size bone defects [3]. Their aetiology is multifactorial, and they seriously affect the quality of life of the patients, as well as leading to a severe economic burden [4, 5, 6]. The current gold standards to treat critical-size bone defects are autologous and allogenic grafts, with critical limitations such as donor site morbidity, injury and limited source [4].

Therefore, recent advances in biofabrication have led to the development of new approaches for bone tissue regeneration and several additive manufacturing (AM) techniques have caught the attention for generating potential treatments [42]. The current technologies employed for the fabrication of bone constructs include layer-by-layer techniques as are extrusion-based and inkjet technologies and light-based 3D printing such as digital light processing (DLP) or stereolithography (SLA). Extrusion-based printing was shown to be suitable for the fabrication of scaffolds with a controlled architecture and porosity with a wide variety of biocompatible materials [7]. However, this technique presents various disadvantages that can be summarized in the lack of biocompatibility of crosslinking mechanisms, mechanical forces that the cells undergo during the process which seriously affect the cell viability, and the nutrient and waste exchange in the fabricated scaffold [8]. Some of these drawbacks are reflected also in inkjet printing, a non-contact printing technique that enables the fabrication of construct layer-by-layer [9]. Although its high resolution and efficiency, this technique is more limited in terms of employed materials, as the bioinks need to be low viscous [10, 11]. Some of the limitations concerning the two previously mentioned techniques can be overcome by DLP printing. This nozzle-free technique allows the manufacture of highly viscous materials without letting the cells undergo mechanical stress, in addition to printing high-resolution constructs via a layer-by-layer photopolymerization [12]. However, the suitable materials are expensive and restricted to photopolymers and the cytotoxicity of uncured photoinitiators represents a main limitation for its use in 3D bioprinting [13]. That is why the importance of post-processing in light-based 3D printing.

In this context, volumetric 3D printing has recently been investigated, first mentioned in 2017, for the fabrication of complex living-tissue constructs [14]. It consists in directing visible light from different angles to a vat full of resin using several projections [17]. The desired geometry can be designed and built at once by furnishing a cumulative energy dose higher than the photocrosslinking threshold of the resin [17].

This layerless technique gives the possibility to develop free-form 3D structure with μm scale resolution within seconds [15]. Furthermore, design based on Peripheral quantitative computed tomography (pQCT) scans allow the production of centimetre-scale constructs tailored according to the patient-specific defect [14, 50].

Hydrogels are highly porous polymers with a three-dimensional network able to retain large amount of water [44]. They are widely used in bone tissue engineering as they ensure biocompatibility, biodegradation ability and their 3D porous structure can possibly match the porosity of the native tissue [16]. Volumetric additive manufacturing has been proved to be suitable for cell-laden hydrogels, and the nozzle-free peculiarity dramatically decreases the shear stress that the cells undergo in other 3D printing techniques, like extrusion-based bioprinting, improving cell viability [17].

In this study, the selected hydrogel is poly (ethylene glycol) diacrylate (PEGDA), a polymer able to induce bone regeneration in addition to showing good biocompatibility and high stretchability [18]. Rodríguez-Pombo et al. have successfully explored the suitability of PEGDA for volumetric printing [19]. However, this cell-friendly hydrogel shows low mechanical stability [20]. Here, is the necessity of the combination of volumetric bioprinting with electrospinning which is going to be explored.

Electrospinning enables the fabrication of fibers with porosity and morphology able to resemble the native extracellular matrix (ECM) via a simple setup and operation low cost [21]. As osteogenesis can be boosted by materials that can mimic the ECM of the native tissue and in nature the protein fibers in the ECM are in the form of fibrillar collagen, electrospinning represents an attractive technique to resemble the hierarchical native tissue architecture [22]. The nanoscale resolution that can be achieved via electrospinning is unrivaled and among natural and synthetic materials, the use of silk fibroin (SF) has been proved to have many advantages [23, 24, 49]. The high biocompatibility, good mechanical properties and cell adhesion performance of the fibrous structure were proved to be similar to that of type I collagen together with showing higher resistance to breaking compared to synthetic fibers [25]. Furthermore, the presence of apatite in the fibers enhances the formation of strong bonds in the bone and improves early bone formation around implants [26]. Li et al. showed a process of apatite mineral growth on silk fibroin associated with acidic poly-Asp that biomimetically resembles the structure of the native tissue [27].

