The EuroBiotech Journal
RESEARCH ARTICLE
Medicine and Biotechnology

Silk Fibroin Hybrids for Biological Scaffolds with Adhesive Surface and Adaptability to the Target Tissue Change

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Abstract

Background

Regenerative Medicine (RM) is a branch of medicine that aims to regenerate tissues and organs to overcome the problems transplants entail (poor availability, risk of rejection and intense immunosuppression). To do this, RM makes use of tissue engineering (TE). This fundamental branch deals with creating biological scaffolds capable of performing the role that physiologically belongs to the extracellular matrix (ECM). In this review, we report how specific characteristics of the scaffolds (biocompatibility, biodegradability and mechanical and conformal properties) can be obtained using 3D printing, which facilitates the emulation of physiological tissues and organs.

Purpose and scope

This review reports recent advances in the fabrication method of bioactive scaffolds that can be used clinically, providing support for cell seeding and proliferation. To this end, silk fibroin, tannin and graphene were used to improve the scaffold's electro-bio-mechanical properties. These materials in different compositions are studied to demonstrate their potential use as bio-ink in bioadhesives and cellularized and implantable 3D-printed scaffolds.

Summary of new synthesis and conclusions reached in the review

Silk fibroin is a natural biopolymer; tannin, on the other hand, is a biological polyphenol, highly reactive with other molecules by nature and with promising antioxidant capabilities. Finally, graphene is nothing more than a monolayer of graphite that has been shown to implement the mechanics and electrical conductivity of the compounds in which it is inserted; it also has excellent biocompatibility and surface area, qualities that promote cell adhesion and growth.

Conclusion

Polyphenols and graphene have been shown to work in synergy in improving the electro-mechanical properties of silk fibroin scaffolds. We reported optimal and potentially market-competitive bioadhesives, but above all, the proliferation of neuronal precursor cells in vitro was successfully demonstrated.

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DOI: 10.2478/ebtj-2023-0005

Introduction

1.1 Regenerative medicine

Regenerative Medicine (RM) is a medical branch aiming to recreate damaged tissues and organs instead of replacing them by developing biological substitutes that restore, maintain or improve tissue function.

This could be possible through using and manipulating the patient's cells, a frontier of medicine brought forward by continuous innovations in tissue engineering (TE).

RM may represent a solution to the critical challenges of organ transplantation: indeed, organ transplantation has significant problems like the overwhelming demand (which also occurs in racial, sex, age and geographical disparity) and the immunosuppression of the recipient, with all the risks it entails for the patient.

With these assumptions, we can consider RM as the third of three phases in organ transplantation, with the first two corresponding to mere surgery and the advent of immunosuppressants (first and foremost cyclosporine), respectively.

Immunosuppression has been a key to transplantation success. Still, it does so at the cost of side effects, which is why research has focused on achieving an immunosuppression-free status (IFS) for the recipient in the last decade.

In this context, it becomes clear how RM, thanks to recent breakthroughs in bioen-

gineering and organ regeneration technologies, could produce organs from the patient's cells while ensuring IFS.

The feasibility of this approach has been proven with the implantation of sections of the urinary tract or upper airways. Still, it is only before complex organs are bioengineered (1).
In recent decades, TERM (Tissue Engineering for Regenerative Medicine) has shown that it can improve patients' lives, thanks to the achievements in tissue and organ construction (2).

However, not all organs or tissues have the same degree of design complexity, of course: flat organs such as the skin are certainly the easiest to develop, followed in increasing difficulty by tubular structures such as trachea and vessels, hollow organs and finally solid organs, that are the most complicated to create. It is challenging to find biomaterials and build scaffolds capable of supporting the growth and function of all the cell types simultaneously present in an organ (2).

Tubular structures have also been produced and applied clinically with excellent results: for example, a trachea transplant in a paediatric patient showed full functionality at a four-year follow-up (3). For trachea reconstruction, various approaches have been used in different cases, e.g., using a donor's trachea decellularized and then reseeded with respiratory epithelium from the recipient or using 3D printers to create an implantable tracheal splint (4). Hollow organs have also been successfully produced, particularly for the urinary tract, as mentioned above: autologous urethras for paediatric grafts have been successfully created after taking muscle and epithelial cells and culturing them on tubular scaffolds (5,6).

The ageing of the world's population and the consequent increasing prevalence of chronic diseases indeed reveal the success in treating conditions on which acute intervention is needed, but also the need to research how to limit the incidence and progression of chronic diseases.

In this context, regenerative medicine opens up new perspectives of hope and solutions for many diseases considered incurable. Furthermore, it offers an alternative to tissue degeneration and allogeneic transplantation, reducing the socio-economic burden of managing chronic patients and gaining a place at the forefront of healthcare.

RM harnesses the self-healing capacity of tissues and uses TE to produce new tissues thanks to continuous advances in materials and biotechnology.

Despite RM's tantalising promise, it requires changing the healthcare market model and how doctors, patients and payers view medical care.

At the moment, one of the challenges of regenerative medicine is reproducible and routinely usable production; this involves functional raw materials and their cost, automation, scalability and product stability (7).

A key role in TERM is played by three-dimensional (3D) printing; advances in this field have, over time, made it possible to print 3D biocompatible materials, which is therefore applicable to TERM.

3D bioprinting allows it to create functional and structurally viable biomimetic tissues with great versatility. Furthermore, it enables minute control over the construct's composition, architecture and spatial distribution, facilitating emulation of the organs and tissues of interest.

Bioprinting involves some critical points, such as the choice of materials, growth factors and cell type, but also technical difficulties due to the different characteristics and needs of the cells and tissues of interest (8). Therefore, bio-inks are a vital part of 3D bioprinting, and their mechanical and biological properties are also crucial, defining their printability and biocompatibility. To be printable, a bio-ink must be able to create stable and structurally intact constructs, while to be biocompatible, it must promote cell adhesion, proliferation, diffusion and interaction.

1.2 Scaffolds

A fundamental step in tissue engineering is creating substrates for cellular settlement, growth and differentiation, called scaffolds: porous 3D scaffolds provide a suitable microenvironment that closely mimics the host site for the desired cellular responses and so for the regeneration of tissues and organs.

Scaffolds are typically seeded with cells and occasionally growth factors and can be subjected to biophysical stimuli. This combination of cells, signals and platforms is often referred to as a tissue engineering triad (9). Cell-seeded scaffolds can be either cultured in vitro to implant regenerated tissue into the injured site or injected directly into the lesion, promoting tissue regeneration in vivo.

It is easy to understand how scaffolds, in regenerative medicine, take the place of the extracellular matrix (ECM), which in living tissue acts as a natural substrate for cell growth and differentiation and for the remodelling of the tissue in general over time. However, the ECM plays a crucial role in so many cellular processes that there has been an increasing attempt to understand its mechanisms of interaction with cells and make them reproducible in the field of RM.

