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# Circular Seas the great challenge for plastics

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**Keywords:** plastic, compound, moulds, boat fabrication, additive manufacturing.

## Abstract.

**Microalgae biorefinery: high-value products from bioremediation processes Turnkey solution: from sea to sea**

The economic recovery of plastic salvaged from the sea represents a great challenge. The rescue activity itself is arduous, expensive and that is why it is important we look for processes, methods and applications that value this material as much as possible. Plastic from the sea was exposed to salt, and a whole set of conditions that promote degradation phenomena and contamination. The application of large quantities of these plastics in a correct and added valued way will constitute an added motivation for their rescue and use. In the following figure we can see boxes, fishing nets, buoys, and other plastic products. Note the degradation observed as well as the encrustation of shells in the boxes. In addition to the state of degradation of the plastic materials collected, the state in which they are presented also represents greater or lesser difficulty in treatment. Thermoplastic materials are generally recyclable. Thermosetting materials, such as polyurethane foams that make up the inside of buoys, are difficult to recycle. Fishing nets, mostly made of nylon, are thermoplastic and thus have a high recycling potential, however the filament form in which they are presented makes them very difficult to crush and prepare for recycling. The study and classification of plastic materials rescued from the sea looking to their total recycling and application by 3D printing methods is being studied. The most common filament-based 3D printing systems are very sensitive to changes in the filament, to the diameter and homogeneity of the plastic. In this way, in the application of large-scale additive manufacturing, we seek a solution to process even heterogeneous compounds and which will consume large amounts of raw material.



Figure 1  
Plastic  
rescued  
from the  
sea: boxes,  
fishing nets,  
buoys.  
(courtesy  
CDRSP)

Therefore, we are developing a turnkey solution aimed focused to the manufacture of large-scale, fully recyclable moulds to produce boats. This solution includes the formulation of a compound that makes use of PE, PET, Nylon or PP rescued from the sea, where we include some additives and plasticizers as well as significant amounts of calcium carbonate extracted from seashells from fishing activities. Calcium carbonate provides greater stability, less shrinkage and stiffness to the resulting compound, allowing post-treatment operations such as machining and sanding necessary for this type of tool. In the following figure we can see seashells collected from the food processing industry.



Figure 2 Shells: mussels and oysters

The application of thermoplastics to the production of moulds for boats, allows them to be recycled at the end of their life. In this way, we contribute to minimizing the environmental impact of moulds that are usually produced in expanded poliester or poliurethanes resins in the boat manufacturing industry. The moulds and master prototype are normally produced in expanded polystyrene and at the end of their life they are incinerated, used in the construction industry as a light load or end up in landfills.





Figure 3 End-of-life expanded polystyrene moulds .(Courtesy scaleOceans)

The turnkey solution proposed, which is intended to replace moulds and master prototypes normally produced in thermosetting materials and other non-recyclable materials by thermoplastic materials, recyclable and with properties adjusted to the requirements. The materials used have already been presented in general, and the processes and methods as well as the case study are presented below.

### Materials, methods and processes

As a case study to validate the turnkey solution (materials, processes, methods), the company Scale Oceans designed a small electric catamaran that we present in the following figure.

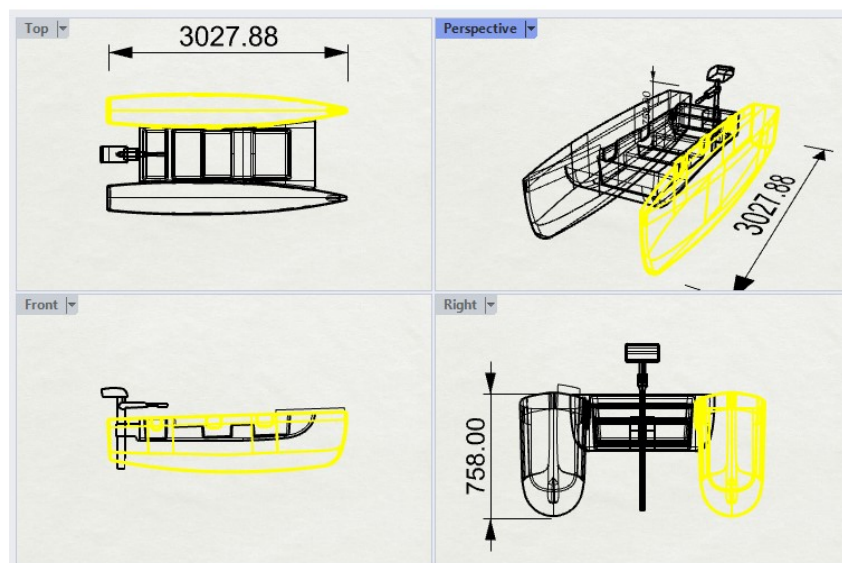


Figure 4 Design of the case study catamaran (Scale Oceans).

This catamaran is about 3 meters long and we are going to produce the master prototype of the floats which are shown in yellow. Each float master will need 150 kg of thermoplastic material compound with calcium carbonate and will take about 15 hours to be produced by 3D printing apparatus and finishing by milling. The production of the thermoplastic compound with a high concentration (up to 75% by weight) of calcium carbonate takes place in several stations which are represented below in cooperation with the company LCR. In the following figure we can see the complete pilot unit. In figure 6, the processing unit for additive manufacturing of large parts is shown. It is important to emphasize the need to strongly fix the part to be produced to the platform to limit the possible warping.

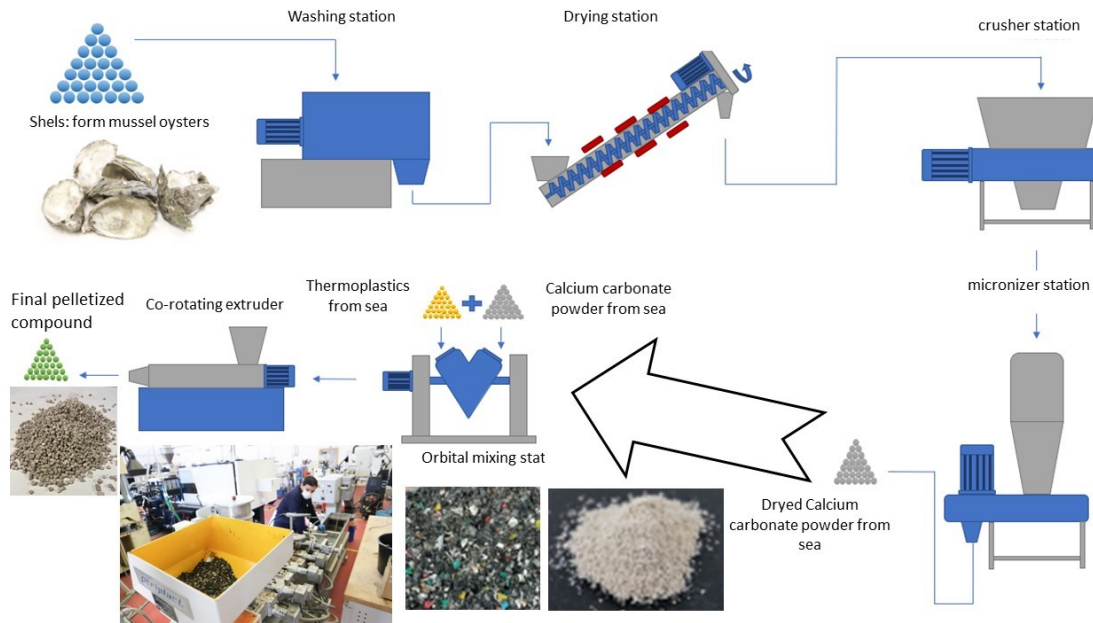


Figure 5 Steps in the process of cleaning, grinding and obtaining calcium carbonate and thermoplastic compound (Luz Costa e Rodrigues, company)

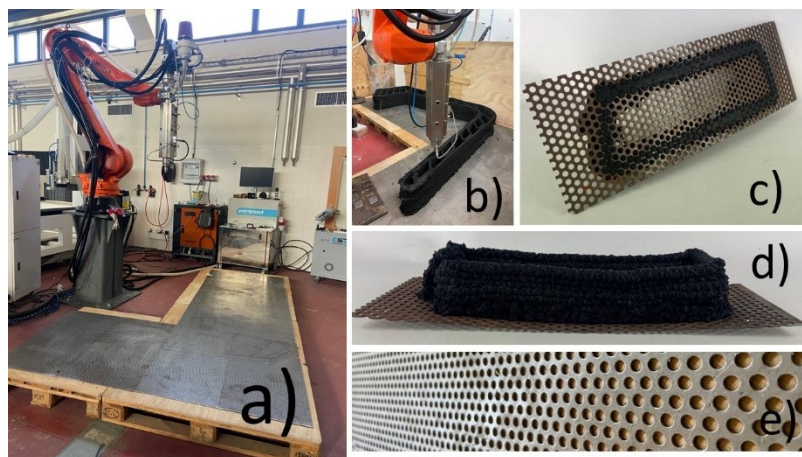


Figure 6 Direct digital manufacturing station (CDRSP): a) robot with vertical extruder; b) detail of extrusion; c) special platform to fix the part; d) warpage problem; e) the platform.

## Conclusions

We have established a complete system for processing and manufacturing moulds for boats, taking advantage of plastic and shells from the sea, seeking to attribute advantageous characteristics to the product obtained. This final product resulting from this turnkey solution is recyclable and eliminates the use of the usual moulds produced in thermosetting materials.

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## Production of Hybrid Structures by Solid-State Welding

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**Keywords:** Hybrid structures; Copper-stainless steel; Explosion welding; Friction Stir Spot Welding; Interface.

**Abstract.** The hybridisation of the components is one of the main challenges for the industry, as it contributes to increase the sustainability of several sectors. Despite the technical interest of the multi-material structures, their production is still difficult because the conventional fusion welding processes are not suitable to join dissimilar materials. The aim of this research was to test the application of two solid-state welding techniques, i.e., explosion welding and friction stir spot welding, for producing dissimilar copper-stainless steel welds. Both processes were found to provide good welding conditions, allowing the production of welds with sound structures. The interaction between the welded materials was found to be stronger in EXW, although no intermetallic phases were formed. The FSS welding conditions were found to depend on the position of the materials in the joint.

## Introduction

It is increasingly more difficult to meet the demanding requirements of many industrial sectors through the production of mono-material components. The recent industrial trends are focused on the development of hybrid components, which conjugate the specific properties of two or more materials. This allows the industry to avoid the use of very expensive materials or to do inevitable trade-offs in properties that may condition the in-service performance of the components. Usually, the interest of the hybrids is as higher as higher is the difference in the specific properties of the materials composing them, such as the density, thermal and electrical conductivities, corrosion resistance, strength, low-temperature toughness, etc. In particular for the transportations industry, the development of hybrid components results in important gains in the efficiency of the vehicles, which strongly contributes to a much more sustainable sector, with a lower carbon footprint (Kumar et al., 2015).

Despite the technical interest of the multi-material structures, their production is still a huge challenge because of the very high complexity of welding materials with quite different physical properties. In fact, the conventional fusion welding processes, which are widely disseminated in the industry, are not a solution for producing these structures. On the other hand, the solid-state welding techniques have a very high potential to join dissimilar materials. These processes can be divided into two big groups, i.e., the low-interaction time techniques, like the impact or ultrasound-based processes, and the low-temperature techniques, like the friction or diffusion-based processes (Galvão et al., 2016; Loureiro et al., 2020).

The aim of this research is to test the application of explosion welding (EXW) and friction stir spot welding (FSSW), which belong to different families of solid-state techniques, for joining two dissimilar materials with quite different physical properties, specifically, copper and stainless steel. The welding of these materials is especially relevant because it allows the production of industrial components that couple the high strength and corrosion resistance of the steel and the high thermal and electrical conductivities of the copper.



## Materials and Methods

Two series of stainless steel-copper (Cu-SS) welds were produced using two different welding processes, i.e., explosion welding (EX series) and friction stir spot welding (FSS series). Table 1 displays the welding parameters tested for each weld series. As can be observed in the table, the alternative position of the base material plates in the joint (Cu/SS and SS/Cu) was tested for both weld series.

Table 1 - Welding parameters used to produce the EX and FSS welds.

Weld series	Welding parameters
EX	<b>Explosive mixture:</b> ANFO <b>Materials position:</b> Cu/SS; SS/Cu <b>Standoff distance:</b> 4.5 mm
FSS	<b>Tool design:</b> Flat pinless tool <b>Materials position:</b> Cu/SS; SS/Cu <b>Rotation speed:</b> 870-1500 rpm <b>Dwell time:</b> 20-60s

After production, the welds were cut and the samples were prepared using conventional metallographic procedures. The morphological and microstructural characterisation of the welds was conducted by optical microscopy (OM) and scanning electron microscopy (SEM). The chemical composition of the welds was characterised by energy dispersive spectroscopy (EDS). Microhardness testing was used to assess the mechanical properties of the welds.

## Results and Discussion

Please The interface morphology of the EX and FSS welds is illustrated in Fig. 1. From Figs. 1a and 1b, it can be observed that the EX welds have a wavy interface, which is a typical morphology of the EXW process (Loureiro et al., 2020). Regardless of the base materials position, the EX welds were consistent and no bonding discontinuities were found at the weld interface. On the other hand, for the FSS series, consistent welds were only achieved by locating the SS plate at the top of the joint. As shown in Fig. 1c, these welds present a flat interface with no signs of materials mixing, which agrees-well with the use of a pinless tool (Andrade et al., 2021). For the welds produced with the Cu plate at the top, no bonding was achieved, which points to quite different FSS welding conditions for the alternative positions of the base materials.

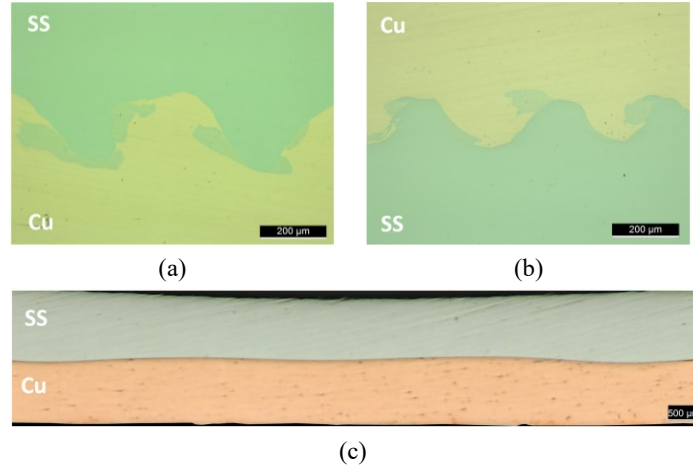


Figure 1 OM micrographs of the weld interface: (a) SS/Cu EXW; (b) Cu/SS EXW; SS/Cu FSSW.

Fig. 2 shows high-magnification SEM micrographs of the weld interfaces. The figure shows that the wavy interface of the EX welds contributes to the mechanical interlocking of the materials (Fig. 2a), which is often reported to have a positive effect on the weld mechanical behaviour (Athar and Tolaminejad, 2016). In turn, the interlocking was much less evident in the FSS welds, but micro-interpenetration was found to occur (Fig. 2b), which may also have contributed to the non-separation of the plates after the welding process. As opposed to the FSS welds, the EX welds were found to present an interaction region of both base materials. This region corresponds to the vortex zones, where the peaks in temperature are usually reported to be reached (Bataev et al., 2014).

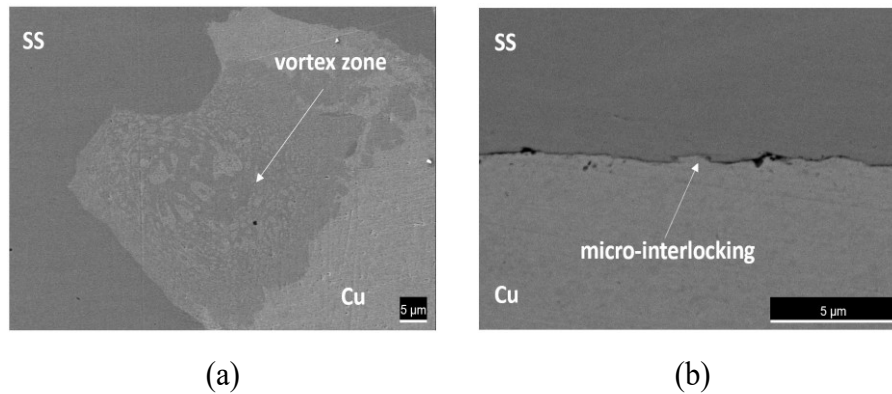


Figure 2 High-magnification SEM micrographs of the weld interface: (a) EXW; FSSW.

