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Chapter 13

Bioengineering the Pancreas: Cell-on-Scaffold Technology

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Additional information is available at the end of the chapter

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Abstract

Nowadays, type I diabetes mellitus is a pathology afflicting millions of people globally with a dramatic assessment in the next future. Current treatments including exogenous insulin, pancreas transplantation and islets transplantation, are not free from important lifelong side effects. In the last decade, tissue engineering and regenerative medicine have shown encouraging results about the possibility to produce a functional bioengineered pancreas. Among many technologies, decellularization offers the opportunity to produce an organ-specific acellular matrix that could subsequently repopulate with endocrine cellular population. Herein, we aim to review the state-of-art and this technology highlighting the diabetes burden for the healthcare system and the major achievements toward the manufacturing of a bioengineered pancreas obtained by cell-on-scaffold technology.

Keywords: regenerative medicine, pancreas, extracellular matrix, scaffold, bioengineered pancreas

1. Introduction: definition of diabetes and diabetes' impact on the economy and future projections

In 2014, the World Health Organization (WHO) has estimated diabetes as a worldwide disease afflicting 422 million people with an increasing, globally, heavy burden both for the health-care system and the economic policies [1]. As stated in 2014 by National Diabetes Statistics Report [2] T1D, the so-called diabetes mellitus, is a chronic metabolic disorder, which is afflicting around 9.3% of worldwide adult population widely counting 29.1 million people worldwide. Moreover this data could even be worse if considering prediabetes or

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borderline diabetes global prevalence of around 7.8% (343 M) [3]. In this regards, according to ADA expert panel, in 20 years, without any kind of therapeutic intervention, it is reasonable to estimate that up to 70% of prediabetic individuals will develop a "real" diabetic status [4], whereas, individuals with lifestyle modifications and drug intervention, normoglycemic conversion will take place ranging from 35 to 50% of cases [5] in a 10-years follow-up cohort. Starting from these data, the prospective future scenario is dramatic.

In the 7th Edition of the Diabetes Atlas, the International Diabetes Federation (IDF) has recently estimated that by 2040, 1 out 10 adult individuals will suffer of diabetes (actually the estimation is set to 1 out 11 adults) with a global picture of more than 640 M of people (raised from actual 415 M) [5]. This scenario will also have huge impact on economic health-care policies. Nowadays, the direct cost of diabetes hits 825 billion dollars a year [6] to whom should be added all the indirect costs deriving from diabetes consequences.

Diabetes is currently recognized as a group of different metabolic disorders, which led to the same final outcome: the organism incapacity to manage intracorporeal glucose levels.

This inability can be referred to two main physiological alterations and consequently to two principal diabetes categories: type 1 and type 2 diabetes.

Type 1 diabetes (T1D) (also recognized as autoimmune or juvenile diabetes) is a lifelong chronic autoimmune disease characterized by insulin deficiency due to pancreatic β cells loss and, therefore to a subsequent hyperglycemia status [7].

Type 2 diabetes (T2D) (formerly called non-insulin-dependent or adult-onset) represents the most common type of diabetes grossly counting 90% of all cases worldwide. T2D pathophysiology is related to genetic and epigenetic factors, environmental conditions and lifestyle (obesity, physical activity and diet) that bring to a mixture of hyper-insulinemia, insulin resistance and pancreatic β cells failure [8, 9].

If insulin resistance cannot be treated by bioengineering approach, insulin deficiency, typical of T1D, could surely benefit bioengineered pancreas.

T1D is a chronic autoimmune metabolic disease characterized by hyperglycemia, which is secondary to insulin deficiency that develops as consequence of the numerical loss of the pancreatic islet β cells. This loss is due in 70–90% of cases by an autoimmunity disorder that brings to a cascade characterized by β cells destruction, dysglycemia and finally hyperglycemia. It represents 5–10% of whole diabetes cases with more than 20 million people worldwide [10]. This disease is also marked by numerous short- or long-term complications. Acute complications include life-threatening crisis ranging from severe hypoglycemic episodes to diabetic ketoacidosis. Long-term complications comprise chronic micro- and macro-vascular diseases including retinopathy, nephropathy and neuropathy due to stroke and ischemic heart attack. This panorama leads, in almost all cases, to lifelong disabilities reducing, at the same time, quality and expectancy of life and involving immense health-care expenditures.

As deeply analyzed by Dall et al. in 2007, at that time, the estimated cost for T1D patient for year was around 3000 pounds (versus around 2000 pounds estimated for T2D patient for

year), which includes direct medical costs and indirect costs (such as productivity loss or premature mortality) [11]. These data dramatically changed in recent years accompanying to a huge increase both to the worldwide number of diabetic patients as well as the deriving costs. The World Health Organization [12] has estimated for the 2014 a total of 422 million diabetic people which, compared to 108 millions counted in 1980, have more than triplicated the total global health-care spending for diabetes reaching 827 USD billion/year. [13, 14]. Forecasting models and projections define a situation where diabetes will be pandemic. Recently Krohe et al. has published their future projection for 2030 indicating an increase of the American prevalence (type 1 and type 2 diabetes) of around 54% with a 54.9 M people afflicted by the disease and a total cost increase of 53%, 622 billion USD just for the US population [15].

2. Historical development and current therapeutic options for diabetes' treatment

2.1. Insulin approach

Diabetes was born in 1910 when the English physiologist Sir Edward Albert Sharpey-Schafer discovers a substance normally produced by non-diabetic patients: insulin. The name derives from the Latin word "insula" means island, referring to the islets of Langerhans (www.diabetes.org), is a specific structure that is able to produce insulin in the pancreas. Since then, all the efforts have focused on the possibility to isolate this substance in order to replace for diabetic patients (so far described as simple, non-producing insulin patients) [16]. This need was driven by the fact that, without a substitute, diabetes was a pathologic condition that would surely have led to death. In 1921, Banting and Macleod (Nobel prize in Physiology or Medicine in 1923) succeeded in isolating insulin from a canine diabetic model, literally revolutionizing the entire health world. One year later, with this pioneering finding, the first diabetic patient was treated. Since then, several modifications have been completed, making the insulin replacement more refined, from slower acting insulin (the first form was introduced in 1936) to genetically engineered artificial "human" insulin that was produced in 1978 exploiting *Escherichia coli* bacteria able to avoid frequent allergic reactions derived from the use of cattle or porcine insulin. Nowadays, exogenous insulin administration is considered as an essential therapy for patients affected by T1D, being able to avoid, when accurately managed, acute metabolic compensations.

