# An Emerging Paradigm in Tissue Engineering: From Chemical Engineering to Developmental Engineering for Bioartificial Tissue Formation through a Series of Unit Operations that Simulate the In Vivo Successive Developmental Stages†

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The field of Tissue Engineering is in a critical stage, with the need to replace the trial-and-error methods (which, so far, are the most-often used) with rational methodologies. The opinions of several research groups expressed in the literature, as well as recent research efforts, seem to converge on a change of direction toward in vitro recapitulation of the in vivo process of tissue formation (biomimetic processes), replacing the three-dimensional (3D) cell growth and differentiation paradigm that is currently practiced. However, what the fundamental principles for the ex vivo creation of a biological implant are and how these could be translated to process design criteria for biomimetic processes is still unclear. Chemical Engineering has already offered to Tissue Engineering various tools, such as biomaterials and bioreactors, but its contribution to the development of a scientific basis for Tissue Engineering for the design of biomimetic process now has become critical. The intellectual core of chemical engineering in process design, based on the synthesis of processes from the assembly of unit operations, can provide a methodological framework suitable for the development of a rational biomimetic methodology in Tissue Engineering, with the unit operations recapitulating the successive stages of in vivo tissue development. In this article, we describe (i) why and how biomimetic processes should be designed to synchronize the evolution of variables that describe the biological transformations during the tissue development, (ii) how information from developmental biology can be used for the design of biomimetic processes, and (iii) from where the scientific and technical feasibility of designing such processes arises.

*"Confusion, ignoratio elenchi, is itself the most fatal of errors, and that it occurs whenever argument or inference passes from one world of experience to another."*
*Michael Oakeshott, Experience and Its Modes*

## 1. The Falling Three-Dimensional Cell Growth and Differentiation Paradigm of Tissue Engineering

**1.1. The Role of Paradigms in Determining Research Directions.** Tissue engineering was introduced few decades ago as a distinct research field, with the objective of generating bioartificial tissues in the laboratory to solve the problem of organ shortage for transplantations. According to the theory of the evolution of science developed by the historian of science and philosopher Thomas Kuhn, the research practice in each scientific field is determined by a "set of theories, beliefs, values, instruments and methods" that Kuhn called "paradigm".[1] The paradigm is based on past scientific achievements that are acknowledged by a particular scientific community as the foundation of its research practice. It defines, as Kuhn mentioned, a coherent scientific tradition as such, for example, is the Ptolemaic or Copernican astronomy and the Aristotelian or Newtonian dynamics, or, more recently, the Systems Biology, which studies gene and protein interaction networks.[2] The new paradigm of systems biology focuses on the interactions between the components of biological systems that give rise to the function of these systems (either cells, tissues, organisms, or ecosystems), contrary to the previous genocentric paradigm that attributed the behavior of biological systems to single genes. As we now know, after the numerous attempts of genecentric paradigms to identify genes that are responsible for diseases, the disturbances of cell or organism normal functions are not determined by single genes; this is true only in rare cases, accounting for ~2% of the total disease load.[3] Instead, they are determined by networks of interacting gene and proteins that influence each other's expression. When this network is not operating properly, the expression of various proteins is modified and the normal system state is transformed to a diseased one. For example, more than 100 genes have been identified as contributing to coronary artery disease,[4] rendering meaningless to give any privilege to any gene outside the context of the others and their interactions that determine its expression.

The above statements do not mean that the paradigms are correct or incorrect. Rather, it signifies that the role of the paradigms is to focus the research activities on specific areas, so that data will be accumulated rapidly by the collective efforts of a particular scientific community that will accept the paradigm. However, at some point, the accumulated data will indicate that the paradigm's concepts and methods have reached their limit in the explanation of these data. A change in focus then is needed to other phenomena that the accumulated data

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uncovered. For example, the genocentric paradigm of checking the role of genes and their interactions produced data which indicated that their interactions in networks are an important factor to be studied, more than the function of single genes, which, as became evident, depends on such interactions. Then, the new paradigm of Systems Biology, focusing the research activities in the study of the behavior of networks of genes and proteins as the determinants of the cell or organism functions, was unavoidably introduced with the assistance of data accumulated under the concepts of the previous paradigm. As we will see below, an exhaustive study of the phenomena related to the growth and differentiation of cells in three dimensions will lead Tissue Engineering to refocus its attention onto other more complex phenomena of tissue growth that involve the natural way tissues develop in vivo during development.

**1.2. The First Paradigm of Tissue Engineering: From the Two- to Three-Dimensional Cell Growth and Differentiation.** The first paradigm of Tissue Engineering is "three-dimensional (3D) cell growth and differentiation". This paradigm originated from the achievement of the groups of Langer and Vacanti, who succeeded to grow in vitro cells that were attached to porous biomaterials, called "scaffolds".[5] The authors embedded the cell-bearing scaffold in media that contained nutrients, oxygen, and growth factors (various proteins or hormones that stimulate the cell growth and determine the cell differentiation state), so that the cells could grow and spread attached to the walls of the pores of the scaffold. In this way, the cells were distributed in three dimensions compared to two-dimensional cultures traditionally applied in biology on the surface of tissue flasks. The pore network of the scaffolds played the role of the in vivo vascular network. It allowed for cell nutrition and oxygenation in three dimensions, since the cells were in constant contact with the medium that was filling the pores of the scaffold and was replaced (by convection, diffusion, or flow, depending on the bioreactor system in which the scaffold with the attached cells was placed) with fresh medium of the bulk medium surrounding the scaffold.

**1.3. The Relationship between the Concepts of Biochemical and Tissue Engineering.** Without such porous biomaterials, the cells could grow only to a limited extent in three dimensions, attached to each other and forming aggregates. As more isolated cells floating in the medium attach to the aggregate surface, the aggregate size increases and the center of the aggregates becomes necrotic. Cells there have no access to nutrients and oxygen, because the medium cannot easily penetrate the cell aggregate (via diffusion) to bring them inside the aggregate and, at the same time, remove the toxic byproducts of cell metabolism (such as lactate and ammonia). This not only leads to cell apoptosis (suicidal cell death mediated by an intracellular program implemented in a series of steps to control the formation of cell debris, so that other cells will not be damaged, while, at the same time, sending the appropriate signals for the removal of cell debris by neighboring phagocytic cells[6,7]) and necrosis (traumatic cell death that results from cellular injury in which, however, no specific signals are sent to phagocytic cells, with the result of a buildup of dead cells[8]), but also proliferation and differentiation is spatially affected differentially, because of the gradient of nutrients, oxygen, and growth or differentiation factors toward the center of the aggregate.

For example, from mammalian cell cultures in bioreactors in biochemical engineering, it is known that the deprivation of nutrients (such as glutamine, cystine, glucose, or oxygen) induces apoptosis.[9,10] In some cultures, the deprivation of any single amino acid leads to apoptotic death.[11] On the other hand, the accumulation of toxic byproducts from the metabolism, such as ammonia and lactate, induces cell necrosis.[12] Fed-batch or perfusion medium strategies then were developed to provide, to the cells, an appropriate environment for their growth, one that could keep the nutrient concentration sufficient but low, to minimize the production of cell metabolic byproduct.[13−16]

Therefore, scaffolds were introduced to overcome such types of problems, allowing the formation of large 3D cell constructs with viable cells; thus, the concept of scaffolds for cell growth is similar to the concepts of Biochemical Engineering for mammalian cell cultures. For example, packed-bed bioreactors for the culture of mammalian cells, with the bed consisting of porous particles for cell immobilization, presents the same physical phenomena as observed in cells distributed in scaffolds. In a packed-bed filled with cells attached to porous microcarriers, two types of medium flow can be encountered: intermicrocarrier flow of the medium, which brings the fresh medium in contact with the microcarriers, and intramicrocarrier convenctional flow of the medium, which brings nutrients, growth factors, and oxygen inside the microcarriers and in contact with the cells within. It has been shown that convenction becomes an important factor for the prevention of anoxic areas inside the microcarrier.[17] The solution of flow and mass-transport equations for the optimization of such processes provided the gradient of concentrations along the bed and inside the microcarriers.[18] Such types of bioreactors have been transferred as a tool to applications of Tissue Engineering. For example, the continuous flow of medium has been shown to improve cell functions, such as the production of collagen and proteoglycans (an increase of 50%−70% has been observed), in comparison to bioreactors without flow, where the replenishment of substances was only based on convection and diffusion.[19]

The conclusion from the above analysis is that (i) Tissue Engineering is conceptually a continuation of Biochemical Engineering and (ii) various tools and methods already in use in biochemical engineering have been modified for the needs of Tissue Engineering research. This also means that many of the processes of Tissue Engineering for the cell growth in three dimensions were already in place. Their feasibility had been thoroughly tested in biochemical engineering, so that, as we will see below, such processes can be considered as unit operations in more complex Tissue Engineering processes whose objective is bioartificial tissue development to a construct with tissue structure and functions, instead of only cell growth and differentiation.

**1.4. Cell-to-Cell Interactions Determine the Functions of 3D Cell Constructs.** It is only inside the organisms in tumors that their cancer cells survive, forming large aggregates, because most cancer cells induce angiogenesis (formation of new blood vessels) via paracrine mechanisms (cell signaling between neighboring cells of different types). For example, prostate cancer cells inside tumors express the angiogenic factors VEGF and interleukin-8 (IL-8) to stimulate the proliferation of endothelial cells that form new blood vessels. The blood vessels penetrate the tumor mass, carrying nutrients and oxygen inside the tumor and keeping the cancer cells alive.[20] Therefore, tumors cannot be considered simply as random cell aggregates, because interrelationships are developed between cancer and normal cells, such as the endothelial ones, that are beneficial to both cell types. It is these types of interactions that make the treatment of cancer difficult, because single cancer cells that have been isolated from tumors and cultured in two dimensions in tissue flasks lacking such interactions can be easily killed by drugs. However, these drugs are not similarly effective in tumors in

organisms because tumors are complex ecosystems in three dimensions, with the normal cells surrounding the tumor, providing support to the cancer cells. Below, we will see that normal tissues also are complex ecosystems of heterogeneous cell populations that develop interrelationships through secreted protein signals. This fact should make the goal of Tissue Engineering to fabricate such ecosystems, restoring, in vitro, the cell-to-cell interactions among different cell types, instead of making constructs of cells randomly distributed in three dimensions inside a scaffold. A random cell distribution prevents any in vivo-like cell-to-cell interaction and, consequently, any function at the tissue level that arises from these interactions. The resistance of tumors to treatments is based on such functions; therefore, it is a property at the tumor level, not at the cancer cell level.

**1.5. The Lack of a Methodology in Tissue Engineering.** The work of the above-mentioned groups of Langer and Vacanti[5] has been identified as probably the most influential for the initiation of Tissue Engineering as a distinct field.[21] Therefore, according to Kuhn's theory, it played the role of a paradigm in determining the eligible problems and accepted solutions, as well as in determining objectives and methods of research practice in making bioartificial tissues in vitro. In accordance with this paradigm, Tissue Engineering started to collect expertise in tools needed to grow cells in scaffolds placed inside bioreactors from various fields on a need-to-know basis, in an effort to solve practical problems, some of these mentioned below as examples:

*Material Science* was employed to determine how the scaffold surface could be modified with extracellular matrix molecules (a complex mixture of proteins around the cell that provides structural support to them), so that the cells could better attach to the pore walls of the scaffold,[22] finding an environment similar to that which they had in vivo, before their isolation from the organism tissues. The scaffold surface was also modified with molecules that control the cell differentiation,[23] although it is known that cell differentiation is a multistage process with various signaling molecules involved in each stage, such that it is impossible for all of them to be delivered to the cells in the time schedule of the in vivo cell differentiation.

*Chemical and Biochemical Engineering* were employed to determine how the bioreactors could be redesigned so that the cells would avoid shear stress from the fluidics of the medium surrounding the scaffolds or how the cells could be better oxygenated by perfusion (diffusional gradients of oxygen reduce the formation of viable tissue from primary cells to a layer that is only $\sim$100 $\mu$m thick).[24] For transformed cells, the oxygen demands are low compared with that of primary cells as hepatocytes.[25] On the other hand, for other processes with other cell types (such as the expansion of embryonic stem cells), high oxygen has detrimental effects on cell growth and cellular state stability.[26,27]

*Molecular Biology* was employed to provide the recipes of growth and differentiation factors needed for cell growth and their differentiation to the destined tissue cell types. However, we now know that different factors are needed at the different stages of cell differentiation. D'Amour et al., for example, have shown that an efficient generation of beta cells with high insulin secretion ability was produced in a process composed of five stages.[28] The multiple stages of cell differentiation implemented in vitro allowed the use of a different combination of growth and differentiation factors for each stage, according to information available from Developmental Biology.[28] It also allowed the determination of biochemical properties of cells to assess the cell state at each stage of the process. We could not then expect that the one-stage processes of Tissue Engineering that start with undifferentiated stem cells can lead to authentic in vivo tissue cells.

*Medicine and Surgery* were employed to determine how diseases could be treated with bioartificial tissue implants and what properties the implants should have, so that they could be integrated to the recipient tissue.[29] However, now the implantation of bioartificial tissues with cells that differentiate in vitro and are still in the differentiation process when the scaffold is implanted has been questioned, because the cellular microenvironment in the adult tissue is different from that of the embryonic one.[30] Therefore, it is not surprising that cells generated in vitro from stem cells are not equivalent to those arising in vivo, given the extensive cellular interactions and "education" that occur during the in vivo development.[30] This is reflected in the non-optimality of implants, such as the instability and weakness of a bioartificial thumb.[31]

Despite the involvement of various research fields under the common goal of fabricating bioartificial tissues in the laboratory, Tissue Engineering remains the only engineering field that has not yet succeeded in unifying the methods and tools developed by the diverse fields involved. Tissue Engineering still means different things to researchers from different fields that specialize and focus on different aspects of the in vitro tissue formation, rather than considering all of the aspects needed to make bioartificial tissue.[32] None of the research fields has as a goal to make bioartificial tissue: instead, they all try to solve specific problems of their expertise in an "Edisonian" way, without having a methodology based on principles of how tissues develop, as it is mentioned in the study of the National Science Foundation (NSF), which had the purpose of assessing the status of the field by interviewing tissue engineers.[21] Consequently, the entire procedure that is followed in the laboratory—from cell isolation from organism tissues or from embryonic stem cells to bioartificial tissue formation and finally to implantation of the cell-bearing scaffold—is broken into subfields that are handled by different disciplines.