The results of the EDS analysis conducted in the vortex regions of the EX welds are displayed in Fig. 3a. From the figure, it can be observed that these zones present a hybrid chemical composition, rich in Fe and Cu. However, Fig. 3b shows that the hardness values registered in these regions are lower than the values registered in the deformed stainless steel. This way, formation of brittle intermetallic phases, which are usually reported as a major problem in dissimilar welding (Galvão et al., 2016), is not expected to have occurred in these zones.

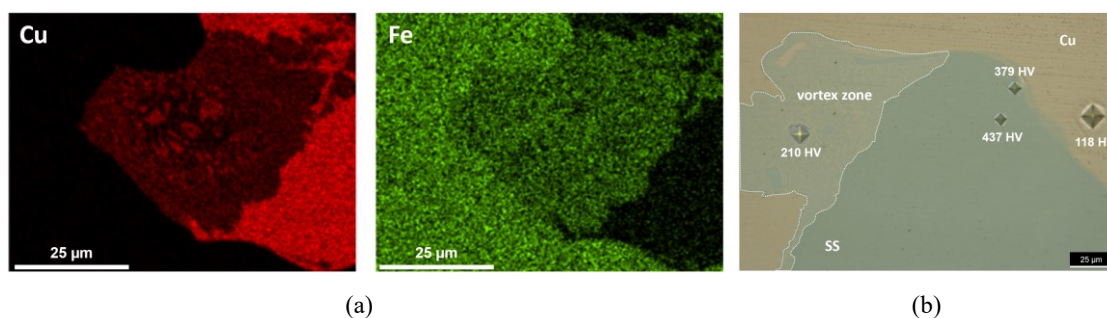


Figure 3 Characterisation of the vortex regions of the EX welds: (a) EDS map; (b) Microhardness.

## Conclusions or Summary

The aim of this research was to test the application of EXW and FSSW for producing dissimilar Cu-SS welds. Both processes were found to provide good welding conditions, allowing the production of welds with sound microstructure. However, FSSW was significantly influenced by the position of the base materials in the joint, as good bonding conditions were not achieved when copper was the top plate. Regarding the base materials interaction, it was stronger in EXW, promoting the formation of zones with hybrid chemical composition. However, formation of detrimental intermetallic phases was not found to occur. Although further research on this issue is required, these processes showed high potential for joining copper and stainless steel.

## Acknowledgements

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# Computational evaluation of sustainable water-cooled heat sinks manufactured by powder bed fusion

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**Keywords:** heat sink; computational fluid dynamics; powder bed fusion; thermal management.

**Abstract.** This work shows the performance of water-cooled heat sinks (HS) with different designs via computational fluid dynamics simulations. Initially, a complete and detailed analysis of the thermal performance of various HS designs was taken. Afterwards, HS designs were modified following some additive manufacturing (AM) approaches. Results showed that, in all cases, the use of these AM designs were advantageous to decrease the HS surface temperature. Furthermore, the best AM design was compared to a previously optimized air-cooled HS with equivalent weight. The water-cooled HS allowed a volume reduction and material savings to achieve the same performance. Hence, this study further acknowledges the potential of AM technologies in the production of sustainable thermal management components.

## Introduction

All electronic devices generate heat during their operation. By providing heat dissipation, a heat sink (HS) prevents overheating and plays a crucial role for temperature control. Nowadays, cooling techniques must be improved as the increase in electronic complexity generates more heat in more dimensional constrain enclosures. Due to its geometric freedom and the capability to build complex internal structures and with higher total area to volume ratio, additive manufacturing (AM) can be a useful way to produce HS that outperform the thermal performance of traditional ones, while using less amount of raw materials (Chinthavali and Wang, 2018).

Therefore, the main goal of this study is to evaluate the performance of different water-cooled HS to be produced by powder bed fusion (PBF), an AM technology, with AlSi10Mg aluminium alloy, reaching HS temperatures as low as possible and minimizing heat emissions to air.

## Methods and Materials

The computational domain (Fig. 1) includes a microchannel HS (main dimensions  $50 \times 50 \times 20 \text{ mm}^3$ ) with a 4.5 mm shell and pressure and temperature sensors.



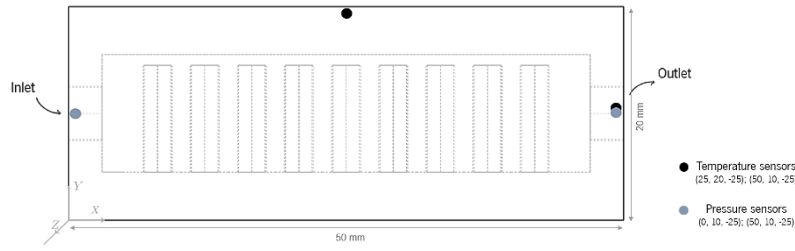


Figure 1. Computational domain for studying water-cooled heat sinks.

The geometry was meshed by applying body sizing operation and, depending on the HS model under study, were considered tetrahedral or hexahedral elements. Element size varied in a range between 0.5 mm and 1.5 mm and the total number of elements was the one whose results converged, with minimal computational effort, i.e., sufficient to ensure mesh independence. Solver settings were defined, including material properties, appropriate physical models, operating and boundary conditions, and initial values.

For the HS, the material considered was an aluminium alloy widely used in PBF technologies – AlSi10Mg. Most of the properties of this material were set based on previous characterizations (Silva *et al.*, 2022). For the fluid passing inside the HS, ideal water was selected. As initial values, the system was considered at 20 °C. Heat source temperature was set at 90 °C and an inlet water velocity of 1.22 m/s achieving a Reynolds number of 12500. Pressure drop across the HS, its temperature and water outlet temperature were reported after 15 s on the respective sensors (Fig. 1), considering a time step size of one second with 10 maximum iterations.

## Results

Considering the critical knowledge obtained on a previous study (Silva *et al.*, 2021), an analysis of the influence of fins/pins thickness/diameter (Figure 2) and spacing (Figure 3) inside the microchannel HS was done. According to the data, for a lower HS temperature, the thickness of the fins should be as small as possible, with bigger spacings. In the case of pins HS, better results were achieved for higher pins diameter and spacing. In almost all HS studied featured with fins or pins, the HS temperature is smaller than when a simple HS (i.e., without fins or pins inside) is used (orange line in Fig. 2 and Fig. 3 indicates the temperature of a simple HS). This proves that the inclusion of small features inside water-cooled HS is beneficial to improve their performance.

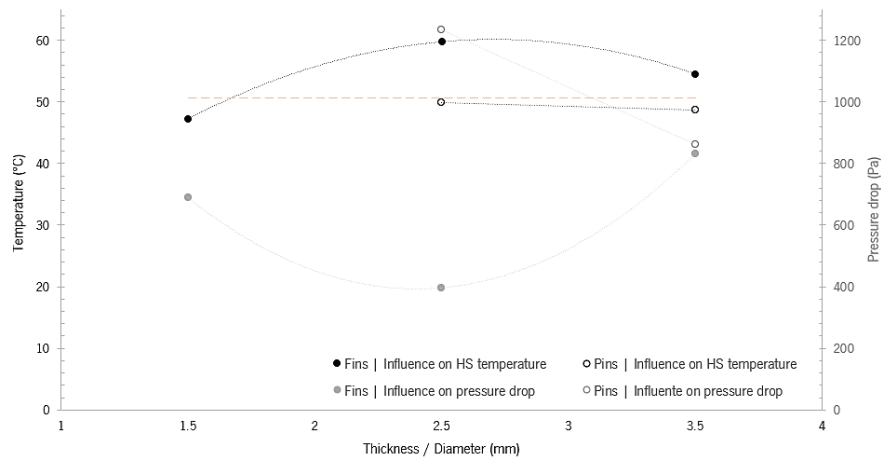


Figure 2. Influence of pins diameter or fins thickness on HS temperature and pressure drop.

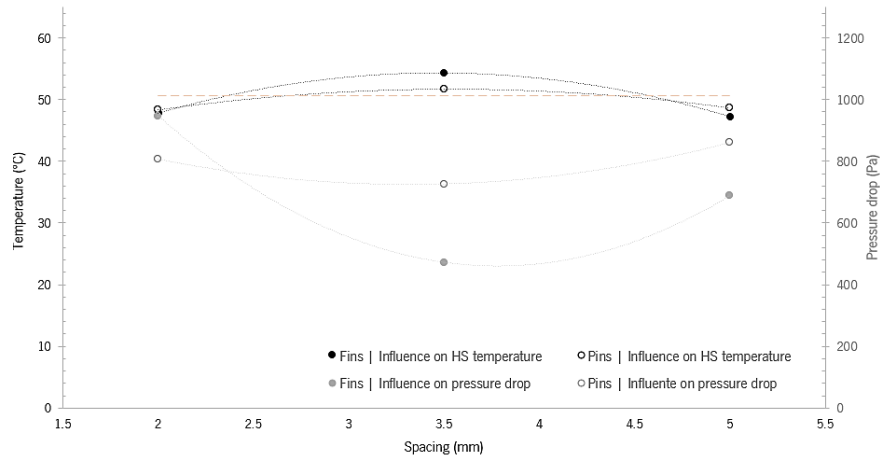


Figure 3. Influence of pins/fins spacing on HS temperature and pressure drop.

After these conclusions, the same studies were done considering the best fins and pins HS reported before (Fig. 4) (Silva *et al.*, 2021). Comparing with the simplest fins or pins HS, there was an improvement in the thermal performance up to 9.5 %. Hence, AM design approaches are also advantageous in water-cooled heat sinks.

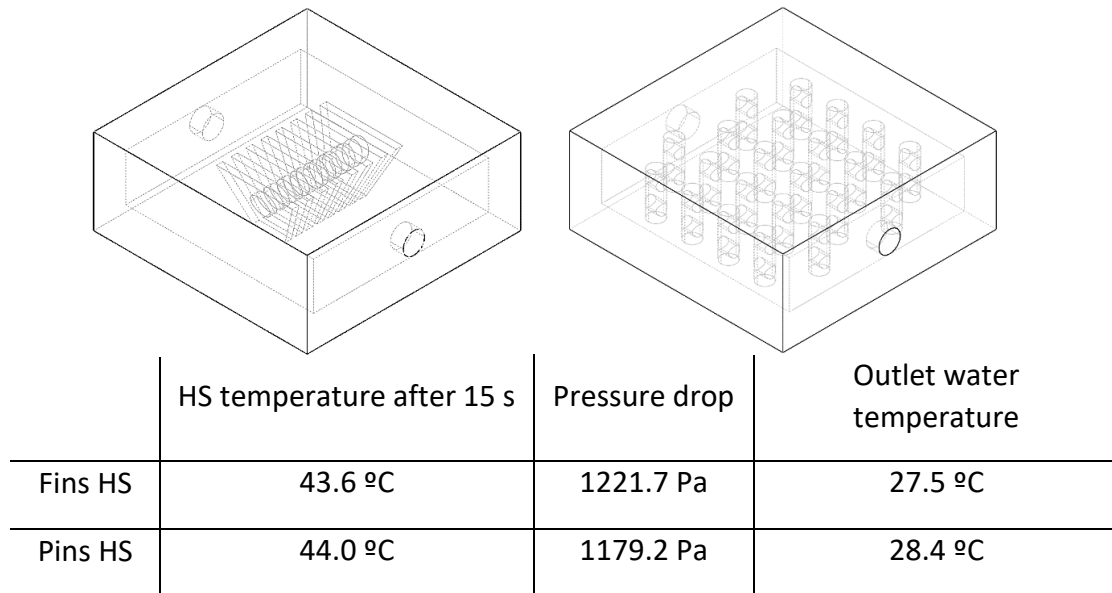

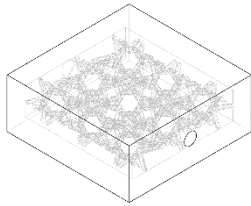


Figure 4. Water-cooled heat sinks based on the best air-cooled ones.

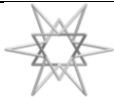
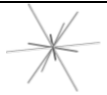
Considering the potential of AM technologies, lattice HS with Star, W, 3D, Tetra and Cross Pattee unit cells were studied. Cell size and thickness were fixed at  $15 \times 15 \times 15 \text{ mm}^3$  and 1.5 mm, respectively. Among the models studied, the cross pattee HS showed the lowest temperature (Table 1). The unit cell used in this model, is the one with the higher surface area to volume ratio.

Table 1. Performance of different microchannel lattice HS.

Model	HS temperature after 15s	Pressure drop	Outlet water temperature
 Star	40.8 °C	1517.4 Pa	28.1 °C



W	40.8 °C	1321.6 Pa	26.7 °C
Tetra	42.7 °C	1737.6 Pa	27.5 °C
Cross Pattee	<u>35.4 °C</u>	<u>1578.2 Pa</u>	<u>26.1 °C</u>



## Discussion

After the analysis of water-cooled HS, where the considered height is 20 mm, a study was carried out to understand what would be the height of a previously optimized lattice air-cooled HS (Silva *et al.*, 2021) needed to reach the same temperature that was reached with water (35.4°C), considering the same Reynolds number (12500). Computational fluid dynamics results for lattice air-cooled HS temperature vs height are recorded in Fig. 5. It was found that the air-cooled HS temperature tends to a minimum value that, according to the fitted equation, is reached when the HS height is around 50 mm. To reach the temperature that was achieved with a 20 mm water-cooled HS (35.4°C, highlighted with a grey line in Figure 5), a 28.5 mm high air-cooled HS is required, which can be explained by the different values of thermal diffusivity. This proves the advantages of applying AM technologies, namely PBF, in the production of components for thermal management, whether air or water-cooled. In the case of water cooling, a smaller volume of material is needed for the production of the HS, making them more beneficial for tiny and sustainable electronic enclosures.

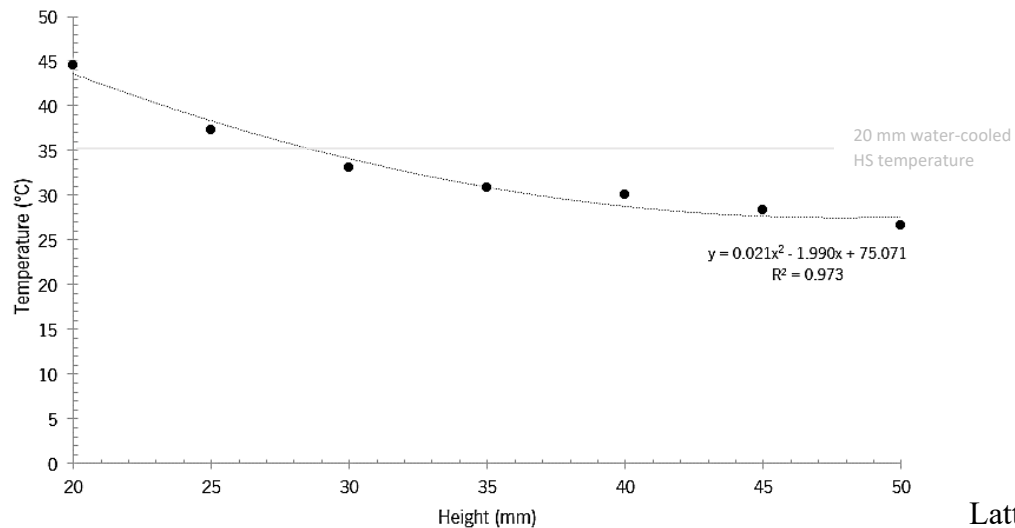


Figure 5.

cooled HS temperature for different heights.

Lattice air-

## Conclusions

The thermal performance of water-cooled HS designs was conducted by computational fluid dynamics simulation, taking advantage of AM freedom of design. Through a direct comparison of HS temperatures, under the same boundary conditions, it was concluded that the inclusion of small features inside water-cooled HS is beneficial to improve their performance. The various HS designs presented in this study can be easily produced by PBF AM technologies with aluminium, reducing the amount of raw material used and the amount of waste generated. Therefore, additively manufactured HS have great potential to be applied in efficient thermal management systems.