It is said that insulin is not a "perfect therapy" as it is being accompanied by several side effects, both in short- and long period and finally considering that just 40% of treated patients achieve and maintain a satisfying glucose range [17]. In fact, there are different factors influencing the effectiveness of insulin therapy such as daily stress, the food intake and the physical activity [18] highlighting the vital importance of a baseline good lifestyle management [19]. Insulin therapy for T1D is based on multiple daily subcutaneous injections of insulin trying to follow a patient-tailored scheme created by considering patient carbohydrate assumption, pre-meal glucose levels and anticipated physical activity [20]. Recently, a continuous intra-venous insulin administration has been considered as a simpler way for exogenous

insulin administration in order to match insulin requirements. This approach can be effectively considered useful for perioperative period of patients who has undergone surgery [21, 22] but it could not be used as a lifelong option due to the modest differences between the two delivering systems [23] in terms of final outcomes.

Even with some limitations, insulin treatment represents, as of now, the most widespread therapy to face T1D diabetes.

2.2. Pancreas transplantation

Pancreas transplantation has been successfully established for the first time in 1966 by the surgical team driven by Prof. W. Kelly [24]. That ground-breaking success has opened a new chapter in the transplantation field bringing almost 50,000 transplants performed worldwide by 1996 [25, 26]. The worldwide diffusion of this procedure has been strengthened by the improvement of surgical technic but, overall, by the discovery around 1980, of immunosuppressive drugs that prevent the otherwise unavoidable immunological rejection. Cyclosporine was the first immunosuppressive drug tested, which led to very significant improvements for long-term patient survival (91% at 1 year and 84% at 3 years, respectively) [25]. Later tacrolimus and mycophenolate mofetil have been developed [27] and then also T cell depleting agents (Alemtuzumab, OKT3 or Minnesota ALG), until the modern immunosuppressive regimes, have permitted to abandon the initial immunosuppressive drugs (tacrolimus and prednisone) and their side effects (hypertension, hyperlipidemia and nephrotoxicity).

When available, nowadays, pancreas transplantation represents the best treatment to offer T1D patient, yielding higher rates of insulin independence compared to insulin treatment or islet encapsulation (which will be discussed below) [28]. Additionally, the quality of life and the complications, deriving from diabetic status, have more benefits from pancreas transplantation than from insulin therapeutic plan or from islets encapsulation [29, 30].

For the above-explained reasons, pancreas transplantation is known as the only definitive long-term treatment for insulin-dependent patients with survival rates of >95% and graft survival rate at almost 85% at 1 year [31]. The major problem that strongly limits a wider diffusion of pancreas transplantation currently is the lack of viable transplantable pancreas. Recently, the total of pancreas transplants has been reduced. Between 2005 and 2014, the number of pancreas transplants declined by more than 30% as well as the number of candidates on the waiting list (−48.0%) [32] with a total of just 954 pancreas transplants performed in the USA [33]. This is secondary to several factors including above all shortage of a primary referral source, difficulties of acceptance by the diabetes scientific society and developments in diabetes management. The final consequence concerning the effective clinical application is that, in the USA, just 3 out 10.000 T1D patients are treated with pancreas or islets transplantation [17].

2.3. Islets transplantation

Islets cell transplantation is one of the most powerful weapons to treat selected T1D patients. It is based on the transplantation of a single selected cluster of cells (islets cells) that mainly contain β cells responsible for the physiological production of insulin. This procedure is

offered only to selected diabetic recipient who did not benefit "standard" insulin treatment with severe hypoglycemic episodes (brittle diabetes) and unstable glycemic profile proposing the whole pancreas transplantation only in case of very poor metabolic control [34, 35]. Islet transplantation has been a solid road to follow just from 1972 via islet isograft performed by Lacy and Ballinger who transplanted pancreatic islet in streptozocin-induced diabetic rats [36]. These revolutionary data have shown how a cell therapy could be effective in the treatment of diabetes even if the translation to the large animal and, finally, to the clinic was still far and full of hurdle to overcome. During the evolution of the whole purification process, Ricordi has furnished a significant improvement [37]. He built an automated chamber to optimize and standardize the isolation procedure thus becoming an indispensable tool both for animal and human models [38] until becoming an internationally accepted procedure for selected candidates. As is done in whole organ pancreas transplantation, the immunosuppressive drugs evolution has been crucial also for the development of islet transplantation. Till date, immunosuppressive regimes are mandatory after islet transplantation due to the source of islets, which need one or more multiple deceased donors in order to be numerically sufficient (it has been calculated that 265,000 islets can be grossly sufficient to achieve and maintain an insulin-independence status [39] up to 7½ years after transplantation). Later, the desired number of transplantable islets cells has been refined and tailored on recipient features, up to 5000–10,000 islets equivalents (IEQ) per kg/body mass that is actually recommended as the minimal β cells mass [40] to transplant. The necessity to obtain this number of viable islets to transplant faces the organ shortage for whole pancreas transplantation. The clinical islet use follows rigid pre-transplant preparation rules, which depend on multi-step approach with the final goal to extract, from the whole pancreas, only the islet fraction (that represents just 1–2% of the total cellular volume).

The entire process is based on enzymatic digestion, controlled mechanical shear and final purification until to enrich a satisfying amount of pure pancreatic islets ready to be injected into the recipient [41]. Even if much less invasive compared to pancreas transplantation (mainly because of its intravascular portal approach), islets transplantation is not free from limitations [42]. Some acute effects comprehend bleeding [43], portal vein thrombosis [44] or a transient increase of hepatic inflammatory markers [45]. All these issues most of the times are transient and much more important long-term side effects related to immunosuppressive regimes (sharing in this way the immunological problems of pancreas transplant).

3. Biomaterials

Biomaterials science is an interdisciplinary field focused on the physical and biological activity of materials and, furthermore, on their interactions if used in a biological environment.

Conventionally, the most intense development and investigation have been oriented toward biomaterials synthesis, optimization, characterization, testing and the biology of host-material relations [46]. The global health care has enormously benefited this field through the creation of heart valve prostheses, artificial hip joints, dental implants, intraocular lenses and many other dispositive, becoming the fundamental cornerstones of many modern therapies.