Using natural and reconstituted ECM in gel form has helped fulfil the requirement of modelling, investigating tissue differentiation and architecture and distinguishing normal from malignant tissue at a behavioural level, but IBM (e.g., Matrigel (10)) remain rudimentary analogues of natural ECM.

Nevertheless, in trying to reproduce some of the biological characteristics of ECM, type I collagen is a molecule that has demonstrated utility, even more so when combined with rBM, fibronectin (FN) or purified laminin, and is also easily modulated enzymatically or chemically in terms of its fibril orientation, functionality and stiffness.

The limitation of collagen gels is that they are too heterogeneous, which complicates the interpretation of research data because if the architecture changes, so do the pore size, the organisation of the molecule and the concentration of ligands (11).

The next step to overcome these problems was to create scaffolds made from the extracted, isolated and denuded ECM of various tissues, on which stem cells were seeded to fairly closely reconstitute the native tissue. For this reason, the research developed synthetic matrices that had as many of the properties of ECM as possible while at the same time having an adjustable size, stiffness and remodelling (12).

Even if the ECM of each tissue is different from the other, it tries to ensure the proper structure of the tissue itself.
Similarly, scaffolds for different implantation sites can't be identical, but there are some common characteristics that every stand must respect:

- **biocompatibility:** is an essential requirement for cell adhesion, functionality, migration and proliferation. Associated with this is the need for the scaffold, once implanted, not to provoke an exaggerated inflammatory reaction to avoid damage or even failure of the implant itself.
- **biodegradability**: the scaffolds are designed as temporary substrates since the cells housed in them will produce their own ECM over time after implantation. Therefore, platforms and the by-products of their degradation must be biodegradable and safe for the organism and tissue that will receive them.
- **mechanical and architectural properties:** the scaffold should have mechanical properties and architecture compatible with the target tissue, but it has to be strong enough to allow manipulation during surgical implantation. This concept is essential for every tissue, particularly cardiovascular and bone tissues; for the latter, in particular, even more variables have to be taken into account, such as the fact that at different ages, there is an additional healing time.

However, it must be found a balance between mechanical properties and materials used to try to radiate them without overriding the porosity of the scaffold.

Cells primarily interact with scaffolds via chemical groups (ligands) on the material surface. The available surface influences the ligand density within a pore to which cells can adhere. From here, it is clear that another critical factor is the average pore size of the scaffold: they need to be large enough to allow cells to migrate into the structure but small enough to establish a sufficiently high specific surface, leading to a minimal ligand density to allow efficient binding of a critical number of cells to the scaffold. For any scaffold exists a critical range of pore sizes which may vary depending on the cell type used (9). With 3D printing, we can create complex architectures and study their properties (13).

A biomaterial can be defined as "any substance (other than a drug) or combination of substances of synthetic or natural origin, which can be used at any time, either as a whole or as part of a system that treats, increases or replaces any tissue, organ or function of the body" (14).

Over time, the variety of biomaterials applicable to RM has increased, with the specificity and complexity of the desirable characteristics for a material to be printable.

A printable material must have suitable cross-linking mechanisms that make it easier for the printer to deposit. Still, it must also be biocompatible, given long-term transplantation, and have adequate stability and swelling index in the initial phase so that the architecture and mechanical properties of the construct (pores, channels, superstructure) are guaranteed. Still, it must be able to support cellular rooting and growth. Over time, on the other hand, the implanted product must be able to be remodelled by the host tissue, encouraging new matrix deposition and cell proliferation in situ (8).

Another factor to consider when choosing a material for bioprinting is its thermal conductivity, as this can influence the ability of the biomaterial to protect cell viability during printing: thermal ink and laser-assisted printing cause a temperature increase in the material at the deposition site, so a material with a low conductivity may be protective towards cell viability post-printing (15,16).

Different types of materials can be used to create scaffolds. Talking about inorganic biomaterials, such as metals and bioceramics (hydroxyapatite -HA- and tri-calcium phosphate -TCP-), they can be subdivided according to their degree of interaction with the tissue into which they are implanted via scaffolds: they can be bioinert (typically used for structural support implants), bioactive (used to heal small bone and periodontal defects), and bioresorbable (17).

Despite these advantages, their clinical applications for tissue engineering are limited due to their fragility and difficulty in modelling for implantation. Finally, the new bone formed in an HA scaffold cannot sustain the mechanical load necessary for bone remodelling. It is also problematic to control its degradation rate (9).

1.3 Cell lines for tissue engineering

Fibroblasts are dynamic cells of mesenchymal origin, naturally involved in steady-state physiology and regenerative and pathological situations.

They have a fundamental role in the interactions between epithelial and mesenchymal cells due to their ability to secrete growth factors and cytokines that promote cell proliferation, differentiation and the development of the new extracellular matrix (ECM).

Fibroblasts have autocrine and paracrine abilities that can modulate the biochemical and mechanical microenvironment. The advantages of their use are many: they have an immunoregulatory action, reducing the probability of rejection in allogeneic transplants; they are available and easily accessible through skin biopsies for autologous cell therapy, easily cultivated in the laboratory, and with multipotent differentiation features (some characteristics are similar to mesenchymal stem cells - MSC-).

Fibroblasts, as we know, are present in different anatomical compartments, where they retain a similar morphology, expressing different phenotypes and gene profiles that allow them to be site competent.

Cultured fibroblasts express Nanog, a transcription factor typical of stem cells, suggesting their ability to maintain the plasticity feature.

Particularly interesting in TERM are the skin fibroblasts, easily obtained from skin biopsies and cultured on a medium containing fetal calf serum. Unfortunately, this culture medium has repeatedly raised concerns about the risk of bovine spongiform encephalopathy (BSE) transmission; therefore, it is only produced in countries where this disease is not detected.

Fibroblasts' important autocrine and paracrine activity is realised through the growth factors and cytokines they produce. An important role is played by TGF-β (transforming growth factor), which induces the synthesis of connective tissue growth factor, promoting collagen generation and fibroblast proliferation (autocrine action). Other growth factors act on different substrates, such as KFG (keratinocyte growth factor), IL-6, and FGF-10 (fibroblast growth factor): paracrine action. However, fibroblasts produce growth factors and target paracrine-acting factors, such as PTHrP (parathyroid hormone-related peptide) secreted by neighbouring keratinocytes.

Given the crucial role of ECM in tissue homeostasis, healing, remodelling, angiogenesis and lymphangiogenesis, TE is increasingly looking for biomimetic solutions that exploit the

matrix produced by fibroblasts to reproduce the native tissue microenvironment.

A strategy is to cultivate isolated fibroblasts in vitro and make them produce ECM, which is used as a support for the growth of other cell lines, allowing us to study the interactions between cells and matrix better.

As we mentioned, another essential quality of fibroblasts that enhances their potential use in TE is their ability to promote angiogenesis, providing both biochemical support through angiogenesis-promoting factors like vascular endothelial growth factor (VEGF) and structural support, in particular with the deposition of collagen IV, laminin and heparan sulphate creating the basal membrane of the vessels.