Tissue Engineering did not pay attention to the interfaces between the involved disciplines, since researchers involved in Tissue Engineering only had experience in their field. Although they could optimize some of the properties of the bioartificial tissue, they devoted no efforts to develop a unifying methodology that could be able to optimize the bioartificial tissue itself (i.e., the critical properties needed for a successful implant). Therefore, it can be expected that Butler[33] posed the question for bioartificial tissue: "How good is good enough?" This question still remains without an answer. The lack of an engineering methodology that could unify the involved disciplines is a critical difference in the development of Tissue Engineering, when compared to the development of other engineering fields that are based on the principles of Physics and Chemistry. Such a difference has been already mentioned in the literature. Williams argued that the way Tissue Engineering is practiced gives the impression that "the reason why there is the word 'engineering' in the term 'tissue engineering' is not intuitively obvious".[34] While engineering has as a purpose "to solve practical problems and/or the systematic analysis of physical data to yield tangible end-products", no such problems that could lead to products have been yet solved in Tissue Engineering.[34] Profitable end-products for clinical applications are still expected to be made.[35]

Although Tissue Engineering has not yet solved the critical problem of providing bioartificial tissues suitable for clinical

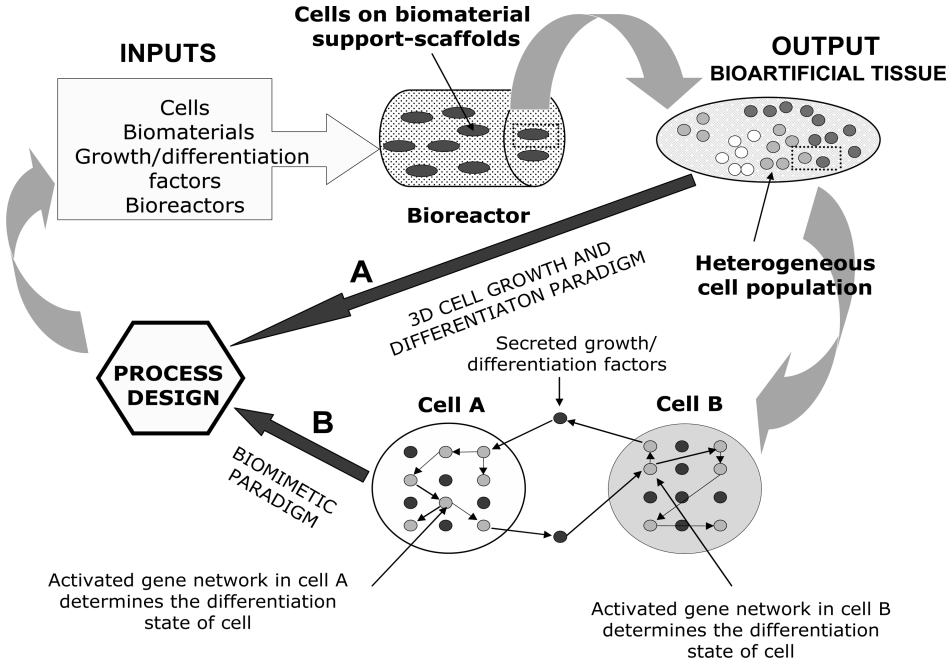


**Figure 1.** According to the three-dimensional (3D) cell growth and differentiation paradigm, bioartificial tissue is a construct resulting from the cells grown inside porous biomaterial scaffolds placed inside a bioreactor. Because of the lack of a vein network that in the body supplies the cells with nutrients, porous scaffolds are used as support for the cells, and the cells spread, move, divide, differentiate, secrete extracellular matrix, and occupy the internal surface of the pores of the scaffold. In this case, the pore network of the scaffold plays the role of the vein network, providing the cells with the necessary nutrients and oxygen from the medium that fills its pores. The cell-populated scaffold is suspended in a medium containing the necessary components for the cells in a bioreactor system: nutrients, oxygen, and growth/differentiation factors. The in vitro process design refers to the appropriate selection of the various inputs of the process to optimize the cell growth and differentiation (following arrow "A"). However, real tissues are not random cell collections; rather, they are complex ecosystems composed of different cell types that develop interactions between them, exchanging protein signals that determine which gene each cell expresses to acquire the properties of a specific cell type. It is the cooperation among different cell types that is responsible for the emergence of tissue functions (i.e., functions of tissues that single cells cannot perform). Consequently, the bioartificial tissue made in one-stage processes that do not take into account the cell interactions do not exhibit tissue functions. It is only if the tissue develops in vitro, rather than in vivo, that the cell-to-cell interactions will be established as in real tissues resulting in tissue functions (arrow "B" of biomimetic (mimic the natural in vivo tissue development) process design).

applications, it is studying 3D cell growth and differentiation extensively. It could not still conclude what the methodology to make bioartificial tissue should be. As Butler mentioned,[33] "many talented researchers are publishing data for such a wide variety of treatment conditions that it becomes difficult to place the results into proper context", and he continues in saying that the numerous treatment factors and response measures of published in vivo or in vitro experiments makes interpretation of the results difficult, so that this complexity finally creates confusion. The confusing situation even has been transferred to the interpretation of Tissue Engineering, with tissue engineers arguing in favor of various versions, such as debate over whether cells in scaffolds or only cells or only scaffolds or even only delivery of growth factors to enhance tissue generation could be or not included in the interpretation of the field.[36]

**1.6. Tissue Engineering Research Practice and Its Problems of Providing Optimal Bioartificial Tissue.** If Tissue Engineering is not yet in a position to provide bioartificial tissue suitable for clinical applications, the question of what type of problems its research is trying to solve arises naturally. According to the 3D cell growth and differentiation paradigm, the studies of the in vitro formation of bioartificial tissue focuses on the development and appropriate selection of the technical tools used in the in vitro process, such as different cell types, growth and differentiation factors recipes, scaffolds with different chemical and physical properties, bioreactor types and modes of operation, etc., which are provided by different fields (these are referred to as "inputs" in Figure 1).

The paradigm of the 3D cell growth and differentiation defines the following questions as being important to be answered:

• How will the cells survive and multiply optimally in three dimensions?

• With the introduction of stem cells in Tissue Engineering (before the stem cells that can differentiate to various cell types, primary cells taken from organisms that had already been differentiated were used), how they will differentiate to cells of specific tissues?

The silent assumption is that these two phenomena, cell growth and differentiation, when they occur in three dimensions instead of two dimensions, are sufficient to lead, through cell self-organization, to tissue structures with tissue functions. However, this perception ignores the complexity of the tissue development in vivo. Developing tissue, as it grows until its final form, passes from different developmental stages with its cells in different differentiation stages. The developing organism provides the optimal conditions that are needed for each stage of the developing tissue and are different for each one. The extension of the study of the basic phenomena of 3D cell growth and differentiation to product development, and especially to extreme promises for complex tissues and organs development[37] was illegitimate: this was a product of the press that oversells stem cells now, while, a few years ago, it was doing the same with Tissue Engineering.[38] The picture of the ear growing in the back of a mouse[39] was not a real ear that developed naturally. Although this was written in the title of the above-mentioned article ("*Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear*"), a different perception had been created by the press. The bioartificial ear was simply a scaffold precut in the shape of a human ear that was seeded with chondrocytes. The chondrocytes grew inside the scaffold but the bioartificial ear

was not developing as in vivo. In the press, this study[39] was extrapolated, defining the near-future goal of Tissue Engineering the organ fabrication (a "whole host of other lab-grown body parts are just around the corner", according to the interview of one of the authors of the previously mentioned study included in the article by Steven J. Milloy in *The Washington Times* (December 10, 1999)).

However, it is misleading that the development of clinical products was the direct goal of the concepts upon which Tissue Engineering research practice was based, according to the 3D cell growth and differentiation paradigm. The goal of the research was to study the basic phenomena relevant to in vitro 3D cell growth and differentiation, such as cell viability and growth, cell attachment to scaffolds, cell spatial distribution, cell differentiation and so on; therefore, it must be characterized as basic research in engineering and not as product development. According to the definitions of the American Institute of Chemical Engineers (AIChE), Engineering R&D includes basic research ("understanding fundamental principles and mechanisms relating to the human-made world"), applied research ("acquiring knowledge to determine how a specific need can be met"), and technology development ("using knowledge to produce materials, devices, and systems, including design, testing, and demonstration").[40] Therefore, it is clear that, until now, the research efforts of Tissue Engineering do not belong to the category of technology development, because the knowledge to fabricate tissues or organs still does not exist. They belong to the category of basic research in engineering with thoroughly investigated methods to grow and differentiate cells in three dimensions. However, the fact that most of the research has been performed in the private sector gives the wrong impression, that Tissue Engineering involves product development as a direct goal of the current knowledge. According to Kuhn's theory of science development, the role of any paradigm is to restrict the studies focusing on particular phenomena dictated by this, so that the scientific community that will adopt the paradigm will massively attack problems in a specific narrow area, rapidly increasing the accumulated knowledge, which is a prerequisite for the development of science,[1] and this is what Tissue Engineering has done until now. It restricted its studies in 3D cell growth and differentiation, which is rather the first step in a long process of clarifying all the phenomena involved in tissue growth and being able to make bioartificial tissue with properties similar to those of the in vivo ones. When the study of these phenomena will be exhausted, a change of the paradigm will define new problems of studies that focus on other relevant phenomena, as we will see below with the paradigm change of Tissue Engineering from 3D cell growth and differentiation to biomimetic process design for in vitro recapitulation of the in vivo processes of tissue development.

The questions of tools suitability (such as cells, scaffolds, or bioreactors) for 3D cell growth and differentiation are investigated empirically; various combinations of these tools are tried. The process is redesigned empirically, modifying some of the properties of the tools/inputs (see arrow "A" in Figure 1). However, since each researcher, coming from a different field, has a different background, he/she addresses only questions in his/her expertise, without considering if an optimization of a particular aspect of the 3D cell growth and differentiation is compatible with the other properties of the bioartificial tissue examined by other fields. For example, an increase in the porosity of a scaffold destined to be used for bioartificial articular cartilage (the cartilage surface at the end of long bone joints, depicted in Figure 8 presented later in this paper) for damaged joint surfaces, which could enhance cell nutrition and oxygenation and is treated by Material Science, may lead to slow structural recovery after unloading or even to permanent destruction of the implant,[41] as indicated by Medicine. As a patient with the implanted bioartificial cartilage in the knee walks, the implant is successively loaded and unloaded and, therefore, must be flexible but also have mechanical strength. However, the mechanical strength decreases with increasing porosity. Such types of incompatibilities, arising from the fact that each researcher in Tissue Engineering looks at the in vitro process and the bioartificial tissue generated by them from his/her point of view,[32] are not exceptions but constitute the normal research practice in Tissue Engineering. As Ikada mentioned, biomaterial scientists, who are responsible for providing the scaffolds, instead of addressing the requirements of surgeons "seem to be busy with their own work".[42] For example, Ikada argued that much work has been devoted to the fabrication of scaffolds with nanofibers. Although sufficient porosity is exhibited by thin nanofiber sheets, when the thickness of the nanofiber sheets is increased to a level adequate for bioartificial tissue for use in implantation, the pore size decreases to levels that are unable to seed cells in such scaffolds.[42]

In conclusion, therefore, we could say that the fragmented nature of the Tissue Engineering science and methods used does not allow the optimization of bioartificial tissues but only the modification of the tools used in the in vitro process, to optimize some of the properties of the 3D cell constructs, which are related to each one of these tools. However, in most cases, the partial optimizations of some properties are in contrast with the optimization of other properties of bioartificial tissue, which are dependent on other tools provided by other fields. As we will see below, the problem of optimizing bioartificial tissue is even much more difficult, as described above, because the criteria of optimization are conceptually wrong, referring to the cell and not to tissue-level properties.

**1.7. Tissue Engineering Does Not Have a Working Definition of What a Tissue Is but Neither Biology Has One.** The major problem of Tissue Engineering is that the criteria for the evaluation of the properties of bioartificial tissue, which are used for the optimization of the tools involved in the in vitro process, do not refer to tissue but only to cell properties, such as cell viability, cell functions, etc., because these criteria have been provided by Molecular Biology which has as a reference system the cell and looks at sub-cellular processes. This means that a scaffold or bioreactor that can better support the cell viability is not a sufficient condition to generate a tissue in vitro, because a tissue is not a collection of viable cells in a scaffold, just as a tumor is not a collection of cancer cells but rather a complex ecosystem of different types of interacting cells. Tissue-specific instead of cell-specific indices must be examined to evaluate the suitability of the tools used in the in vitro process to provide conditions needed for bioartificial tissue with properties similar to those of the in vivo ones. The important concept that is missing from the 3D cell growth and differentiation paradigm is that what makes a cell collection a tissue are the cell-to-cell interactions that are responsible for the correct cell differentiation and cell organization to tissue structures, as it happens in vivo during the organism development (see arrow "B" in Figure 1). Only if the native structure is recapitulated in the bioartificial tissue, allowing the cell-to-cell interactions to occur, can the cell construct behave as a tissue, having tissue properties instead of only cell properties.

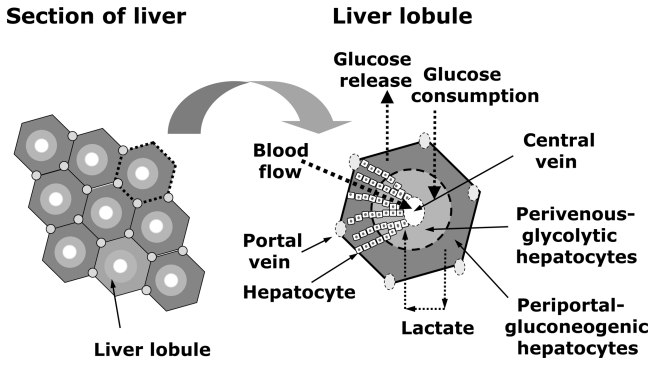


**Figure 2.** According to the "metabolic zonation" theory, the enzymatic activities of metabolic functions (oxidative and carbohydrate metabolism, amino acid and ammonia metabolism, cholesterol synthesis, xenobiotic metabolism, protective metabolism, and plasma protein formation) are unequally distributed among the periportal (afferent) and perivenous (efferent) zones of liver lobules. Concerning the glucose metabolism in the liver, the periportal (afferent) hepatocytes specialize in glucose release and glycogen formation via gluconeogenesis, and the perivenous cells specialize in glucose uptake for glycogen synthesis and glycolysis. The metabolic activities of periportal and perivenous hepatocytes is the best characterized example of zonation and allows the liver to operate as a "glucostat" regulating the blood glucose concentration at relatively constant levels (80−120 mg/100 mL). The liver removes glucose from the circulation during the absorptive phase after a carbohydrate-rich meal via glycogen synthesis, glycolysis, and liponeogenesis and releases glucose during the post-absorptive phase via glycogen degradation and gluconeogenesis, to supply the central nervous system and the erythrocytes. The glucose taken up during the absorptive phase is incorporated into glycogen, and then when the glycogen stores are replenished, it is degraded to lactate. Lactate then is transported by the circulation to the periportal cells, where it is converted via gluconeogenesis to glycogen. Glycogen is first degraded to glucose in the periportal cells during the post-absorptive phase, and later it is degraded mainly to lactate in the perivenous cells. Lactate transported by the circulation to the periportal cells is converted to glucose via gluconeogenesis. In this way, the liver solves a regulatory problem, the glucose homeostasis, allocating the glucose release or uptake to different cells that constantly maintain the same direction of substrate flux, altering only the rate in different phases. For example, the periportal cells during the 24-h feeding cycle always release glucose: at low rates during feeding, at intermediate rates shortly after feeding, and at high rates during fasting.