As reported by the first Consensus of the European Society for Biomaterials in 1976, a biomaterial can be defined as "a nonviable material used in a medical device, intended to interact with biological system" but, alongside the evolution of the field, this definition has been evolved into "a material intended to interface with biological system to evaluate, treat, augment or replace any tissue, organ or function of the body" [46].

Typically we can recapitulate three important big types of biomaterials:

- Ceramics
- Synthetic polymers
- Natural polymers

The correct choice depends on which physiologic function is aimed to augment/replace. For example, in case of bone part replacement, a ceramic scaffold (with major strength and higher mechanical stiffness) will be preferred, otherwise, for cellular therapies, synthetic/polymers will be chosen, favored by their major sustainability in cell behavior.

Scaffolds can be considered as the missing link between biomaterial science and the tissue engineering approach and are defined as biomaterial-based tridimensional structures that should be able to support cellular viability.

According to the biomaterial from which they are manufactured, scaffolds can be divided as

- Ceramics scaffolds
- Synthetic scaffolds
- Natural scaffolds

Regardless of the material, a scaffold must have precise characteristics and important requirements. Biocompatibility is the first mandatory characteristic for every scaffold intended to be used in a biological environment. Generally defined as the capacity of a material to be in contact with a living tissue or integrated in a living environment by not being toxic, injurious or physiologically reactive and not causing immunological rejection, biocompatibility's definition has been modified into "the ability of materials to locally trigger and guide wound healing, reconstruction and tissue integration" [47].

This new definition moves the focus from an outlook where biomaterials must be simply inherent and not causing damages, to a new perspective where they have an active role being a dynamic element. Practically, this is exploited by the capacity of biomaterials to receive cells, allow their attachment and guarantee their proliferation and differentiation at the same time.

Obviously, after implantation the scaffold must be well integrated and not being the target for immunologic reaction.

Mechanical properties are a second important aspect to analyze.

The "ideal" scaffold must present specific mechanical properties suitable both for the anatomical site of implantation (they have also kept mechanical characteristics that make the

scaffold handle for surgical implantation) and for the type of cells intended to use. In particular, using natural scaffolds, mechanotransduction regards the transformation of cellular stress into electrochemical responses, crucial for the survival and the right function of both cells and higher organisms [48]. Additionally, it has been shown how mechanosensitivity could as well facilitate mesenchymal stem cells differentiation [49]. Finally, biological features and biological activity include all the cues that the scaffold could reciprocally interchange with seeded cells and the biological environment. These kinds of properties, moreover in terms of growth factors, chemokines and cytokines, are typical of natural scaffolds suitable to regulate cellular functions.

According to these properties four major categorizes of scaffolds are known:

1. Pre-made scaffold for cell seeding [50].
2. Cell sheets with new-secreted extracellular matrix.
3. Cell encapsulation and hydrogel scaffold.
4. Organ extracellular matrix-derived scaffold.

3.1. Pre-made scaffold for cell seeding.

As deeply reviewed by Chan et al., pre-made scaffolds represent the first structure that have been seeded with the birth of "tissue engineering" [51]. The idea is to produce a tridimensional structure that are able to furnish a non-toxic environment for seeded cells providing a gentle transition by which the scaffold degrades together with the functional enhancement and engraftment of seeded cells. Initial pre-made scaffolds were made in the attempt to overcome limitations due to the "classic" 2D cell culturing, offering all the advantages deriving from the third dimension adding a more physiological environment and more predictive data.

The porosity of the scaffold is always been considered as a crucial property for cellular vitality for the possibility to guarantee the effective delivering of oxygen and nutrients to cells.

Porosity of the scaffold is also today seen as paradigmatic for an ideal-scaffold. In the review of "Porous scaffold design for tissue engineering" [52], the author paraphrased the state of an important architect, Robert leRicolais "The art of structure is where to put the holes" transforming it into "The art of scaffolding is where to put the holes and the biofactors".

This approach has numerous advantages: the choice of biomaterials to use is wide (both considering natural or synthetic polymers) and it can exploit a relative precise and repetitive assembling to micrometer size and the material used can be loaded or cross-linked with numerous molecules in the attempt to augment cellular functionality [53, 54].

3.2. Cellular sheets approach for tissue engineering

Cell sheets approach, even if considered as scaffold-free technology, must be included in the tissue-engineered approach.

Cell sheets technology has been recently developed by tissue engineering and regenerative medicine community has a potential technology able to manufacture layer-by-layer transplantable cellular constructs [55, 56]. The entire principle is based on a particular temperature-reversible polymer, the poly(N-isopropylacrylamide) (PIPAAm) able to change from an hydrophilic to an hydrophobic state at 37 and 32°C, respectively [57].

Cell sheet approach has been tested as a potential approach for pancreatic islets and β cell transplantation exploring alternative site of transplantation as well as the intraportal injection. Subcutaneous site is one of the new sites of islets injection under evaluation (in animal model) [58] and the most important difference is the absence of the vascular connection between transplanted cells and the blood flow. Moreover, in this regard, other advantages can be numbered including less-invasiveness (the hypothetical subcutaneous implantation could be performed under local anesthesia), the possibility of repeated-procedures in case of immunological rejection and, finally, the opportunity to safely remove transplanted islets.

In 2009, the group driven by Okano has manufactured a transplantable cell-sheet made by rat islets [59]. In their research, rat β cells have been cultured on temperature-responsive dishes (with or without adding extracellular matrix) to form a transplantable β cells layer. The resulting cellular structure was subcutaneously implanted in streptozotocin-induced diabetic immunodeficient mice achieving a euglicemia state after 1 week, which last over 100 days. Subcutaneous space is still under analysis in order to be translated to clinics principally due a reduced quantity of oxygen and nutrients that can reduce cell viability at long-term [60]. Recently Pepper et al. gave place to a newly born use of the subcutaneous site for islet implantation. In order to overcome the limitation of the avascular condition of the subcutaneous site, the authors have previously vascularized the implantation site to later infuse in a well-oxygenated site the islet mass. The final described result showed the possibility to revert the hyperglycemic state of severely diabetic induced mice. This innovative approach is recently proposed as alternative vascularized site of implantation that can be combined with device or scaffolds for beta cell replacement in type 1 diabetes.