The cell component is a crucial aspect of bone tissue engineering. The inclusion of cells was proven to boost the regeneration of critical-size bone defects [28]. MCT3T3-E1 cells have raised interest due to their wide use in bone tissue engineering, in addition to showing great phenotypic stability [29]. To include this preosteoblast cell line, the scaffold needs to meet specific requirements such as creating a favorable environment able to properly guide the differentiation of MCT3T3-E1 cells

towards the osteoblast lineage. These dynamics depend on scaffold parameters such as the use of biocompatible materials and mechanical properties.

The idea of this study is to converge electrospinning and volumetric bioprinting to enhance the regeneration of critical-size bone defects (Figure 1). The cell-laden PEGDA structure will be used to provide a three-dimensional environment for the MCT3T3-E1 cells, with porosity resembling that of the native tissue and reducing the inflammatory response due to its soft texture. The electrospun SF fibers combined with apatite aim to reproduce the nanohierarchical structure of the bone tissue representing the reinforcing component of the scaffold potentially able to properly guide the cell differentiation.

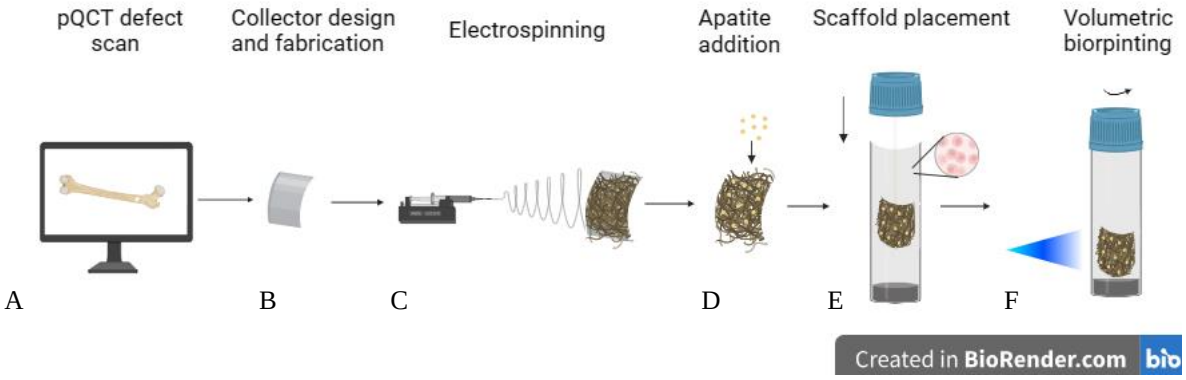

Figure 1: Convergence of electrospinning and volumetric (bio)printing. pQCT scan acquisition (A) and subsequent fabrication of the aluminum collector (B). Deposition of SF fibers on the collector (C) and addition of apatite crystals (D). Placement and alignment of the scaffold on the resin support (dark grey) by means of the PCL wire (E). Volumetric bioprinting over the electrospun scaffold (F) with a 385 nm light beam (light blue).

Preliminary characterizations will be performed with regard to the electrospinning and volumetric bioprinting processes. The mechanical integrity and the biocompatibility of the electrospun scaffold need to be assessed in addition to characterizing the fibers in terms of resolution, apatite deposition and mineralization.

Regarding the volumetric printing, preliminary investigations will concern the bioink resin, in terms of optimal concentration of photoinitiator, mechanical and rheological properties, resolution and shrinkage. It will be necessary to make sure that all the points reach the desired light dose and that they are cured simultaneously.

For the final product, that is the electrospun scaffold combined with cell-laden PEGDA, the shape fidelity, mechanical properties, cell viability and differentiation need to be monitored over a period of at least 21 days.