They also synthesise fibronectin and collagen I, a known stimulator of angiogenesis both in vitro and in vivo.

Experiments in mice have confirmed that fibroblasts can modulate the angiogenic process without inducing a significant inflammatory response.

A stable and functional vascular network is one of the main challenges of TE, and only through its development tissue regeneration grafts can be successful.

Fibroblasts have been shown to modulate the behaviour of other cells if they create direct contact with them. This allows us to grow fibroblasts and other cell lines together in vitro to produce tissue engineering products.

In addition, as they are the leading producers of ECM, the use of fibroblast-derived matrices that are subsequently decellularized to obtain acellular substrates to support tissue regeneration is also being researched.

As we mentioned, these cells are widely abundant in our organism; in particular, the dermis is a rich source of them, easily accessible using a simple, minimally invasive skin biopsy (18,19).

Neuronal Precursor Cells (NPCs) are considered neural stem and progenitor cells. Stem cells can self-renewal and give rise to differentiated progeny. NSCs generate multipotent cells that can differentiate into neurons, oligodendrocytes or astrocytes; on the other hand, neural progenitor cells can only self-renew and differentiate to a limited extent.

Neurogenesis remains active in some small areas of the mammalian CNS, including humans. In particular, adult NPCs were detected in two regions: the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus (sub-granular zone). The former is a zone that lines the walls of the lateral ventricles (ependymal and sub-ependymal layers). NPCs are mainly in the sub-ependymal layer.

However, hippocampal NPCs appear to have little self-renewal and multipotency in vitro, which casts doubt on their true stem nature.

NPCs are slow proliferating cells (type B cells, GFAP+) that give rise to fast proliferating progenitor cells (type C cells), from which neuroblasts (type A cells) will arise.

The newly formed neurons then move radially, tangentially or in a chain towards their intended regions, e.g., they may travel along the rostral migratory stream (RMS) towards the olfactory bulb, then differentiate into interneurons and integrate into the neuronal network.

The discovery of NPCs in the adult brain has given new hope for the possibility of regeneration in adult nerve tissue. Therefore, numerous studies and experiments were conducted to explore the potential of neuronal precursor cells detected in the adult brain's subventricular zone (SVZ).

Research on the SVZ, a region where there are neural stem cells, is essential to study the applicability of neuronal replacement. The autologous replacement is preferably used, given the undoubted advantages over the use of allogeneic foetal stem cells, which always imply problems of rejection risk and ethical issues, such as embryonic cells.

Neural stem cells can be obtained from various sources: foetal neuroectoderm or adult SVZ and DG. Once isolated, these primary NSCs will be cultured with essential fibroblast growth factor (bFGF) and epidermal growth factor (EGF).

Another way to obtain NSCs is to generate them from embryonic stem cells (ESCs) or induced pluripotent cells (iPSCs), a procedure requires numerous manipulations and steps. Indeed, it has been confirmed that iPSC-NSCs and ESC-NSCs are very similar but without an identical genetic profile.

The direct conversion of somatic cells into NSCs may be a further gain, and research is progressing in this direction.

The transformation of murine fibroblasts into iNSCs using the pluripotency factors has already been successfully implemented, with the possibility of limiting Oct4 activity to the early stage of reprogramming. Still, other ways to achieve direct reprogramming are known (20).

It's also interesting to understand the mechanisms underlying the differentiation, survival, integration and migration processes of these NSCs, and how the surrounding tissue interacts with them.

While embryonic cells in the adult recipient are easily detectable post-implantation and remain compact in a nuclear-like structure, NSCs are less easily localised as they can migrate over long distances.

The first problem is identifying markers (cytoplasmic, nuclear, membrane, metabolic) that make the graft cells distinguishable from the native cells of the recipient.

Implanted SVZ cells can be discriminated against since they produce GABAergic and dopaminergic neurons, so that is these neurons are not physiologically present at the graft site they can be easily distinguished. However, whether the graft cells can induce dopaminergic differentiation in the surrounding native cells is still unknown.

The nuclear markers are considered the best among the various cellular features because they interact less with the cells at the implantation site and even remain after cell division (21,22). NSCs can be pre-labelled with a thymidine analogue, bromodeoxyuridine (BrdU), which is incorporated at the nuclear level and allows quantification of NSC cell divisions after transplantation.

Another important objective in NSCs transplantation is their phototropism, an innate tendency to home into the lesion, partly made possible by the fact that NSCs express on their surface receptors for chemokines that attract them to the damaged region. For example, the chemokine stromal cell-derived factor-1α (SDF-1α/CXCL12) is increased in stroke-affected brain tissue and can bind to the CXCR4 receptor on NSCs.

NSCs migrate following concentration gradients of both native and synthetic SDF-1α, which outlines a possible promising strategy to increase the ability of NCSs to go towards the site of

injury (20).

To optimise the potential of these cells already present in our brain, endogenous activation strategies to stimulate tissue repair and functional recovery, using growth factors, cytokines and hormones (e.g., EPO), have been simultaneously worked on.

Hormones seem safer in the clinic as they physiologically undergo considerable fluctuations with minimal carcinogenic effects. On the other hand, stimulating NPC proliferation with other factors may quickly increase the risk of tumour formation (21).

The autologous transplantation of NPCs is favoured by their presence in a precise area of the CNS. In addition, NPCs are an excellent cell line compared to others since their neural targeting limits the possible production of undesirable non-neural cell phenotypes.

However, the problem remains to obtain endogenous NPCs, which has promoted studies focusing on NPCs derived from other stem cells (foetal and embryonic). In post-stroke trials on mice, the latter has given good results regarding the reduction of ischaemic area, anti-apoptotic and immunomodulatory effects, and behavioural improvements. These promising results have encouraged phase I clinical trials of intracranial transplantation in patients with subcortical or basal ganglia stroke.

In addition to intracranial administration of NPCs, intravascular administration has emerged as a clinically viable alternative. NSCs can reach the pathological niche after injection into the tail vein or artery. However, the intravenous route may result in partial entrapment of the cells in the filter organs (lung and liver), which is why the intra-arterial course, being more direct, is clinically the best choice.

Administering NCI during the subacute phase of a stroke may be a proven strategy to reduce brain damage.

Human studies focus on post-stroke rehabilitation improvement in acute and chronic stroke, with more critical results if NCI is administered in the subacute phase of stroke (20).

2. Purpose of the research

This review is part of a much larger research project that aims to create hybrid scaffolds that can be implanted and therefore used clinically: structures that can provide support for cell seeding and proliferation, thus allowing tissue reproduction in vivo.

To this end, we have introduced the use of two materials in creating silk fibroin scaffolds, a material whose qualities have already been amply demonstrated.

The two materials under study are tannin and graphene, two natural materials that are particularly promising in improving the scaffold's electro-mechanical properties and, above all, their antioxidant capacity for cells.