3.3. Cell encapsulation and hydrogel scaffold

As previously described, islets transplantation procured from deceased donors is one of the most used technology in order reverse a T1D state. Although this procedure is able to produce a euglicemia state, is not free from side effects and limited principally by donor shortage, lifelong immunosuppression and the immunological rejection of transplanted islets. The use of encapsulation device, able to separate the transplanted β cells from the surrounding recipients' environment, has emerged as a promising approach with the attempt to eliminate the immunological issue and, consequently, the need of immunosuppression [61]. The first attempt for encapsulation of human insulinoma tissue dated 1933 by Bisceglie et al. who encapsulated human tissue into membranous bags and transplanted into rats [62]. From that moment, several encapsulation technologies have been developed and implemented and actually they can be sorted into micro- and macroencapsulation technologies depending on the size [63] with the recent addition of the nano-encapsulation technology.

Regardless of the capsule size, the whole approach has a common rationale: enveloping pancreatic islets in biocompatible membrane able to permit the diffusion inside the capsule of molecules including oxygen and nutrition and, at least in theory, to shield it from larger molecules such as antibodies or immune cells [64].

In this way, if successful, an immunological physical barrier, through a perm-selective coating, could prevent the systemic administration of immunosuppressive drugs, which are actually essential to avoid graft rejection.

Materials for islets encapsulation are deeply studied always searching for better performances. It is recognized that two important properties must be developed: firstly, capsule must permit the admission inside the capsule (and so on in contact with encapsulated islets) of small molecules and the diffusion out of waste material and insulin; secondly, they must isolate the content from immune competent cells (B or T cells or macrophages).

These abilities are secondary to the material/s used to produce the capsule.

Alginate, a colloidal substance derived from brown seaweed, is the most famous and the first biomaterial suitable to produce capsules. The main advantage of using alginate relies on its capacity of not interfering with islets (and with insulin release) while guaranteeing a good stability [65]. Several materials are then be added as multiple layers in order to improve alginate functionality. On this subject, poly(ethylene glycol) (PEG) and poly-L-lysine (PLL) are the more established aiming to reduce plasma absorption and increasing long-term capsule stability. In 1999 Chandy et al. reported a modified encapsulation technique with alginate and PEG and showing an improved stability [66]. One year later, Desai et al. showed islets good viability and insulin release with the same encapsulation protocol [67]. Alginate/PLL is the most utilized combination for cell encapsulation in a multi-layer composition. A three layer encapsulation protocol was proposed by Goosen et al., which was based on a alginate/PLL/alginate composition providing a good shield from immune system and a limited diffusion of serum immunoglobulin albumin and hemoglobin [68].

3.3.1. Microencapsulation

In matter of dimension, the size belonging to the microencapsulation group has been the first to be developed and examined. In 1984, O'Shea et al. manufactured an alginate-based microcapsule [69] open up a new era in islets encapsulation that, after almost 40 years counts more than 100 studies in preclinical model (approximately 96% involving small animals). In all these settings several strategies have been tested including capsule customization with three particular molecules such as the alginate, the glucoronic and mannuronic acids, which if used (or combined with other mono/polymer(s)) [70] can confer more strength and stability. Rodent preclinical models are the most studied, as reviewed by Souza et al. who have evaluated more than 60 encapsulation strategies and have founded that the most effective approach is based on the use of intraperitoneal alginate-base microencapsulation without immunosuppressive strategies (islets mean survival rate 100 days) and intraportal injection with immunosuppression (islets mean survival rate 164 days) [71]. Indeed, high mannuronic acid, as biomaterial for islets encapsulation, has shown prolonged survival rate for more than 350 days [72].

3.3.2. Macroencapsulation

Macroencapsulation (or transplantation systems) principle depends on macro-extravascular chambers containing the transplanted tissue. This approach has been developed by Algire et al. and exploits the presence of a semi-permeable membrane to block immune cells but preserving the diffusion of oxygen, nutrients, glucose, insulin, glucagon and somatostatin [73]. Algire's study has paved the way for the creation of a commercially available device that can be transplanted [74]. The peculiarity of this chamber resides in the porosity dimension, 450 nm, able to avoid the direct contact between islets and immunocompetent cells. Follow-up of this device reported a euglicemia state up to 8 months in rodents [75]. Macroencapsulation devices have also shown same interesting results for large animal also even if still not consistent [76]. Baxter Healthcare is the author of one of the first prototype of macroencapsulation device. Two sealed membranes constituted the designed structure, with an autonomous inlet gate. The outside was defined in order to have a gradient membrane to immune-isolate the transplanted cells but also to allow the vascular growth. Small animal experiments displayed a significantly high level of device vascularization in the subcutaneous site 1 year after the implant. This promising approach could be considered the first generation of the actually known TheraCyte Device.

3.4. Organ extracellular matrix-derived scaffold

Extracellular matrix (ECM) can be defined as a heterogeneous, connective network composed of several fibrous glycoproteins able to coordinate cellular functions providing a physical architecture, mechanical stability and biochemical cues necessary for tissue morphogenesis and homeostasis [77]. These qualities will make ECM a potential successful strategy in order to obtain a bioactive scaffold supporting *in-vivo* cellular viability. Recently decellularization protocols have been implemented in order to achieve an ECM-based scaffold that recapitulate the tridimensional architecture of the organ from which, cell intended to be seeded, had been harvested, as well as its biological-specific features [78]. The importance of using an organ-specific ECM relies on the opportunity of working with a natural, biocompatible, bioactive and structural scaffold. Specific properties of extracellular matrix and its application in the diabetes treatment will be discussed below.

4. Extracellular matrix as a template for cell culturing

The extracellular matrix ECM is an organ-specific complex system composed by a plethora of molecules (structural and non-structural) created by the tissue-/organ-specific cells, and deposited into the nearby medium to provide biophysical and biochemical support to the surrounding cells. Originally identified as an inert and passive structural architecture, today its role has been completely transformed and the entire scientific community indicates ECM as an active environment able to support viability, growth and differentiations both stem [79] and differentiated cells. In particular it has been reported how ECM plays a crucial position, being essential part of micro environmental stem cell niches [80] and being a substantial part of any given tissue. ECM is composed of a multitude of supporting bioactive molecules that

are secreted, in different quantities and composition by resident cells in order to support tissue-/organ-specific cellular function(s) specific of each tissue or organ.