## Acknowledgements

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# Microalgae biorefinery: high-value products from bioremediation processes

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**Keywords:** microalgae; biobased products; sustainability; circular economy; bioeconomy

## Abstract

Climate change, resources scarcity and chemical pollution are creating a global crisis, affecting the entire planet. The current production and consumption patterns have been exhausting the natural resources and increasing pollution in the environment. An integrated system for producing biobased products while rehabilitating the environment could be a potential solution. A biorefinery concept based on a circular economy and using microalgae, is presented in this study. The performance and efficiency of microalgae *Nannochloropsis* sp. to remove industrial pollutants, while producing biomass for different purposes were assessed. The marine microalgae *Nannochloropsis* sp. was found to remain alive in the presence of industrial pollutants and was able to remove them from water. Also, the high lipid content of those microalgae can be used as a feedstock for innovative applications.

## Introduction

The concept of the microalgae biorefinery is similar to the established oil refinery or petroleum refinery: it converts feedstock into energy and several chemicals. These unicellular microorganisms contain high amounts of lipids, proteins, and carbohydrates, which can be used for several purposes. Microalgae biomass can produce a variety of high value-added products for many different applications, including fuels, cosmetics, pharmaceuticals, chemicals, food, and feed (Encarnação et al. 2015). One of the many advantages of a microalgae biorefinery is that it can be integrated into different industrial production units such as textile, paper, tannery, dairy, and cement. By integrating microalgae production



into these industries and in water treatment plants, it is possible to mitigate environmental damage while adding value to the waste generated.

Many of the pollutants found in water are considered endocrine disrupting chemicals. Through the urban cycle of water, pollutants such as phthalates, bisphenols, organochlorine pesticides, heavy metals, and so many others are found in the ground, surface, and drinking waters. Conventional drinking and wastewater treatment do not completely remove most of these pollutants. Therefore, an integrated system represents a potential contribution to significantly reducing pollutants from water. In addition, microalgae reduce CO<sub>2</sub> emissions.

In our research, we assessed the performance and efficiency of microalgae *Nannochloropsis* sp. to remove industrial pollutants, while producing biomass for different purposes using a biorefinery model. By producing multiple product streams and increasing the value of the biomass with innovative applications, we can make the all process economically feasible.

## Methods and Materials

Several methods were employed to carry out relevant tasks in our research. These include extraction techniques and chromatographic, spectroscopic, and hyphenated techniques. A rapid reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated. The methods were validated according to the guidelines of the US Food and Drug Administration (FDA), the International Conference on Harmonization (ICH), and Eurachem. The industrial wastewater profile was performed using electronic, optical, and fluorescence microscopies, Fourier-transform infrared (FTIR), and Raman spectroscopies.

The wastewater suspended solids were incorporated as a filler material into the formulation of cementitious mortars and polymer-based composites. They were assessed in terms of their impact on mechanical performance. The chemical composition of the polymer-based composites was analysed by FTIR spectroscopy. The microalgae biomass generated in the bioremediation process can be converted into feedstock for biobased products for advanced applications. After the bioremediation process, strategies for the maximisation of lipids were performed. For that the method of lipid monitoring BD-C12 is a fundamental tool (Encarnação et al. 2018). Lipids can be used as a feedstock to produce various chemicals. After lipids extraction, it is possible to obtain feedstock for producing biopolymers; we evaluated the feasibility of polylactic acid (PLA) for applications in advanced optical products through thermal and optical analyses using Differential Scanning Calorimetry (DSC), Polarised light thermo-microscopy (PLTM), Refractive index and Abbe number measurements.

## Results and Discussion

In studies of the bioremediation potential of *Nannochloropsis* sp., results of UV–Vis spectroscopy analysis revealed a high percentage of pollutants removal from wastewater. Epifluorescence microscopy images of *Nannochloropsis* sp. cells (Figure 1) showed the fluorescence of pollutants in whole cells and lipid bodies indicating their absorption.

The industrial wastewater profile analyses revealed the presence of different polymeric matrices such as polycarbonate, polyurethanes, and allyl diglycol carbonate. It also showed the presence of persistent organic molecules.

The wastewater separated solids were used as fillers to produce mortar and polymer-based composites. The replacement of limestone filler with plastic waste material in the formulation of the cementitious mortar led to a decrease of about 20% in compressive strength at seven days, from 36.2 to 28.7 MPa. This result may be related to a negative impact of the plastic material on the hydration process of cement and the increase in water/cement ratio that was observed when the plastic waste powder was used in place of limestone filler.



After the extraction of lipids, the lipid-free residues can be neutralised and concentrated into sugars, which can be fermented by a wide range of microorganisms, such as bacteria, yeasts and fungi. The product obtained from the fermentation is lactic acid, which is an important biobased block for producing PLA. Feasibility studies on the use of PLA in Ophthalmic Lenses were performed. For comparative purposes, a CR39 polymer lens was used. CR39 is a commercial material that presents the best optical quality on the market. We compared the mechanical properties of two grades of PLA with CR-39. With these, PLA presents better performance when compared with CR-39 and is suitable for its application in ophthalmic lenses. The thermal behaviour of PLA was also studied and showed that the glass transition temperatures are around 59.0 °C, while the degradation temperatures are observed between 140 and 160°C. PLA has suitable thermal and mechanical properties that can be processed by various production methods, such as injection moulding, casting, or 3D printing. Refractive index and Abbe number were also measured to show similar values for the two lenses (CR39 and PLA). Refractive index and Abbe number of PLA lenses were 1.46 and 55.24, respectively.

## Conclusions

From the results obtained in our research, it became apparent that the microalga *Nannochloropsis* sp. could be considered a promising specie in the removal of pollutants from effluents. The microalgae *Nannochloropsis* sp. was found to remain alive in the presence of pollutants and were able to remove them from water. The high biomass and oil obtained also demonstrate that *Nannochloropsis* sp. is a promising biobased feedstock for innovative applications.

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## The circular business models as strategic tools for a sustainable development

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**Keywords:** Circular business models, circular economy, sustainability, manufacturing, bioeconomy

### Abstract.

Nowadays, the future of companies and organizations is strictly dependent on their sustainable development and fast adaptation to the numerous changes that industry is facing. Consumers, as well, are becoming more aware of environmental issues and are claiming for green products. Therefore, enterprises

need to innovate their business models to respond to the sustainable development goals set by the United Nations for the 2030. The circular economy is one of the main drivers of sustainability, and it is changing the way of making business from a linear to a closed-loop perspective, using residues to produce new products. Thus, transition to circularity is considered essential for the manufacturing players, which need to innovate their business models into circular ones. In this context, this work aims to describe and understand the circular business models as sustainable tools for companies. Performing a bibliographical review, it is evident that the circular business model is itself a sustainable business model with the application of circular strategies, although the boundaries between the two models are not strictly defined. Moreover, it resulted that there is not just one kind of circular business model, but different ones, according to the various circular actions which industries are adopting. In fact, waste reuse, waste reduction, product life-cycle increase, consuming habits' reduction, property's sharing, and industry 4.0 innovation are among some of the circular actions that can be embraced. Nevertheless, further studies about implementation and adoption of circular business models in companies are needed to help their transition toward a circular and sustainable future.

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# Application of circularity micro-indicators to plastic products in various industrial sectors

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**Keywords:** Circular Economy; Circularity indicators; Sustainability

The global consumption of plastics has been continuously increasing for decades in several industrial sectors, and while in recent years there are many voices opposing the use of plastics in general, the actual problem does not reside in the materials as in their application in a sustainable manner. Thus, changes are undoubtedly needed, as some plastics products are either superfluous or should have different design solutions based on their full life cycle thinking (a clear example being some single-use plastics products, and the recent legislation imposing restrictions and in some specific cases a complete ban of those products). However, most of the applications of plastics have brought tremendous societal progress, through increased safety and improved quality of life. One of the means to improve the sustainable use of plastics is by looking at their circularity. Circular design with plastics has become a valuable methodology to improve the circularity of plastic products. To assess circularity there is no standard method, and the scientific community has proposed a myriad of tools for this purpose, such as circularity indicators. However, circularity indicators range widely in complexity, philosophy, method of calculation, and type of required information, and most focus only on some aspects of the entire product life cycle.

In this work, we analyse the different circularity indicators proposed in the literature in terms of their applicability to different industrial sectors, always in the scope of plastic materials. We have selected 9 major sectors of application of plastics: packaging, building & construction, automotive & transportation, food & beverage, medical, textiles & accessories, electrical & electronic, agriculture / horticulture, and general consumer goods. For each sector, we discuss and argue the potential application of each micro-indicator, and we highlight the most relevant indicators overall for that sector. This is done obviously in a broad sense, as specific products can sometimes fall outside the typical characteristics and features of their application area. This information aims to promote the transition to a more circular economy of plastic products in the various industrial sectors.

# Edible coatings and films for sustainable food preservation

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**Keywords:** seaweed extract, packaging, shelf-life, fruit, seafood.

## Abstract

Several seaweed species can be used to obtain valuable by-products for food industry. Seaweed valorization for food applications constitutes a three-folded opportunity: high biomass availability for bioactive components extraction; conversion of the residual biomass waste into added-value products; and mitigation of negative environmental impacts caused by alien species, restoring the marine ecosystem integrity and sustainability. Combining the sequential extraction of high-value components and lower value products can create a sustainable production system where little to no waste flow is generated, therefore decreasing negative environmental impacts and improving economic viability. The earlier concept that seaweeds' benefits were due only to their manorial value or to their micronutrient suites is currently a limited perspective given seaweeds array of identified functionalities.

Seaweeds composition varies significantly across species depending on the type of habitat, making this natural resource a valuable source of bioactive compounds and other components to be incorporated in edible coatings and films. The use of synthetic materials for food packaging is one of the factors with greater environmental impact in the processing of food products, which makes the development of edible materials of greater importance. The use of edible coatings and films to extend the keeping quality of food products is a very common practice in the food industry. Two examples in different food matrices will be discussed.

The use *Codium tomentosum* seaweed extract in the formulation of edible coatings for the shelf-life extension of fresh-cut apple and pears is validated at industrial scale. *C. tomentosum* based coating application results in a lower superficial browning development in fresh-cut apple and pear.

The incorporation of seaweed extracts in edible films formulations for frozen seafood has also been proved to extend the product shelf-life minimizing freeze burn, weight loss and lipid peroxidation. This novel material composed only by natural compounds of marine origin, will therefore contribute towards seafood products differentiation, reduction of food waste and the reduction of single-use plastics applied in frozen seafood production.

These examples illustrate distinct successful application of seaweeds in the sustainability of food value chain.

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# Sustainable Biomaterials of bacterial origin and their use in Biomedical and Environmental Applications

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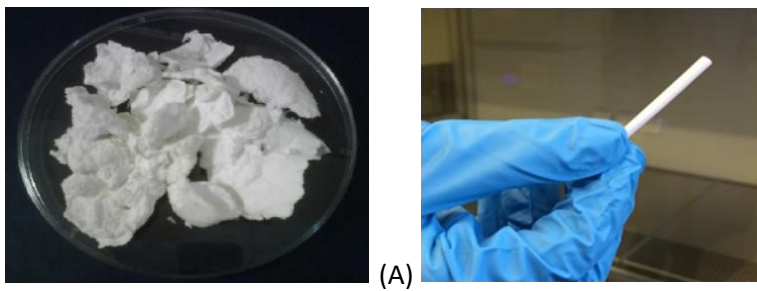
**Keywords:** biomaterials, polyhydroxyalkanoates, bacterial cellulose, biomedical applications, environmental applications

## Abstract.

Currently there is a huge need to find replacements for petrochemical-derived plastics which are not sustainable, degradable and lead to high concentrations of recalcitrant plastics in the soil and in the sea. In addition, in the medical arena, currently there is a lot of use of plastics for packaging, implants, tissue engineering and drug delivery. However, there is hardly any attention paid to their sustainability and environmentally friendly aspects. This group of plastics also lead to a huge environmental impact.

In this work we have focused on the production and use of bacteria-derived sustainable biomaterials for use in biomedical and environmental applications. Two main types of biomaterials have been focused on, including polyhydroxyalkanoates (PHAs)<sup>1</sup> and bacterial cellulose (BC)<sup>2</sup>. PHAs are polyesters produced by a range of bacteria including *Ralstonia eutropha*, *Pseudomonas putida* and *Bacillus subtilis*. These polymers are biodegradable in the soil and in the sea. In addition, they are also resorbable in the human body and are highly biocompatible. Hence the PHAs can be used for the development of green packaging materials and coatings. In addition, they can be used for biomedical applications such as the development of scaffolds for hard and soft tissue engineering and drug delivery. BC can also be produced by a range of bacteria including *Gluconobacter xylinus* and *Sarcinia ventriculi*. BC is also a green polymer, is sustainable and degradable in the soil. It is also highly biocompatible and can be used in biomedical applications.

Polyhydroxyalkanoates are polyesters with monomer chain length ranging between C<sub>4</sub>- C<sub>16</sub>. They are divided in to two main types, short chain length PHAs (scl-PHAs) with monomer chain length C<sub>4</sub>-C<sub>5</sub> and medium chain length PHAs (mcl-PHAs) with monomer chain length C<sub>6</sub>-C<sub>16</sub>. The scl-PHAs are normally hard and brittle whereas the mcl-PHAs are soft and elastomeric in nature. Hence, we have mainly used the scl-PHA, Poly(3-hydroxybutyrate for bone tissue engineering<sup>3</sup>, drug delivery<sup>4</sup>, medical devices such as coronary artery stents, and the mcl-PHAs for cardiac<sup>5</sup>, nerve<sup>6</sup>, pancreas, kidney and skin regeneration (Figure 1). For bone tissue engineering we have used neat P(3HB) and composites of P(3HB) with Bioglass<sup>®</sup><sup>7</sup>, hydroxyapatite<sup>8</sup> and carbon nanotubes. The mcl-PHAs are being used for development of cardiac patches<sup>6</sup>, nerve guidance conduits<sup>5</sup>, wound healing patch, bioartificial pancreas and bioartificial kidney (Figure 1). Processing techniques used include additive manufacturing, electrospinning and melt electrospinning.





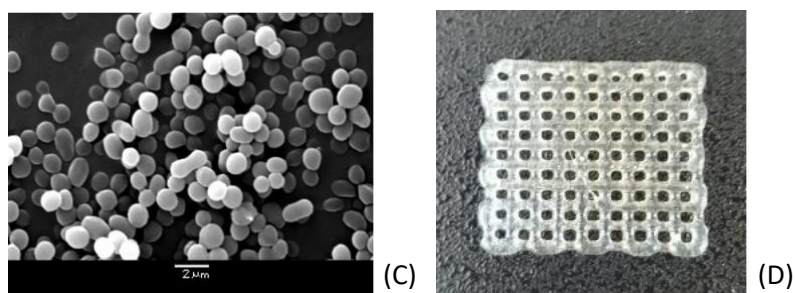


Figure 1: (A) P(3HB) produced using *Bacillus subtilis*; PHA based (B) Nerve guidance conduit (C) Microspheres for drug delivery (D) Wound healing patch

Bacterial cellulose has also been produced under static culture conditions using *G. xylinus*. This is a highly nano-fibrillated structure and hence is a great substrate for cell attachment and growth (Figure 2). We have surface modified bacterial cellulose to create antibacterial bacterial cellulose<sup>9</sup>. We have also used BC as a filler for P(3HB) based composites since BC is one of the stiffest known materials. In the context of environmentally friendly applications, we have used BC as a green and sustainable coating on plastic substrates.

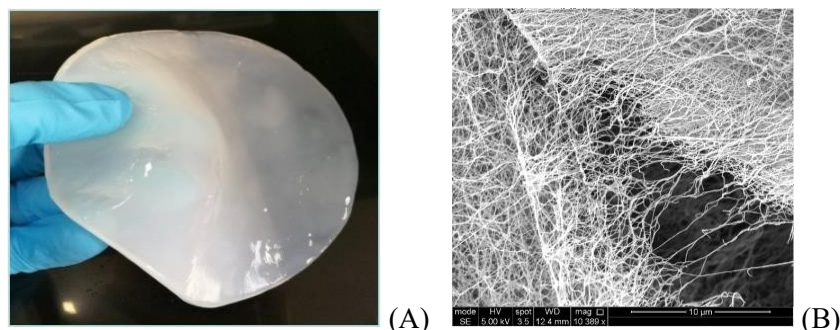


Figure 2: (A) Bacterial cellulose pellicle (B) SEM of the Bacterial cellulose

In conclusion, we have successfully used bacteria-derived sustainable biomaterials for a variety of medical applications and have initiated their use in environmentally friendly applications. Both PHAs and bacterial cellulose have a lot of potential in the future as sustainable materials of choice.

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# Electrostatic spraying for health and hygiene

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**Keywords:** SARS-CoV-2, Microorganism, Charged sprays, Aerodynamics, Bacteriophage MS2.