**1.8. Glucose Control by the Liver: An Example of Cell Cooperation.** The concept of component interactions to establish the system properties is not different from the interactions between electrons of molecules in chemical reactions that generate new molecules with properties different from any of the reactant molecules, or in polymers where an examination of the length of chains is not sufficient to determine the macroscopic properties of the polymer, which are of interest to applications, unless other aspects at a higher level, such as the chain intercalation, will be considered. Similarly, the liver function of glucose homeostasis, as well as all the liver functions, such as detoxification of substances, is not a hepatocyte function. In the liver, there are two types of hepatocytes, with each one either releasing or consuming glucose (see Figure 2). Therefore, they must function in coordination in time in order to control the glucose level of the blood for the erythrocytes and brain cells to not be without glucose between meals. Hepatocytes are structurally arranged in zones (periportal and perivenous one) in the liver lobules along the walls of the sinusoids through which the blood passes, and they acquire different metabolic character according to their position, being able to cooperate to control the glucose concentration. More importantly, they determine each other's metabolic character: which one will release or will consume glucose, according to their relative spatial position. They operate alternatively at different phases, according to the glucose level, either decreasing (consuming) or increasing (releasing) glucose in the blood circulation, which is high after a meal and low later.[43]

The reason for the existence of the two metabolically different hepatocyte types is that the expression of different enzymes by a hepatocyte or any cell to perform different metabolic functions, either consuming or releasing glucose, takes considerable time and it cannot follow the faster-changing conditions that the organism faces, such as the frequency of meals or the presence of a toxin; therefore, the liver must be ready at any moment to respond quickly to such rapid changes imposed by the rapidly changing environmental conditions. The existence of metabolically different hepatocyte types means that a single hepatocyte is not a liver in microscale, neither is a random distribution of hepatocytes inside a scaffold a small piece of liver tissue, as a random mixture of polymer chains cannot provide the spectrum of required polymer properties without further consideration on how these chains are arranged relatively in space. Chan et al. have already discussed the importance of the metabolic zonation (which is defined as the spatial arrangement of metabolically different hepatocyte types, relative to their distance from the veins that bring the blood to the liver lobules) in the bioartificial liver, with regard to ammonia and toxin metabolism,[44] which are two critical functions that should be provided to patients whose liver does not work until a donated liver for transplantation will become available. The authors suggested that these metabolic functions, such as glucose homeostasis, cannot be optimized at the hepatocyte-cell level but only at the liver-tissue level, since the liver detoxifies toxins using a series of metabolic reactions performed by hepatocytes that have different metabolic character, because of their different position in the liver lobules. Therefore, it is expected that, despite the number of different bioartificial liver devices that have been designed, currently there is no agreement with regard to which design is optimal,[45] and the exhibited functionality by the bioartificial liver devices also is not expected to result in clear benefits for the treatment of the patients.[46]

There is nothing specific for liver tissue: all the tissues have properties different from the cell properties (emergent properties in complex systems) arising from cell-to-cell interactions (such as, for example, fine-tuning the control of insulin release by the pancreas or, as we will see below, the control of the cartilage and bone development).[47]

**1.9. Adoption of the Genocentric Paradigm of Biology by Tissue Engineering Leads to Insufficient Criteria for Bioartificial Tissue Evaluation and Optimization.** Therefore, it is surprising for an engineering field that claims to make tissues in vitro to not have any rigorous or even working definition of what constitutes a tissue and in what aspects it is different from a 3D cell construct. However, there is a reasonable explanation for this omission. When Tissue Engineering started as a distinct field, besides the engineering expertise and tools, it needed support from Biology, since, at that time, engineers were not familiar with biological concepts and methods. Tissue Engineering looked at Biology to complete the tools and criteria needed in the design of in vitro processes. However, Biology, at that time, did have the cell as a reference system and looked to elucidate phenomena inside the cells, following the genocentic paradigm of genetic determinism, according to which specific genes determined the behavior of cells or organisms or their healthy or diseased state.[2] Consequently, and unavoidably, Tissue Engineering adopted the same reference system and methods developed by Biology for its particular studies at the subcellular level. The genocentric paradigm determined that the parameters that tissue engineers

must check in bioartificial tissue are at the cell and subcellular level and not at the level of interacting cell communities. However, this is an error of logic called "ignoratio elenchi".[48] According to this error, arguments cannot be transferred from one world of experience to another. The irrelevance arising from transferring arguments between two different worlds of experience means that the arguments that are used may be valid but they prove a different proposition. Applying, for example, a differentiation protocol of Biology in three dimensions with cells inside a scaffold placed in a bioreactor and using, as an argument, the expression of gene markers (genes whose expression characterize particular cell types) to show that the cells differentiated to tissue-specific cells, does not prove that the resulting cell-populated scaffold is bioartificial tissue with tissue properties. It only proves that specific pathways activated by the growth factors used (activation or repression of gene expression or protein function with each gene and protein that has been activated or repressed influencing the activation and repression of other genes and proteins in the cascade, leading to a network of interactions, until some tissue-specific genes will be expressed) belong to those of a cell in the process of differentiation to a tissue-specific cell type. That was what Biology wanted to show, choosing the particular conceptual framework of the genocentric paradigm being preoccupied in deciphering the role of genes in cellular processes. This means that what is true or false in the world of Biology under the genocentric paradigm is meaningless in the world of Tissue Engineering, which has the goal to make tissues, instead of providing explanations of gene activations and repressions at the subcellular level, either with cells growing in two or three dimensions. Molecular Biology may not need a definition of tissue under the genocentric paradigm, because it looks at the subcellular level and, for this reason, cultures cells in 2D tissue flasks. However, Tissue Engineering must examine the level of interacting and cooperating cell communities, which is what causes a tissue to have different properties from a random collection of cells, as we saw previously in the example of hepatocytes in the liver. This renders any evaluation of bioartificial tissues at the cell or subcellular level irrelevant for Tissue Engineering. Therefore, Williams is correct with the statement that there is "a misunderstanding of what it (Tissue Engineering) actually is".[34] It seems that Tissue Engineering became a branch of Molecular Biology, studying the cell behavior and gene expression of cells when the cells grow in three dimensions inside scaffolds placed in bioreactors, as Molecular Biology is doing the same with cell cultures in two-dimensional tissue flasks. This strong relation of Tissue Engineering with Biology, instead of physical sciences as the other engineering fields, has already been mentioned in the literature.[34] Williams finally concluded that "not only might the paradigms be wrong, but also some of the concepts and even the definition (of Tissue Engineering)", suggesting a systematic approach from the cell isolation to bioartificial tissue implantation.

**1.10. The Lack of Studies in Tissue Engineering for the Compatibility of In Vitro Conditions with the In Vivo Conditions of Natural Tissue Development.** Why, however, is Chemical Engineering not a branch of Chemistry while Tissue Engineering can be considered, in some aspects mentioned above, as a Biology field? First, the goal of Chemical Engineering is not to study Chemistry in large scale. Chemical Engineering studies the physical and chemical factors and their compatibility, which are different in the large scale than in the test tubes of Chemistry. Similarly different are the biological and physical factors between the in vivo environment of the organism, inside which tissues naturally develop, and the in vitro one, in which Tissue Engineering tries to make bioartificial tissue. However, such differences between the in vivo and in vitro conditions are not included in the studies of Tissue Engineering. It is an obvious question how a bioartificial tissue can be made if one does not consider in the in vitro process design how it is made in the organism. Only now does Tissue Engineering try to address this question, with the adoption of the biomimetic paradigm. Not only is the goal of Chemical Engineering in large-scale production clear, but the definition of what the product is and what its properties should be, are also clear and are taken explicitly into consideration in the process design. On the other hand, Tissue Engineering lacks a definition for what tissue is and what properties it should have that are different from the cell properties. It does not refer explicitly to the tissue functions; it only refers to cell functions. Obviously, then, the bioartificial tissue that results from such in vitro process cannot be similar to a real tissue but it is rather a random collection of viable cells in three dimensions that do not cooperate to establish tissue functions.

More important, as we see below, Chemical Engineering transfers the synthesis of chemical products from the test tube to the large scale, where numerous different factors, such as those arising from transport phenomena, are involved, and it tries to determine the necessary conditions, referring to factors not encountered in test tubes, so that the chemical reaction will proceed as indicated by the studies of Chemistry. This means that Tissue Engineering must operate in a similar manner. Since tissues are made in vivo in the organism during development and Tissue Engineering must make tissues in an artificial environment, it has to ensure in vitro conditions that do not contradict the in vivo conditions, so that the tissue formation can happen in vitro as in vivo, to the degree possible, given the current knowledge provided by Developmental Biology and the current limitations of the technical tools used. This has not happened yet in Tissue Engineering. Developmental Biology was not one of the fields that Tissue Engineering had considered as belonging to its basis, because Developmental Biology does not provide tools as the other fields; instead, it provides concepts that are difficult to translate to engineering terms of process design. However, as we will see below, the Tissue Engineering community is prepared to move toward this direction, incorporating information from Developmental Biology into the biomimetic in vitro process. However, this field of study is still far from determining the conceptual background needed to design processes similar to those occurring inside the organism during tissue development. As Mow mentioned in ref 33, the currently practiced methods in Tissue Engineering are "perhaps naive". Moreover, indeed, a method that involves a one-stage in vitro process of placing cells in scaffolds and embedding them in bioreactors to make tissues without any consideration of the compatibility of the conditions provided in the artificial environment with those existing in the organisms seems naïve, rendering such methods completely unrelated with the real tissue development and, very likely, unsuccessful. Such simplistic methods cannot work, as a protocol for a chemical reaction performed in a test tube in industry could not work only with an increase of scale from the tube size to the bioreactor size, without any consideration of the new phenomena appearing at the increased scale and the selection of large-scale conditions that are compatible with basic phenomena occurring in the test tube, instead of preventing them. It was found finally, after two decades of research in Tissue Engineering, that using scaffolds in a simplistic one-stage process, with the cell seeding in

scaffolds and placement in a bioreactor, is inadequate to make tissues. Such a method could only make the cells to grow in three dimensions in constructs perceived wrongly as tissues, because tissues are not random collections of cells in three dimensions. The cells are actually attached to the 2D surface of the pores of the scaffold giving a 3D cell construct but are unable to make tissue structures, because they are not allowed to be in contact exchanging signaling molecules and arrange their relative positions by themselves, because of the obstacles posed by the material walls of the pores.

**1.11. The Incompatibility of Scaffolds with the Cell Survival after Scaffold Implantation.** In addition to this mechanical incompatibility, another more subtle and critical incompatibility of the scaffolds with the conditions encountered during the in vivo tissue development was found. It refers to the prevention of vascularization by the scaffold when this is implanted. This means that an implanted scaffold with attached cells causes massive cell death, which has been observed in studies with the implantation of a scaffold with osteoblasts for the treatment of cancellous bone[49] (cancellous bone is spongy, instead of the compact bone found at the end of long bones in joints.) The cells cannot survive after implantation, because no new blood vessels can be formed inside the scaffold. Although the necessity of the scaffold after implantation is provisional, because it is gradually dissolved (biodegradable) as the cells grow, it was expected that the organism will generate new blood vessels that will penetrate the scaffold and feed the implanted cells. However, the excessive tension generated by cells that are attached to the scaffold surface changes the conformation and deactivates fibronectin, a protein that is needed for vascularization.[50]

This phenomenon, which is observed in implanted scaffolds, is particularly interesting, because it directly opposes the use of scaffolds for the same reason these had been initially introduced, i.e., to facilitate cell growth, providing empty space between the cells through which the medium in a bioreactor or the blood when the scaffold is implanted will flow being constantly in contact with cells. According to Kuhn,[1] this is another indirect role of the paradigm. Restricting the studies on some phenomena and accumulating knowledge rapidly, it reaches a stage in which it contradicts itself and must be replaced by another paradigm, focusing on other phenomena or aspects of phenomena.[1] In addition, since Tissue Engineering has reached that point, it is now ready to adopt a new paradigm. However, it is not ready yet to transform it to practical methodologies of process design. For this to happen, Tissue Engineering requires the assistance of the conceptual background of Chemical Engineering, as we will examine below.

## 2. The New Tissue Engineering Paradigm of Process Biomimetics

**2.1. Tissue Engineers Acknowledged the Limitations of the 3D Cell Growth and Differentiation Paradigm.** The adoption of a "simple and appealing" but "perhaps naïve" method of seeding cells in scaffolds and placing them in bioreactors (Mow in ref 33) made the Tissue Engineering methodology simply "tissue try this", since no methodological design of the in vitro process was involved (as Niklason noted in ref 33) or, as Ingber mentioned in ref 33, the method was just "put together your best ideas and materials, throw them in an animal, and pray for the best". The lack of a concrete methodology of process design had an investment cost of more than 4 billion dollars, in terms of government funding and Wall Street investments,[51] which, however, was not sufficient to lead to a profitable clinical product.[52] We could attribute the facts that "most tissue-engineering efforts have not come to fruition", and "almost all venture capital-funded biotechnology companies in tissue engineering have failed" (according to Mow in ref 33) to the lack of appropriate methodology. As Williams said, "after some 15 years, (Tissue Engineering) has yet to really make its mark, either clinically or commercially".[34]

Therefore, today, we are at a point in which the lack of "basic understanding" and "basic science" studies in Tissue Engineering must be addressed, as recommended explicitly at the Symposium of Reparative Medicine at the National Institutes of Health (NIH), so that the "inherently limited trial-and-error approaches can be replaced by rational ones".[53] Since basic studies that could lead to a methodology have not been performed until now, it is expected, as Niklason mentioned in ref 33, that Tissue Engineering lacks "true tissue engineers" who understand all of the fields involved in designing the in vitro process, so that all the components/tools could be assembled properly, leading to the structurally and functionally integral entity that is tissue. Below, we will see that process design in Tissue Engineering must rely on the conceptual background of Chemical Engineering methodology. It is this methodology that makes Chemical Engineering different from Chemistry or Mechanical Engineering. Chemical engineers who have background in several fields are involved in process design and have developed a methodology to place the tools provided by different disciplines in the process in the correct way.