The feasibility of the study will be assessed by performing in vivo studies on small animal models with a view to a potential clinical translation.

B.2.2 Approach (How)

(a) The aim of the project is to prove the hypothesis presented in the previous section.

The first step of the study consists in the design and fabrication of the electrospun scaffold. The setup of the electrospinning process can be customized according to the final shape of the construct. An aluminum collector will be designed and subsequently fabricated via metal 3D printer according to the desired shape. The fabrication of a collector with a morphology sized according to the patient defect allows to collect the nanofibers in a shape that resembles the bone defect and therefore allows the production of a patient-specific device. SF will be produced as previously described [24, 30]. Briefly, silk cocoons will be opened and cleaned inside, they will be degummed and extensively washed with distilled water. The fibers will be dried for several hours and after one day, the fibers will be dissolved in a $CaCl_2/H_2O/CH_3CH_2OH$ solution. The following step will regard the dialysis and the preparation of the solid mass. To prepare the fibers for the electrospinning process, they will be dissolved in formic acid (FA). The fibers will be electrospun with a random orientation to ensure isotropic mechanical properties. The electrospinning process will be optimized in terms of capillary tip and collector distance, applied voltage and flow rate of the blending solution starting from the previously reported parameters [27]. To mimic the native bone tissue composition, the alternate soaking process reported by Furuzono et al. [26] will be applied to deposit apatite on the electrospun silk fibers [26]. Briefly, the fibers will be immersed in Calcium (Ca) solution and subsequently on a Phosphorus (P) solution at 37°C for 1 hour alternating the process for about 30 cycles [26]. After the soaking cycles, the fibers will be washed in distilled water and air-dried at room temperature for one day [26].

The morphology and nanostructure of the electrospun fibers in terms of average diameter of the fibers will be investigated via Scanning Electron Microscopy (SEM) observations, coupled with Energy Dispersion X-ray Spectroscopy (EDS) [32]. The EDS analysis is expected to confirm the presence of mineral component on the mineralized electrospun fibers. Additionally, the thermal behavior of the samples will be investigated by thermogravimetric analysis (TGA) and subsequent differential scanning calorimetry (DSC). Firstly, TGA will be performed to provide quantitatively analysis of thermal stability of materials through the investigation of the change in mass/weight of the fibers depending on the temperature, time and atmosphere [45, 46]. The TGA will be performed in nitrogen

(N_2) ambient, range of temperature will vary from RT to 600°C under the heating rate of 10°C min^{-1} [48] to evaluate the total weight-loss. DSC will be used to investigate conformational changes in the polymer fibers by determining the amount of energy absorbed or released by a material when it is exposed to different temperatures [45]. Thus, the samples will be subjected to a heating cycle from 30°C to 400°C, at a heating rate of 10°C/min, under N_2 atmosphere and flow rate of 20 ml min^{-1} [24]. Notably, the mechanical properties of the fiber mates are strongly dependent on fiber orientation, fabrication technique and measurement technique [31]. To predict the mechanical performance of the electrospun fibers, they will be characterized in terms of tensile mechanical properties via a universal material testing machine to assess whether the mechanical behaviour of the silk fibers matches the properties of native collagen type I fibers [33]. The tensile properties of the fibers will be evaluated deforming the fiber using a load cell of 10 N, applying a constant strain rate of 1 mm min^{-1}. To observe the long-term deformation of the viscoelastic electrospun fibers, creep test will be carried out [38]. The fibers will be stretched until reaching 33% of the ultimate strength, the load will be kept constant for 10 minutes to evaluate the deformation of the fibers under constant load [51]. Mechanical properties and thermal behavior of the silk fibroin electrospun fibers will be evaluated prior and following the mineralization of the samples.