**Abstract.** The sudden outbreak of novel coronavirus SARS-CoV-2E also termed as COVID-19 has become a global threat for human being and it has put the world in tremendous crisis. The virus attacks on human respiratory system and transmit through human-to-human via droplets, hand-shaking, and physical touch of surfaces. Disinfection and sanitization has become one of the most essential tasks to stop the spread of novel Corona virus. Fruits and vegetables, poultry, livestock, food commodities, healthcare, public transport, airports and railways, hotels and catering, work place and offices are the objects/places, where harmful microorganisms makes people vulnerable to diseases. The conventional methods of disinfection such as manual washing and cleaning consumes more material with lesser efficiency and increased load of chemical waste in the environment.

Electrostatic spraying methods, based on the electrostatic charging principles, produces uniform and fine spray droplets of disinfection material in the size range of microorganism. Due to the small sized and uniformly distributed droplets, the surface area of spray droplets increases which enhances the interaction with the harmful microorganisms. Charged droplets cover the directly exposed and obscured surfaces uniformly with increased efficiency and efficacy. Therefore, it kills or inhibits the growth of pathogens. The electrostatic spraying uses very less disinfection material as compared to conventional methods, which helps to save natural resources and negligible increase of chemical waste in the environment.

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## Membranes Technologies for Water and Energy

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Water is the most vital resource for all kinds of life on this planet. Unfortunately, this vital resource is adversely affected both qualitatively and quantitatively by all kinds of human activities, such as industrialization, urbanization and other developmental activities. To address these, membrane-based technologies play a vital role. Among the different technologies, Electrodialysis is the most viable technology. The technical feasibility of this technology for mass production largely depends on the membrane and its properties. Therefore, an emphasis will be made to describe the preparation of different types of ion exchange membranes developed in our laboratory. During the presentation, principle of Electrodialysis and its application will also be discussed.

Similarly, energy is the key input in economic growth and there is a close link between the availability of energy and the growth of a nation. Since energy is essential to conduct the process of production, the process of economic development requires the use of higher levels of energy consumption. Therefore, there is an urgent need to develop technologies to produce clean and reliable energy as well as its storage. Proton exchange membrane fuel cell is a promising energy producing technology. However, supercapacitors are the promising devices to store the energy. Realizing this, our group is actively involved in developing the membranes for the fabrication of both energy producing and energy storage technologies. Thus, a brief account on both fuel cell and supercapacitor technologies developed in our laboratory will be discussed and compared with the technologies available in the market.

# Orthopedic Applications of Additive Manufacturing

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**Keywords:** Implants, Customized, Stress Shielding, Porous, Biocompatible

**Abstract.** Over the last three decades, Additive Manufacturing (AM) also known as 3D Printing or Rapid Prototyping has evolved remarkably. The definition of AM given by ASTM-F42 Committee is “*a process of joining materials to make objects from 3D model data, usually layer on layer*”, unlike the conventional manufacturing technologies. Once only used for rapid prototyping, its potential to create functional parts has been already recognized by the researchers. A major advantage of AM technologies is its ability to fabricate complex structures in a single operation with a great degree of freedom at high speed and without having a requirement of part-specific tooling. The attempt to imply AM in every manufacturing sector is still going on and hence technological advancement in the AM techniques and machines is a continuous process. The biomedical industry is one of the first sectors to adopt AM technologies for its advantages. Some of the applications include the printing of biodegradable tissues, planning of surgical operations, and, most significantly, the fabrication of orthopedic implants. Conventionally made implants come in a restricted range of sizes, which might lead to improper bone remodeling as anatomy of each patient is different. By replicating the patient's bone with an implant, AM enables for the fabrication of patient-specific implants. Simultaneously, tailored porosity can be added to solid metal implants, lowering the elastic modulus of the implant and improving tissue integration, eliminating stress shielding. Another benefit of AM technology is the design freedom they allow for improving the performance of orthopedic implants.

Metallic orthopedic implants are commonly manufactured using Selective Laser Melting (SLM), Electron Beam Melting (EBM), and Direct Laser deposition (DLD) based AM techniques. These techniques are able to manufacture implants with attributes equal to those manufactured conventionally, as well as to improve standard implants. These implants can be customized for individual patients using medical data, and design features like hierarchical scaffolds, latticing, or features that compliment patient anatomy can be incorporated via additive manufacturing to make highly functional patient specific implants. The design-to-process lead time and associated post-processing can also be drastically shortened using AM. The use of reverse engineering in AM technology allows for the customization of fabricated implants. The process begins with data acquisition from scanning techniques, typically from computed tomography (CT) or magnetic resonance imaging (MRI) of the patient, collected and stored in the Data Imaging and Communications in Medicine (DICOM) file format to produce a custom-made additive implant. To create a digital computer-aided design (CAD) model, the data is processed by image segmentation software like MIMICS, Simpleware, etc. These CAD models can be used for the generation of implant CAD at the required anatomical sites using software.

Following that, the CAD model of the implant is converted to Standard Tessellation Language (STL) file format. The STL file is transferred to the printer for manufacturing.

Because of their excellent mechanical strength, lack of cytotoxicity, and good corrosion resistance, biocompatible metals such as titanium (Ti) alloys, cobalt-chromium (Co–Cr) alloys, and stainless steel (SS) are main choices for implant applications using AM. However, major share of AM based orthopedic implants is dominated by Ti and its alloys mainly Ti6Al4V. Ti alloys with properties like good corrosion resistance, good biocompatibility, high strength to weight ratio, low stiffness, and fatigue resistance in physiological media makes it one of the best suitable materials for implant applications. But solid Ti alloy implants have the disadvantage of high stiffness than the human bone leading to stress shielding which results in implant bearing most of the load, leaving the bones with less load. According to Wolff's Law, bone requires continuous mechanical stimulation to renew otherwise it will begin to lose mass and becomes thinner. This effect causes implant loosening and finally the failure of the implants. Using AM, it is possible to manufacture porous titanium implants having better load bearing characteristics, less micromotion, high compressive strength with good osteoconductivity, and bone-bonding ability. The lattice structures incorporated in the AM fabricated porous implants lowers the overall stiffness of the implant which in result reduces the stress shielding effect and simultaneously provide region for bone ingrowth which leads to biological fixation of the implants, reducing the complications related to mechanical loosening of the implants.



# Exploration of soil-chitosan mixtures for additive earthen construction

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**Keywords:** 3D printing, earthen construction, biopolymers, sustainable construction.

**Abstract.** A more sustainable construction industry should move away from cement-based materials due to its high-energy consumption and greenhouse gases emission, earthen construction is an alternative but requires improving both the material properties and the manufacturing efficiency. Polymer-modified soil mixtures can be used in additive construction to improve efficiency and produce materials with increased mechanical properties and water erosion resistance and, at the same time, low environmental impact.

## Introduction

The cement-based construction industry is highly pollutant and is one of the main greenhouse gas generators, it consumes more raw materials (3000 Tm/year) than any other economic activity (Pacheco and Jalali, 2012; Medineckiene et al., 2010). More sustainable technologies are needed to achieve goals 11 and 13 of the “17 Sustainable Development Goals” of the United Nations (2015). Earthen construction is an alternative since soil is 100% recyclable, non-toxic, economic and widely available, which means, low transportation costs and environmental impact. Besides this, excellent acoustic and thermal isolation are characteristics of earthen construction, main limitations are low resistance to erosion by water and wind and limited mechanical properties. Chitosan is a polysaccharide derived from chitin (a waste product from the fishing industry). Chitosan’s chemical structure, solubility in aqueous media and soil particles’ binding capacity make it a good additive to improve soil mixture flowing and binding properties

to be used in additive manufacturing in construction (Perrot et al., 2018) and, at the same time, increasing the water and wind erosion resistance of the dried soil.

The main goal of this work is to develop a soil mixture with proper workability (flow and stability) when wet and good mechanical resistance when dried, that can be used for 3D printing in the construction industry. Fiber reinforcement can also improve mechanical properties.

## Methods

Chitosan structure characterization was accomplished by NMR spectroscopy, molecular weights were determined by gel permeation chromatography (GPC) and capillary viscometry. Chitosan was dissolved in 1% (v/v) acetic acid aqueous solution in concentrations of 1%, 2% and 3% (w/v). Fiber content was fixed at 1% (w/w) with respect to soil content. Unreinforced and fiber-reinforced earthen-based matrices (Table 1) were prepared by mixing soil (and fibers) with the liquid (water or chitosan solution) between 26% and 30% liquid content (weight of liquid/total weight without fibers). For example, EC27(2%) corresponds to a mixture of 73% (w/w) soil and 27% (w/w) chitosan solution (of 2% w/v concentration); ECF27(2%) corresponds to the previous mixture with addition of fibers.

Qualitative extrusion tests of the mixtures evaluated the manual effort needed to deposit a 15 cm extruded filament. Some of the mixtures were also subjected to shear vane and cylinder stability tests to evaluate their workability. The drying process of 50-mm cubic samples was evaluated and also their compressive strengths and wettability (water contact angle) when hardened.

## Materials

Food grade chitosan from SHQ (Mexico) with 597.8 kDa (Mw by GPC), 360.2 kDa by capillary viscometry and 91% degree of deacetylation (by NMR) was used. Raw soil from a quarry located in Carabayllo, northern Lima, Peru, was sieved through ASTM #20 mesh. Sisal fibers were obtained from ropes supplied by Lanyard SAC and cut into pieces of 10 mm in length.

## Results

Table 1 shows the mixture compositions and their qualitative extrusion behavior in the fresh state. Samples were also subjected to shear vane and cylinder stability tests, the results are shown in Fig. 1.

Table 1: Extrusion qualitative evaluation of unreinforced and fiber-reinforced matrices.

Mixtures	Description	Result
EC27(1%), EFC28(1%)	High manual effort. Material could not be extruded.	NE

EC28(1%), EFC29(1%)	Sufficient manual effort. Non-continuous extruded filament.	EL
EC29(1%), EFC30(1%)	Low manual effort. Non-continuous extruded filament for unreinforced, but continuous and non uniform extruded filament for fiber-reinforced.	E
EC26(2%), EFC26(2%)	High manual effort. Material could not be extruded.	NE
EC27(2%), EFC27(2%)	Sufficient manual effort. Continuous and uniform extruded filament.	EL
EC28(2%), EFC28(2%)	Low manual effort. Non-uniform extruded filament.	E
EC26(3%), EFC27(3%)	High manual effort. Material could not be extruded.	NE
EC27(3%), EFC28(3%)	Sufficient manual effort. Continuous extruded filament.	EL
EC28(3%), EFC29(3%)	Low manual effort. Continuous and uniform extruded filament	E

NE: non-extrudable. EL: extrudable limit. E: extrudable.

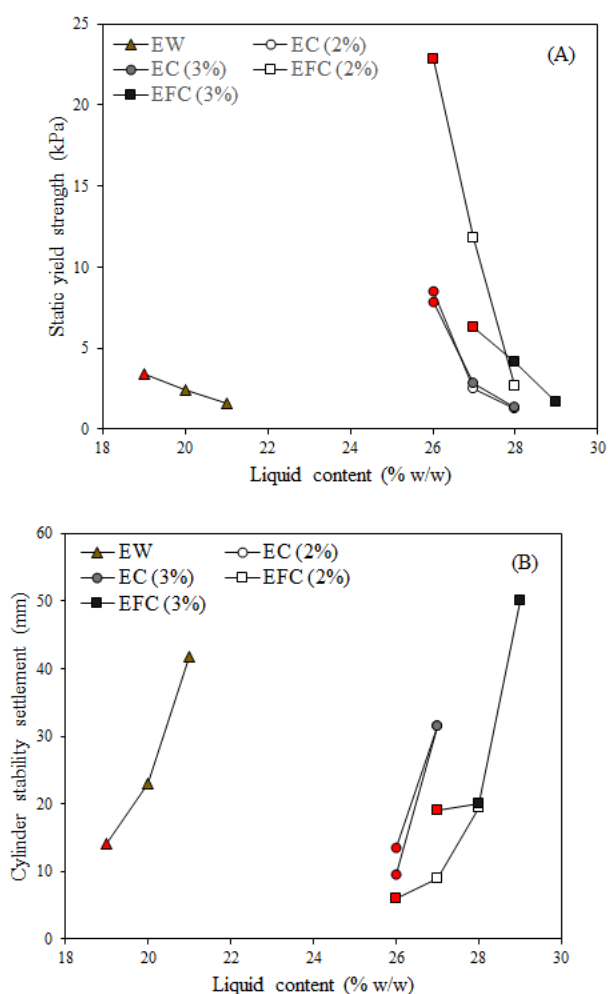


Figure 1. (A) Static yield strength with respect to liquid content in the mixture. (B) Cylinder stability with respect to liquid content. Red marks indicate non-extrudable samples.

Fig. 2 shows water loss during the drying process of the samples (control and samples with chitosan, with and without fibers) and the compressive strength of samples with chitosan after drying. Samples with chitosan (with and without fibers) showed water repellency, as can be seen in Fig. 3.

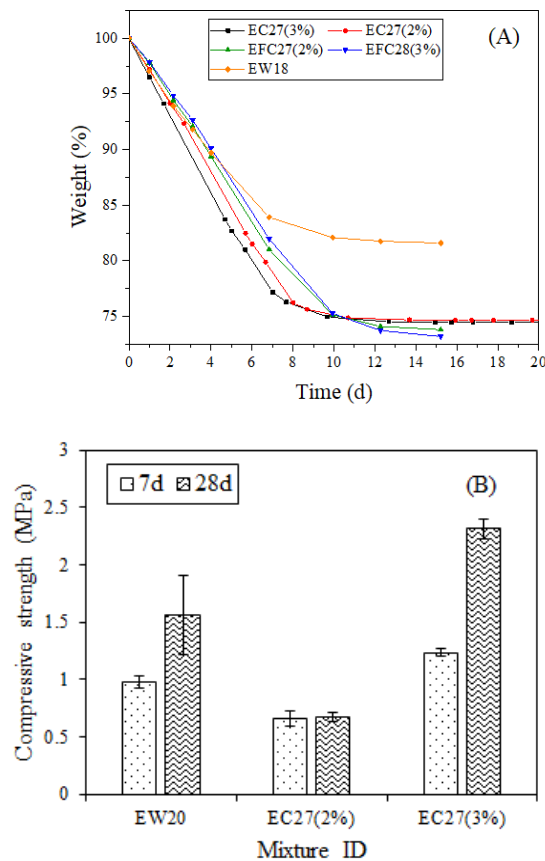


Figure 2. (A) Weight loss of samples during drying under ambient conditions. (B) Compressive strengths of samples after 7 and 28 days.

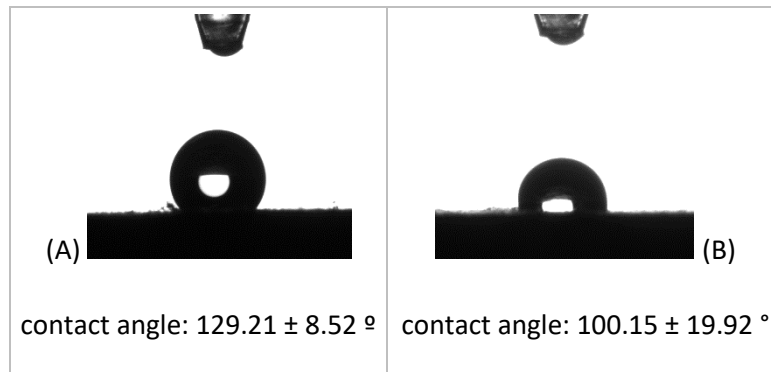


Figure 3. Drop of water over dried sample EC27(2%), A) when the drop was deposited on the surface and B) after 60 s.

## Discussion

### *Fresh mixture: Qualitative extrusion evaluation, shear vane and cylinder stability tests*

The extrudable limits of mixtures with various liquid contents were qualitatively determined (Table 1), mixtures with 1% higher and lower liquid contents were also examined. Samples with 1% chitosan solutions did not form continuous nor uniform filaments and, therefore, were discarded.

As expected, Fig. 1-A shows that a low liquid content is related to a higher static yield strength, while, on the other hand, a low liquid content is related to a lower cylinder stability settlement (Fig. 1-B). An inverse relationship between static yield strength and cylinder stability settlement becomes apparent. Although a low liquid content seems to be better to extrude continuous and uniform filaments because of low settlements, this also increases the static yield strength until the mixture is no longer extrudable (red marks). High liquid content is also undesirable because it results in a high settlement (higher than 30 mm). Unreinforced matrices have similar static yield strengths: EW20 with EC27(2%, 3%) and EW21 with EC27(2%, 3%). Also, unreinforced matrices EC(2%) and EC(3%) behave similarly in both, the shear vane and the cylinder stability tests.