Why has Tissue Engineering moved to product development without having the appropriate methodology? Perhaps because "the lure of sudden Wall Street and venture capital riches clouded the clear thinking of normally cautious people", as Mow mentioned in ref 33, arguing "not to abandon our normal cautious scientific approach". It seems that "it was a gentle conspiracy shared by the scientific bureaucracy and self-interested academics and businessmen—that is everyone who needs to raise money", as Teitelman mentioned for the rise and fall of biotechnology firms that promised miracle proteins for the cure of diseases such as cancer when the technology of recombinant proteins (proteins expressed in cells by genes introduced into them) had been developed.[54] Similarly, in Tissue Engineering, it has been admitted that some of the promises, such as, for example, the fabrication of a bioartificial heart in 10 years, was an extrapolation without substantial scientific and technological grounds. As one of the researchers participating in this 10-year program mentioned, "we were trying to capture the attention of the public".[37] The extrapolation of the central dogma of Molecular Biology for the protein coding from genes (based on the achievement of Watson and Crick of the double-helix structure of DNA), to the role of single genes in diseases under the genocentric paradigm and the promise that preventing or restoring the function of single genes could lead to disease treatments, was "illegitimate", according to Strohman,[2] as illegitimate as the promise of a bioartificial heart by Tissue Engineering. The media promoted the idea that "everyone will get better as their biotherapists become richer",[2] and this role of media has continued, until now, in biomedical fields. As Ingber and Levin mentioned, "if the press seems to be overselling stem cells now, one only needs to go back a few years to find that they had done the same for tissue engineering".[38] Until now, Molecular Biology has failed to provide a treatment of cancer for the same reason Tissue Engineering has failed to provide clinical products. We know today that most of the diseases such as cancer are

not caused by a single gene but by a malfunctioning network of interacting genes,[2,3,55,56] whose expression in the tumor depends on the interaction with other cells. Similarly, it is known in Tissue Engineering that tissue functions arise only from different and complementary cell functions that the cells perform when they interact together in very specific spatial arrangements.

**2.2. The Biomimetics Paradigm Requires the Design of Multistage Processes That Recapitulate the Successive In Vivo Stages of Tissue Development.** After the acknowledgment by the Tissue Engineering research community of the failure of the first paradigm of Tissue Engineering as an "automatic", one-stage process that is able to organize the cells into tissue structures as long as the cells grow and differentiate, as well as after the lack of tissue properties of bioartificial tissue made in such simplistic processes of mixing cells and scaffolds in a bioreactor and expecting that the cells, under these conditions, will do what they do during the in vivo development, Tissue Engineering started looking to Developmental Biology to determine the critical factors that are involved in the in vivo processes have not been taken yet into account in the in vitro ones and which do not allow the cells to be organized as in real tissues in vivo. The Tissue Engineering research community has recently initiated discussions with developmental biologists to facilitate the incorporation of information from the in vivo tissue development into the in vitro processes for bioartificial tissue formation.[33] The previously mentioned article summarizes the results of a meeting devoted to the relation of Tissue Engineering with Developmental Biology, (the Tissue Engineering—The Next Generation workshop, Boston, MA, USA, May 2−4, 2005, sponsored by NIH[57,58]). Another discussion between tissue engineers and developmental biologists took place in the Keystone Symposium on "Developmental Biology and Tissue Engineering" in Snowbird, UT, USA, April 2007, with the results summarized by Ingber and Levin.[38] As Ingber mentioned in ref 33, a paradigm shift is needed in Tissue Engineering research practice and "tissue engineers should try to fabricate scaffolds or create microenvironments that mimic these metastable tissues and promote complex cell−cell and cell-matrix interactions, rather than precisely engineer the final tissue form". This means that Tissue Engineering must repeat the in vivo process of tissue development in such a way that the in vitro process will provide a series of tissue transformations from the cells to immature-metastable tissues structures, to final tissue as in vivo, therefore adopting the new paradigm of the "in vitro biomimetics of in vivo tissue development". However, as Ingber and Levin mentioned, "more questions were generated than answered at the symposium" in Snowbird.[38] Answers to the questions of which Tissue Engineering tools and methods, and how they should be used in an in vitro process to accommodate the needs of complex in vivo developmental processes that evolve in space and time in an orchestrated way, are far from clear at this moment, since Tissue Engineering has no experience in multistage process design, as such are the in vivo processes of tissue development. The limited experience in multistage processes refers to nonbiomimetic processes that are restricted to one or few stages. For example, a process of bioartificial cartilage formation is restricted in two stages (culture of chondrocytes in alginate beads under conditions that maintain their phenotype and continuation of the culture on a porous membrane)[59] or another in three stages (formation of embryoid bodies (an aggregate of cells formed when embryonic stem cells are cultured in suspension that induces their early differentiation to the three germ layers, ectoderm, mesoderm, and endoderm with each one giving with further differentiation different set of the body cells), chondrogenic differentiation of their cells, and seeding of cells suspended in alginate into polylactic-co-glycolic acid (PLGA) scaffolds.[60] Although not related to tissue development, as this is described in Developmental Biology, such processes can be considered as being composed of unit operations. Similar unit operations can be used for biomimetic processes, as we will see below, arguing in favor of their feasibility in recapitulating the stages of tissue development.

While Tissue Engineering found finally that its basic science could be built only from Developmental Biology, recognizing Developmental Biology as "a key to future success in the field",[33] it still needs to develop a methodology to use the appropriate information concerning developmental phenomena in the in vitro processes. What should such information be? Developmental Biology, as any biological field, had adopted the genocentric paradigm and accumulated a vast amount of information about the role of specific genes in developmental processes. "Unfortunately, knowledge that gene A or mitogen B triggers a particular morphogenetic process in the embryo is not helpful to engineers interested in identifying critical design criteria necessary to construct 3-dimensional (3D) materials with defined shape, physical properties, and biochemical functions necessary to replace a living organ in an adult", as Ingber mentioned in ref 33. Particular genes play the role indicated by Developmental Biology only in the in vivo context. In the in vitro process, however, the context is different, because the process is not given unless it will be first designed. How can a similar context be recapitulated in vitro, so that the particular genes of interest to the developmental biologists will have the chance to play the same role as in vivo? Outside this context, the role of any gene cannot be used as a design criterion simply because its normal function has, as a prerequisite, the existence of this context. Therefore, even if the principles of the in vivo tissue development would be understood by tissue engineers, it still remains a "deep chasm" to translate them into process design criteria (according to Ingber in ref 33).

Below, we will refer to an example of in vivo tissue development that shows that biological and physical factors are inter-related in tissue development. Protein signals exchanged between different cells guide the cell organization only in the appropriate physical space with the appropriate cell number and distances between cells. These conditions must be implemented in vitro with the proper process design that will take into account the mechanics of tissue development, such as, for example, the gradual increase of the tissue size synchronized with the progress of cell differentiation. For such process design to be achieved, Tissue Engineering needs the concepts developed for the design of processes in Chemical Engineering. As Chemical Engineering developed a conceptual framework that integrates the concepts of Process Engineering with those of Chemistry, Physical Chemistry, Physics, Mathematics, etc., similarly, Tissue Engineering must find concepts that can incorporate the appropriate information from Developmental Biology into the in vitro process design. A conceptual framework such as this will facilitate the exchange of information between tissue engineers and developmental biologists. The discussion between them will then be focused on issues of in vivo developmental processes that are critical for the in vitro process design. Such a discussion can provide the new rules of in vitro biomimetic process design and the new criteria of process and bioartificial tissue evaluation, selecting from the vast information provided by Developmental Biology the one that is relevant to process design and transforming Tissue Engineering to "Developmental Engineering".

However, it took a long time after the introduction of embryonic stem cells (cells that are, in principle, able to differentiate to any of the tissue cell types) to Tissue Engineering[61,62] for the field to realize that stem cells participate in the in vivo process of tissue development under specific conditions that guide them not only to their correct differentiation but also to their organization into tissue structures. Actually, neither authentic cell differentiation can be achieved in vitro unless conditions to facilitate the cell organization will be established as those in vivo. These two phenomena—cell differentiation and organization into tissue structures—are inter-related, because it is the cell-to-cell interactions that determine the cell differentiation and these cannot happen as long as the cells are not allowed to be spatially organized. The equivalence, for example, of cell populations generated in vitro from the stem cell differentiation to those arising in vivo has been questioned in the literature.[63] The differences between in vitro and in vivo seem to be predominantly due to the lack, in vitro, of extensive in vivo cellular interactions that determine cellular characteristics and, in turn, the way cells interact to establish their properties, following their natural differentiation pathway.[63] In the example presented below for the development of long bones, we will see the role of cell interactions through exchanged protein signals in determining the genes expressed by the cells that, in turn, determine the cell differentiation state, as well as how the cell differentiation unfolds over time, following the increase in the size of the developing tissue for the cells to be finally organized into tissue structures.

**2.3. The Interdependence of Physical and Biological Factors in the In Vivo Tissue Development. 2.3.1. Endochondral Ossification: The Developmental Process of Long Bone Formation.** Bone formation occurs during development following two different processes, both of which involve the transformation of mesenchymal tissue to bone tissue. One process occurs primarily in the flat bones of the skull. It involves the direct conversion of mesenchymal tissue to bone, and it is called *intramembranous ossification*. For other bones, such the skeletal components of the vertebral column, the pelvis, and the limbs, the osteogenetic process is indirect. The embryonic skeleton of these bones early in development is composed of cartilage. The cartilage is formed by the differentiation of mesenchymal tissue and is used as a template, or a "model" for the shape of the bone that will be formed later. This indirect bone formation process is called *endochondral ossification*.[64,65]

The process of endochondral ossification follows several consecutive stages. Initially, the mesenchymal cells receive paracrine signals that commit them to differentiate into chondrocytes, or cartilage cells, activating the expression of cartilage-specific genes. Subsequently, the committed mesenchymal cells condense (aggregate), forming compact nodules (tight clusters of cells) (see Figure 3a), and differentiate into chondrocytes.[66] The cell condensation is induced by growth factors of the TGF-$\beta$ family produced by the mesenchymal cells. These factors increase the secretion of the adhesive extracellular glycoprotein fibronectin.[67,68] Fibronectin has multiple domains that interact with many extracellular matrix substances, such as collagen, fibrin, and heparin, as well as with membrane receptors of cells. Through this mechanism, fibronectin becomes the link of cells with the extracellular matrix anchoring them to the matrix molecules. The areas where fibronectin molecules are secreted by the cells create adhesive patches that trap them as they diffuse through the extracellular matrix, thereby forming the spatiotemporal pattern of the skeleton.[69] Cells first accumulated in regions of increased cell−fibronectin adhesive interactions express cell-to-cell adhesion molecules (molecules located on the cell surface involved in the binding with other cells), such as N-cadherin and N-CAM, that keep them together, stabilizing the cell condensate.[70,71] The condensations are restricted in space, because they exert a laterally acting inhibitory effect on chondrogenesis.[72] These two mechanisms—activation through diffusible TGF-$\beta$ and inhibition—constitute a minimal pattern-forming mechanism to generate the basic form of a vertebrate skeleton,[73−75] as has been shown in reaction−diffusion models (see, for example, refs 76 and 77), in agreement with the theory of Turing for pattern formation.[78]

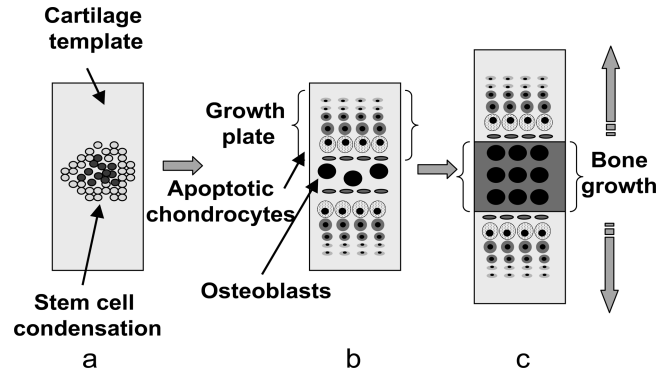


**Figure 3.** In the in vitro biomimetic process, the design must follow the stages of the tissue development, because these occur inside the organism. In the in vivo developmental process of endochondral bone formation, bone is formed by replacing a cartilage template that is the natural scaffold for the recruitment and differentiation of bone-forming cells. The process passes through different stages. Initially, the mesenchymal stem cells condense, forming a cell aggregate, restricting their exchanged signals in a small area (this step is represented by panel a). These signals induce the expression or repression of some genes that initiate a cascade of alterations of gene expression related with the induction of the differentiation of stem cells to chondrocytes. With the invasion of blood vessels, the condensate is divided in two areas, from which the bone formation toward the two direction of the cartilage template long axis will start (this step is represented by panel b). As the chondrocytes that still have a capacity for proliferation stop their proliferation and start their differentiation, they pass from different differentiation states up to the hypertrophic and apoptotic one, moving synchronously, following the long axis of the cartilage template and forming, in this way, columns of progressively more mature (in a later differentiation state) chondrocytes (see panel b). The apoptotic chondrocytes are dying and are replaced by osteoblasts that further mature to bone cells when they secrete the bone matrix and are enclosed in this material. However, new hypertophic cells that die from apoptosis are continuously coming to the end of the column from above those that have already been replaced by osteoblasts, since the chondrocytes continue their differentiation, moving from one state to the other along the column. Consequently, osteoblasts replace the successively dying chondrocytes and they finally replace the cartilage template by bone (this step is represented by panel c). The in vitro process for bone formation in the 3D cell growth and differentiation paradigm of Tissue Engineering with osteoblast seeding in an artificial scaffold is irrelevant to the in vivo process of endochondral ossification. The artificial scaffold is unable to direct the bone formation, because the hypertrophic chondrocytes of the growth plate that is the natural scaffold for bone formation attract osteoblasts, induce their differentiation, and induce vein formation that is needed to keep the osteoblasts alive in large constructs.

The chondrocytes proliferate, increasing the size of the cartilage templates of the bone. However, the cells at the middle of the cartilaginous template stop dividing and proceed further in their differentiation, passing from the stages of prehypertrophy, hypertrophy, and finally apoptosis, forming the so-called "primary ossification center" (secondary ossification centers are formed later in the epiphyses at the ends of long bones (see Figure 8, presented later in this paper). Hypertrophic chondrocytes secrete alkaline phosphatase, which is an enzyme capable of releasing inorganic phosphate from organic phosphate and pyrophosphate. The phosphate combines with calcium, forming the mineral hydroxyapatite, which is first deposited in the lower

hypertrophic zone, initiating the mineralization process within the cartilaginous matrix.[79,80] Nutrients can no longer diffuse if the matrix becomes sufficiently calcified and the chondrocytes subsequently die from apoptosis. At this stage, the hypertrophic chondrocytes, which reside at one end of the area of differentiating chondrocytes, induce vascularization. The hypertrophic chondrocytes that die are replaced by osteoblasts (immature bone cells). The ostoblasts form the bone matrix, which is deposited on the partially degraded cartilage.[81,82] Hypertrophic chondrocytes are simultaneously osteoinductive[83] and angiogenic.[84] Therefore, the cartilage template controls osteoblast recruitment and differentiation, and it sustains cell viability through enhanced vascularization.