The most important constituents of ECM are represented by macromolecules: polypeptide chain of collagen, laminin, fibronectin and glycosaminoglycans. It is important to highlight how this composition could change not only during physiological aging but also under pathological conditions that are able to really upset the whole ECM structure (e.g. liver fibrosis). In native pancreas, ECM lives a sort of "environmental dynamic reciprocity" with pancreatic cells (belonging both to exocrine and the endocrine part).

Islets isolation is a process where Langerhans islets are detached from ECM by the progressive enzyme-based ECM degradation [81]. In other words, the entire cellular compartment is stripped of its surrounding environment leading, in case of islets transplantation, to a very low survival rate around 10% of grafted islets [82]. Obviously the low graft success is not just the consequence of ECM absence being also related to the transplant site characteristics and to a hypoxic state in short- and long-term period, as well as to immunological rejection. Matrix composition is particular important for islets homeostasis and stability.

Pancreatic ECM is an intricate tridimensional network enriched by multiple carbohydrate and protein which, through a system protein-integrin orchestras islets stability, growth, differentiation and death. In this regards, the presence of a specific structure, known as "basement membrane", is essential. This cytological structure, strictly attached to cells is the final activator of specific pathways via interactions between ECM proteins and dedicated integrins. With all these intrinsic properties it is easy to understand how ECM could represent the "ideal" scaffold recapitulating most of the qualities needed such as presence of bioactive molecules, mechanical strength and tridimensional environment.

Moreover these potentials have the additional advantages to be organ specific.

With these bases, the use of an extracellular matrix scaffold as a template for cell seeding can be perfectly in line with the paradigm of tissue engineering.

4.1. Decellularization technology

Decellularization is defined as a multi-step process able to separate the organ/tissue extracellular matrix from its inhabitant cellular component, leaving ECM relatively intact with regard to tridimensional characteristics and biological properties. Resultant structure is an acellular scaffold, which recapitulates the native tissue/organ features furnishing an ideal template to be seeded with new cell families. In 2011 Crapo et al. has profoundly examined all the techniques that can be applied to remove cells from the surrounding ECM [83].

They have divided decellularization techniques in three major types that are still valid today:

1. Chemical decellularization
2. Biological decellularization
3. Physical decellularization

4.1.1. Chemical decellularization

Chemical decellularization is based on the use of chemical agents in order to detach the cells from ECM, catalyzing hydrolytic biomolecular degradation.

Usually, these substances can be acids or bases.

Most common acids used for this purpose are acetic and paracetic acids that have demonstrated good capacity for cellular removal but they also seemed to be too aggressive to ECM structure with an excessive loss of ECM mechanical properties [84]. Calcium hydroxide, sodium sulfide and sodium hydroxide are the most common bases utilized as decellularization agents. It is generally accepted that their use, during decellularization, leads to the elimination of growth factors that enrich the ECM resulting in a loss of bioactivity [85]. For above described reasons, acids and bases are not yet universally used.

Detergents (ionic, nonionic and zwitterionic) may represent the most important chemical agents used for decellularization. Solubilizing cell membranes (cytoplasmatic and nuclear) [86] and separating DNA from proteins, they are therefore effective in removing cellular material from the tissue or the organ treated [77].

Sodium dodecyl sulfate (SDS) and Triton X-100 are the most common used detergents present in decellularization protocols [87].

SDS is an ionic, synthetic, organic compound with an established experience in tissue engineering of decellularized tissues. Dedicated scientific literature offers many protocol based on the use of SDS for organ or tissue decellularization [88–90].

Triton X-100 (TNX-100) is a nonionic surfactant composed by a hydrophilic polyethylene oxide chain and an aromatic hydrocarbon lipophilic or hydrophobic group [91]. By its chemical properties, Triton X-100 can effectively remove cells from tissue but seems to be less aggressive compared to SDS (even bringing to an ECM degradation) and so more useful for thicker tissue [92]. Furthermore, several reports [93, 94] demonstrated how SDS is more effective in removal nuclear material (and consequently, shortening the risk related to residual presence of immunological material).

Finally zwitterionic detergents including 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), sulfobetaine-10 (SB-10) and SB-16 have shown encouraging results, with an important preservation of ECM biological cues but final results need to be more studied [95].

4.1.2. Biological decellularization

Biological decellularization involves the use of biologic enzymatic and non-enzymatic agents able to specifically remove of cell residues or undesirable ECM constituents. Enzyme such nucleases (DNases or RNases) are the perfect archetype centered on their capacity to cleave nucleic acid sequences aiding, in such this way, to eliminate nucleotides after cell lysis [96]. Non-enzymatic agents are mainly represented by chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA). Through metal ions,

segregating EDTA and EGTA can separate cells from ECM. Unfortunately these agents are not particularly effective if used alone. For this, most of the time, they are added in a multi-step protocol [97–99].

4.1.3. Physical decellularization

Physical decellularization protocols include several procedures that exploit physical strategies in order to remove cell from ECM, counting temperature-protocols (freeze-thaw cycles), mechanical-protocols (via the agitation and the immersion of samples) and pressure-based protocols [100]. Temperature-based decellularization protocols are relatively simple procedures that only necessitate of multiple freeze-thaw cycles to be effective in cellular removal [101]. If, on one hand, these protocols seem to be satisfying for cellular removal, on the other one, multiple temperature changes cause important damages on final tridimensional ECM ultrastructure [102]. Freeze-thaw cycles technology is especially appealing for the decellularization of simple structure such as tendon or cartilage-base organs but results hardly applicable on more structural complex architectures (pancreas, kidney or liver). Samples shaking and immersion have instead a strategic role and they are often used for tissue engineering. Most of the times these protocols are used when samples to decellularized are small and without dedicated vascular inlet of outlet such as pre-cut parenchyma cubes of tissue, blood vessels [103] or bone fragments [104]. These techniques provide numerous benefits, such as the possibility to simply change the duration of protocol (and therefore the time-of-contact between detergent and sample) or the shaking force. The choice of the liquid immersion, the total duration time or the shaking force (expressed by rpm in case of orbicular shaking) permit a variety of different protocols targeted on the density of native tissue.

However, the achievement of an homogeneous decellularization state remains one of the main limit of immersion and shacking techniques, also considering that sample external surfaces will be more treated (with a major probability of ECM degradation) compared to the inner parts (that could contain cellular residuals at the end of the process).