### *Drying process and hardened-state: Weight loss during drying, compressive strength and wetting resistance*

Water loss in the fresh samples is affected by the presence of chitosan and fibers, as can be seen in Fig. 2-A. Note that each mixture starts with a different water content that is reflected in its final weight loss. Sisal fibers are hydrophilic, therefore, they slow down water evaporation. Drying time for unreinforced samples is around 10-12 days; for fiber-reinforced samples, around 14-16 days; and for soil-water samples, around 12-14 days, under ambient conditions.

Fig. 2-B shows the significant effect of chitosan as a soil stabilizer on the compressive strength of the samples. When dried, the sample with high chitosan content, EC27(3%), improved its mechanical strength substantially to 2.31 MPa, compared to 1.56 MPa for the control, this improvement in mechanical strength with chitosan has also been

reported by Aguilar et al. (2017). The presence of fibers might also contribute to better mechanical properties of the samples.

A contact angle of a drop of water could not be measured for the control sample (soil+water) because water was absorbed immediately. As shown in Fig. 3, chitosan decreases the wettability significantly, the contact angles ranged between 112 and 138°, similar to what Donayre et al. (2018) reported. It should be noted that contact angles decreased with time. Water-resistance improvement is important for earthen constructions.

## Conclusions

The liquid content needed for mixtures with chitosan solutions is higher than with water alone (26-30% compared to 20%). And, as expected, a low liquid content causes a higher static yield strength but a lower cylinder stability settlement. Static yield strength and cylinder stability settlement have an inverse relationship, therefore, the liquid content at the extrudable limit must be an intermediate value between both properties.

Weight loss during drying is in accordance with the liquid content of mixtures. Fiber-reinforced samples dry slower than other samples due to the hydrophilic nature of the fibers. Mechanical strength increased substantially in samples with high chitosan content (almost 50%) when compared to the control sample. Furthermore, chitosan also reduces significantly the wettability of the samples making them more resistant to water.

## Acknowledgements

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# Nanoconjugates of metal and polymers with improved antimicrobial properties

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**Keywords:** antimicrobial agents; chitosan; dopamine; nanoconjugates; nanoparticles; silver

**Abstract.** Multidrug-resistant pathogenic microorganisms have become a serious threat to public health making it difficult to prevent, treat and fight infections. This drug resistance is often associated with the misuse of antibiotics [1]. Silver nanoparticles (Ag-NPs) arise as potential antimicrobial agents for their microbicidal effects against a wide range of pathogens [2]. Besides, Ag-NPs can cooperate with other biomolecules like polymers with antimicrobial properties, to increase their biocidal activity. Among these polymers, dopamine (DA) and chitosan (CH) could be good candidates to be combined with silver, since DA presents great adhesive properties and strong biocompatibility and CH is a natural polymer with antibacterial, antifungal and antiviral properties, whilst being non-toxic to human cells, biocompatible and biodegradable making it excellent fits for numerous applications [3,4].

Hence, this work presents the first steps towards the formulation and characterization of nanoconjugates based on CH and DA with an Ag-NPs core (Ag-NPs+CH/Ag-NPs+DA). These conjugations are intended to enhance the antimicrobial properties of each material alone, producing a more effective antimicrobial effect. After the formulation of nanoconjugates by electrostatic interactions, different characterization methodologies were applied: UV-visible spectra to trace the efficiency of Ag-NPs coating with CH/DA; Scanning electron microscopy (SEM) to analyze the nanoparticles diameter across the coating, and Fourier transformed infrared spectroscopy (FTIR) to chemically verify the Ag-NPs coating with CH/DA. Additionally, the antibacterial effects of these nanoconjugates against the Gram-positive *Staphylococcus aureus* (*S. aureus*) and the Gram-negative *Escherichia coli* (*E. coli*) bacteria, as well as the antifungal effect against *Candida albicans* (*C. albicans*) were also assessed and compared do Ag-NPs, CH and DA alone.

In general, the results revealed that it was possible to conjugate the Ag-NPs with both polymers: UV-vis spectra measurements showed the decrease of the silver peak when covered with CH and DA; by SEM analysis an increase in size was observed from 10 nm (Ag-NPs) to a mean diameter of 123 nm (Ag-NPs+CH/Ag-NPs+DA); and in FTIR analysis the correspondence between peaks was seen, being further confirmation of the CH and DA coating in the Ag-NPs surface. Additionally, from the antimicrobial evaluation, it was possible to observe that the Ag-NPs+polymer nanocomplexes presented better results than the Ag-NPs alone.

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# Development of Novel Electroactive Nanofibers for Osteochondral Tissue Engineering Applications

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**Keywords:** Electrospinning; Electroconductivity; Osteochondral Tissue Engineering; Polyacrylonitrile; PEDOT:PSS

**Abstract.** Osteochondral tissue (OCT) related diseases, particularly osteoarthritis, are among the most prevalent in the adult population worldwide. However, no satisfactory clinical therapies have been developed so far to address this issue. Osteochondral tissue engineering (OCTE) strategies involving the fabrication of OCT-mimicking scaffold structures, capable of temporarily replacing damaged tissue and promoting its regeneration, are currently under development. While the electrical properties of the OCT (dielectric and piezoelectric properties) have been extensively reported in different studies, they keep being neglected in the design of novel OCT scaffolds, which tend to focus primarily on the tissue's mechanical properties. Therefore, the aim of this study was to bridge this gap in the literature by developing electroactive polyacrylonitrile/poly(3,4-ethylenedioxythiophene):polystyrene sulfonate (PAN/PEDOT:PSS) nanofibers via electrospinning capable of not only emulating the native tissue's fibrous nature but also its electroconductivity. The resulting nanofibers were modified through different post-processing techniques to improve their electrical properties and were functionalized with apatite-like structures to mimic the inorganic phase of bone extracellular matrix (ECM).

## Introduction

The OCT is an important interfacial tissue present at the end of long bones responsible for facilitating joint movement and supporting the loads being applied within the joint space. The OCT is constituted by two specialized forms of connective tissue – articular cartilage (AC) and subchondral bone (SB) – with distinct biochemical, mechanical, structural and electrical properties. As a result, the OCT is comprised by multiple gradients within its heterogenous structure that shift between its cartilaginous and osseous layers (Ansari, Khorshidi and Karkhaneh, 2019).

The electrical properties of the OCT have been extensively described in the literature. The AC's electrical properties are mostly related with the presence of negatively charged proteoglycans in the cartilage ECM, which generate different electrical potentials. The

predominance of type II collagen on cartilage ECM is also responsible for this layer's piezoelectricity. On the other hand, the bone tissue is characterized primarily by its dielectric and piezoelectric features that stem from the presence of hydroxyapatite and type I collagen, which are main components of the inorganic and organic phases of bone ECM, respectively (Barbosa, Ferreira and Silva, 2022). Overall, these electrical properties have a significant impact on tissue function, being involved in tissue regeneration mechanisms and modulating cell behavior (da Silva *et al.*, 2020).

Osteochondral diseases, particularly osteoarthritis, have a significant prevalence among the adult population worldwide. Current clinical treatments have failed to properly address this major healthcare issue, given their poor efficacy and long-term outcomes (Gorbachova *et al.*, 2018). For this reason, OCTE strategies focusing on the development of scaffolds for replacing damaged OCT and promoting its regeneration have been gaining interest. Despite the promising potential of developing electroactive scaffolds capable of mimicking the native electrical properties of the OCT as well as providing direct electrical stimuli to damaged tissue, promoting its regeneration, this strategy has been overlooked in current OCTE settings (Zhou *et al.*, 2020).

The aim of this study was to develop PEDOT:PSS-based electroconductive nanofibers via electrospinning capable of mimicking some of the main electrical, structural and compositional features of the OCT's fibrous ECM. Given the transitional quality of these properties within the OCT, the focus was placed solely on developing scaffolds addressing the SB layer of the OCT. The main physicochemical features of these fibers were analyzed. The ability of the electrospun fibers of supporting mesenchymal stem cell (MSC) proliferation was evaluated.

## Methods

### 1. Fabrication of PAN/PEDOT:PSS Nanofibers via Electrospinning

**Electrospinning Casting Solution Preparation.** Doped PEDOT:PSS solutions were prepared by adapting the protocol described by Lu *et al* (2019) (Lu *et al.*, 2019). The resulting annealed doped PEDOT:PSS pellets were grinded into a powder which was, in turn, dispersed in DMF:DMSO (9:1) and DMF:Cyrene (9:1). Solutions with different PEDOT:PSS concentrations were produced: 1%, 2%, 3% and 5% (w/v). The resulting solutions were agitated for at least 5 days. Before electrospinning, PAN was added at a fixed concentration of 10% (w/v) and the mixtures were heated in a hot plate for approximately 20 minutes at 85°C.

**Electrospinning Parameters.** The following operational conditions were used to produce the PAN and PAN/PEDOT:PSS nanofibers: Voltage - 20 kV; Working Distance - 25 cm; Flow Rate - 1 mL/h; Needle - 21G; Temperature – 20–25°C; Humidity: 35–55%.

**Fiber Post-Processing: Heat and Sulfuric Acid Treatment (HAT).** The resulting electrospun PAN/PEDOT:PSS fibers were doped with sulfuric acid. First, the fibers were placed in an oven at 210°C for 24 hours. After cooling, the fibers were positioned in glass plates. The fibers were then immersed in sulfuric acid for 30 minutes. Afterwards, the acid was removed from the glass plates, which were placed in hot plates at 130°C for 30 minutes. Several washes with distilled water were then performed.

**Mineralization of Post-Processed Fibers.** After being heat treated and doped with acid (HAT), the PAN/PEDOT:PSS fibers were also mineralized. Two different mineralization strategies were considered. One of the strategies, involved the immersion of the fibers in a PBS solution supplemented with 100 mg/L of calcium chloride pellets (Yang *et al.*, 2018). The second mineralization technique involved the immersion of the fibers in simulated body fluid (SBF).

## 2. Characterization of PAN/PEDOT:PSS Electrospun Nanofibers

**Scanning Electron Microscopy (SEM).** The structural characterization of the scaffolds was performed using a field emission gun SEM (FEG-SEM) (Model JSM-7001F; JEOL). Prior to imaging, the samples were mounted on a holder using carbon tape and were coated with a 30 nm gold/palladium (60:40) layer (Model E5100 Sputter Coater; Polaron/Quorum Technologies). The average fiber diameter of the different fibrous mats was computed using the ImageJ software.

**Attenuated Total Reflectance Fourier-Transform Infrared (ATR-FTIR) Spectroscopy.** The ATR-FTIR analysis was performed using a Spectrum Two FT-IR Spectrometer (Perkin-Elmer). Transmittance spectra were obtained over the spectral region from 400  $\text{cm}^{-1}$  to 4000  $\text{cm}^{-1}$ , with a resolution of 4  $\text{cm}^{-1}$ .

**Contact Angle.** The contact angle of the electroconductive nanofibers was quantified by a DSA25B goniometer (Kruss) using the sessile drop method with distilled water.

**Four-Point Probe Electroconductivity Measurements.** Four 50 nm thick gold stripes were deposited on the surface of PAN/PEDOT:PSS fibers using a thermal evaporator (Model E306A; Edwards). The electroconductivity was measured using the 4-point probe method using a current source Keithley DC power source (Keithley Instruments) and a multimeter (Model 34401A; Agilent Technologies). Experimental data was collected and processed using the LabVIEW 7.1 software.

**3. In Vitro Assays.** Previously expanded BM-MSCs (Lonza), were seeded on the fibers at a density of 100,000 cells per scaffold. Cell culture was conducted for a 7-day period using standard cell culture growth media, which was exchanged every 2 to 3 days. The proliferation of the BM-MSCs on the surface of the electroconductive scaffolds was evaluated using the Alamar Blue assay.

## Materials

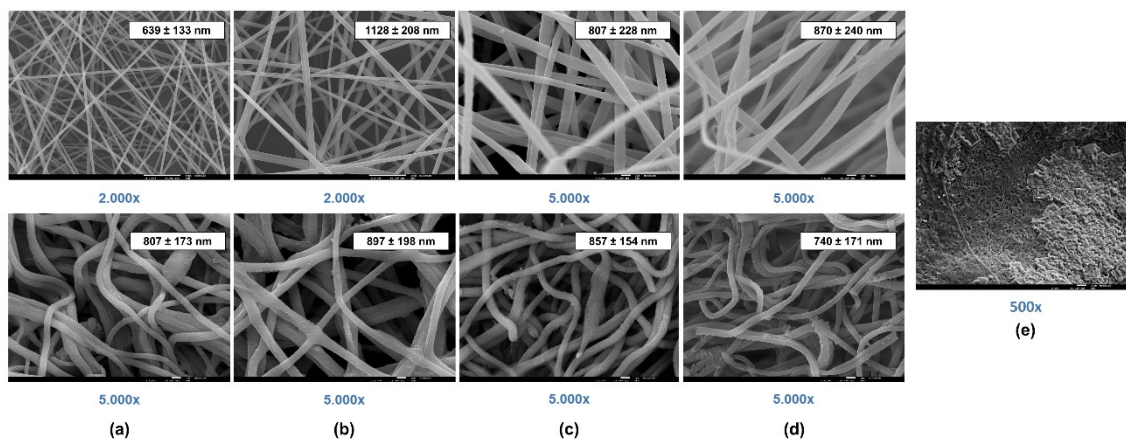
PAN (MW 200,000 Da) was acquired from Polysciences. PEDOT:PSS (Clevios PH 1000) was purchased from Heraeus.

## Results

Different amounts of DMSO doped PEDOT:PSS pellets were dispersed in DMF:DMSO (9:1) and DMF:Cyrene (9:1) to study the effect of the concentration of the conductive polymer on the overall conductivity of the PAN/PEDOT:PSS fibers. The solutions with 5% (w/v) PEDOT:PSS, and the DMF:Cyrene (9:1) based solutions with 2% and 3% (w/v) PEDOT:PSS were found to be non-electrospinnable. All other conditions were able to produce fibers and their main physicochemical features were analyzed (**fig. 1**).

The as-spun PAN/PEDOT:PSS scaffolds were then subjected to a PAN stabilization heat treatment to develop fibrous structures resistant to the subsequent sulfuric acid treatment used to further improve their conductivity. As intended, the applied HAT procedure was able to generate conductive scaffolds, which maintained their fibrous architecture (**fig. 1**).

As a proof of concept for the production of bioactive and electroconductive PAN/PEDOT:PSS nanofibers appropriate for mimicking the bone region of the OCT, HAT treated PAN/PEDOT:PSS fibers comprised by 1% (w/v) PEDOT:PSS and electrospun from casting solutions with DMF:DMSO (9:1) were mineralized using two distinct mineralization strategies: SBF mineralization was selected as the most



**Figure 1** SEM images of the different as-spun (top) and HAT treated (bottom) PAN/PEDOT:PSS fibers comprised by 1% (a), 2% (c) and 3% (d) (w/v) DMSO doped PEDOT:PSS (DMF:DMSO (9:1)), and PAN/PEDOT:PSS fibers comprised by 1% (b) (w/v) DMSO doped PEDOT:PSS (DMF:Cyrene (9:1)). The magnification of each SEM image is presented on the bottom of each corresponding image. SEM image of SBF mineralized HAT treated PAN/PEDOT:PSS fibers (e) comprised by 1% (w/v) DMSO doped PEDOT:PSS.

appropriate functionalization strategy (**fig. 1 (e)**).

ATR-FTIR analysis of the as-spun fibers enabled the detection of PAN and PEDOT:PSS. Important shifts in the ATR-FTIR spectra of the PAN/PEDOT:PSS fibers were observed with HAT treatment: significant PSS loss was observed, while important modifications to the pristine PAN chemical structure occurred, namely cyclization. The addition of PEDOT:PSS to the PAN casting solutions was found to largely improve the wettability of the fibers. While all as-spun fibers and HAT treated PAN fibers were found to be non-conductive, most HAT treated PAN/PEDOT:PSS fibers were conductive: the HAT treated PAN/PEDOT:PSS fibers with 1% (w/v) PEDOT:PSS electrospun from DMF:DMSO (9:1) yielded the highest conductivity.

Successful BM-MSC proliferation was observed for all experimental conditions tested, particularly for the mineralized fibers.