For the volumetric printing, poly (ethylene glycol) diacrylate average Mn 700 (PEGDA-700) will be used. Previous studies showed that the average molecular wight for the formulation led to the best results when printing using a commercial stereolithography (SLA) system [34]. In addition, PEGDA-700 has been shown to be effective for volumetric printing approaches [19]. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) in different concentrations will be selected as the photoinitiator due to its maximum absorption wavelength at ~385 nm and its high absorbance contribute to a rapid polymerization [35]. Additionally, it is high soluble in water and has a high initiation efficiency [47]. Prior the combination of the electrospun scaffold with volumetric printing, it is crucial to understand how the SF fibers attenuate or refract the light beams. A study reported that the presence of polymeric micrometric fibers can impair the tomographic illumination thus leading to a lower resolution [36]. The light intensity needs to be low enough to go through the resin but at the same it must be sufficient to activate the photocrosslinker [36]. Previous studies reported that the light intensity value needed start the LAP photocrosslinking reaction, is above 37%, thus a device like the one reported by Größbacher et al. [36] could be set up to investigate this aspect [36]. The light intensity that will be used to activate the photocrosslinking of LAP is 385 nm and the optimal photocrosslinker concentration will be achieved [37]. The crosslinking ability will be evaluated together with the photorheological and mechanical characterization of the resin. In particular, the storage (G') and loss moduli (G'') will be assessed in response to shear rate and light exposure.

Furthermore, the compression modulus will be evaluated via uniaxial unconfined compression tests to investigate the behavior of the scaffold under physiological stress.
As the idea is to fabricate a scaffold tailored according to the defect morphology, the resolution and shape fidelity play a pivotal role. Therefore, the PEGDA construct will undergo swelling and shrinking tests by immersion in water and subsequent exposure to heat [39]. Following the optimization of the electrospinning and the volumetric printing processes, the behavior final scaffold made of silk electrospun fibers and volumetric printed PEGDA will be evaluated using a uniaxial testing machine. The compression will be applied with a loading profile of a linear ramp with a constant nominal strain rate of 0.005 s^{-1}.

As mentioned in the previous section, MC3T3-E1 cells will be laden in the resin to enhance the bone regeneration process. The cells will be derived from Mus musculus (mouse) calvaria and expanded. As the inclusion of cells in the ink affects the scattering of the light beam, the transmission properties of the resin must be investigated. The cell density will be optimized according to the crosslinking properties of LAP and the outcomes of the volumetric bioprinting process. Bernanez et al. [17] showed that the addition of iodixanol can tune the optical performance of gelMA-based bioresins in terms of refractive index, decreasing the scattering of the light and leading to a better printing resolution [17].

After the characterization of the electrospun scaffold and the (bio)resin, the electrospun fibers will eventually be combined with volumetric printing. First, a support will be designed with the diameter of the vial and it will be printed by using a DLP printer and a standard resin, such as Phrozen ABS-like Resin (Phrozen technology). The support will be placed at the bottom of the vial. The electrospun scaffold will be gently placed and aligned to the vial by means of a polycaprolactone (PCL) wire securing the extremity of the construct to the centre of the rotation mount. Then, the (bio)resin will be poured in the vial containing the scaffold.
After the bioprinting process, the constructs will be kept in osteogenic medium, and the viability of the cells will be monitored over a period of 21 days by performing live/dead test. The performance of the cells in terms of metabolic activity will be checked via Alamar blue assay over the same period. In addition, the differentiation of the cells will be evaluated via histological analysis of the samples, while immunohistochemistry tests will assess whether the cells are able to produce a collagen structure resembling that of the native tissue. The markers that will be taken into account to assess the osteogenic differentiation of the MC3T3-E1 cells will be the turnover markers related to the bone formation, such as collagen type I, Serum Alkaline Phosphatase (ALP) and osteocalcin [52]. The

adhesion and spatial distribution of the MC3T3 cells in the scaffold will be visualized using SEM to check whether the electrospun fibers are able to guide the cells.