4.2. Pancreas-specific ECM

Pancreas ECM results composed by proteins belonging to the basement membrane (collagen, laminin, fibronectin, nidogen/entactin, vitronectin and perlecan) and by proteoglycans (HS proteoglycan, syndecan, glypican, betaglycan and chondroitin sulfate proteoglycans).

4.2.1. Basement membrane ECM components

Collagen fibers are responsible both for structural strength of pancreatic ECM as well as for biological action including cellular adhesion and morphogenesis: collagen I, II, III, IV V and VI are specifically represented in the islets ECM [104, 105]. Their functions are still not completely clear moreover if considering their role on the pancreatic endocrine pathways. As acutely reviewed by Poole-Warren et al. [106], Collagen I molecules seem to promote islets survival [107] but decreasing at the same time insulin release. Furthermore collagen properties can be exploited only with the presence of specific proteins of surface: the integrins

(Int) [108, 109]. These small particles, classified as Int-α1β1, Int-α2β1, Int-α10β1 and Int-α11β1 have been shown to act as receptor supporting matrix-cellular interactions via the subunit α of collagen 1 fibers [110].

Beyond the collagen, laminin proteins are plenty involved in the structural ECM pancreatic architecture. Laminin takes part in the structural integrity of the extracellular matrix by bounding to collagen, nidogen and glycosaminoglycan fibers [111]. Laminin proteins have been localized in the islets cells permeating their islet's microvasculature, but their actions are still under debate. Laminin-islets relationship is controlled by the presence of following integrins: Int-α1β1, Int-α2β1, Int-α3β1, Int-α6β1, Int-α7β1, Int-α9β1, Int-αvβ3, Int-αvβ5, Int-αvβ8 and Int-α6β4 with islets morphogenesis functions (α3 and β1) [112] and pancreas development (α6) [113]. Finally, vitronectin is an ECM protein expressed during human fetal islets development. Its correct pathway is essential for the appropriate growth of islets to "mature" β cells and moreover for their migration acting as mobility promoter [114].

4.2.2. Proteoglycans ECM components

Proteoglycans are heavily glycosylated proteins that take ubiquitously part in most of all organs and tissues both with a structural and deposit activity. Their composition is based on a core protein with several different, negatively charged, protein chains (GAGs) attached. Different protein chains bring to different proteoglycans [115]. Negative charge is crucial for the deposit role of this protein being able to conserve specific growth factors, cytokines and chemokines (that differ from tissue to tissue in terms of composition) releasing them just if necessary [116].

4.3. Whole organ decellularization and regenerative medicine

Whole organ perfusion is the most commonly used technology, if the target sample is an entire organ. This technique exploits the organ native vascular system to homogenously perfuse the organ with selected detergent(s) [117]. Perfusion-decellularization is the most consistent method to obtain decellularized whole organ scaffolds due to the native vascular architecture, which is naturally designed to permit the delivery of oxygen and nutrients by blood flux. This allows the same time-to-contact between detergent(s) and cells in the entire organ by an anterograde or a retrograde perfusion. Likewise, the presence of a vascular outlet (most of the times represented by the native venous outflow system) allows an efficient strategy to eliminate cellular debris deriving from decellularization. Whole organ perfusion has been already established for several organs or tissues that differ in terms of size, origin (small or large animal model and human) and physiopathological conditions [118]. Obviously, as described for immersion/shaking technology, the appropriateness of the perfusion protocol must be pointed on the organ properties (moreover size and resident cell density).

This technology has been also applied in the attempt of creating a bioengineered pancreas.

Basically the idea is to customize clinical-relevant size acellular pancreas that can be secondly repopulated with patient-own endocrine cellular population and then transplanted in the same patient. If successful, this approach could address the limitations that today affect diabetes

treatment. It could be created a non-immunological recellularized scaffold with functional islets, β cells or human-induced pluripotent stem cells (hiPSC) derived to β cells and endothelial cells subsequently orthotopically transplanted in the patient whose cells were harvested. In this scenario, animal source could provide a hypothetical unlimited pool of scaffold to repopulate and stem cells (mesenchymal or iPS) can be used for the repopulation.

A bioengineered approach could so provide an unlimited source of transplantable pancreas, eliminating the organ shortage, and, at the same time, the use of patient-own cells could avoid (or limit) the use of immunosuppressive drug regimes.

The entire decellularization development must be balanced according to the final desired quality of the extracellular matrix. This quality is currently evaluated by histological staining, DNA content, collagens and proteoglycans assessment and tridimensional imaging technologies (scanning electron microscopy). This assessment provides crucial data not only on the effective cellular removal but also about the biological and structural properties of the decellularized matrix intended to seed.

If decellularization technology has provided satisfying results in term of pancreas decellularization and ECM maintenance, recellularization strategies seems to be the most important hurdle, still not yet overcome.

These, together with engineering significant advances for the manufacturing of dedicated bioreactors, are the next steps to go.

4.4. Recellularization technology

Recellularization strategies play a key role for the creation of a functional bioengineered organoid. Working recellularization requires a proper cell source (considering both mature or stem cell origin), an optimal seeding method and a long-term culture system (available with appropriate bioreactors). Scaffold recellularization needs tree different cell group, belonging to parenchymal, vascular and supporting types respectively with different tasks and responsibilities.

Parenchymal cells are responsible for the effective organ function whereas vascular cells must entirely cover the vascular extracellular matrix providing a suitable blood flux (after transplantation) both as inlet (with oxygen and nutrients delivering) as well as in outlet for metabolic wastes spill.

In this regards, vascular coverage during recellularization is essential for the success, and it must be achieved covering as much ECM as possible. Missing ECM parts, which are not protected by vascular cells, will directly expose ECM to the blood flow, resulting in an almost instant activation of the coagulative cascade and in the formation of blood clots. This situation will bring to a subsequently blood stoppage directed to the entire portion downstream the clot, with final seeded cells death [119].

Finally supporting cells must provide an active sustain for parenchymal and vascular cellular families.

Pancreas scaffold recellularization can be performed via vascular perfusion (using the same principle utilized to remove cells). Inlet pancreatic vasculature can be mainly accessed by splenic and pancreaticoduodenal artery.

Pancreatic inlet can also exploit the presence of the pancreatic duct via a retrograde flow. Pancreatic outlet vasculature relies on the portal and the splenic vein.