Therefore, we combined these materials in different compositions and studied their characteristics, testing their adhesive properties, support for cell growth and 3D printability to demonstrate their potential use in bioadhesives and cellularised and implantable 3D printed scaffolds.

Materials and methods

3.1 Silk fibroin

Proteins are often used in TE, as they are a component of natural tissues. That is why they are already used in the medical field as suture threads, haemostatic agents, scaffolds etc. (23).

Among these proteins, we find silk fibroin, a natural biopolymer produced by some insects such as silkworms, which has long been used as a suture material (Figure 1).

Over the years, however, its field of use has become increasingly broader, thanks to the excellent properties of this material, which are so suitable for TE: purified silk fibroin (SF) has a perfect biocompatibility profile, the ability to support cell and tissue growth, high tensile strength, controllable biodegradability, haemostatic properties, non-cytotoxicity, low antigenicity and non-inflammatory characteristics.

Indeed, compared to other protein biomaterials, silk has a lower risk of infection and, as a consequence, has cheaper production protocols: nearly 1000 tonnes of silk are produced each year.

The silk produced by the silkworm is the most widely used compared to that of other arthropods. The advantages of its production over spider silk, for example, are clear: a single silk cocoon produces about 600-1500 m, while about 137 m are obtained from the gland of a spider, to which 12 m can be added from spider webs.

Silk is synthesised at the level of the insect glands in a liquid state and converted to a solid form (fibres) only when exposed to the outside air as it is assembled into a cocoon (23).

There are also various types of silkworms; undoubtedly, the most widely used is the *Bombyx mori* of the family *Bombycidae*, whose silk is commonly called "mulberry silk", differing from the "non-mulberry silk" produced by another family of silkworms, the *Saturniidae*.

3.1.1 Composition and structure

Silk has a significant molecular weight (around 200-350 kDa) with large repetitive hydrophobic domains alternating with small hydrophilic groups.

It is a protein composed mainly of fibroin (72-81%), a fibrous protein, and sericin, an amorphous gummy protein (24). However, the biomedical use of silk can only occur after sericin elimination because complete silk can trigger hypersensitivity and immunogenicity. Therefore, the purification procedure is called degumming. This process in our study is carried out by soaking the silk cocoons in $NaHCO_3$ (5 g in 200 mL of water) and boiling water for 30 min, and then rinsing them with deionised water; this is repeated twice, and the degummed fibres are then left to dry at room temperature (25). The fibroin of Bombyx mori silk consists of two chains, a heavy one (H) and a light one (L), to which a glycoprotein (P25) is also bound.

The H-chain's hydrophobic domains contain Gly-X repeats (X = Ala, Ser, Thr, Val). They can form antiparallel β-sheets (nano-crystalline structures); the L-chain is hydrophilic and relatively elastic, while the P25 protein appears to have a structural role.

In terms of secondary structure, next to a crystalline portion of the protein (β-sheets), there is also an amorphous (random-coil) one consisting of sizeable polar side chains which act as hydrophilic bonds between the hydrophobic domains. These random-coil portions give the silk its elasticity. The antiparallel β-sheet structure is predominant in silk fibroin and is called silk II. At the same time, amorphous systems such as random

coil (also α-helix and β-turn) are present in smaller quantities and are called silk I. The conformation silk II is stable and insoluble in water, and silk I am metastable and soluble in water. Therefore, the mechanical properties of silk are directly related to the size, number, arrangement, distribution and orientation of the crystalline and non-crystalline domains (26).

The crystalline conformation is responsible for the excellent stability and strength of the material under consideration. Still, our aim is not to obtain a structure with the greatest possible crystalline fraction (by crystalline fraction, we mean the percentage of crystalline structure in the whole molecule), as this would create a conformation that is too rigid and, therefore, inevitably unstable. The amorphous components must be present in a certain quantity to give the structure elasticity and stability. The compact and ordered architecture resulting from this crystalline secondary structure of fibroin must necessarily be disintegrated if we wish to use fibroin in creating scaffolds with a different morphology from the native fibroin. This can be achieved by using a solvent (like formic acid, FA) capable of breaking the hydrogen bonds of the molecule without degrading it. After this procedure, we will obtain a fibroin in the silk I conformation, from which we will reconstitute silk II (e.g. regenerated silk, RS), more suitable for biomedical use as it is insoluble in physiological solution (27).

3.2 Tannin and graphene

Tannins are ecological and biological polyphenols that are natural and highly reactive. They have versatile properties that are continuously being studied and expanded. In particular, they can interact with other species and compounds, including metals, ceramics and organic species, resulting in hybrid systems with advanced properties.

This results in various types of advanced and continuously improved nanomaterials.

Plant polyphenolic tannins and cellulose, hemicellulose and lignin, are among the most abundant compounds extracted from biomass. After lignin, tannins are the primary source of natural aromatic macromolecules.

In nature, tannins work as defence agents for vascular plants against herbivores, fungi and micro-organisms, making the plant less assimilable; they are also produced as a chemical shield to protect the wood structure from radical oxidative processes and play various metabolic roles in non-vascular plants (algae). They are primarily present in the soft tissues of plants (needles, leaves or bark), reaching the highest concentration in the bark of pine, mimosa or oak trees.

Tannin derives from tanning, which converts animal skin into leather through aqueous plant extracts. In recent decades, of course, the application of these compounds has expanded from the tanning industry to embrace oenology, medicine and materials engineering.

Based on their chemical structure, tannins can be categorised mainly into hydrolysable tannins and condensed tannins. In addition, complex tannins discovered later and with more sophisticated systems are not very abundant and, for these reasons, are of limited application.

Polyphenols can also exfoliate graphene, a property that makes them more exciting and further expands their field of application.

Tannin has also demonstrated its antioxidant effect in TE; this quality seems to be supported and reinforced by graphene in a dose-dependent manner (25).

Graphene is a promising material consisting of a monolayer graphite (carbon atoms arranged in a honeycomb network) that we have used as an additive for bioadhesives, as it can improve mechanical properties such as tensile strength, particularly when combined with RS.

It has excellent electrochemical properties, including high thermal conductivity, low redox potential, large surface area and mechanical strength, which make it attractive for the fields of electronics, optics and biomedicine, both in drug delivery systems and in improving contrast agents and gene therapy.

Various effective techniques can produce graphene: chemical vapour deposition, micromechanical exfoliation, epaxial growth, and chemical synthesis from graphite, graphene, or other graphite derivatives are examples. The last of these techniques listed are particularly interesting in terms of results, and the graphene obtained from it is called chemically converted graphene (CCG) or simply graphene.

CCGs are usually obtained by wet processes (such as chemical exfoliation of graphite) that are reproducible on a large scale and at a relatively low cost (28).

To prepare CCG, the chemical reduction of graphene oxide is among the most widely used techniques, the latter being usually synthesised by exfoliation of graphite oxide, which in turn is derived from the oxidation of graphite powder with various oxidants in acidic media.