In terms of the applicability, following the investigation of the *in vitro* performance, the scaffold will be tested *in vivo* on a mouse model, following the procedures provided by The Experiments on Animals Act and the regulations of the Animal Welfare Body Utrecht. Three groups of mice will be selected for a total of 27 test subjects. In the first group consisting of 9 of mice the scaffold will be implanted subcutaneously to investigate the cytotoxicity. For the second (9 mice) and third group (9 mice) critical-sized defect will be created with the desired dimension of the scaffold. In the second group of mice the defect will be left empty as a control. The third group will have the wound filled with the scaffold with the desired dimension. The scaffold implanted in the mouse defect will be validated in terms of biocompatibility, and each group will be monitored over a period of at least 21 days. At specific time points, mice will be sacrificed to evaluate the viability, metabolic activity and osteogenic differentiation of the MC3T3-E1 cells.

In terms of accessibility, the present study can enhance the understanding of the dynamics underlying the bone tissue regeneration process, through an attentive follow up. The results of the study aim to be shared with the scientific community through papers and subsequent conferences. In this study, materials and techniques used to fabricate the scaffolds will be economic and accessible and the outcomes will reveal the suitability of the approach for the regeneration of critical size defects.

(b) The study is planned to last 4 years and the following Gantt chart suggests the duration of the different phases (Table 1). The point (A) regards the characterization of the electrospun scaffold in terms of morphology and nanostructure, thermal behaviour and mechanical performance. The point (B) concerns the characterization of the scaffold that combines the electrospun construct with the resin after the volumetric printing process without the inclusion of cells. The aforementioned techniques will be used to characterize the scaffold mechanically and rheologically. The point (C) involves the characterization of the final scaffold with the inclusion of cells in the resin, therefore performing the assays mentioned in the previous paragraph.

Table 1: Gantt Chart.

	Year 1				Year 2				Year 3				Year 4			
Months	1-3	4-6	7-9	10-12	13-15	16-18	19-21	22-24	25-27	28-30	31-33	34-36	37-39	40-42	43-45	46-48
Literature review																
Collector design/fabrication																
Electrospun scaffold design/fabrication																
(A) Scaffold characterization																
Resin optimization/characterization																
Volumetric printing																
(B) Scaffold characterization																
Bioresin optimization/characterization																
(C) Scaffold characterization																
Small animal model																
Scientific divulgation																

B.2.3 Feasibility / Risk assessment

The present study aims to show that the convergence of electrospinning and volumetric bioprinting represents a novel revolutionary approach to enhance the regeneration of the bone tissue in critical-size defects. The strength of the idea is given by the *in vitro* use of biocompatible materials potentially able to resemble the native tissue ECM, for the fabrication of scaffolds tailored according to the defect size.

In order to predict the practicality and success of the project, an attentive feasibility evaluation must be conducted.

The feasibility of the project will be evaluated regarding two main areas:

1. Economic feasibility
2. Technical feasibility

1. Economic feasibility

The feasibility in terms of materials and equipment relies on the use of economic resources and techniques based on a cost-effective setup. The main techniques used to fabricate the implant are electrospinning and volumetric printing. The relatively basic setup of electrospinning makes it a quite affordable technique, despite the fact that the cost may vary according to the used materials. Although silk fibroin might not be the cheapest material for nanofiber applications, its unique properties outweigh the quite high production costs. Another aspect to take into consideration is the quality control of the fibers. To ensure the desired fiber diameter and uniformity visualization equipment such as SEM microscope must be used, adding production costs. On the other hand, collaboration

with institutes such as the Institute for Technology-Inspired Regenerative Medicine (MERLN), which already own this machinery can significantly cut the production costs.
Regarding the volumetric printing, its set up is not the cheapest around if we consider 3D printing techniques such as FDM. This is mainly due to its novelty, which is reflected in the lack of availability of the machine on the market. However, studies showed the possibility to design a personalized setup [43,53]. In terms of materials, among the factors previously mentioned, the choice of PEGDA-700 relied also on its affordability and the easy photopolymerization setup [40]. Therefore, for the desired application, volumetric printing combined with PEGDA-700 presents unrivalled advantages that more than justify its choice. Furthermore, partnership with academic institutions such as Utrecht University that already work with volumetric printing can help mitigate the costs of materials and set up.