## Discussion

The obtained results appear to suggest that not only the addition of the conductive polymer, but also the HAT treatment are essential conditions for producing conductive PAN based fibers. While most studies reported increased conductivity with increased PEDOT:PSS presence, we obtained the highest conductivity for the fibers with the lowest PEDOT:PSS concentration: this could be related with a potential loss of PEDOT:PSS during the water washes (lixiviating effect after HAT treatment-mediated migration of PEDOT:PSS to the fiber surface). Successful mineralization of electroconductive fibers with SBF appears to confirm the ability of these scaffolds of being mineralized in an *in vivo* setting, an important feature for bone biomimetic substitutes. While HAT treatment appeared to negatively impact the mechanical properties of the fibrous scaffolds, which

became brittle, the contact angle of the scaffolds remained low, which is a good property for TE related applications, since it facilitates cell adhesion. As expected, increased MSC proliferation was obtained for the mineralized scaffolds given their improved bioactivity. The addition of PEDOT:PSS to PAN based casting solutions did not yield significant improvements to the biological features of the resulting HAT treated nanofibers. Future work will include periodical electrical stimulation of the PAN/PEDOT:PSS scaffolds during cell culture.

## Summary

In this work, electroconductive PAN/PEDOT:PSS fibrous scaffolds capable of mimicking some of the main electrical, structural and compositional features of the SB region of the OCT were successfully fabricated. Besides being conductive, the generated fibers were found to be highly hydrophilic and brittle. The mineral coated scaffolds displayed improved biocompatibility, which translated to an augmented MSC proliferation. The generated fibers could potentially be integrated in an OCT hierarchical scaffold, envisaging novel therapies for the treatment of OCT defects.

## Acknowledgements

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# Boosting the osteogenic potential of additive manufactured polycaprolactone scaffolds through functionalization with cell-derived extracellular matrix

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**Keywords:** Additive Manufacturing; Bone Tissue Engineering; Cell-derived Extracellular Matrix; Mesenchymal Stem/Stromal Cells; Polycaprolactone Scaffolds

**Abstract.** The demand for novel strategies for bone regeneration is growing due to the increase of the number of non-union fractures and ineffective healing in an aging population. In this work, we present a method to improve the biological performance of additive manufactured (AM) bone tissue engineering (BTE) scaffolds through their decoration with human mesenchymal stem/stromal cell (MSC)-derived extracellular matrix (ECM) produced in situ. The successful functionalization of 3D porous polycaprolactone (PCL) scaffolds with MSC-derived ECM (PCL-MSC ECM) was confirmed after decellularization using scanning electron microscopy and immunofluorescence/elemental analyses. Additionally, when re-seeded with MSCs, PCL-MSC ECM scaffolds showed significantly improved cell proliferation and osteogenic differentiation potential in comparison to pristine PCL scaffolds. Overall, our findings highlight the potential of combining bioactive/osteoinductive cell-derived ECM and AM patient-tailored scaffolds towards the development of new personalized BTE strategies to address the clinical demand for high quality tissue-engineered bone.

## Introduction

The growing clinical demand for bone tissue implants observed in recent years due to the increased number of non-union fractures that require medical intervention in an aging population combined with the side effects and scarcity of bone grafts motivated the search for new solutions for bone repair (Holmes, 2015). Bone tissue engineering (BTE)

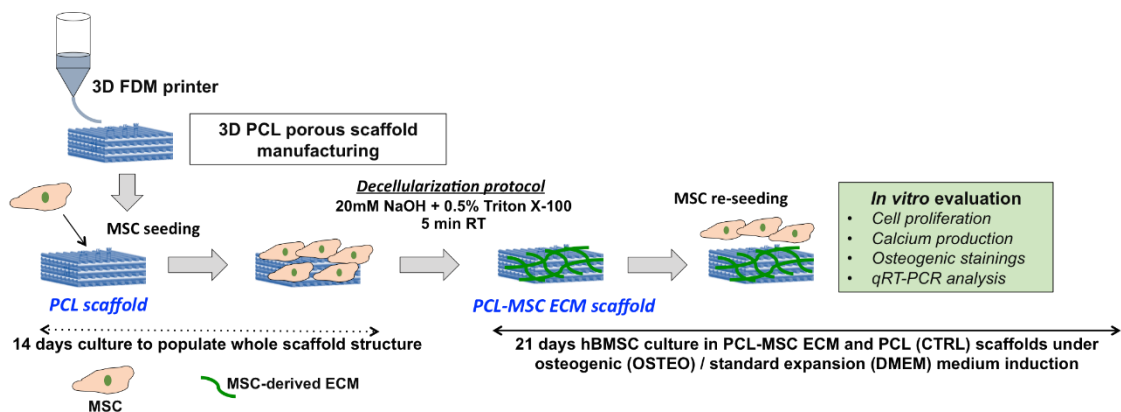


emerged as a promising strategy to generate new functional bone tissue by combining cells (stem cells or osteoprogenitors), 3D biomaterial scaffolds and differentiation inducing factors. Additive manufacturing (AM) techniques such as fused deposition modeling (FDM) have been widely explored in BTE strategies due to their ability to produce scaffolds in a fast, reproducible and scalable manner, and with a precise control over the scaffold's structural features (e.g., porosity and mechanical properties). Moreover, AM technologies are fully complied with the more recent trends of personalized BTE (Roseti *et al.*, 2015).

AM-based scaffolds are often produced using biocompatible and biodegradable synthetic polymers such as polycaprolactone (PCL). PCL, which was previously approved by US FDA for clinical use, has been widely used in TE due to its facile processing and favorable mechanical, thermal and chemical properties (Silva *et al.*, 2021; Woodruff & Hutmacher, 2010). Nevertheless, PCL lacks bioadhesive/bioactive motifs, which might impair its successful application in BTE strategies. Therefore, despite strategies focusing on the modification of the scaffold surface with extracellular matrix (ECM) individual components (e.g., collagen, fibronectin) have obtained positive results, such proteins are difficult to process and are still limited to recapitulate the complexity of native bone ECM (Bracaglia & Fisher, 2015).

Decellularized cell-derived ECM appeared as a promising alternative to enhance the bioactivity of synthetic scaffolds as it serves as a more complete reservoir of multiple growth factors/cytokines and provides a closer mimicry of the chemical and physical cues occurring in the bone microenvironment *in vivo*. In fact, our group has previously demonstrated that decellularized ECM from mesenchymal stem/stromal cells (MSC) deposited in standard polystyrene cell culture plates is able to efficiently promote the proliferation and osteogenesis of MSCs (Carvalho *et al.*, 2019).

The aim of this study was to develop FDM-based 3D porous PCL scaffolds with enhanced bioactivity/osteogenicity through functionalization with MSC-derived ECM produced *in situ*. We hypothesized that by providing a scaffold with controlled architecture (high porosity and interconnectivity) and good mechanical support that contains bone *in vivo*-like environmental cues from MSC-derived ECM, we could generate an advanced *in vitro* platform to improve the outcomes of BTE strategies. The PCL-MSC ECM scaffolds were characterized in terms of their structure and presence of ECM components. In addition, their capacity to improve the proliferation and osteogenic differentiation of re-seeded MSCs was evaluated in comparison to pristine PCL scaffolds (Silva & Carvalho *et al.*, 2020).



**Figure 1** Schematic representation of the experimental plan for the fabrication of decellularized PCL-MSC ECM scaffolds and assessment of their capacity to improve the

proliferation and osteogenic differentiation of hBMSCs (adapted from Silva & Carvalho *et al.*, 2020).

## Methods

**Fabrication of 3D extruded porous PCL-MSC ECM scaffolds:** PCL scaffolds with a 0-90° lay-down pattern were fabricated in a layer-by-layer approach using the Bioextruder, an in-house developed FDM equipment, as previously reported (Silva *et al.*, 2017). After sterilization, PCL scaffolds were seeded with human bone marrow mesenchymal stem/stromal cells (hBMSCs) and cultured for 14 days in standard cell culture growth media to promote cell proliferation and migration through the entire scaffold structure. Afterwards, the cell-scaffold samples were decellularized (PCL-MSC ECM) by exposure to a 20 mM ammonium hydroxide + 0.5% Triton X-100 solution following a previously published protocol (Kang *et al.*, 2012).

**Characterization of PCL-MSC ECM scaffolds:** The efficiency of the decellularization protocol was assessed by scanning electron microscopy (SEM), energy dispersive X-ray (EDX) analysis and fluorescence microscopy upon DAPI/Phalloidin staining (before and after sample decellularization). Moreover, the presence and distribution of key ECM protein components (fibronectin and laminin) on the decellularized PCL-MSC ECM scaffolds was evaluated by immunofluorescence analysis.

**Assessment of the *in vitro* biological performance of PCL-MSC ECM scaffolds:** PCL-MSC ECM and pristine PCL scaffolds were seeded with 100,000 hBMSCs per scaffold and cultured for 21 days both under standard expansion media and osteogenic differentiation induction media. The culture media was fully renewed twice a week. The proliferation of hBMSCs on the different scaffold experimental groups was evaluated on days 1, 7, 14 and 21 using the AlamarBlue® cell viability reagent following the manufacturer's guidelines. The morphology of the cells as well as the mineral deposition after 21 days of culture on PCL-MSC ECM and PCL scaffolds under the different culture media conditions was observed by SEM. EDX analysis was performed to identify the presence of mineral elements on the final constructs. The osteogenic differentiation of hBMSCs in the different scaffold experimental groups was assessed qualitatively by the typical osteogenic stainings (alkaline phosphatase (ALP)/Von Kossa and Xylenol Orange) at day 21, and quantitatively through calcium content quantification assay (days 14 and 21) and by RT-qPCR analysis of typical bone-related gene markers (collagen type I (*COL I*), *Runx2*, *ALP* and osteopontin (*OPN*)) evaluated at the end of the experiment (day 21).

## Materials

The 3D-extruded porous scaffolds were fabricated in PCL (MW 50,000 Da, CAPA™) acquired from Perstorp Caprolactones UK Ltd (Warrington, UK).

## Results

The morphology and presence of MSC-derived ECM on the surface of PCL scaffolds was clearly confirmed by SEM and EDX analysis. The EDX spectra of PCL-MSC ECM scaffolds presented a nitrogen peak, which was not observed in the spectra of pristine PCL. Moreover, the positive staining for fibronectin and laminin observed for the PCL-MSC ECM scaffolds demonstrates the efficiency of the decellularization protocol adopted. The presence of ECM on the scaffolds (PCL-MSC ECM) promoted significantly the proliferation of hBMSCs in comparison to the pristine PCL scaffolds when cultured both under standard expansion and osteogenic differentiation conditions. Despite the

mineralization of the scaffolds was observed in all conditions, it was much more significant on the PCL-MSC ECM scaffolds cultured under osteogenic induction. RT-qPCR analysis showed the positive effect of MSC-derived ECM on the upregulation of osteogenic marker genes (*COL 1*, *ALP*, *Runx2*) both under standard expansion and osteogenic induction conditions. Importantly, a significant enhancement in the expression of *OPN* gene was only observed when hBMSCs were cultured on PCL-MSC ECM scaffolds under osteogenic differentiation conditions.

## Discussion

Our results demonstrated that PCL-MSC ECM scaffolds considerably improved the osteogenic differentiation of hBMSCs, and therefore, are highly promising for use in personalized BTE strategies. The successful decoration of the PCL scaffolds with MSC-derived ECM was clearly shown by SEM/EDX and immunofluorescent staining of typical ECM proteins fibronectin and laminin. However, these proteins were not homogeneously spread along the scaffold structure, which is in accordance with the work from Kim and colleagues that reported a similar observation for fibronectin distribution in human lung fibroblasts-derived ECM coated polymer mesh scaffolds (Kim *et al.*, 2015). PCL-MSC ECM scaffolds improved significantly the attachment and proliferation of hBMSCs in comparison to PCL scaffolds both under standard growth and osteogenic induction conditions. These results are in line with previous studies that have also reported increased cell growth resulting from the incorporation of cell-derived ECM in synthetic polymer scaffolds (Kim *et al.*, 2015; Noh *et al.*, 2016). Overall, our data suggest a positive role of MSC-derived ECM decorated PCL scaffolds on the osteogenic differentiation of hBMSCs as evidenced by the expression of bone-specific marker genes, ALP activity, calcium production, osteogenic stainings and SEM/EDX analysis. Notably, it was observed that hBMSC osteogenic differentiation was improved by a synergistic effect of PCL-MSC ECM scaffolds and osteogenic induction medium, which was clearly evidenced by elevated calcium contents and upregulation of the osteogenic genes, especially of *OPN*, which is produced at the late stages of osteoblastic maturation (Denhart & Guo, 1993) and plays a key role on mineralization (Zurick *et al.*, 2013).

## Conclusions

In summary, we successfully developed a methodology to produce 3D porous MSC-derived ECM coated PCL scaffolds with defined architecture, proper mechanical properties and enhanced bioactivity/osteogenicity. The proposed strategy of combining AM technologies with decellularized ECM matrices holds great promise for BTE applications as it fosters the fabrication of “patient defect-tailored” scaffolds with an improved biological performance as a result of a more reliable mimicry of the *in vivo* bone niche.

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# CIRCULARSEAS, the next challenge for Plastics

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**Keywords:** plastics, microplastics, ocean pollution, reuse and recycling, 3d printing

## Abstract.

The 20th century was dominated by the explosion of the development of plastics. Walker has described polymer based materials as the building blocks of the 20th century [1]. The creativity and ingenuity of polymer chemists to prepare polymers tailored for particular applications ranging from the safe transport of electricity and water, through affordable and durable household goods and lightweight cars to applications in medicines, coupled with development of high throughput fabrication technologies such as film blowing, fibre spinning, and injection molding have powered these developments. In the late twentieth century, there was a growing realization of the downside of widely available durable plastic goods. There are two strands to this downside. The first is focused on the impact of the production, use and disposal of plastics on the atmosphere, in particular the contribution to Global Warming and Climate Change. It is interesting to note that Global Warming and Plastics have a rather similar timeline in terms of scientific discovery. The start of the plastics revolution is associated with the work of Baekeland in developing Bakelite [2] and not long afterwards Staudinger published his groundbreaking work on the concept of long chain polymers [3,4]. At the start of the 20th Arrhenius had highlighted in his Nobel Laureate Address that carbon dioxide may play a part in fixing the temperature of the earth [5]. In 1938 Guy Callendar published a paper “The artificial production of carbon dioxide and its influence on temperature”, he also attempted to attribute the rise in global temperatures in the first part of the century to increase levels of carbon dioxide [6]. In the 1950s there were great strides in modelling the atmosphere of the planet Earth and by 1975 Wallace Broecker published a scientific paper titled “Are We on the Brink of a Pronounced Global Warming?” [7]. This introduced the phrase “Global Warming” for the first time which led to an acceleration in the focus on climate change. In 1988 the Intergovernmental Panel on Climate Change was launched at the United Nations, and it has been publishing reports ever since then and it is now its sixth assessment exercise to inform the 2023 Global Stock take by the UN on progress towards the Paris Accord to limit Global Warming to 2.0 °C [8]. A recent publication by Cabernard et al. [9] shows that carbon dioxide equivalent emissions from plastic production and use continue to grow and in 1995 they contributed 4.5% of all emissions. There is much public call for the reduction in the use of plastics, but in part the reduction in carbon dioxide equivalent emissions can be reduced by reversing the shift to coal-based power and move towards the use of clean energy such as direct solar power. Although much attention has been directed at reducing the carbon footprint, the last third of the

20th Century saw the revelation of a major catastrophe for the oceans. In the late 1960s the first evidence for marine pollution by plastics was reported in the scientific literature [10], in the form of plastic parts recovered from the stomach of an albatross. This was followed by much anecdotal evidence but Thompson et al. [11] revealed the extent of plastic pollution of the world's oceans. We now know that this is not just restricted to the plastics island in the Pacific Ocean, but the micro-remnants of plastic degradation are everywhere and form part of the food chain of the oceans and therefore of humans. It is claimed that "Without significant action, there may be more plastic than fish in the ocean, by weight, by 2050" [12]. Whereas the challenge of the emissions from the production and use of plastics has led to a number of solutions, the plastic waste in the world's oceans seems an insurmountable challenge. We estimate the volume of the oceans as  $1.3 \times 10^{21}$  litres [13]. The most powerful of filtration systems can process  $57 \times 10^6$  litres/day meaning a filtration time of  $6 \times 10^{11}$  years. To reduce this to a single person's lifetime means  $8 \times 10^9$  filtration systems! This gives rise to huge material and power requirements.