Originally, the center of ossification expands in all directions. Later, it divides into two peripheral extensions that have the form of platelike structures called "growth plates" (see Figures 3b, 3c, and 4a). The growth plate consists of parallel columns composed of chondrocytes that are in gradually advancing differentiation stages along the columns.[85] The columns are formed because each chondrocyte that stops its proliferation and starts the differentiation enters a column formed by the previously differentiating chondrocytes.

From this point, the endochondral bone formation is restricted to the growth plates. As the growth plates grow away from each other toward the epiphyses, the endochondral ossification at their end of hypertrophic chondrocytes spreads in both directions from the center of the bone. The chondrocytes that are opposite to the hypertrophic side proliferate prior to undergoing hypertrophy, pushing out the cartilaginous ends of the bone. As long as the growth plates are able to produce chondrocytes that follow their differentiation up to hypertrophy, the bone continues to grow longitudinally (see Figure 3c). Finally, the gradual replacement of cartilage by bone happens, because the process of column elongation up to apoptotic cells and replacement of these cells by osteoblasts is continuous, starting from the beginning of the column where proliferating cells reside and continuously stop their proliferation and start differentiating entering the columns. Eventually, all the cartilage is replaced by bone.

It is important to mention here that the natural "scaffold" for bone formation is the pattern of columns of differentiating chondrocytes (or parallel horizontal zones, since chondrocytes in the same differentiation stage reside at similar heights of the columns). Therefore, the growth plate should be the goal of the first process that Tissue Engineering must design, if it is to follow the biomimetic paradigm for long bone formation, instead of developing an artificial scaffold that cannot behave as a living scaffold with dynamic properties, evolving over time as the growth plate and providing the osteoblasts with the optimal conditions to form bone.

**2.3.2. Inter-relation of Physical and Biological Factors of the Dynamic Structure of the Growth Plate.** Column elongation in the growth plate depends on the chondrocyte differentiation rate, because new chondrocytes leave the proliferation zone, entering the column and moving along this route as their differentiation state proceeds to prehypertrophy, hypertrophy, and apoptosis. However, on the other hand, the chondrocyte differentiation rate (i.e., how fast the chondrocytes move from the one differentiation state to the next, moving along the column) depends on the column length, because it is determined by cell-to-cell signaling that occurs between chondrocytes situated at different column heights/zones that exchange proteins that must diffuse along the columns to reach their target cells. Therefore, the efficiency of signaling through proteins

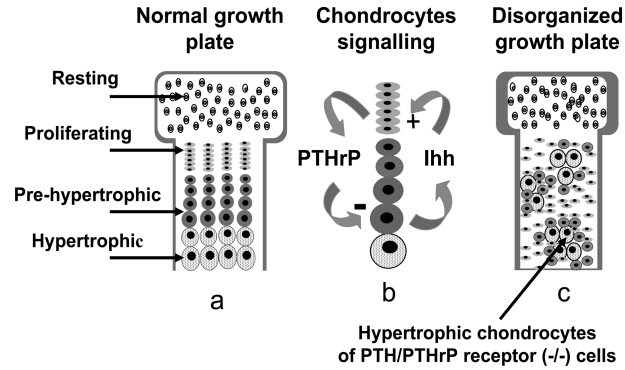


**Figure 4.** The structure of the growth plate that is the natural scaffold for the formation of long bones is dynamic and is formed with an interplay between physical and biological factors. It is composed of columns of differentiating chondorytes (panel a). As proliferating chondrocytes stop their proliferation and enter their differentiation program, they move along the long axis of the cartilage template, forming columns. The columns originating from different cells are parallel with the chondrocytes in different columns but the same differentiation state aligned at the same column height, forming zones. Along the columns, a continuous movement of chondrocytes occurs as they proceed from the one differentiation state to the next. The rate at which the chondrocytes differentiate, which is also the rate at which the column elongates, depends on protein signals exchanged between chondrocytes residing in different zones along the column. To a first approximation, signals initiated by Ihh secreted by prehypertrophic chondrocytes diffuse upward and reach the proliferating chondrocytes (panel b). In response to the signal initiated by Ihh, the proliferating chondrocytes secrete PTHrP, which diffuses downward, reaching the prehypertrophic chondocytes. It then prevents them from continuing their differentiation toward the hypertrophic state. In other words, because the physical and biological phenomena are coupled, the differentiation rate depends on the distances between different chondrocyte zones, proliferating and prehypertrophic; however, these distances are dependent on the chondrocyte differentiation rate, since, as chondrocytes proceed in their differentiation, they move along the columns. Only if the differentiation rate (and, consequently, the column elongation rate) is inside the limits set by the negative feedback loop of Ihh/PTHrP can the columnar structure be stabilized, because the elongation rate is synchronized with other processes, such as the extracellular matrix secretion that retains the stability of the columns, facilitating the chondrocytes to move along them and restricting them to move in other directions. If the negative feedback loop is disturbed, a disorganization of the columnar structure occurs, because of the lack of process synchronization (panel c). A disorganized growth plate could have hypertrophic chondrocytes dispersed inside the mass of cartilage template, instead of being aligned in zones if the prehypertrophic chondrocytes are not prevented from quickly moving to the hypertrophic state. If such a construct will be used as a bioartificial aritcular cartilage implant, bone will be formed randomly inside the cartilage, instead of only at the end of the hypertrophic zone. The same phenomenon of spatial randomization of chondrocytes at different differentiation stages can occur when chondrocytes are placed in scaffolds, according to the 3D cell growth and differentiation paradigm. The scaffold, with its pores acting as barriers, restricts the operation of the negative feedback loop in small separated areas, so that no global (within the whole) scaffold space cell signaling can occur and, consequently, no global organization to the growth plate pattern can be established.

secreted by the cells and diffusing to find their target cells is directly related to the column length though diffusion, although the diffusion through the extracellular matrix is facilitated by other molecules produced by the cells.[86−88] The protein signals exchanged between chondrocytes at distinct differentiation states and consequently located at different zones or column heights, which determines the differentiation rate and, consequently, the column elongation rate, is due to a first approximation to a negative feedback loop of Indian Hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) (see Figure 4b). These protein signals are exchanged between proliferating and prehypertrophic chondrocytes located at distinct zones.[89−91] The inter-relation of physical and biological factors then becomes evident. On one hand, the column length that determines the

cell distances depends on the rate of chondrocyte differentiation that controls the number of cells residing in the column; however, on the other hand, the cell distances determine the chondrocyte differentiation rate, since this rate depends on the transport of protein signals along the column.

PTHrP, in response to Ihh signaling originated by the prehypertrophic chondrocytes, is secreted by the proliferating chondrocytes and diffuses along the column, reaching the prehypertrophic chondrocytes. It then binds to cell surface receptors of these cells and changes the expression of genes, with the result of retardation of the progression of the differentiation of prehypertrophic chondrocytes to the hypertrophic state and, thereby, causing a delay in the column elongation. This negative feedback loop sets the limits of the chondrocyte differentiation rate and, consequently, column elongation. These limits are needed for the synchronization of column elongation, with other processes occurring in parallel, such as secretion and organization of the extracellular matrix by the chondrocytes, which are needed for the structural integrity of the columns. Disturbances of the negative feedback loop, such as inhibition or induction of the involved proteins with genetic modifications, changes the differentiation rate of chondrocytes and make it incompatible with the processes of column stabilization, finally disorganizing the columnar structure. These disturbances may either cause a rapid elongation of columns that cannot be followed by the process of their structural stabilization through the secreted extracellular matrix molecules and therefore the columns lose their one-dimensional elongation to accommodate an excess of chondrocytes, or they may cause a slow elongation of the columns, because of an excess of extracellular matrix secretion, compared to the column length, which makes the column less permeable to new chondrocytes that start their differentiation leaving the proliferating zone, and, therefore, these cells must move in various directions, where they do not find resistance. For example, Chung et al., using chimeric mice containing both wild-type and PTH/PTHrP receptor (−/−) cells, showed that the mutant cells, whose differentiation cannot be retarded because they do not receive the signal of PTHrP (because of the lack of cell surface receptors for this protein), prematurely differentiated into cells resembling the hypertrophic chondrocytes while surrounded by wild-type proliferating cells, whose differentiation was retarded by having the receptor of PTHrP[92] (see Figure 4c). The disturbance of the growth plate organization in this case leads to random bone formation at the sites of hypertrophy, since the osteoblasts are attracted and differentiate to bone cells near hypertrophic cells that are osteoinductive[83] and angiogenic.[84]

Now, we will determine if the concept of using a scaffold as the substrate for the 3D cell growth and differentiation does or does not facilitate the above-described phenomenon that happens inside the organism. It has been mentioned in the literature that artificial geometrical constraints imposed by scaffolds suppress the dynamics of cartilage development.[93] Restriction of the chondrocytes and, consequently, their communication locally by the physical boundaries (such as the walls of the pores inside a scaffold) disperses the negative feedback loop signaling into several unconnected local areas of variable size, and no signaling can occur through the entire scaffold. On the other hand, the differentiating chondrocytes cannot move freely as in vivo forming columns, since their movements are restricted by the pore walls of the scaffold. Consequently, because of the mismatch between physical and biological phenomena, the appropriate natural architectural organization in the pattern of the growth plate inside a scaffold is unlikely to be formed, which shows the inadequacy of the currently used paradigm of 3D cell growth and differentiation to design biomimetic process.

**2.4. The Methodological Process Design in Chemical Engineering Solved Problems Similar to Those of Tissue Engineering.** The success of Chemical Engineering to establish the compatibility of mechanical and chemical factors in processes was achieved with the introduction of "unit operations". The unit operations, as units from which a process is composed, arose from the focus of Chemical Engineering to the analysis and synthesis of processes instead of components of reactors and other equipment or components of chemical reactions, in one-stage processes. In such one-stage processes, there is a high number of physical and chemical factors, and, consequently, many inter-relations are created among them that do not allow studies of their compatibility. Unit operations are elementary physical and/or chemical operations (such as distillation, crystallization, catalysis, etc.) and are based on distinct principles. Davis was the originator of the idea of unit operations in the second edition of his publication, *Handbook of Chemical Engineering*, although the term was coined by Arthur D. Little at the Massachusetts Institute of Technology (MIT) in 1915.[94] Therefore, chemical engineers incorporated the minimum number of physical and chemical phenomena for which clear descriptions were available from Physics, Physical Chemistry, and Chemistry. Such unit operations could be studied thoroughly from their thermodynamic and kinetic aspects. The data from these studies can be incorporated into mathematical models to clarify quantitatively the inter-relation of the various factors involved and the effect of each one on the others. The knowledge gained from the detailed description of unit operations allowed the process optimization. Synchronously unit operations were used in several processes with slight, well-informed, and computationally performed modifications, because the underlying principles of physics and chemistry applied to these elementary operations (e.g., distillation of several type of solutions) were the same. Therefore, unit operations, with their assembly to the entire process, gave the ability for a modular design of processes (in a modular architecture, each of the modules of a product or process is autonomous in its function, which arises from the functional connections among the components of the module and not from connection with other modules). Instead of redesigning the entire process for optimization purposes, chemical engineers only had to redesign some of the modules/unit operations, since the phenomena in any unit operation do not depend directly on the phenomena occurring in other unit operations but rather only indirectly at the level of the entire process. The methodology of process assembly from unit operations has been proven historically as a successful way to develop efficient, quickly designed, optimized, and controlled manufacturing processes.[95]

First, let us examine what types of problems Tissue Engineering has at the level of process design to clarify why the Chemical Engineering intellectual core in process design from the assembly of unit operations is relevant to in vitro process for bioartificial tissue formation and critically important in the design of biomimetic processes. The lack of unit operations in the one-stage processes of Tissue Engineering under the 3D cell growth and differentiation paradigm, which is in contrast with the natural in vivo tissue development that occurs through distinct successive stages (e.g., Figure 4, the stages of bone formation) can give us an initial idea of the inadequacy of one-stage processes to recapitulate natural developmental phenomena. Since one-stage processes are used, researchers have a tendency to modify several factors/inputs, which, in this way,

also increases their inter-relations. These "numerous treatment factors and response measures, making it difficult for investigators to interpret, much less replicate, experimental results. The complexity creates confusion" (according to Butler in ref 33). The inputs of the process, such as cells, growth and differentiation factors, scaffolds, bioreactors, etc., are all placed together in the one-stage process. "However, these inputs likely exhibit strong interactions with each other, clouding the picture and slowing our ability to sort out those that deserve further study" (again, according to Butler in ref 33). Therefore, it is not strange that "there was so little understanding of why something did or did not work" (according to Ingber in ref 33). Re-engineering a candidate product to improve some properties of bioartificial tissues in a one-stage process with the strong and still largely undefined inter-relations of the inputs proved to be a very difficult problem. This problem, according to Arrow AK in ref 96, resulted in the high risk of product development of the Tissue Engineering companies, which turned out to be a prophetic comment, as was proven few years later with the failure of Organogenesis and Advanced Tissue Sciences, after several years devoted by them in product development, without, however, the appropriate methodology.[97]

Therefore, as mentioned previously, there are very practical methodological reasons why unit operations should be introduced in Tissue Engineering. On the other hand, since Tissue Engineering now wants to recapitulate the in vivo tissue development that happens in successive stages with each one under different conditions (e.g., stem cell condensation, then growth plate formation, and then bone formation at the end of growth plate; see Figure 4), it cannot develop an in vitro cell culture environment offered by a bioreactor-biomaterial system that has the capability of the human body to be self-adapted in a continuous way to accommodate the different requirements of cells during the sequential stages of in vivo tissue development, such as, for example, the continuously synchronization of the column elongation and chondrocyte differentiation rate. Therefore, the entire process of tissue development should be simulated in vitro by a series of subprocesses/unit operations that involve different bioreactor−biomaterial systems that best serve the different stages of the different developmental stages. For a biomimetic process design, the key variables that change during the developmental process from the cells to the tissue must be indentified.