To assess whether the cost-benefit analysis is favourable with respect to the outcomes of the study, the estimation of the total amount of money spent on the project is made (Table 2).

Table 2: Direct and indirect costs predicted in the research project.

Direct costs (€)	
Salary	287.565
Materials and supplies	10.000
Equipment	0
Bench fees	20.000
Travels to conferences	15.000
Indirect costs (€)	
Manteinance of equipment	20.000
Software licenses	12.000
TOTAL (€)	**364.565**

The direct costs include the PhD salary, calculated according to the directives of the Dutch Government for a 4-years project. The evaluation of the materials and supplies costs is more challenging because of the variability of the project changes, therefore an overall estimation is made. As the bench fees typically range around €1.000-€10.000, the average is considered and multiplied for the number of years, for a total of €20.000. The money spent in conferences is one of the most

variables points of the prediction of the costs as it depends on the frequency and the location of the conferences.

In a research project, also indirect costs must be included, among the main there are the maintenance of the equipment used over the 4 years and the software licenses for the elaboration of the acquired data. Every year academic institutions reserve part of the budget to ensure the functionality of the machinery and to renew licenses accessible to employees, the total amount is calculated over the whole period.

2. Technical feasibility

Another crucial point to consider in the discussion is the technical feasibility of the project. The technical feasibility includes evaluation of the risks linked to the use of the materials, techniques and operators (Table 3).

Table 3: Risk assessment table.

Risk description	Likelihood	Solution
Microbial contamination of silk fibroin during the electrospinning process.		Use of synthetic widely used polymers such as the easily produced and economic PCL.
Presence of toxic solvents in the electrospun fibers.		Use of organic solvents.
Volumetric printed PEGDA does not match the desired structure and properties.		Use of widely used, biocompatible and tunable gelMA as a bioresin.
No proper differentiation of MC3T3-E1 cells into osteoblastic lineage.		Use of MSCs or cytokines to recruit the local cells in the host tissue and still achieve tissue regeneration.
Volumetric printing may not reach the desired resolutions and exposure of the operator to high intensity light source		Use of other techniques to deposit the bioink, such as extrusion-based bioprinting, inkjet printing or laser-induced forward transfer.
The (bio)resin might not adhere to the electrospun scaffold as desired.		Use of coatings to enhance the biocompatibility of the electrospun scaffold (eg. collagen).

The relatively short life span of mouse used as *in vivo* model limits the investigation of the long-term outcomes.		After the assessment of a standardized procedure and consistent results, the procedure may be translated to medium/big animal models.

B.2.4 Scientific and societal impact

The use of medical devices with a strong societal impact is of interest in the scientific community. The use of cutting-edge technologies and the possibility of restoring the functionality of the bones in people affected by critical-size bone defects can potentially increase the quality of life of the patients. In this context, the project is designed towards the improvement of bone regeneration strategies thanks to the combination of both biofabrication techniques and selected biomaterials.

The main deal is concerning the funding to develop the presented project. This year, the European Union has been supporting the development of critical technologies through the Strategic Technologies for Europe Platform ('STEP'). The investment reaches €160 billion euros and enhances existing projects such as InvestEU, Innovation Fund, Horizon Europe, EU4Health, Digital Europe Programme, European Defence Fund, Recovery and Resilience Facility programmes. The current project can be included in one of the mentioned fields, the *biotechnologies*, with a focus on medical technologies and biomanufacturing. Additionally, in The Netherlands, the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) grants funds through the Open Technology Programme NWO Domain AES directed to researchers able to achieve knowledge transfer between technical sciences and users. A maximum of €850,000 is invested per project and therefore, the current research project falls into the provided budget.