In the last decade, graphene-derived compounds have also been studied, including graphene oxide (GO) and its reduced form (rGO). Various studies have shown that GO and rGO are two materials that have demonstrated a remarkable influence on stem cells in culture.

They have excellent chemical, physical, mechanical and electrical properties, high biocompatibility and surface area, all of which implement intercellular or cell-scaffold interactions and promote cell adhesion and proliferation while promoting cell differentiation, particularly towards osteogenic, cardiac and neuronal lines (29,30).

Thus, incorporating GO and rGO into the scaffolds is advantageous: it improves their resistance, cell adhesion, electrical and mechanical properties, protein stability, cell proliferation, hydrophilic state, uptake and stability of growth factors.

The electrical conductivity of GO and rGO-enriched scaffolds was significant, especially in cardiac and neural cultures, as it helps establish good signal conduction between cells and scaffolds.

On the other hand, the high protein uptake and surface area of GO and rGO promote bioconjugation with proteins, antibodies and growth factors within the scaffold, which contributes to an environment conducive to cell adhesion and proliferation.

GO and rGO do not have only positive sides: there are conflicting data on their biotransformation, biodistribution, cytotoxicity and immune response.

These variables are undoubtedly influenced by the method of preparation of the molecules. Still, dose, size and surface charge also seem to play a role: doses >50 μg/mL increase the cytotoxic effect, as do small lateral dimensions and high charge

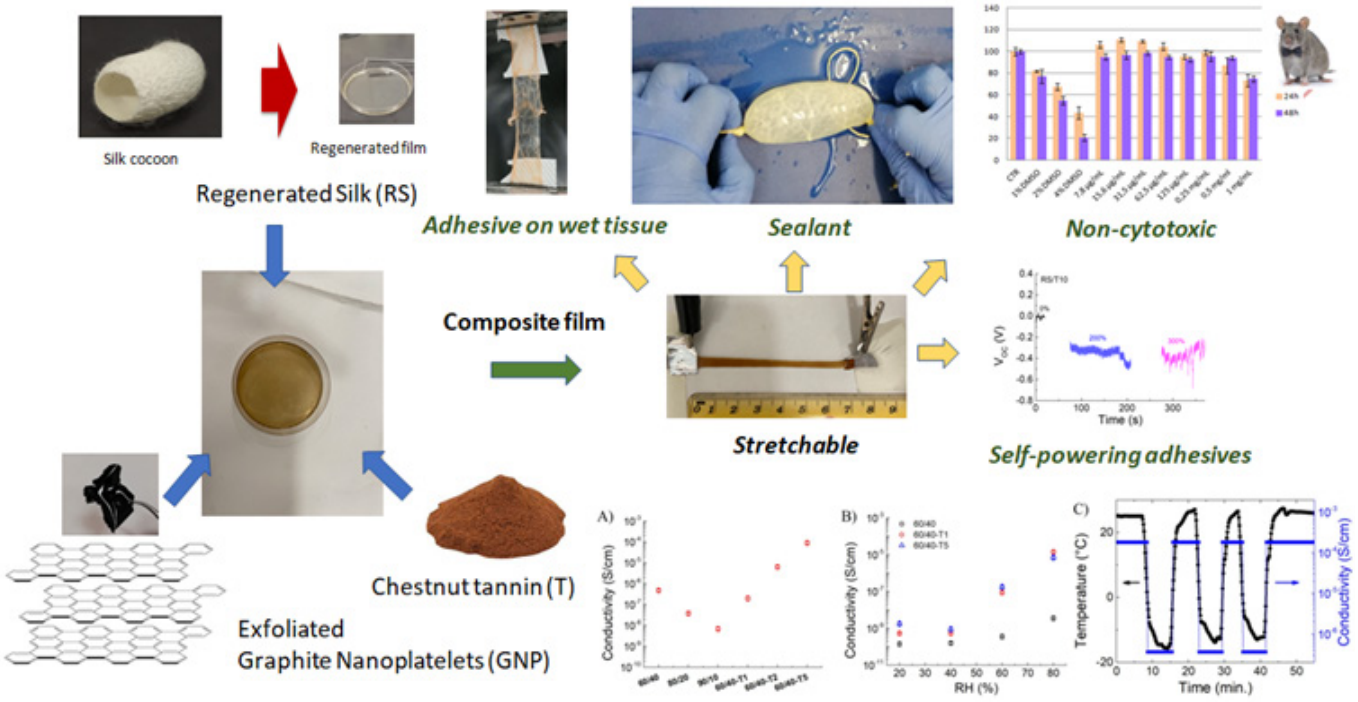

Figure 1. Strain programmable and printable devices based on silk fibroin and nanomaterials for designing ideal interfaces with biological tissues.

density.

About biodistribution, these compounds can remain in various organs (e.g., lung, spleen, marrow and liver) for long periods. Nevertheless, graphene and its derivatives are suitable additives to scaffolds to improve their mechanical and functional properties with excellent results. Of course, challenges remain, but their inclusion in scaffolds improves the inconvenient aspects of these molecules (31).

Results

4.1 Materials combination

Combining a material such as a graphene with biomaterials (in this case, RS and tannin) makes it possible to create intelligent constructs that comply with the characteristics required to develop an adhesive that is sympathetic to tissue movement and self-sensitive to interface stresses.

To create such a construct, we exploited the tannin-assisted exfoliation of graphite nanoplates in the same solvent used to regenerate silk.

Graphene and tannin act in synergy to significantly improve the mechanical and electromechanical properties of the adhesive and adhesiveness on wet surfaces, making it promising for RM applications.

As previously mentioned, the silk cocoons were scoured in boiling water with NaHCO3 (5 g in 200 mL of water) for 30 minutes and rinsed with deionised water; this was repeated twice, after which the fibres were left to dry at room temperature.

The scoured silk fibres thus obtained were dispersed in a solution with FA/CaCl2 by magnetic stirring at room temperature for five minutes to get homogeneous RS solutions. Specifically, 0.65g of silk was dissolved in 5 mL of FA and $CaCl_2$ in a silk:$CaCl_2$ ratio of 60:40.

Subsequently, concerning the silk content, first 1 wt% and 10 wt% of tannin were added, allowing dissolution to progress at 50°C, and then 1 wt% of GNPs; the GNPs powder was dissolved with bath sonication at 100W and 40 kHz for 30 min, left to stand for 24 h to allow the insoluble graphite to precipitate and then centrifuged for 30 min at 500 rpm.

The resulting solutions were deposited in 5 cm diameter Petri dishes and then evaporated overnight under a laminar flow hood.

As shown in Table 1 below, films of pure RS, RS/T and G-RS/T with various percentages of tannin (both 1 wt% and 10 wt%) were compared (25).

4.2 Printability and adhesive properties

Six different solutions (Figure 2A) were fabricated with a cus-

Table 1. Composition of the prepared samples.