## 3. The Three Variables of Developing Tissues Evolve Gradually and Synchronously

**3.1. Cell Organization to Tissue Structures through Cell-to-Cell Communication in a Continuously Changing Physical Environment.** If we examine the descriptions of Developmental Biology, we see several stages from stem cells that condense and form aggregates and differentiate, gradually forming mature tissue that has an organized structure, as in the example of bone formation shown in Figure 2. This description is necessary, but a parameter is missing. This parameter is the size of the tissue, which changes continuously during the developmental process. The increase in the size of the developing tissue is synchronized during development with the cell differentiation and cell organization, so that the involved biological phenomena occur under different and optimal conditions for each size. However, this is not the case for a one-stage in vitro process of the 3D cell growth and differentiation paradigm of Tissue Engineering. In such processes, the size of the bioartificial tissue is prefixed at the beginning of the process by the size of the scaffold. However, at this size, the distances are large for the not-yet-differentiated cells to have acquired the molecular machinery needed to secrete their proteins in an amount sufficient to diffuse and reach their target cells. In other words, the cell-to-cell communication is difficult or totally prevented already from the beginning of the process. In vivo, at each developmental stage, the size of the tissue is increased by the cell growth, the recruitment of cells from surrounding tissues, the increase of the cell size, (e.g., hypertrophic chondrocytes can be 10−15 times bigger that nonhypertrophic ones[98]), and the secretion of extracellular matrix. At the same time, cell differentiation proceeds, thereby changing the cell properties, such as the ability to produce and secrete protein signals or to receive such signals through surface receptors. The cell capabilities in signaling are compatible with the new size of the developing tissue, as we saw in the example of the growth plate. Therefore, the new phenomena that occur in the next developmental stage inside the growing tissue are compatible with its new size. The growth factor secretion is adjusted to the progress of cell differentiation, and, therefore, the cell properties are also adjusted to the developing tissue size, to circumvent diffusional limitations. In other words, everything is coupled and synchronized; time and space become one variable.

Since the correct cell differentiation cannot be externally induced with growth and differentiation factors, because the cell-to-cell interactions that play a major role in determining the cell fate in the differentiation process cannot be reproduced in vitro,[63] the same is true for the cell organization into tissue structures that also depend on cell-to-cell interactions and, in turn, depend on physical factors. There is no direct way, using, for example, growth and differentiation factors, to spatially organize the heterogeneous cell population of developing tissues in vitro. Even if an initial spatial arrangement will be attempted, positioning cells on a substrate with great precision with a method such as micropatterning[99] and cell printing,[100] this is only one arrangement from a series of different arrangements that the tissue cells will adopt as the tissue develops and grows in size (as its cells multiply) and their differentiation state changes, as we have seen in the example of the growth plate, where the scaffold prevents, instead of facilitates, the cell organization to columns. It also has been observed in nonbiomimetic processes that strict compliance with a prespecified geometry has a negative impact on the development of neurons and the formation of synapses.[101,102] Therefore, it seems a feasible strategy to achieve the cell organization in vitro only in an indirect way (i.e., not direct with the use of growth and differentiation factors), with the objective of providing to the cells conditions in which they could be self-organized, exchanging between them the required signals in an in vivo-like way. However, it means little that the correct signals through secretion and reception of the correct in vivo proteins are exchanged between cells, because if this signaling does not occur in the proper and gradually increasing physical space of the developing tissue, it will be dissipated in space, instead of spatially organized along the directions that define the directions of cell organization. The cell-to-cell signaling responsible for cell organization depends, of course, on the ability of cells to secrete and respond to such signals, which is determined by their differentiation state, with each one being associated with a particular pattern of gene expression. However, quantitative factors are equally important for this signaling to occur properly and have the expected effect. Such factors include the cell capacity of signal secretion and reception (which depends on the cell differentiation state), the cell number (which determines

the total quantity of the exchanged proteins), the cell-to-cell distances (so that the proteins will reach their targets, which, in turn, depends on the way the cells have been distributed in the scaffold), the size of the scaffold or cell aggregate, the cell proliferation rate, to mention some of them. If one of these factors is not compatible with the others (e.g., cells in a differentiation state not yet able to produce a sufficient quantity of a protein signal and large distances between cells in a scaffold, so that this protein has no chance to reach its target cells), it is unlikely that the cell-to-cell signaling will be reproduced effectively as in vivo and, consequently, the cell organization that depends on this signaling likely will not occur. In addition, we must consider that all of the above-mentioned factors change continuously during the tissue development and, therefore, their compatibility should be readjusted at each developmental stage, which is impossible to achieve in one-stage processes of the 3D cell growth and differentiation paradigm. In vivo, however, an orchestrated and gradual progression of tissue size, (related to the number of cells and their differentiation state (e.g., hypertrophic chondrocytes are large, compared to other chondrocytes)) and cell differentiation (relative to the ability for signal secretion and reception) occurs, providing, at each stage, the optimal conditions for cell-to-cell signaling to happen properly, gradually leading, as the tissue develops, to the spatial organization of the cells. We will see below why Tissue Engineering has historically followed a different road, ignoring the above-mentioned factors.

**3.2. From Primary (Differentiated) to Stem (Undifferentiated) Cells: The Role of Scaffolds.** Tissue Engineering has many differences from Biology, with regard to the way phenomena are perceived. However, the important difference with Biology that initiated Tissue Engineering as a new discipline (and is still the central theme of its research, although not always realized) is the parameter of "size". The difference in quantity between the cell number or the total mass of cells that are contained in a tissue flask in biological studies where the cells form 2D layers, and the cell mass required to have a suitable for transplantation bioartificial tissue, imposes directly, as a requirement in Tissue Engineering, an increase in the number of cells held together, or, in other words, the size of a 3D cell construct. In vitro, however, in tissue flasks, there is no sustained increase of the size of the cell mass. The cells may increase in number during their growth and form few layers or small aggregates, but no accompanying vasculature is provided, which eventually sets a limit on the size to a few hundreds of micrometers. For example, take a porous biomaterial where the cells can attach and remain together. At least the mass and size of a tissue could be determined under conditions that allow the cells to survive, exchanging nutrients, oxygen, and metabolites through the pores of the biomaterial. That idea came to Joseph Vacanti of Harvard Medical School of Boston on a "balmy summer afternoon" in 1986, while "he was sitting on a stone breakwater near his Cape Cod vacation house watching his four children play on the beach".[103] Looking at a seaweed waving its branches, Vacanti got the idea that "branching is the nature's way of maximizing surface area to supply thick tissues with nutrients". As Vacanti said, he raced up the road to a pay phone to call his colleague, Robert Langer. "He asked if we could design polymers that had a branching structure". Langer replied, "well, we could probably do that", and "we tried and we did" as Vacanti mentioned.[103]

The above arguments for the introduction of scaffolds, together with the start of Tissue Engineering, look obvious today, but the understanding of the situation is superficial. Let us resolve a misconception that is critical in deciding the way that we could design biomimetic processes. In 1986, Vacanti did not have stem cells. He had primary cells, already differentiated, and very little knowledge (like everybody else at that time) about how he could keep these cells alive (requirements in media, growth factors, etc.) and able to perform some of their differentiation functions that used to perform in vivo. Cell viability and function were perceived to be synonymous; it was determined later that the cells may be kept alive for longer periods with the improvement of media compositions, but they were loosing their differentiated functions. Vacanti and Langer performed, with the attachment of the cells to scaffolds, a very simple transformation: an increase of the size of the cell mass, without changing any other parameter. They did not change the differentiation of cells intentionally, because they were already differentiated; the organization of cells to tissue structures also was unchanged, because they could barely keep the cells alive and growing to consider the issue of organization. Therefore, they could not simultaneously address the question if the organization was reversible or not and could be repeated in vitro after the cell isolation from the organism tissues and their placement in a scaffold. We now know that only stem cells have the ability to be organized in tissue structures, but they exhibit it only in vivo, under the appropriate conditions. In rare cases, such as of embryonic avian retina, its cells can be organized in vitro in spheres, reconstituting the arrangement of retinal layers.[104] In most cases, however, such as the initiation of the mesenchymal stem cell differentiation to chondrocytes, it is the imposed specific in vitro conditions that recapitulate the in vivo conditions, such as compacting the cells in micromass or pellet form,[105,106] that allow the initiation of differentiation or perform the subsequent stages of development in vivo as in vivo to assure the cell organization as we will see below in the example of bioartificial growth plate formation in a biomimetic process.

We are now in 2010, and everybody in Tissue Engineering works with stem cells, instead of, primarily, already-differentiated cells. The goal is to start with a few stem cells and form in vitro a 3D construct of cells that has a size suitable for clinical applications. This construct should contain at the end of the process cells that are differentiated and organized to tissue structures. If somebody does not know the above story with Vacanti and Langer (i.e., that they had a specific question to answer with the scaffolds, which was how to keep many cells alive in a large size construct, and which could not be the question they could had if they had to do with stem cells), it is easy to be misled, believing that scaffolds are the key components in Tissue Engineering and what is needed to make bioartificial tissue is to place the stem cells in a scaffold and try to differentiate them with the addition of growth and differentiation factors. Scaffolds were such critical components in Tissue Engineering in 1986, of primarily already differentiated cells and, therefore, no differentiation needed to occur in vitro, so that developmental phenomena, which was always connected with cell differentiation, did not have to be considered. There is no Tissue Engineering in the expression "this growth factor induces the differentiation of cells", although this is encountered in studies with stem cell differentiation inside scaffolds, because cells do not constitute tissues unless they are fully differentiated and spatially organized, since there is no meaning in the expression "complete differentiation of chondroblasts up to mineralization (deposition of minerals in the extracellular space) is observed in micromass (cell aggregate) culture", because the size is not that of bioartificial tissue suitable for transplantation.

These expressions are meaningful in Biology, which does not have as a goal the increase of the size of the developing tissues, as Tissue Engineering does, but, instead, its goals are studies at the cell and subcellular level after their isolation from organisms, they constitute the logic error of "ignoratio elenchi"[48] in Tissue Engineering.

Because of the lack of knowledge of tissue development, Tissue Engineering followed the easy path: fixing the size of the bioartificial tissue from the beginning at the size of the scaffold, as Langer and Vacanti did, to be at least certain that the research is related to Tissue Engineering and not to Biology. However, this is the size scale where development has finished in vivo. It is rather overoptimistic to fix one variable at the end of development and expect that you can repeat the in vivo developmental phenomena in vitro, since the three variables—cell differentiation, tissue size, and cell organization—must evolve simultaneously and gradually to sustain the compatibility of size with the cell signaling capabilities that determine the correct cell differentiation and organization to tissue structures. We will see below how we can move in the space of the three variables, evolving them gradually in a synchronized way, assembling an in vitro biomimetic process from unit operations using as example the formation of growth plate. According to Developmental Biology, the growth plate composed of differentiating chondrocytes (developing cartilage) is the natural "scaffold" that osteoblasts use in bone development. It can be used for the formation of bioartificial cartilage, bone, or osteochondral bioartificial tissue, and therefore has particular significance for Tissue Engineering as the basis for the formation of other tissues.

## 4. The Unit Operations of Biomimetic Processes, in a Series of Sequential Steps in Variable Space

### 4.1. A Biomimetic Process for Growth Plate Development.

**4.1.1. The Robustness of Developmental Processes.** The design of a biomimetic process in Tissue Engineering has an important difference from this in Chemical Engineering. Developmental Biology is still a "law-less science".[107] Therefore, it cannot yet provide a concrete and detailed theoretical basis for the foundation of a biomimetic process design methodology, such as that which physical and chemical laws have provided to other engineering fields. However, the robustness of developmental processes to perturbations, either internal (such as mutations) or external (such as environmental conditions), was already well-appreciated by 19th century developmental biologists.[108] Such a robustness suggests that laws that govern developmental phenomena exist. The concept of robustness, which determines the biological transformations that are feasible during development, can be perceived as analogous to thermodynamic laws that determine which physical or chemical transformations are permissible. In an in vitro developmental process, the robustness will be reflected in stability to external perturbations that are unavoidable in an artificial environment. A stable process has feasibility of implementation, because, if temporarily disturbed, it will return to its normal course, providing cells or tissues with reproducible properties. However, are nonbiomimetic processes robust? This question has no meaning if we refer to biological instead of chemical and physical transformations, because the only example of robust biological transformations, with long-term observational validation, are those which occur during the in vivo development. It is then obvious that there is no scientific grounds for the design of nonbiomimetic processes with the 3D cell growth and differentiation paradigm. This looks like designing, by trial-and-error, a chemical process without considering the thermodynamics or kinetics of the reactions. The possibility to succeed accidentally in obtaining bioartificial tissue with its cells organized in a nonbiomimetic process is null. Some in vitro processes that exhibit high robustness recapitulate developmental mechanisms, such as, for example, the micromass or pellet culture system for the induction of differentiation of mesenchymal stem cells to chondrocytes. Because of its robustness, this culture has become a reference system in Biology. However, this system, through recapitulation of the in vivo mesenchymal cell condensation that induces the chondrocyte differentiation, is inadequate for the purpose of making, in vitro, a bioartificial growth plate, which necessitates, except the cell differentiation, the simultaneous increase of the tissue size and cell organization. In these systems, the size of the cell aggregate is small and fixed from the beginning and, therefore, they can only simulate, in vitro, the first stage of in vivo growth plate development, although, at some times, cell organization could be also observed but not in the correct direction and certainly in limited size, determined by the size of the micromass or pellet. For example, in the pellet system, the cells can be arranged in columns that, however, are not parallel but radiate outward from the center of the pellet.[109] Therefore, although the feasibility of a designed biomimetic process can be checked, to some extent a priori, incorporating, in the design, the now-known conditions provided from the studies of Developmental Biology (the closer to development, the more robust the process), as well as during the implementation of the designed process, examining if developmental mechanisms have been established as in vivo and making the necessary modifications, there is no meaning in the term "feasibility" for a nonbiomimetic process whose design cannot reach the evolutionary optimized design of the in vivo developmental processes. In addition, if there is no robustness, there is no feasibility, and if there is no developmental mechanism, there is no criterion of process optimization. It is then expected that "there was so little understanding of why something did or did not work" in the implementation of simplified unrelated with the developmental phenomena Tissue Engineering processes (according to Ingber in ref 33).