Plastics are not the direct cause of this pollution, human beings are. We assert that the route to begin reducing pollution is to increase the value of recycled plastics so as to make such materials highly valued items. To add value to recycled waste requires the use of innovative product design and material processing technology. 3d printing has the ability to meet such demands [15]. Figure 1 shows some of the steps in the transformation of marine waste to marine products by 3d printing. The catamaran (0.5m length) shown here is a prototype prepared using 3d printing technology, the final product will be produced from recycled marine waste and will be 3m in length. The large size of the product underlines the volume of recycled material required. In summary, to limit the continued pollution of the oceans, we need to greatly increase the value of recycled plastics and developing manufacturing streams which involve significant volumes of recycled plastics. 3d printing can deliver the innovations required in design and fabrication to produce added value products



Figure 1: A graphical representation of the steps in transforming marine plastic waste into high value products. Left: Marine industry waste centre granulated waste. Right: A prototype catamaran 0.5m in length. The final 3d printed product will be 3m in length and manufactured from plastic waste.

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# Electrical Stimulating Regimes to Influence Stem Cell Proliferation and Differentiation for Tissue Engineering

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The growth of new bone tissue *in vitro* requires a variety of different factors that need to be controlled and optimised. One of these factors that has previously not been considered for bone tissue engineering is electrical stimuli. Given that bone is piezoelectric in nature, it is feasible to assume that local electrical regimes have an effect on osteogenesis. There are clinical products currently on the market that deliver electrical currents locally via a cathode to fracture sites. These products demonstrate significant clinical improvements in bone repair.

We have recently designed a variety of different bioreactors to both house the developing tissue and also control the applied electrical stimuli in either capacitive or direct contact methods to *in vitro* cultures. These bioreactors have enabled us to assess the potential use of this stimuli for *in vitro* bone tissue engineering purposes. It has also allowed us to further study the mechanism by which the activity of primary human mesenchymal stem cells are altered both in terms of cell proliferation and differentiation. A novel finding of the importance of the faradic by product of  $\text{H}_2\text{O}_2$  proximal to the cathode as result of the direct electrical stimulus will be presented and its subsequent role in influencing primary mesenchymal stem cell proliferation. The morphology of primary cilia on these cells after electrical stimulation has been applied will also be discussed, in addition to the effect of varying electrical regime on cell response. The use of conducting polymers and piezoelectric materials to apply electrical regimes to the cells will be discussed.

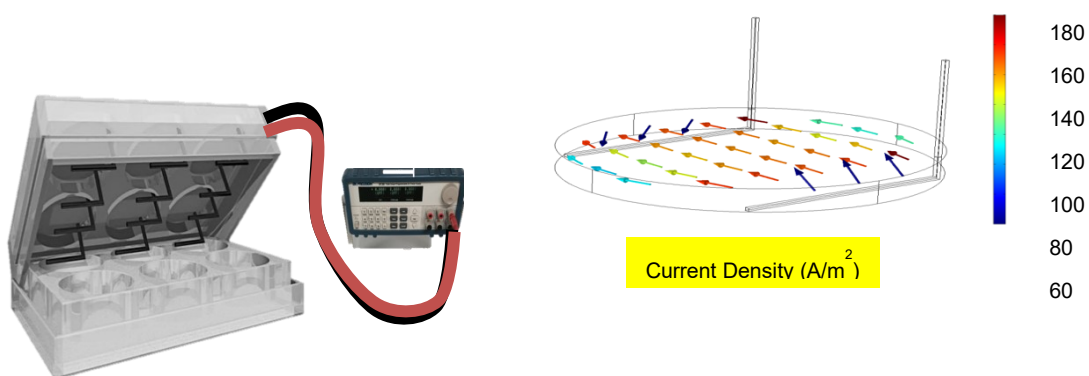


Figure1: LEFT Image demonstrating one of the direct electrode bioreactors; RIGHT steady state model of current density in in vitro set up.



# Numerical Modelling Impact in the Design and Development of Tissue Engineering Systems

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**Keywords:** Bioreactor; Mathematical Models; Digital Twins; Osteochondral Tissue Engineering; Curved Scaffolds

## Abstract.

Defects in the osteochondral (OC) tissue are a major cause of joint malfunction. Due to limitations of articular cartilage (the superficial layer of the OC tissue) to heal and regenerate, the defects tend to aggravate with time, leading to degenerative diseases like osteoarthritis, and loss in mobility with consequent difficulties performing daily activities. Research has led to the development of methods to repair OC defects, still none has been able to mimic native tissue properties and structure. More recently, tissue engineering (TE) has been showing promising results to overcome limitations of current therapies, and in particular extensive research has been devoted to scaffold design and manufacturing. The impact of using numerical models in TE applications is enormous helping in optimizing scaffolds and bioreactors designs and contributing to design guidelines for optimised experimental protocols, reducing the need of experimental work, time and money involved and helping in reducing or eliminating animals' tests. A limitation of current scaffolds is the shape disparity in relation to native tissue. As a first approach in the direction of creating more native like structures, a method to create scaffolds with a spherical shape was developed, aiming to reproduce the curvatures in the OC regions of the human femur. These scaffolds were manufactured by fused filament

fabrication (FFF) using poly (lactic acid), an FDA approved material. To assess the potential of using this technology to create scaffolds, a maximum printable curvature of the scaffold was determined, for a scaffold with a projected square side of 20.1000 mm, corresponding to use a sphere with a radius of 17.0638 mm as a template. Scaffolds with radius of 14.0000 mm, 17.0638 mm, and 20.0000 mm were fabricated and the structural integrity analysed by micro-computed tomography ( $\mu$ -CT), confirming the expected weaker structural integrity of the scaffolds with the 14.0000 mm and 17.0638 mm. Additionally, finite element analysis (FEA) was used to assess the behaviour of the scaffolds to compressive loads, with higher stresses being predicted in the contacts of scaffold fibres between adjacent layers. Considering these results there are promising prospects for the manufacturing of constructs mimicking the native curvature of OC tissue.

Bioreactors requires a complex multiparameter control process capable of delivering an adequate cellular environment to promote cell growth and differentiation into the desired tissue. To surpass difficulties on cell monitoring and to improve the prediction of the cellular surrounding environment, we propose a numerical framework involving a digital twin model of the bioreactor to better guide researchers, when choosing the environmental conditions, and adjusting their hypothesis to the real bioreactor system. This framework will also contribute to avoid protocol mistakes during the *in vitro* experiments and may help with the definition of the design, setting construction parameters like component sizes, channel dimensions, or input variables magnitude to reach the desired environment surrounding the region of interest for a particular cell type.

In this presentation we will discuss the potential of digital twins (numerical models) of electrical stimulation protocols explored considering scaffolds and bioreactors systems and its capacity of predict with a good degree of confidence the delivery of multiple electrical stimulation systems and the impact of scaffold properties in the delivery of electrical stimulation. Also, by running computer fluid dynamics models the environmental conditions generated by perfusion lab equipment are better understood and can be tuned to desirable levels of mechanical stimulation.

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# Development of a High-Density System for the Expansion of Human Induced Pluripotent Stem Cells as Aggregates in Single-Use Vertical-Wheel™ Bioreactors

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**Keywords:** human induced pluripotent stem cells; expansion; aggregates; Vertical-Wheel bioreactors; dextran sulfate

**Abstract.** Usage of human induced pluripotent stem cells (hiPSCs) for Regenerative Medicine applications requires their expansion to clinically-relevant quantities. The novel Vertical-Wheel bioreactors (VWBRs) allow for homogeneous mixing while conveying less shear stress to cells compared to traditional alternatives. This work reports strategies to establish and optimize expansion of hiPSCs as aggregates in VWBRs. Cultures were performed in the PBS MINI 0.1 bioreactor with 60 mL of working volume. Two different expansion media were tested, mTeSR1 and mTeSR3D, as well as dextran sulfate (DS) supplementation. The generated hiPSCs were analyzed by flow cytometry and qRT-PCR and their differentiation potential was assayed via EB formation and directed differentiation. A maximum cell density of  $(2.3 \pm 0.2) \times 10^6$  cells·mL<sup>-1</sup> was obtained after 5 days with mTeSR1+DS, resulting in aggregates with an average diameter of  $346 \pm 11$  μm, without loss of pluripotency. The results here presented suggest the VWBR as a promising technology for the development of hiPSC-derived products under Good Manufacturing Practices for biomedical applications.

## Introduction

The advent of human induced pluripotent stem cells (hiPSCs) in 2007 has taken the medical field by storm (Takahashi et al, 2007; Yu et al, 2007). These cells can self-renew indefinitely, generating identical copies of themselves, as well as differentiate into all cell types of the human body. Furthermore, they can be derived from the patient's own cells, avoiding many of the ethical issues commonly associated with human embryonic stem cells and avoiding rejection upon transplantation (Sayed et al, 2016). Overall, all of these

characteristics grant hiPSCs an immense potential for drug screening, disease modelling and even Regenerative Medicine (Rowe & Daley, 2019; Shi et al, 2017). Any of these applications, however, will require methods for controlled and reproducible expansion of hiPSCs at a clinical scale.

The extensive know-how on the usage of bioreactors for traditional biomanufacturing processes can be applied to stem cell bioengineering. Bioreactors provide a 3D, scalable and controlled environment for cell culture, potentially allowing to obtain the large cell numbers required for clinical applications (Zweigerdt, 2009). However, traditional stirred tank bioreactors are not suitable for the culture of shear-sensitive cells such as hiPSCs due to the high shear stress next to the impeller. For this reason, the novel Vertical-Wheel bioreactors (VWBRs), by PBS Biotech, have been developed (Fig 1A). These bioreactors employ a large, vertical impeller, which, along with the U-shaped bottom of the vessel, allow for an efficient mixing of the vessel contents with minimal power input and, consequently, minimal damage to the cells (Croughan et al, 2016). VWBRs have already been shown to sustain growth of hiPSCs attached to microcarriers (Rodrigues et al, 2018). However, an aggregate-based system would forgo external matrices and be potentially more compatible with Good Manufacturing Practices (GMP).

This work aimed at developing a high-density system for hiPSC expansion as aggregates in Vertical-Wheel bioreactors. Cell expansion was tested in a repeated batch and fed-batch format, and supplementation of dextran sulfate (DS), a molecule commonly used in the biopharmaceutical industry for aggregate size control, was also assayed (Lipsitz et al, 2018). The results obtained in this study show the viability of large-scale expansion of hiPSCs in VWBRs and were an important step for the development of hiPSC expansion, and, prospectively, differentiation protocols under GMP, envisaging their use for Regenerative Medicine.

## Methods

This work was performed using the F002.1A.13 hiPSC line (TCLab – Tecnologias Celulares para Aplicação Médica, Portugal). hiPSCs were maintained on Matrigel (1:100)-coated tissue culture plates, with daily mTeSR1 medium changes, and kept at 37 °C and 5% CO<sub>2</sub>. Cells were routinely passaged upon attaining 80% confluence at a split ratio of 1:4 and using EDTA.

Prior to bioreactor inoculation, cells were incubated for 1 h with mTeSR1 supplemented with 10 µmol·L<sup>-1</sup> ROCK inhibitor Y-27632, harvested with Accutase and mechanically dissociated to single cells with a micropipette. PBS MINI 0.1 bioreactors were seeded with  $1.5 \times 10^7$  cells in 60 mL of mTeSR1 or mTeSR3D seed medium with Y-27632. For repeated batch cultures, starting from 48 h post-inoculation, 80% of mTeSR1 medium was changed daily. For fed batch conditions, after 48 h of culture, 6.7 mL of feed medium were added daily, except for day 4, when the medium was replaced with fresh seed medium. When used, dextran sulfate (DS) was supplemented at day 0 at a concentration of 10 µg·mL<sup>-1</sup>. All bioreactor cultures were continuously maintained at a 30 rpm stirring speed. Culture sampling was performed daily for cell counting, aggregate measurement and supernatant analysis. After 7 days of culture, cells were collected for flow cytometry, qRT-PCR, immunocytochemistry and differentiation assays.

## Results

For all conditions, hiPSCs formed aggregates, which grew throughout the culture time (Fig 1B). A maximum cell density of  $(1.2 \pm 0.1) \times 10^6$  cells·mL<sup>-1</sup> was obtained using

mTeSR1. The fed-batch protocol using mTeSR3D led to a lower maximum cell of  $(8.8 \pm 1.6) \times 10^5 \text{ cells} \cdot \text{mL}^{-1}$ . Supplementation with DS was shown to increase the maximum cell density about two-fold for both media ( $(2.3 \pm 0.2) \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$  for mTeSR1+DS and  $(1.79 \pm 0.03) \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$  for mTeSR3D+DS) and to reduce the culture time required for this maximum cell density to be obtained (5 and 6 days, respectively). The different conditions did not significantly impact the maximum aggregate diameter obtained, which varied between conditions, from  $\sim 340$  to  $\sim 410 \mu\text{m}$ .

For repeated batch conditions a yield of lactate from glucose of about 1.6 was observed at the end of culture, indicating a prevalence of glycolysis, characteristic of proliferative cells. For fed-batch conditions, however, this yield was lower,  $\sim 1.4$  without and  $\sim 0.7$  with DS, suggesting a shift towards oxidative phosphorylation (OXPHOS).

Nevertheless, despite the possible metabolic shift, the cells showed a maintenance of expression of pluripotency markers and of differentiation potential throughout the expansion process. As such, the VWBRs do not compromise cell pluripotency throughout expansion.

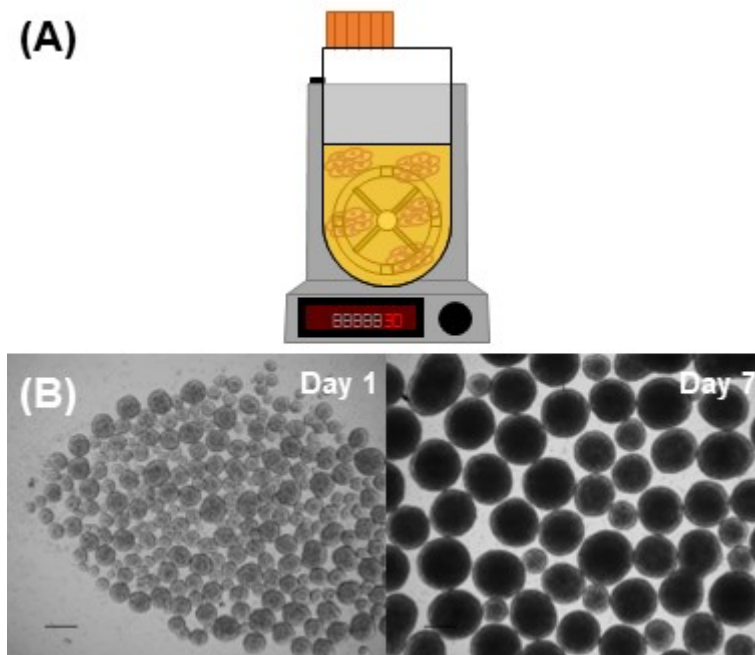


Figure 1 (A) Schematics of a PBS MINI 0.1 bioreactor system. (B) Brightfield images of hiPSC aggregates following 1 and 7 days of expansion in the PBS MINI bioreactor (scale bars =  $250 \mu\text{m}$ ).

## Discussion

This work aimed at establishing an aggregate-based culture system for expansion of hiPSCs in VWBRs. The cell densities obtained were in the range of values obtained in other studies, in spinner flasks (Hunt et al, 2014) and stirred tank bioreactors (Kropp et al, 2016), as well as microcarrier-based cultures in VWBRs (Rodrigues et al, 2018).

mTeSR3D was employed in a fed-batch regime as an attempt to mitigate the drastic variations (“zig-zag” profiles) of culture parameters observed in repeated batch cultures. However, this medium performed below mTeSR1 due to a low refresh rate of nutrients and metabolites. Furthermore, it may have caused some metabolic alteration of the cells.

DS has been commonly used in the biopharmaceutical industry due to its anti-apoptotic effect and for its aggregate size control due to surface charge modulation (Lipsitz et al,

2018). This molecule increased cell yield about two-fold without any apparent drawback, as such, it can provide an important advantage for large-scale production of hiPSCs.