This proposal takes the first steps toward the resolution of the urgent social challenges clearly depicted by the United Nations (UN). The present project addresses 2 of the 17 Sustainable Development Goals (SDGs) of the 2030 Agenda for Sustainable Development adopted by all United Nations Member States in 2015. The first reference is made to the SDG3, "Ensure healthy lives and promote well-being for all at all ages", in particular to the target 3.8, "Achieve universal health coverage, including financial risk protection, access to quality essential health-care services and access to safe, effective, quality and affordable essential medicines and vaccines for all". The project is also in line with the SDG12, "Ensure sustainable consumption and production patterns" because of the accurate selection of the materials and for the optimization of the combination of biofabrication techniques, such as via topology optimization.

To increase the societal impact and to make the knowledge open access to a wide audience, the scientific output of the project will be reflected in the publication of several papers.

1. First, a review of the state-of-the-art of biofabrication technologies will be published to obtain an overview of the strategies for the regeneration of bone tissue and evaluate the advantages and disadvantages of the current techniques and the materials used in this field.
2. Mechanical characterization of the electrospun scaffold will be performed and the scientific output will be reported in a paper. The possible change in terms of mechanical properties in the mineralized scaffolds will be discussed with respect to the non mineralized scaffolds. The impact on the differentiation of MC3T3-E1 cells towards the osteoblast lineage will be investigated to prove the expected advantages of the inclusion of apatite crystals in the scaffold.
3. The novelty and complexity of the the volumetric (bio)printing process, means a long time spent on the investigation of mechanical, rheological and biological material properties. As a result, one papers regarding the use of resin and the bioresin can be expected.
4. Once the approach has been optimized to combine the electrospun scaffold and the volumetric (bio)printing, *in vitro* studies will be conducted on the MC3T3 cells. The biological outcomes of the approach and the finalization of the product will be reported in at least one paper.
5. Moving towards the small animal trials, the behaviour of the implant in a *in vivo* model will be investigated over several weeks. Scientific outputs of the implant in a biological environment will be examined, paving the way to the investigation of the behaviour of the implant in large animal models and humans. At least one paper accounting for the monitoring of the implant during the animal trials can be expected.

The writing of the papers will be accompanied by conferences and events to share the project will be attended to share the scientific output as well as create connections for possible collaborations. Among the several national and international conferences that can be attended are European Biomechanics Summit (EBS) https://esbiomaterials.eu/, Tissue Engineering and Regenerative Medicine International Society (TERMIS), International Society for Biofabrication (ISBF) and International Conference on Biomaterials Science and Tissue Engineering (ICBST). In The Netherlands many institutes such as the NWO encourage national and internationals collaborations to increase the social impact or the research. Taking part in the conferences and similar events gives the opportunity to expand, share and develop the research. However, to improve the societal impact, the knowledge must be shared with a wider audience. In this context, scientific dissemination takes hold as a powerful tool to make a science accessible to an extended target of people, especially to

young students. Therefore, seminars in schools and lab open-days for high-school and college students will boost the interest of young minds towards this innovative approach.

Industrial and academic collaborations are pivotal to creating a bridge between research and commerce has become mandatory to transfer the knowledge to a large target audience. That is why in The Netherlands associations such as Pint of Science or organize presentations and eventsthat give the opportunity to show the research approach and create and reinforce connections between scientists and industrials in The Netherlands. These occasions can be used to provide increased awareness of bone fractures and communicate the advances that this approach brings to the field. As the project involves cutting-edge techniques, showing the proposed procedure and sharing the output can possibly improve the development of the research approach. Thus, in the long term, the collaborations with insitutes as well as national and international scientists are expected to lead to an optimized process to fabricate scaffolds clinically translatable. Subsequently, the patent will be released and the final product will be subjected to European conformity (CE) and Food and Drug Administration (FDA) controls in order to commercialize it globally, and the accessibility will consequently increase. The industrial translation of the product will be finalized with the commission of the scaffold to companies like 3D SYSTEMS or 3Dreyns. Furthermore, on the long term, the research project also addresses the previously mentioned SDGs of the UN, by improving the quality of life of patients affected by critical-sized bone defects.