**4.1.2. Feasibility of a Biomimetic In Vitro Process for Growth Plate Development.** The minimal requirements for a biomimetic process design for the formation of a bioartificial growth plate and an a priori examination of process feasibility can be searched in the information of in vivo growth plate development provided by the studies of Developmental Biology. According to this information, the following critical issues should be considered in the process design:

- Only mesenchymal stem cells are required for the generation of the chondrocyte types of the growth plate.[110] This greatly facilitates the design of the in vitro process.
- Vascular invasion is not required for the differentiation of the mesenchymal stem cells to chondrocytes and for the subsequent differentiation of chondrocytes until the stage of hypertrophy. Cartilage is highly resistant to vascularization, producing angiogenic inhibitors,[111,112] except in the zone of hypertrophic chondrocytes. This further facilitates the process design because no endothelial cells or systemic factors transferred by the blood will be required in the in vitro process of growth plate formation.
- Vascular invasion is not required for growth plate mineralization.[113] According to Gerber et al., mineralization and vascular invasion are uncoupled.[84]
- Apoptosis, which is the last stage of chondrocyte differentiation before their replacement by osteoblasts, is a controversial issue. It happens after vascularization in vivo but

it has been observed in several in vitro systems. Vu et al. proposed that apoptosis of the hypertrophic chondrocytes does not belong to the automatic sequence of previous differentiations stages, but, instead, it is functionally coupled to vascularization and ossification with only the cells that are in contact with the vascular network front undergoing apoptosis.[114] However, other studies provide evidence that hypertrophic chondrocytes inherently die from apoptosis with no need for additional factors that are carried by the blood. For example, it has been shown that, in embryonic limb mesenchyme micromass cultures, the chondrocytes proceed sequentially through all the steps of cell differentiation until apoptosis.[115] The same progression, up to apoptosis, was observed in vitro with chick limb-bud mesenchymal cells.[116]

• The structure and function of the growth plate is determined in vivo by intrinsic factors, the negative feedback loop of Ihh/PTHrP mentioned above, which determines the chondrocyte differentiation rate and pattern formation. Abad et al., for example, showed that the polarity of the growth plate, with the resting and proliferating chondrocytes on one side of the growth plate and the hypertrophic and apoptotic ones on the opposite side, does not depend on the surrounding tissues and remains the same after the growth plate is excised, inverted, and reimplanted, suggesting that the growth plate organization is due to intrinsic factors.[117] Embryologists have long since observed that some parts of developing organisms, such as limb buds or tooth germs, are robust embryonic regions that could be displaced or induced ectopically. Therefore, they exhibit an internal coherence and relative independence from other parts of the organism. These types of units of embryonic development are called "modules"[118] and such a developmental module is the growth plate. This means that, if we succeed in forming a growth plate in an in vitro biomimetic process, its structure will not depend on other factors, either cells or signals, besides those inside the growth plate, which means that the growth plate pattern will be stable with minimal external control.

According to the above information, we could say that, in principle, starting with mesenchymal stem cells, we could design a biomimetic process that will lead to the formation of the growth plate, certainly up to the hypertrophic chondrocyte state, and we could retain this structure in vitro with minimal external control, because of its robustness. As Ingber mentioned, tissues are "robust systems composed of sloppy parts".[33] It is the intrinsic control mechanism of the chondrocyte (sloppy parts) interactions that make the growth plate robust, and, therefore, such interactions should be established in vitro, instead of making only sloppy parts of tissues with a random distribution of differentiating chondrocytes inside a scaffold.

**4.1.3. Importance of the First Unit Operation of a Biomimetic Process in Establishing Optimal Conditions from the Subsequent One.** The purpose of a biomimetic process design is to assemble the process that leads from stem cells to bioartificial tissue from unit operations that are defined with scientific (information from Developmental Biology mentioned above) and technological reasoning (mentioned below in the feasibility of the unit operations). The unit operations should perform small steps in the variable space, from stem cells to bioartifical tissue, following the stages of the in vivo tissue development, instead of increasing the size from the beginning of the process, as it is practiced today. Cell and cell assemblies (3D cell constructs) transformations, biological as well as mechanical, are performed in each unit, and these transformations set the optimal conditions for the next unit, as happens in vivo, to the degree possible, given the current knowledge of the developmental phenomena and the technical limitations. The in vivo tissue development is "path-dependent",[119] which means that the progression of each developmental stage depend on the conditions set by the previous ones. Consequently, optimal conditions for the continuation of a biomimetic process are self-established by the process itself, with each unit operation establishing the optimal conditions for the next one. For example, D'Amour et al.[28] started their study for the design of an in vitro process to generate pancreatic beta cells only after optimizing the first stage, generation of definitive endodermal cells from human embryonic stem cells, in a study published previously.[120] Since the pancreas originates during in vivo development from the endodermal gut epithelium,[121] endodermal cells had to be used in a biomimetic in vitro process for the generation of beta cells, as had been proposed previously by Sipione et al.[122] Such an optimization of the first stage was needed because its optimal conditions were not known. When the authors used endodermal cells to generate beta cells, they determined that further optimization of the first stage of endodermal cell generation was needed. They observed that if the first stage of generation of endodermal cells was suboptimal, the beta-cell productivity of the last stage of the process was low. For example, when low activin A was used in the first stage, the quantity and the anterior pattern of the definitive endodermal cells produced was reduced, which, in turn, substantially diminished the production of endodermal intermediates and endocrine cells in the second stage.[28] This shows that the design of a biomimetic process is not demanding if the major focus will be on the design of the first unit-operation developmental stage, so that we could make use of internally imposed optimal conditions for the subsequent stages, because of path dependence of developmental stages.

**4.2. Unit Operations of a Biomimetic Process for Bioartificial Growth Formation.** Trying to design a simplified biomimetic process for the growth plate development and defining distinct stages corresponding to different unit operations, initially, we roughly could consider only the geometry of tissue forms that appear during the development, as seen in Figure 2. We could then distinguish two stages: the first one is of spherical geometry and refers to the cell condensation−aggregation in the middle of cartilage (see Figure 3a), which initiates the chondrocyte differentiation to hypertrophy, and the other one is of planar geometry and refers to the columnar/zonal pattern of growth plate (see Figures 3b, 3c, and 4a). If the in vitro process has as a goal the bone development instead of only growth plate formation, we must add another stage to complete the process; this of the contact of osteoblasts with the zone of hypertrophic chondrocytes of the developing growth plate (Figure 3c). The two stages mentioned above for the formation of growth plate from mesenchymal stem cells necessitate different bioreactor−biomaterial systems to be implemented in vitro. The cell condensation is the earliest morphological event of cartilage development in vivo; it establishes the cell-to-cell communication that is necessary for the induction of chondrocyte differentiation,[123] and it is a prerequisite for the differentiation of mesenchymal stem cells to chondrocytes.[66]

However, before this unit operation, which corresponds to cell condensation and, for technical reasons not strictly related to a developmental stage, we must amplify the mesenchymal stem cells that are isolated from an adult tissue. The mesenchymal stem cells are a type of adult stem cells that are isolated from bone marrow or other tissues and are the progenitors of multiple cell lineages. These cells can be expanded (multiplied)

and differentiate in vitro with the use of different growth and differentiation factors into several tissue-forming cells, such as bone, cartilage, fat, muscle, tendons, and other cells of connective tissues.[124,125] Since the number of such stem cells in tissues is small, the unit operation of the in vitro amplification (expansion-multiplication of cells), after their isolation to ensure that enough cells are available to make bioartificial tissue at a size suitable for implantation, is imposed by the properties of these cells and the current methods of their isolation from tissues. It should be mentioned that this type of stem cell, isolated from adult tissues, has restricted differentiation potential toward some cell types and not all types as the embryonic stem cells. However, since their differentiation state is close to that of the final completely differentiated tissue cells, the process design becomes easier to handle, because of the limited number of states that they must pass through to reach their final differentiation state. However, it is not the same with embryonic stem cells, which are, although, in principle, capable of being differentiated to any tissue cell (pluripotent cells can differentiate to any cell type but do not participate in the formation of extra-embryonic membranes or the placenta, they are taken from the blastocyst, an early stage embryo 4−5 days old in humans, consisting of 50−150 cells) of the more than 200 that exist in the human body, they must pass through many differentiation states until they will reach the fully differentiated tissue cells. This renders the process design very complex, given that information from Developmental Biology is not available for all the developmental stages from embryonic stem cells to a tissue to a degree needed to design a reliable biomimetic process without introducing empirical trial-and-error unit operations for the stages with little or no information concerning the in vivo conditions for such stages that have not been yet fully explored. For example, D'Amour et al. used, for the generation of beta cells from endodermal cells (which are very similar to embryonic stem cells), a strategy that, as the authors mentioned, "combines an informed approach based on developmental biology and an empirical approach".[28] The authors guided cell differentiation through a series of five stages of endodermal intermediates that are similar to those appearing in vivo: definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm, and endocrine precursors and hormone-expressing endocrine cells. The informed approach refers to recapitulation of successive stages of pancreas development, as these are described in the studies of Developmental Biology and the characterization of the cells appearing at each stage, identifying appropriate patterns of marker expression and extrapolating this information to the complete description of cell types included in Developmental Biology studies. However, some of the information needed was not available from developmental studies, such as, for example, that related to the growth and differentiation factors that had to be used in each stage. An empirical approach was then followed testing various combinations and application time schedules of growth and differentiation factors recipes for each stage of the process, to optimize the properties of the cells at each particular stage. However, such empirical approaches are not suitable for Tissue Engineering, where additional factors, such as scaffolds and bioreactors with their wide spectrum of properties, are introduced, making a factorial analysis unreasonable, as mentioned by Butler in ref 33, even if the optimal recipe of growth and differentiation factors is known by the studies of Biology in 2D systems. In other words, this shows that the worlds of experience of biologists and tissue engineers are different, when biologists consider the ability of stem cells to differentiate to as many as possible directions, giving different tissue cell types, as a success, and they consider any unsuspected transformation from one cell type to another as a breakthrough, engineers consider this to be a serious problem in process design, because they must design the process to provide conditions for the stability of cell differentiation toward decided directions. An unsuspected cell phenotype transformation is the transdifferentiation;[126] this refers to the conversion from one differentiated cell type to another. It occurs in nature, however, only in some cases, such as, for example, when missing parts regenerate in animals;[127] therefore, no such developmental process exists in humans for which a biomimetic process should be designed. Consequently, the process described below starts with adult mesenchymal stem cells (multipotent) of limited capacity to give only some cell types, so that conditions could be chosen easily compared to those for embryonic stem cells, to lead these cells toward the chondrogenic differentiation, instead of, for example, adipogenic differentiation.

**4.2.1. First Unit Operation−Cell Expansion (Cell Multiplication).** The purpose of this unit operation is to increase the number of mesenchymal stem cells without changing their differentiation state in order to provide enough cells for the formation of a large size bioartificial tissue suitable for implantation. A culture system for the first unit operation could be, for example, the microgravity (rotating) bioreactor with the cells in suspension. This is a bioreactor that has been designed specifically for the formation of aggregates. A major problem that prevents the formation of the cell aggregates in conventional bioreactors is the agitation that is required to keep the cells and the cell aggregates in suspension. The agitation creates turbulence that damages the cells and breaks the aggregates, because of the created shear stress.[128] Especially for cells cultured on microcarriers, the cell damage can be caused by collisions of a cell-covered microcarrier with other beads, collisions with parts of the reactor (such as the impeller), and interaction with turbulent eddies of the size of the microcarrier beads.[129] To overcome this problem, NASA has designed a bioreactor that simulates microgravity conditions (gravitational forces are randomized). The simplest form of these systems is the rotating vessel. It consists of a disk that is filled with a medium that rotates continuously, keeping the cells in suspension. The cells remain suspended, because they follow the rotation of the medium, which rotates together with the vessel as a solid body. Since the cells do not move relative to the medium or to each other, the shear stress is minimized and the cells are in close contact for sufficient time to develop their connections, forming aggregates.[130]

In this unit operation, mesenchymal stem cells attached on microcarriers grow inside the rotation bioreactor covering the microcarrier surface. The first unit operation achieves the cell growth-multiplication on the microcarrier surface and corresponds to cell expansion, which increases the number of cells without inducing their differentiation, under the assumption that this unit operation will be terminated before the cells-microcarriers will assemble in large clusters of many microcarriers held together, locally increasing the cell concentration at the level of condensation that initiates the cell differentiation. In Biology studies, this operation is performed on the surface of tissue flasks. The difference here is that, instead of the surface of the flasks, the surface of the microcarriers is used. Although microcarriers were introduced for the scaleup of mammalian cell cultures, in the case of the biomimetic process of growth plate formation, it is not only the large-scale production of mesenchymal stem cells of interest. Their use allows the

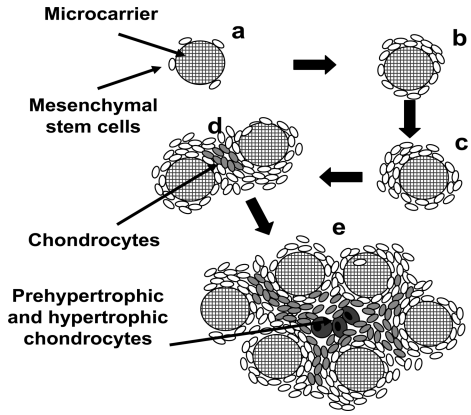


**Figure 5.** In a biomimetic process of growth plate formation, biological and mechanical transformations are performed gradually as we move from the one to the next unit operation, to synchronize the progression of the three variables (cell differentiation, construct size, and cell organization to tissue structures) of the developing in vitro tissue. An important factor for the design of unit operations, besides the fact that they should offer conditions similar to the different successive developmental stages, is the ease of their assembly, which requires that the output of each unit operation be transferred to the next one intact, i.e., retaining its biological and mechanical characteristics, so that the change of the cell differentiation and construct size will progress in the next unit operation, making a small step in the space of the three variables. Therefore, we can choose the culture of mesenchymal stem cells attached on biodegradable microcarrriers inside the rotation bioreactor, which facilitates cell aggregation through the microcarrier clustering to achieve the synchronized progression of cell differentiation and construct size, without the need to dismantle the constructs formed in each unit operation in order to proceed to the next one. The first unit operation has, as a purpose, the amplification of the mesenchymal stem cells that have been isolated from an organism tissue. It performs the cell multiplication on the microcarrier surface (construct states "a" to "b" to "c"). In the second unit operation, the microcarriers form small clusters through bridges between cells on their surfaces (construct states "d" and "e"). As such microcarrers are formed, the cell concentration increases for cells between the microcarriers and it reaches the concentration needed for the induction of differentiation of stem cells to chondrocytes. This unit operation corresponds to the condensation stage of the long bone formation through a cartilage template (see Figure 3a). The third unit operation is the culture of constructs formed in the second one for more time, so that clusters fuse together and, at the same time, the cell differentiation proceeds to subsequent states (construct state "e"). In this way, the two variables, cell differentiation and construct size, advance without however reaching their final state, i.e., cells have not reached the final differentiation states (hypertrophy and apoptosis) and the size will be further increased in the next unit operation (see Figure 9, presented later in this work), in which the cell differentiation will continue together with the onset of cell organization.

continuation of the process to the next differentiation stages of the cells, as well as the increase of tissue size, as we will see in the next unit operations described below. If the cells grow fast and the microcarriers form clusters, it is better to use a stirred tank bioreactor, to prevent the phenomenon of cell-microcarrier assembly in the first unit operation.

The transformation performed in this unit operation is from state "a" (few cells on the microcarrier surface) to state "b" or "c" in Figure 5. No differentiation has been induced, because the cell concentration has not reached the level of condensation.