4.5. Cell-on-scaffold technology toward bioartificial pancreas: state-of-art

Cell-on-scaffold technique has been already examined in order to produce a pancreatic organoid.

One of the first pioneeristic studies that explored the possibility of using ECM matrix as a template for islets seeding has been proposed by De Carlo et al. in 2010. In their report 240-μm slices of rat pancreatic and hepatic acellular were decellularized by a detergent-based protocol (4% sodium deoxicholate was used) and then seeded with islets after static culture conditions (37°C, 95% O_2 and 5% CO_2 in RPMI-1640 medium added with 5.6 mM glucose, 100 IU/ml of penicillin and 100 μg/ml of streptomycin). Furthermore pancreatic islets (N = 50) were seeded on five acellular matrix and cultured under standard conditions. After 7 days, the islet-matrix complexes were inserted into synthetic, tubular PVA/PEG devices and prepared for *in-vivo* implant. Rats were then made diabetic and PVA/PEG devices (containing islets and matrix) were implanted. Results have demonstrated, in an *in-vivo* follow-up up to 6 weeks, how pancreatic devices reacted to glucose acute stimula with insulin delivery and a decreasing dose of daily insulin needed to maintain the euglycaemic condition [120]. To our knowledge, in the same year, Ott's lab proposed for the first time a rat pancreatic whole pancreas scaffold (obtained via detergent perfusion) afterwards seeded with human islets and supporting human MSCs. Results were very fascinating showing effective tridimensional growth of cells on the matrix with good response to glucose stimula [121]. Both these studies have shown important starting lines for the use of this technology to produce a functional bioengineered pancreas. However they have also highlighted significant limitations, some of which have been already overtaken.

Herein we describe the most important findings regarding the use of a whole organ pancreatic scaffold from different models with the aim to manufacture a transplantable bioengineered pancreas.

4.5.1. Small animal models

Small animal model represent the baseline for basic science in terms of costs and prospective translational results. For this reason small animal model has been the first to be investigated.

One of the first complete proof-of-concepts of a murine whole organ pancreas decellularization has been offered by Goh et al. in 2013 [122]. After a midline laparotomy the pancreas was carefully harvested preserving intact vascular inlets that have been then cannulated and used as inlets for retrograde perfusion with a flow of 8 ml/min. A multistep protocol with 0.5% SDS and 1% Triton X-100 has been chosen. Cells for repopulation (AR42J acinar cell line for the

exocrine component and MIN-6 β cell for the endocrine one) were cultured in standard static condition and then seeded on the acellular scaffold. AR42J cells were injected through the main pancreatic duct (30×10^6) cells whereas MIN-6 β cells (30×10^6) via the hepatic vein. The seeded pancreas was cultured under static conditions at 37°C with a 95% air/5% CO_2 atmosphere for 5 days and then implanted by a dorsal subcutaneous pocket of an adult mouse for biocompatible preliminary tests. Results have demonstrated an optimal feasibility about decellularization with preservation of ECM composition. Secondly recellularization was evaluated in terms of cellular engraftment, survival and functionality by Immunohistochemistry (IHC). Reconstructed pancreas showed a homogenous distribution of cell types with a minimal apoptosis rate (detected by TUNEL staining) less than 18% and robust cellular functionality expressed by C-peptide staining positivity and finally confirmed by up-regulation of insulin genes. After 14 days, subcutaneous implantation pancreatic organoid was harvested and established as biocompatible organoid able to positive regulate a neoangiogenetic action. This paper has set the bases for all the subsequent experiments.

Recently a group headed by Struecker has refined the entire process providing, for the first time, a proof-of-concept for the repopulation of the decellularized rat pancreas with functional islets of Langerhans [123]. Briefly rat pancreas was decellularized via vascular perfusion (1% Triton X: 0.5% SDS and 1% Triton X-100) with a flow-rate around 10 ml/min and then repopulated with approximately 2000 islets via the pancreatic duct to test viability and functionality of the islets after the process. *Ex-vivo* TUNEL staining and glucose-stimulated insulin secretion (GSIS) revealed how islets were viable and functional after the injection inside the acellular scaffold.

Both the presented studies have analyzed the opportunity to use mature and already differentiated cells to repopulate pancreas whole organ scaffold.

A further progress toward the creation of an ECM-pancreas can be accomplished exploiting the use of stem cells driven in their growth and specific differentiation by precise ECM biological cues. Thus stem cells can be considered a potent and encouraging cell source for tissue engineering [124]. Accordingly to this concept, Wan et al. has just proposed a study about the possibility to culturing iPSCs derived pancreatic β cells on decellularized ECM [125]. After peristaltic artery perfusion with detergents, decellularized ECM-based pancreas were seeded with already differentiated into β cells like iPSCs (approx. 3×10^6) by two different methods (vascular perfusion and multipositional parenchymal injection). In-vitro continuous monitoring exhibits maintained insulin, C-peptide and glucagon expression. Additionally, insulin level expression in the perfusated media was twofold higher than those levels obtained by traditional bidimensional culturing.

4.5.2. Large animal models

Even if encouraging results have been reported for small animal model, it is mandatory to aim to a functional bioengineered pancreas with clinically relevant size.

That way the use of a large animal model has been investigated.

The main difficulty in the switching from small to large animal is mainly due to the higher stiffness of the pancreatic parenchyma and to the larger volume that has to be decellularized. In this regards, decellularized protocols could not be too aggressive in order to preserve ECM native characteristics. In 2013, research group headed by Orlando published the proof-of-concept for the achievement of a porcine decellularized whole organ scaffold [126]. Vascular-based retrograde peristaltic perfusion via superior mesenteric vein and pancreatic duct with nonionic detergent (1% Triton X-100) guarantied pancreatic cellular removal. Pancreatic extracellular matrix biocompatibility was tested by the static short-term (7 days) cellular seeding with human amniotic fluid stem cells. This type of cells has been also proposed as a potential source of insulin-secreting cells [127]. To validate the hypothetical capacity of acellular ECM-based pancreas like scaffold to sustain endocrine pancreatic function, porcine pancreas scaffold was repopulated with porcine islets and insulin secretion was *ex-vivo* measured under different glucose stimula and at several time points. Results proposed showed a significant similarity to physiological circumstances with higher insulin release in response to higher glucose concentration. Katsuki et al. have also proposed analogous results in 2016 [128] with the creation of a portioned repopulated porcine pancreas.