## Conclusions

Expansion of hiPSCs at a clinical scale is still a bottleneck, however, bioreactors may be the solution. VWBRs were developed for shear-sensitive cells and may be the most appropriate systems for scalable culture of hiPSCs. This work is one of the first descriptions of the usage of the PBS MINI 0.1 bioreactor for hiPSC expansion as aggregates. Expanding the cells in mTeSR1 medium with DS allowed to obtain about 140 million cells in only 5 days of culture without compromising cell pluripotency. As such, this study provides compelling evidence for the use of VWBRs for the production of hiPSCs and their derivatives to be used for pharmacology and/or for Regenerative Medicine.

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# A Novel Decision Support Tool for Optimally Designing Large-Scale Stem Cell Expansion Bioprocesses

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**Keywords:** bioprocess economic modeling; decision support tools; stem cells; vertical-wheel bioreactors; dextran sulfate supplementation.

**Abstract.** Stem cells have played a leading role in the up-and-coming field of cell and gene therapy and are poised to revolutionize clinical practice. However, for their full potential to be unlocked, robust and cost-efficient stem cell expansion bioprocesses must be established. The design and development of such bioprocesses is naturally arduous and costly, making decision support tools capable of aiding aspiring manufactures in this endeavor indispensable. Bioprocess economic models, which constitute an *in silico* approach to bioprocess design, are a prime example of such tools. In this work, a novel bioprocess economic model is presented. To demonstrate its applicability, a case study was undertaken within the context of a large-scale human induced pluripotent stem cell expansion bioprocess carried out in vertical-wheel bioreactors. The case study consisted of evaluating the technical and economic impact of dextran sulfate supplementation on a default bioprocess. Dextran sulfate supplementation was estimated to reduce bioprocess duration from 43 to 30 days and cost by 38%. This case study demonstrates the usefulness of the presented model for bioprocess design and emphasizes the importance of implementing beneficial manufacturing elements in order to develop successful stem cell expansion bioprocesses.

## Introduction

Stem cells are currently at the vanguard of the emerging field of cell and gene therapy, which is expected to revolutionize the treatment of a variety of diseases and has been moving ever closer toward becoming an essential part of standard clinical practice in recent years (Wang et al., 2021). Their intrinsic ability to self-renew and to differentiate into other cell types, along with their highly proliferative nature, makes them the ideal instruments for a myriad of promising therapeutic applications, such as treating autoimmune, ophthalmic and cardiovascular diseases, kidney and neurological disorders and several forms of cancer, just to name a few (Levy et al., 2020; Deinsberger et al., 2020; Kim et al., 2021). However, a very large number of cells is required for most of



these applications to be implemented in actuality, often in the magnitude of several billion cells (Lechanteur et al., 2016; Kropp et al., 2017). Such vast quantities of cells are difficult to obtain with conventional protocols, which suffer from in-process heterogeneity and hefty costs (Colter et al., 2021). Because of this, the development of consistent and economically viable large-scale stem cell expansion bioprocesses, compatible with current good manufacturing practices, is of paramount importance (Lipsitz et al., 2016; Nath et al., 2020).

In designing said bioprocesses, manufacturing elements must be selected from a pool of available technologies and production strategies, with the right choice rarely being an obvious one. Ideally, these would be compared and validated experimentally so as to choose the elements best suited to a given bioprocess, but such an approach can quickly become overly cumbersome and expensive. As such, bioprocess design should be guided by an *in silico* modeling approach, capable of generating surrogate simulation data which can serve as a foundation for identifying *a priori* the most promising elements for further testing and optimization (Luo et al., 2021; Torres-Acosta et al., 2021). Should a certain bioprocess be carried out in planar culture platforms or bioreactors? How many stages of production would be required and how should they be organized? Which culture medium formulation should be employed? Is it feasible for the final product of the bioprocess to be autologous, or does cost-efficiency require it to be allogeneic? All of these are questions that must be carefully considered when designing a bioprocess, and bioprocess economic models are absolutely indispensable for providing early but well-grounded answers.

With this in mind, a novel bioprocess economic model was developed with the express purpose of aiding aspiring manufacturers to justify their investments and allocation of resources when designing a large-scale stem cell expansion bioprocess. This model is a versatile decision support tool which estimates the duration, necessary resources and costs associated with the bioprocess being modeled and suggests an optimized workflow for its practical execution. In this work, the usefulness of the model is demonstrated by exploring a case study performed within the context of a large-scale human induced pluripotent stem cell (hiPSC) expansion bioprocess intended to be part of the production pipeline of an autologous cell therapy product.

## Methods

The model herein presented was developed using the Python programming language. It was structured so as to receive a series of inputs based on which it computes a number of outputs. Examples of the model's inputs are the culture platforms chosen for the bioprocess being modeled, the culture medium to be used, the initial and target cell numbers and the biological variables relevant to the expansion and harvest of the cell type in question.

The main output of the model is an optimal workflow broken down into its several logistical and economic aspects. This optimal workflow indicates how many expansion stages are estimated to be required to produce the target cell number, what resources are required for each stage, how long each stage will last, how much each stage will cost and how the cell number evolves over time.

Notably, the model is stochastic in nature, performing Monte Carlo simulation. Thus, its output is accompanied by a confidence level which translates how likely it is that the suggested workflow will meet or exceed the target cell number when biological

variability is taken into account. It does this by simulating a large number of bioprocess runs and checking how many of these are terminated successfully.

The model simulates expansion bioprocesses founded on the concepts of serial passaging and gradual scale-up. In other words, an initially small cell population is converted into the target cell population by undergoing successive expansion stages in culture platforms of increasing magnitude.

To better evaluate the contribution of different factors toward the overall cost of a single production batch of the bioprocess being simulated, the model subdivides this value into four distinct categories, namely reagent, consumable, facility and labor costs. When calculating costs the model relies on a database with entries gleaned from the catalogues of suppliers of scientific materials and published case studies.

## Results

The model was employed to simulate a default large-scale expansion bioprocess with a minimum confidence level of 95% which sought to produce  $1.5 \times 10^9$  hiPSCs starting from  $1.0 \times 10^6$  hiPSCs, using cell culture plates and vertical-wheel bioreactors (VWBRs) as culture platforms and mTeSR1 as the culture medium. A variation of this default bioprocess where mTeSR1 was supplemented with dextran sulfate (DS) was then simulated.

The biological variables involved were based on previously published experimental results which tested the effects of DS on hiPSC expansion in VWBRs (Nogueira et al., 2019). DS is a simple chemical substance which reduces cell aggregation and exerts an anti-apoptotic effect. It has been shown to roughly double hiPSC fold expansion when added to the culture medium on the first day of bioreactor culture.

The model estimated that the execution of the default bioprocess would entail a batch cost of €32,220 and duration of 43 days, providing an optimal workflow consisting of 3 planar passages and 4 expansion cycles in VWBRs. Reagent costs were the predominant cost category, constituting 42% of the total cost, followed by facility, labor and consumable costs, which comprise respectively 35%, 12% and 11% of the total cost. The culture medium was identified as the major cost driver, being responsible for 39% of the total cost.

According to the model, the impact of DS supplementation on the default bioprocess is that one less expansion cycle is required and that each cycle can be 2 days shorter, reducing bioprocess duration by 13 days. This in turn leads to a substantial reduction across all cost categories, with reagent costs seeing the most significant decrease, now accounting for 36% of the total cost, compared to facility, labor and consumable costs, which contribute respectively 38%, 13% and 13% of the total cost. All in all, the overall batch cost drops from €32,220 to €19,821, a drop of 38%. The culture medium continues to be the major cost driver, being responsible for 32% of the total cost.

## Discussion

DS supplementation presents itself as an ideal strategy for reducing the batch cost of hiPSC expansion bioprocesses similar to the one simulated in the presented case study. Its anti-apoptotic effect and reduction of cell aggregation lead to higher bioreactor cell densities and faster cell growth rate, culminating in a higher cell fold expansion being obtained in shorter periods of time. Concomitantly, it exerts its beneficial effects while being supplemented at a concentration of 100 mg/L, thus incurring a negligible cost of a few euros per liter of culture medium.

The shorter bioprocess timeframe means that less resources are consumed throughout the bioprocess and that the facility and its staff are occupied for a shorter period of time, therefore reducing costs across all categories. Most crucially, less culture medium, which is the major cost driver, is expended, to the point that the reagent costs cease to be the main contributors to the total cost and are surpassed by the facility costs. Tackling the major cost drivers of a bioprocess is the best way to make it more economically viable, and DS supplementation does this by reducing overall culture medium consumption.

DS supplementation comes with few evident drawbacks, and none can be found from an economic perspective. One could eventually argue that DS might exert some unforeseen adverse effect on hiPSC quality. This possibility cannot be categorically discarded since DS has not been extensively applied to hiPSC culture. It has however been used in mammalian cell culture for some time and the experimental work on which the presented case study was based did not detect any apparent diminishment of hiPSC pluripotency when using DS.

## Conclusions

In this work, a novel bioprocess economic model was established and its applicability as a decision support tool for bioprocess design was demonstrated. This model was employed to perform a case study concerning DS supplementation in the context of a large-scale hiPSC expansion bioprocess, exemplifying how the presented model, or other equivalent tools, can be used to evaluate manufacturing elements *a priori*, thus supporting a manufacturer's decision making.

In terms of the results gleaned from the case study itself, the 38% cost reduction suggested by the model is a very significant decrease and is indicative that DS supplementation could represent a noteworthy step toward establishing large-scale hiPSC expansion bioprocesses which are both robust and cost-efficient.

Although stem cell expansion would certainly be one of the primary components of the production pipeline of any stem cell therapy product, it is important to remember that this pipeline would also include other unit operations, such as stem cell differentiation into specific lineages or downstream processing for final product formulation. Thus, one of the main aspects for the future improvement of the model will be to integrate these other unit operations, allowing it to simulate an integral bioprocess leading from cell-based raw materials to a finalized therapeutic product.

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# Digital Twins for Complex Manufacturing processes – Tissue Engineering Bioreactors

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## Abstract.

Tissue engineering for regenerative medicine to replace damaged or missing tissue is a very promising therapy to cope with the ageing population which is common place in most countries. Although this area of medical technology has much to offer, progress has not been as swift as initially expected and there remains a major challenge in optimizing the complex processes involved in the growth of replacement tissue. Digital Twin Technology in which a virtual model is developed to accurately reflect the physical and biophysical processes also offers much promise to the optimization of complex processes. In this work we explore the state of the art of both tissue engineering and digital twin technology and identify where development is required to enable such optimization. We identify the value of considering hybrid digital twin technologies as a practical solution in the case of complex systems.

## Introduction

A digital twin is a virtual model designed to accurately reflect a physical system. The object being studied is fitted with various sensors related to vital areas of functionality. These sensors produce data about different aspects of the physical object's performance. This data is then relayed to a processing system and applied to the digital copy. Once informed with such data, the virtual model can be used to run simulations, study performance issues and generate possible improvements, all with the goal of generating valuable insights — which can then be applied back to the original physical object. The first realisation of the concept of digital twins first introduced by [Grieves (2019)] and taken forward by NASA and relates to spacecraft [Pascik (2010)]. Since that time, the concept has flourished and now Digital Twins are being developed for all areas of science and technology, including for the planet earth [EMCWF (2022)]. In this work we focus on the challenges for the development of digital twins for the optimisation of a bioreactor used in tissue growth in the field of regenerative medicine [Geris et al (2018)]

Tissue engineering is a rapidly developing area [Langer (1993)] which seeks to produce new tissue to replace damaged, or missing tissue using an engineered scaffold which is either seeded with cells in an *in-vitro* environment and placed in a bioreactor with the

appropriate conditions for cell proliferation and differentiation and then transplanted in the patient [Rouwkema (2011)] or it is implanted in the patient (*in vivo*) and acts as a “getter” for cells within the patients body and a suitable environment for the cells to proliferated and differentiate [Abdulghani 2019].

## Methods

In Figure 1 we show a schematic of a bioreactor design which underlines the complexity of the processes which will occur in a bioreactor used to drive cell proliferation and differentiation.

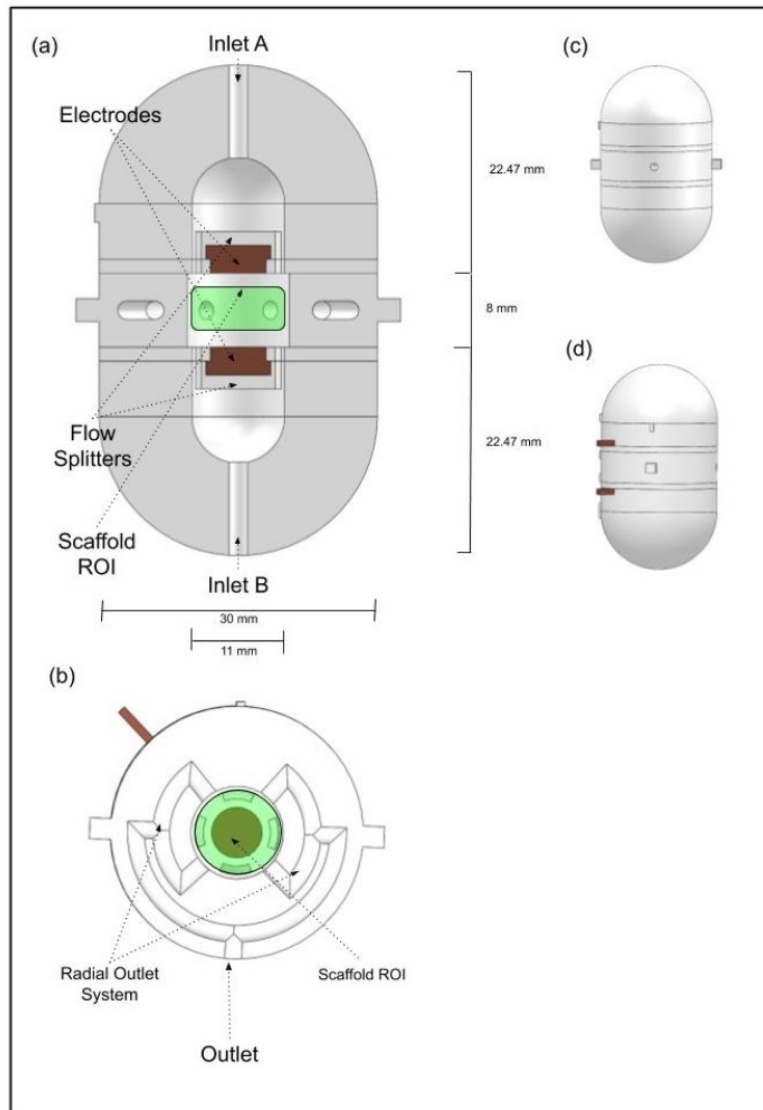


Figure 1. A schematic of a bioreactor design which includes the capability of stimulating cells using electric fields. Reproduced from [Meneses et al (2020)].

There are many types of reactors used in biotechnology and Appl et al have stated “Digital Twins are an ideal tool for the rapid and cost-effective development, realisation and optimisation of control and automation strategies.” [Appl et al (2020)]. Now a key part of this approach to the optimisation of the process is the availability of real time measurements of the process control parameters and measurements on the progress of reactions taking place in the

reactor. It is straightforward to capture data on the temperature, the flow rate in the reactor and the electric fields applied but obtaining measurements which relate to cell proliferation and differentiation are more complex. Indirect measurements such as pH and oxygen level will be facile but evaluation of the extent of cell expansion is much more challenging as identified by Moller and Portner [Moller and Portner (2021)] especially in the case where microcarriers are employed.

Evaluation of the extent of cell proliferation typically takes place through batch based processes, for example as used in Biscaia et al [Biscaia et al (2022)]. More detailed evaluation of genetic markers requires the use of PCR techniques which have a particular cycle time and are of limited value to inline analysis. Now of course we can expect great advances through the development of nanoscale detection and the use of microfluidic technology which will eliminate in this bottleneck. However, as digital twins address more complex, multiscale and multifunction processes, this will lead to the requirement to a more pragmatic approach to providing data on some of these scales and some of these functions. We propose that a hybrid approach could be a positive step where data to drive the digital twin comes from a variety of sources which will include “live data”, data from simulations, data from parameterized off-line experiments and other library sources. Progress has already been made in simulating tissue growth eg [Joldes et al (2015), Egan et al (2018)] and on cell differentiation e.g. [Matziolis et al (2006), Sandino et al (2010)].

## Summary

It is clear that the practice of tissue engineering is complex with many conflicting requirements. The optimization of all factors and parameters is essential to transform this into viable and effective therapy. The use of digital twin technology offers a most promising approach to achieve this. We have identified areas where the use of hybrid technology would provide a much better data flow to drive the digital twin. We anticipate much development in the next few years in sensors and the use of microfluidics for sampling and detection.

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