Proof of concept

The development of the patient-specific scaffold starts with the acquisition of the pQCT scan, which is instrumental in evaluating the morphology of the critical-size bone defect. An STL file is subsequently generated through global thresholding segmentation process. The STL file serves as a model to fabricate the aluminum collector, onto which a silk solution is then electrospun. As a result, the electrospun scaffold is tailored to match the morphology of the defect, with accurate tuning of the scaffold thickness. Afterwards the scaffold undergoes mineralization by immersion in apatite solution.

The next step consists in placing the scaffold in the vat of the volumetric printer supported by a custom made DLP printed support. The bioresin is subsequently poured in the vat of the volumetric printer and the process commences. Once the finalization of the scaffold is accomplished, it will be cautiously taken out of the vat through the PCL wire.

Prior the scaffold implantation, the patient will undergo immunosuppressed to avoid the rejection of the scaffold due to the presence of MC3T3 cells. Dry autoclaving is used to sterilize the electrospun

scaffold and the final implant undergoes additional UV light exposure to sterilize before implantation. After deep discussion with the orthopaedic surgeon, the scaffold will be placed in the patient to fill the void left by the bone defect. Post-implantation follow-up includes the assessment of the physiological and immune response of the host tissue to the scaffold as well as the management of the pain during the first weeks. Rehabilitation will be developed in consultation with a physiatrist to provide the patient the appropriate exercises and instructions to regain the functionality of the femur. Long-term follow-ups aim to evaluate the motility of the limb and the potential adjustments to rehabilitative exercises.

B.2.5 Ethical considerations

This research project proposes a strategy to enhance bone regeneration in critical-size defects combining cutting-edge techniques, thus individuating the ethical issues that may arise from this novel approach is essential prior the translation from bench to bedside.

The main ethical reflection to be pondered in this proposal regards the small animal trials. The choice of the animal model that best suits the aim of the project fell on the mice, as they are widely used for the investigation of bone defect regeneration strategies. However, due to the restrictions applied to *in vivo* studies, the expected outcomes from the animal experimentation must be shown to outweigh the harmful practices applied to the animals.

In this proposal, the use of small animal models is mandatory as reproducing the bone tissue *in vitro* remains a challenge due to the complexity of the native environment. For the purposes of this research project, the investigation of the dynamic interaction between the body and the implant *in vivo* is therefore crucial for the success of the approach. In addition, the use of small animal models would give the opportunity to investigate the effects of the implant in a biological environment before moving towards the scale-up of the implant and the feasibility of the approach on the small scale can potentially be proved.

The Experiments on Animals Act (Wet op de Dierproeven, unofficial translation) delineates the approved reasons for which animal tests can be performed and the good practices that must be followed before, during and after the experiments to minimize animal suffering. The use of mice for bone defect models is justified by their availability, affordability and fast regeneration as well as wide use in the bone tissue regeneration research [41]. On the other hand, this research remains open to the implementation of alternatives that do not directly involve the use of animals, in line with the responsible use of animal sources delineated by the Animal Welfare Body Utrecht.

In accordance with the 3Rs principle, a modest number of mice will be used per group to validate the animal model and to prove the effectiveness of the proposed approach to enhance the regeneration of

the bone in critical-size defects. The studies will be conducted according to the Utrecht Animal Welfare Authority guidelines and the protocols applied for the *in vivo* tests will undergo the approval of the Institutional Animal Care and Use Committee (IACUC). This institution will perform inspections to ensure the proper treatment of the test subjects consistently according to ethical regulations.

The project will ensure the transparency of the expected and unexpected results to avoid the repetition of the experiments. Furthermore, experts such as veterinarians will be involved to provide the proper training and assistance to the research personnel during the trials. The protocols and practices will undergo expert approval to ensure the wellness of the test subjects and the proper post-trial care will be provided.

The physical and physiological needs of the animals will therefore be guaranteed, in line with the National Committee for Research Ethics in Science and Technology (NENT) guidelines. Moreover, the well-being of the animals will be preserved by reducing the use of invasive methods and ensuring a possible human killing of the mice at the end of the experiment, according to the Schedule 1 to the Animals (Scientific Procedures) Act 1986.

B.2.6 Literature/references

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