Sample	Tannin (wt%)	GNPs (wt%)
RS	0	0
RS/T1	1	0
G-RS/T1	1	1
RS/T10	10	0
G-RS/T10	10	1

tomised 3D printer multilayer grid structures on a water-soluble polymer layer (13).

The structures thus printed were left to dry at room temperature for 24 h, and finally, the RS-based grids were removed from the acetate sheet while remaining attached to the hydrofilm (Figures 2B and 2C).

The amount of tannin present in the adhesive must therefore be well calibrated, especially if we consider that, once graphite is added, the tannin primarily engages in exfoliation rather than adhesive properties (25). Nevertheless, in the G-RS/T10 formulation, tannin is sufficient for both peeling and adhesive tightening, giving exceptional results to the biofilm.

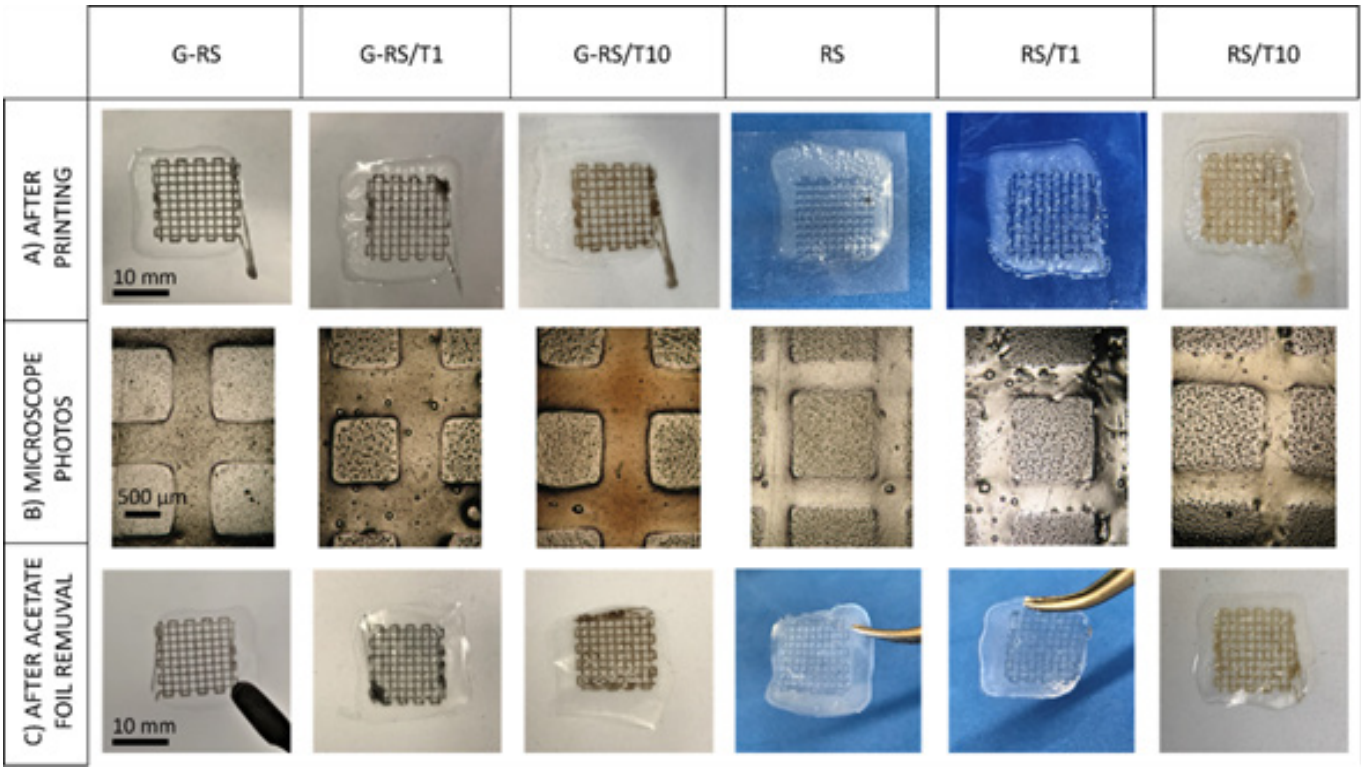

Figure 2. 3D printed grids based on RS, tannin, and GNP. A) Photos were taken as soon as the printing stopped. B) Photos obtained with a brightfield microscope. C) Photos of the structures after the acetate foil removal (13).

Analysing the samples with the various percentages of materials (Figure 3), the adhesive properties were tested. The shear strength observed for RS/T1 is higher than for pure RS because the tannins can primarily engage and tighten the adhesive by creating stable complexes with silk proteins; however, if too much tannin is added, the construct stiffens too much and breaks prematurely.

The possible clinical application of these adhesives is exciting to promote surgical wound closure, reducing hospitalisation time, perforation and infection. The strength of sealing sealants made with RS, tannin and graphene by measuring their bursting pressure indicates that the bursting pressure recorded for the G-RS/T10 glue (57 mmHg) applied to the incised intestine shows an increase of 60% concerning an intestinal anastomosis