4.5.3. Discarded human pancreas as a source of ECM

The use of discarded human organs has been explored as a possible source of organ to decellularize. This opportunity is based on the incredible amount of organ that, annually in the USA, are retrieved for transplantation purposes but finally discarded for various reasons. Orlando et al. described this scenario envisioning [129], in a future not so distant, that a discarded organ will be use to build an acellular human scaffold subsequently repopulated by patients own cells. The final result will be a bioengineered pancreas composed by human-derived extracellular matrix as well as recellularization cell type. The hypothesis to recycle discarded human organs for organ bioengineering projects, hinges on the theory that decellularized human ECM could be the perfect environment for human cells during recellularization.

In 2015, our group has explored the use of discarded pancreas as a source from which obtain acellular whole organ scaffolds [130]. Compared to data achieved on the porcine model, an additional inlet access was demonstrated crucial switching from a two-way perfusion (pancreatic duct and superior mesenteric vein) to a three-way perfusion system (pancreatic duct, superior mesenteric artery and splenic artery). This adjustment brought to a more homogenous distribution of the detergent used (1% Triton X-100) permitting a relative more gentle approach (Triton X-100 is considered less aggressive than SDS). Staining and DNA content confirmed the cellular removal as well as the preservation of the most important pancreatic ECM elements and growth factor that enriched the human native pancreatic parenchyma. Besides important results about static seeding with human islets and dynamic peristaltic seeding with human primary pancreatic endothelial cells, crucial information has been obtained by the analysis of ECM immune properties. In fact, to the best of our knowledge, it is the first time that human pancreas ECM documents an immunosuppressive T-reg promoting properties paving the way for its possible use as immunosuppressant (**Table 1**).

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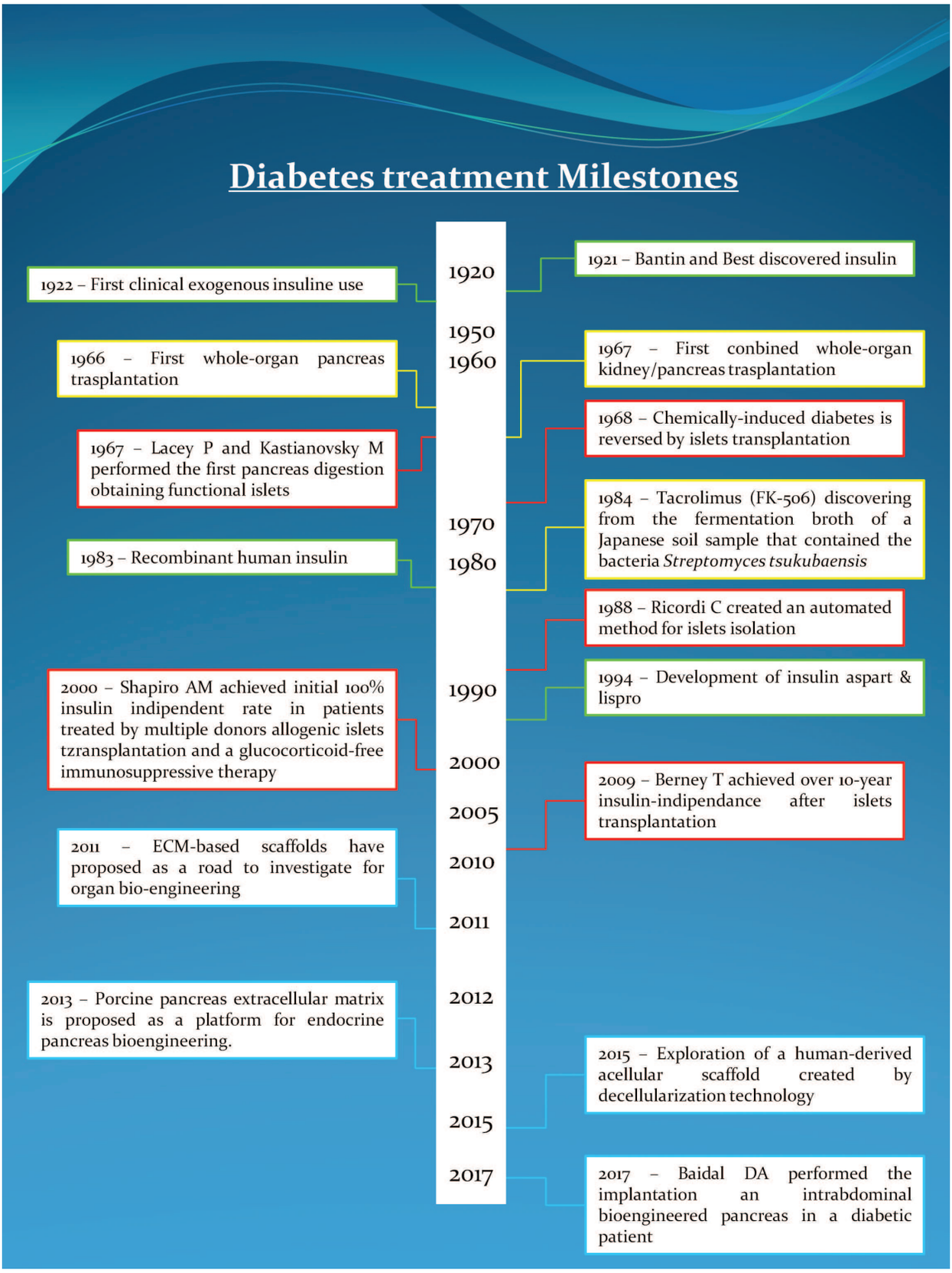


Table 1. Timeline of the major advances in insulin treatment, islets and pancreas transplantation and pancreas bioengineering.

5. Conclusion and future perspectives

In the last decade, regenerative medicine and organ bioengineering have accomplished important progress toward the manufacturing of a functional, bioengineered pancreas, exploring different options both in terms of platform for decellularization as well as for cell type repopulation source. As of now, the emergent use of stem cells appears as an exciting and encouraging field to discover. Several crucial hurdles are not yet overcome and require important advancements, above all *in-vivo* short- and long-term functional testing. Despite these obstacles, cell-on-scaffold technology holds a huge potential in order to solve the problem of pancreas shortage creating a solid transplantable alternative.

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