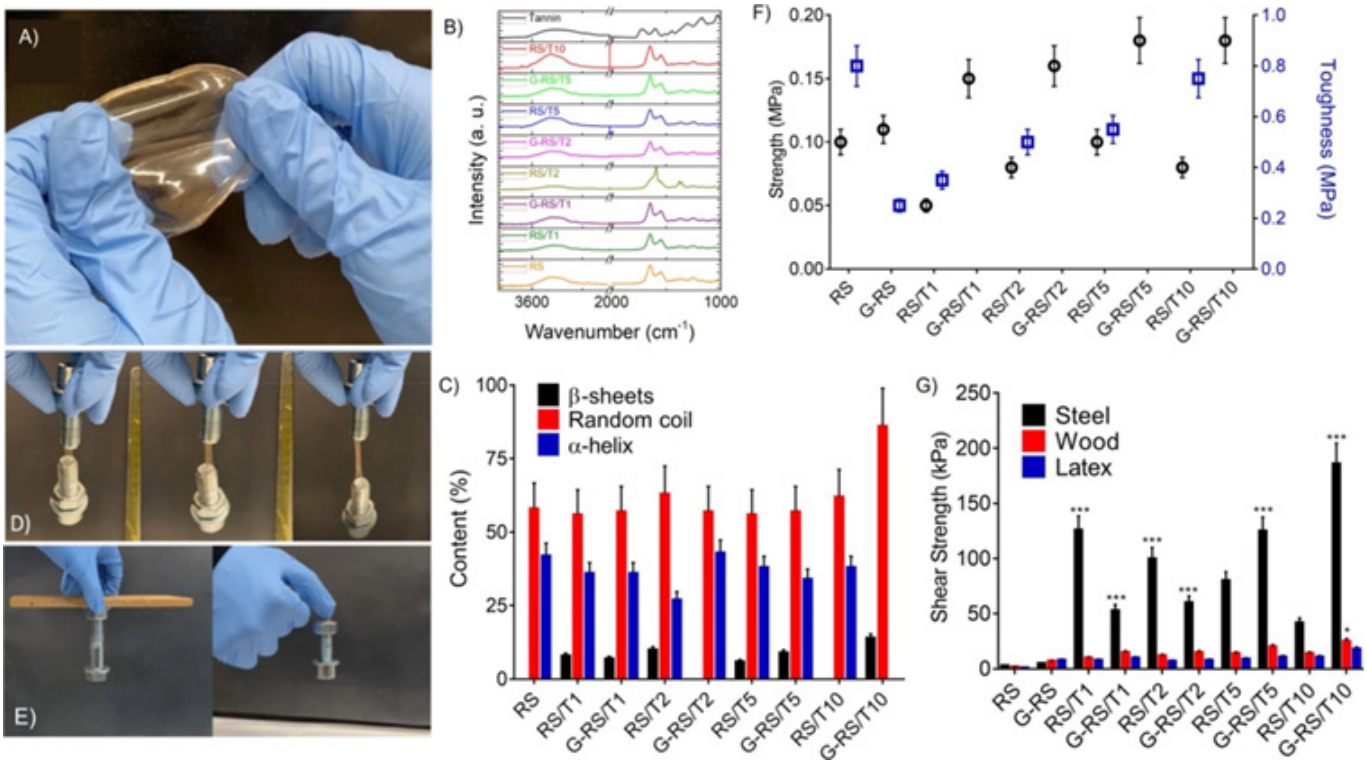

Figure 3. (A) Photograph of the RS/T10 composite. (B) FTIR spectra of neat RS, RS/T, and G-RS/T composites. (C) Structure composition of the prepared specimens. (D) Photographs showing the cohesive adhesion on stainless steel of the RS/T10 composite and tensile strength. (E) Adhesion of RS/T on wood (left) and latex (right). (F) The toughness of the prepared specimens was calculated from the engineering stress–strain curves. (G) Shear strength measurements of the ready models (13).

with mechanical sutures (25).

4.3 Characteristics detected and cell viability

To evaluate biocompatibility, the model used was the HepG2 cell line (a human hepatocyte carcinoma), purchased from ATCC (American Type Culture Collection, Gaithersburg, MD, USA; ATCC HB 8065).

The cells of the HepG2 line were cultured in EMEM (Eagle's minimal essential medium), with the addition of 10% heat inactivated fetal bovine serum (FBS), 1% non-essential amino acids, 1 mM sodium pyruvate, 2 mM of L-glutamine and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin).

After a treatment of 24 h and one of 48 h, to evaluate the biocompatibility of these compounds, the MTT test was used (32), a test that measures cellular metabolic activity based on the reduction of a yellow tetrazolium salt (3- (4, 5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells. For this evaluation, the HepG2 cells were seeded in a 96-well plate at a cell density of 1 × 104 cells/well; after 24 hours, eight different dilutions of stock solutions (1 mg/mL) replaced the complete fresh medium and MTT reagent (0.5 µg/µL) was added to each well for 3 hours at 37° C.

Each experiment was performed three times; cell viability was expressed as a relative percentage (25).

However, the antioxidant capacity of the constructs is vital for future biomedical applicability due to the presence of tannin. Therefore, this property has been studied for various compound formulations.

The cell viability of the various compounds through MMT assay, as previously mentioned.

As shown in Figure 4, HepG2 cells were in vitro treated with six scalar concentrations for 24 h and 48 h. The percentage of viable cells concerning the control was reported as the mean and standard deviation of three independent experiments conducted in triplicate. Positive controls were obtained with DMSO 1%, 2% and 4%.

The lowest concentrations tested (7.8, 15.6, 31.5 and 62.5 µg/mL) are almost safe for the HepG2 cell line. Due to the antioxidant property of tannin, however, these compounds may, in some cases, increase cell viability.

Fibroblast and NPC morphology, confluence, and growth indicate the excellent biocompatibility of RS hybrids (Figures 5A and B). In addition, we observed that low doses of tannin were not cytotoxic to fibroblasts and NPCs, confirming their antioxidant and cytoprotective properties (32).

Conclusion

In this review, we reported the applicability of tannin and graphene to tissue engineering, verifying their qualities and functions, mainly when used in conjunction with RS. These

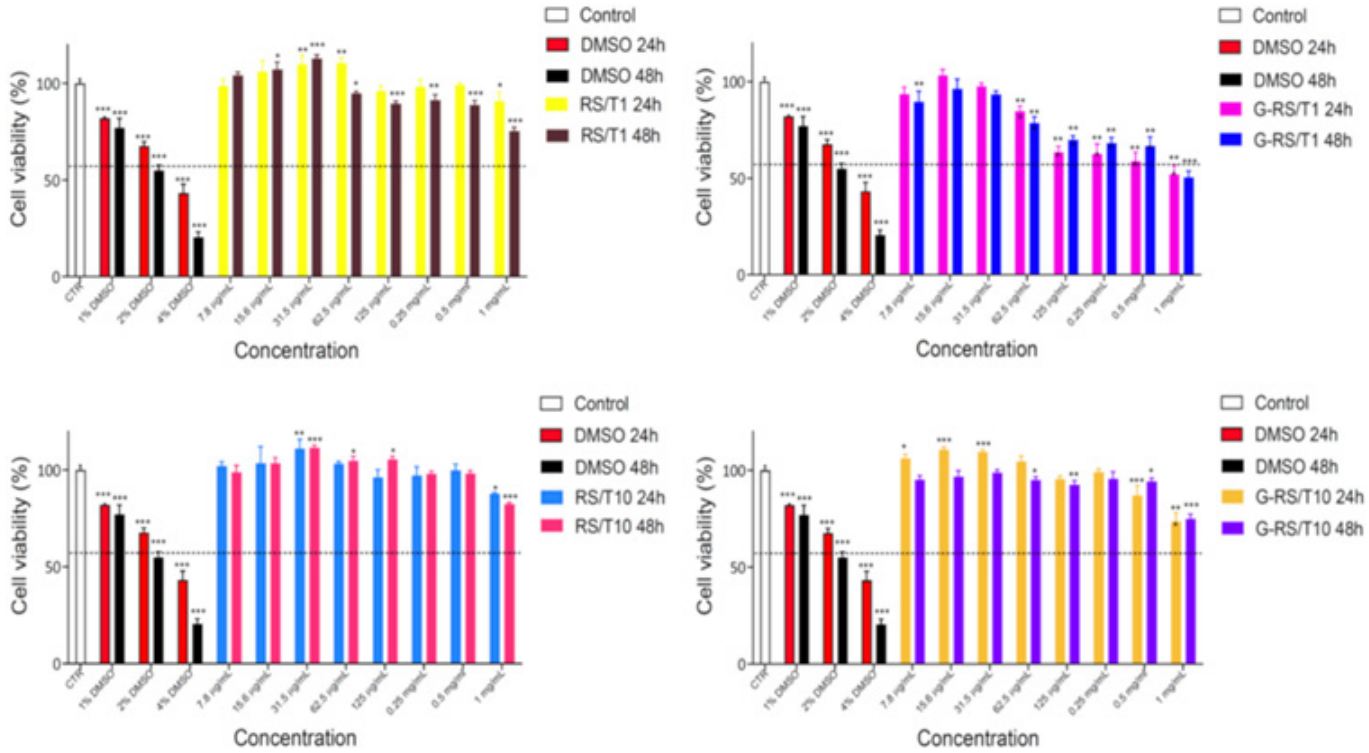

Figure 4. Safety evaluation of the RS/T and G-RS/T samples tested in vitro using the MTT test. HepG2 cells were in vitro treated with six scalar concentrations for 24 h and 48 h. The percentage of viable cells concerning the control was reported as the mean and standard deviation of three independent experiments conducted in triplicate. Positive controls were obtained with DMSO 1%, 2% and 4%. Values were represented as mean ± standard deviation and were compared with one-way ANOVA with $p \leq 0.05$ considered statistically significant; * $p < 0.01$, ** $p < 0.001$ and *** $p < 0.0001$.

biomaterials were presented in different percentages, prioritising the study of their adhesive and the cell viability of the lines seeded on them.

Given the obtained results, a tissue engineer can design the mirror-image organ structure using various materials that simulate the extracellular matrices of the organ tissues (known as scaffolds or models). Then, cells - from a universal cell source specifically engineered for the patient or bank cells cultured from the patient's cells - are seeded onto these scaffolds. These engineered cell-scaffold components are then grown separately and assembled in a special chamber (bioreactor) that provides the proper nutrients, regulatory molecules (such as proteins, growth factors and differentiation factors), physical and mechanical stimuli, temperature, pressure and mass transport conditions for cell proliferation, differentiation and tissue/organ formation. As the tissue/organ regenerates, the scaffold materials degrade and disappear, leaving nothing behind. The regenerated organ or organ precursor will be surgically grafted onto the patient during the second visit to the body shop. The engineered tissues can grow, shape and reshape in concert with the dynamic changes in the body's physiological environment. The grafted organ will integrate into the body. The new organ will grow and age like the body's natural organ. This scenario is an example of what the field of tissue engineering hopes to do in the future.

Scaffold materials and fabrication technologies play a crucial role in tissue engineering and are evolving rapidly. A scaffold must serve as a substrate for cell seeding and proliferation, mimicking as closely as possible the functions that the ECM physiologically performs. Inevitably, it must have biocompatibility, biodegradability (to progressively give way to the new matrix deposited by the seeded cells) and mechanical and structural properties that provide the cells with proper and functional support for their growth.

Tissue engineering is increasingly turning towards using natural rather than synthetic biomaterials, not only for more excellent biocompatibility but also for the green economy. Silk fibroin is used in this context. It is a particularly versatile and promising biomaterial with an excellent biocompatibility profile and promising properties that make it a promising material for future applications. We are obviously still far from achieving the objective of making regenerative medicine as widely and clinically widespread as the example above suggests, but this remains a goal we must strive to achieve.

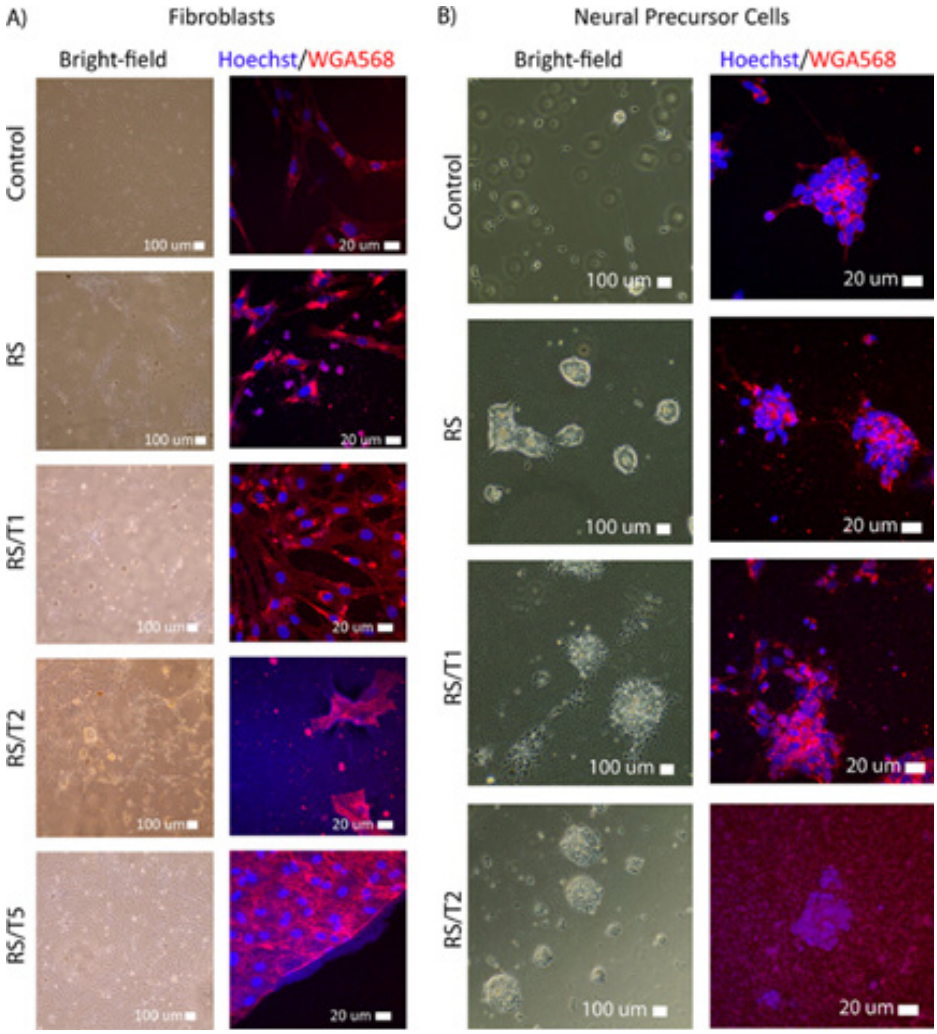

Figure 5. (A) Fibroblast and (B) NPC adhesion and growth on RS and RS/T hybrids. Bright-field (left) and confocal microscopy (right) images of human fibroblasts and NPCs seeded on modified glass surfaces covered with RS Hybrid films after 7 days of incubation in standard conditions. Cells were labelled with the fluorescent Hoechst (blue channel) and WGA568 (red channel) dyes, targeting DNA and sialic acid component of the plasma membrane_respectively. Confocal images correspond to maximum intensity Z axis projections over 10 μm. Scale bars indicate 100 μm for bright-field microscopy and 20 μm for confocal microscopy (32).

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