Since the multiplication potential (i.e., how many times these cells can divide, retaining their ability to progress in their differentiation toward other cell types) is also limited (as limited as their differentiation potential also is, compared to embryonic stem cells that, in principle, multiply in infinity, as long as a fresh medium is provided, retaining their undifferentiated state), special considerations in the design must be taken, relative to the number of cells in each microcarrier at the beginning of the process. Consequently, the cell-to-microcarrier ratio must be optimized, corresponding to the multiplication potential of the cells. For example, a low ratio of cells to microcarriers will result in microcarriers having few cells on their surface, because of the cell-limited number of divisions. This can cause problems in the next unit operation, in which we would like the cells to reach the concentration that corresponds to the concentration of the in vivo condensates that induce the cell differentiation. This will happen when microcarriers will form clusters with many microcarriers held together and having cell aggregates between them (see state "d" or "e" in Figure 5). Therefore, considerations related to the compatibility of biological transformations and the properties of the biological constructs formed in each unit operation are of particular importance for the assembly of unit operation in a series of sequential processes, during which the previous unit facilitates the transformations that occur in the next unit oepration. For example, the above-mentioned bioreactor−biomaterial system of a rotating bioreactor with microcarriers allows the continuation of the process in the next unit operation with the microcarrier clustering that will initiate the cell differentiation without the need to isolate the cells, as we will see below and as we would have to do even if the cell expansion was performed on the surface of tissue flasks.

**Feasibility of the First Unit Operation−Cell Expansion.** The use of microcarriers in bioreactors for cell expansion is a well-established process in Biochemical and Tissue Engineering. However, it has been not considered yet as one of the unit operations in a process: it has only been considered as a method for large-scale production or for the generation of 3D cell constructs. Therefore, the technical feasibility of expanding cells under these conditions has been already tested. Examples from the literature are numerous, such as, for example, the 85-fold expansion of rat multipotent adult progenitor cells with retention of their pluripotency markers and their differentiation potential;[131] the 34- to 45-fold expansion of human embryonic stem cells with the retention of pluripotency markers, such as OCT3/4A, NANOG, and SSEA4;[132] the expansion of ear mesenchymal stem cells, where the control of the percentage of stem cells in S phase was achieved with a suitable growth factor feeding that led to the maintenance of stem cell phenotypes and differentiation potentials;[133] the expansion of mouse and human embryonic stem cells, where different types of microcarriers, such as Solohill Collagen, Solohill FACT, and Cultispher-S, as well as different initial seedings of cells to microcarriers and different agitation conditions, were tested to promote cell attachment to microcarriers and control the size of the aggregates formed, to retain the expression of pluripotency markers, such as SSEA4, Tra-1-60, NANOG, and OCT-4 and the ability of multilineage differentiation;[134] the expansion of human mesenchymal stem cells, where the expansion was optimized by precoating the microcarriers with serum, to increase cell seeding efficiency;[135] the expansion of human bone marrow-derived stromal cells, where optimization was achieved by testing various serum concentrations and feeding regimes;[136] and the expansion of rat bone marrow mesenchymal stromal cells where it was found that direct transplantation of cell-bearing microcarriers reduces the apoptotic death, because of the avoidance of trypsinization, which disrupts cell−extracellular matrix contact,[137] just to mention some of the recent studies.

It is important to be mentioned that this unit operation, which is now used for the expansion of stem cells, had been developed long time ago (in 1967, by van Wezel[138]) for the culture of mammalian cells, for the production of recombinant proteins of viral vectors for gene therapy. Therefore, this process, which is considered here as a unit operation, has been transferred to

different conditions, in terms of cell type and production (production of stem cells, instead of proteins for example), as it happened in the past with the unit operations of Chemical Engineering, greatly facilitating the process design, because critical parameters of this process have been already established, such as microcarrier type,[139] cell seeding,[140] cell growth on microcarriers,[141] feeding regimes, etc.

**4.2.2. Second Unit Operation−Cell Aggregation (Cell Condensation).** The purpose of this unit operation is to induce the chondrogenic differentiation of mesenchymal stem cells that are provided by the first unit operation, achieving a cell concentration similar to the in vivo condensates. The selection of the rotating bioreactor for the second unit operation is compatible with the spherical geometry of the first stage of chondrocyte differentiation, but also provides flexibility in the construct properties. This flexibility is necessary for the optimal switching from the second unit operation to the third unit operation, where the size of the construct will be larger. The increase in size may require the use of biodegradable microcarriers[142] from the beginning (first unit operation) inside the aggregate mass that can provide free space for nutrition and oxygenation of cells and waste removal by the medium that enters from the bulk into the pores of the microcarrier clusters formed by the microcarrier degradation. Such type of microcarriers have been already used in some cases with chondrocytes, either alone[143−147] or embedded in a scaffold,[148] or in the culture of stem cells for their differentiation.[149,150]

The mesenchymal stem cells attached to microcarriers, which is the output of the first unit operation, is the input for the second unit operation, in which we could use the perfused rotation bioreactor system, since the cell concentration is increasing (although the one side of the batch rotation bioreactor vessels contain a membrane that is permeable to oxygen). The oxygen concentration is a critical factor to be controlled, because the induction of chondrocyte differentiation in condensates requires low oxygen.[111,112]

**4.2.2.1. Oxygen in Biomimetic Processes.** The oxygen concentration in biomimetic processes is not as simple an issue as in the cultures of Biochemical Engineering of mammalian cells that are all transformed (having properties of cancerous cells); therefore, there is, more or less, an equal demand for oxygen. In biominetic processes, oxygen plays a major role in cell differentiation, determination of cell metabolic character, and metabolic cooperation of cells in space to define the tissue functions. The oxygen concentration in the second unit operation should be low, to enhance the chondrocyte differentiation,[151,152] as well as also being low for the maintenance of pluripotency of stem cells,[153,154] contrary to primarily already-differentiated cells that have increased needs for oxygen.[155,156] In several cases, however, the oxygen demands of cells are not adequately taken into account. Especially, the situation when cells have different oxygen demands in different positions in a 3D construct, according to which their metabolic role in each position is determined, has not been considered yet. For example, different oxygen concentrations are needed for the zones of the growth plate, with high concentrations being needed in the proliferating and hypertrophic zones (see Figure 10, presented later in this work). Such gradients of oxygen as gradients of growth factors are important in determining the axons of heterogeneities in tissues in a way that the tissue cells will cooperate to establish tissue functions. For example, it is wrongly perceived in several mathematical models that have been developed for bioartificial liver with primary hepatocytes that have high oxygen demand, that the focus should be on the

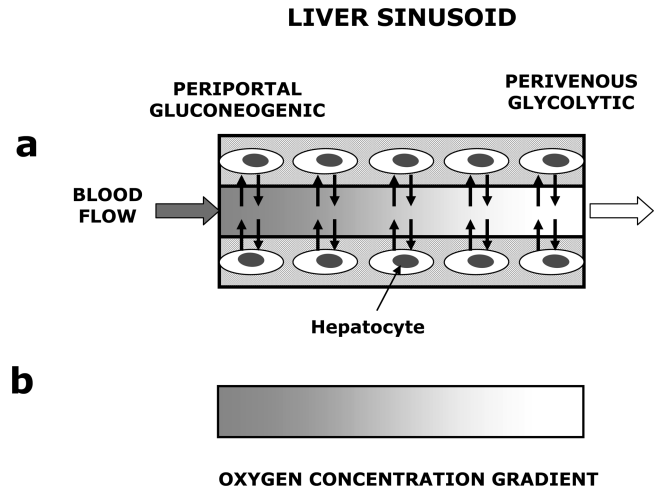


**Figure 6.** The zonal patterns of the gene expression in liver lobules have been categorized as "gradient" instead of "compartment" type, because all the hepatocytes are able to express these genes, but the level of expression is dependent on the position of the cell along the sinusoid. It is the gradients of substances that are created as the hepatocytes exchange metabolites with the blood that flows along the sinusoid that induce the different cell metabolism influencing differentially the gene expression (a). From the in vivo measurements of metabolite concentrations along the sinusoid, a primary role was shown to be played by the oxygen gradient (b). Although the gradients of most major carbon substrates, such as glucose or amino acids, are not high, the oxygen gradient is steeper, falling by 50% from the periportal to the perivenous zone (blood flows from the periportal to the perivenous area), from 70 mmHg (90 $\mu$M) to 35 mmHg (45 $\mu$M).

oxygen transfer optimizing the fluidics of the devices to provide, adequately and uniformly, oxygen to sustain the cell viability.[157−162] Such studies use parameters that refer to the cell level such as the increase of cell viability or the increase of the culture longevity, in terms of some hepatocyte-specific functions, such as albumin and urea production, that are perceived as equivalent to the overall function of the device, according to the 3D cell growth and differentiation paradigm. However, the reference to the hepatocyte instead of to an organized community of hepatocytes contradicts the findings of liver physiology and biochemistry. The elimination of oxygen gradients is, in the above cases, desirable to decrease the danger of hypoxic regions in the device that could impair the cell viability and functionality, since the hepatocytes are oxygen demanding and their functionality is improved in high oxygen concentrations.[163−166] However, with a uniform oxygen concentration, the hepatocyte metabolic heterogeneity, which is the basic factor for their metabolic cooperativity and is necessary for the establishment of liver functions, is lost. Hepatocytes with different enzymatic activities do not belong to different sublineages (i.e., the hepatocyte metabolic character is not determined at the cell level during the cell differentiation, so that could remain the same, irrespective of the microenvironment of hepatocytes). The metabolic heterogeneity of hepatocytes is attributed to the heterogeneity of microenvironments in the liver lobules that have different concentration of regulatory molecules that control the expression of the genes of metabolic enzymes; therefore, the tissue level is responsible for the hepatocyte metabolic differences.[167] The microenvironment of the hepatocytes along the liver sinusoids is different, because several substances in the blood exhibit gradients of concentration along the sinusoids. These gradients arise from the exchange of metabolites between the hepatocytes and the blood as this flows along the sinusoid [43,168,169] (see Figures 2 and 6). For example, the hepatocytes become either glycolytic or gluconeogenic from indirect interactions between them through the blood. These interactions establish an oxygen gradient along the

sinusoids. The gluconeogenic hepatocytes, which are situated in the periportal areas from where the blood enters the sinusoids, are in a microenvironment rich in oxygen. As the blood flows along the sinusoids and the hepatocytes consume the oxygen, the oxygen in the microenvironment of the perivenous hepatocytes is at low concentration. The different oxygen concentration induces the expression of different enzymes in the hepatocytes and makes them metabolically different.[170−175]

Only recently has the hepatocyte metabolic heterogeneity been implemented in vitro (not in a bioartificial liver device). A flat-plate perfusion bioreactor system that allows the formation of a one-dimensional steady-state oxygen gradient has been developed and used to show that the oxygen gradient induces a heterogeneous distribution of hepatocyte enzymatic activities, which is related to the distribution of these enzymes in vivo.[176,177] Although only the metabolic heterogeneity has been achieved in this system and not the metabolic cooperativity for the establishment of liver tissue functions (such as the glucose homeostasis, which requires, except the qualitatively established heterogeneity, the quantitative balance of the two metabolically different hepatocyte zones, gluconeogenic-oxic and glycolyitic-anoxic, and the communication between these zones through lactate), this system can serve as an in vitro model system of liver zonation, where several design parameters for the bioartificial liver device could be tested. It could also constitute the basis for the design of more-complex systems. In this example, we clearly see that the one-stage 3D cell growth and differentiation paradigm is not only inadequate for the design of biomimetic processes that are based on in vivo tissue development, but it is also inadequate and contradictory of the findings of Physiology and Biochemistry of primary cells. We will encounter a similar phenomenon in the fourth unit operation for the formation of the growth plate pattern (see Figure 9, presented later in this paper), where, again, an oxygen gradient should be established between the proliferating and hypertrophic hondrocytes for the establishment of different but cooperative chondrocyte functions that finally lead to the mineralization of the last part of the zone of hypertrophic chondrocytes (see Figure 10), where bone matrix will be deposited.

**4.2.2.2. Description of the Second Unit Operation.** Returning to the description of the second unit operation for the growth plate formation, we can see that small-sized clusters of cell-bearing microcarriers start to form as the cells proliferate and cover the microcarrier surface in multilayers. At the same time, chondrogenic differentiation is induced in high cell density areas, as it is seen in the transformation from "c" to "d" and "e" in Figure 5. The microcarrier clustering occurs because, after the coverage of the surface of microcarriers by cells, the cells promote the formation of larger multimicrocarrier clusters. This happens through microcarrier-to-microcarrier connection from cells protruding from the microcarrier surface that form cell "bridges" between the microcarriers holding them together, which is a phenomenon that has been observed a long time ago in mammalian cell cultures.[178,179] The same phenomenon has been observed in the rotation bioreactor with chondrocytes attached on microcarriers. Impressively large aggregates, ~5−7 cm in diameter, containing 100 microcarriers populated with cells and clumped together, had been formed[180] in this system. However, as we have mentioned in the previous sections, no cell organization could be achieved in such a one-stage process; chondrocytes were randomly distributed between microcarriers.

To clearly see why we should move to a third unit operation, we must follow what will happen if we leave the cell-microcarrier clusters to continue their assembly and chondrocyte differentiation in the second unit operation with the same bioreactor−biomaterial system. As the size of the cluster increases with the addition of more microcarriers in the initial clusters or with the fusion of clusters, a parallel phenomenon that occurs is the cell differentiation that had started in areas inside the clusters, where the cell density is high. If the operation is left to continue, the cluster passes through different stages, becoming larger in size. The chondrocyte differentiation proceeds in some areas toward the final state of chondrocyte differentiation, hypertrophy or apoptosis, as seen in Figure 7. These areas will be mineralized (mineralization is the secretion by the chondrocytes of inorganic phosphate that combines with calcium, forming the mineral hydroxyapatite, which is first deposited in the lower hypertrophic zone) and will be "frozen", since no further differentiation or cell movement occurs in these areas and, therefore, they are not accessible to chondrocytes. The mineralization is, by itself, not a problem, since it is the normal ending of chondrocyte differentiation followed by bone matrix deposition. However, in the in vivo case, mineralization is restricted in the last zone of chondrocytes—the end of hypertrophic one, where bone will be formed. In the above case, however, with the continuation of the process in the second unit operation, the areas of chondrocytes, as well as the mineralized areas, will be randomly distributed inside the large cell-microcarrier cluster. In this case, the cell organization is lost and the only variables of the construct still progressing are the cell differentiation in other not-yet-mineralized areas and construct size with the addition of further isolated or clustered microcarriers. The mineralized areas prevent any organization of chondrocytes in the entire cluster mass. At some point during

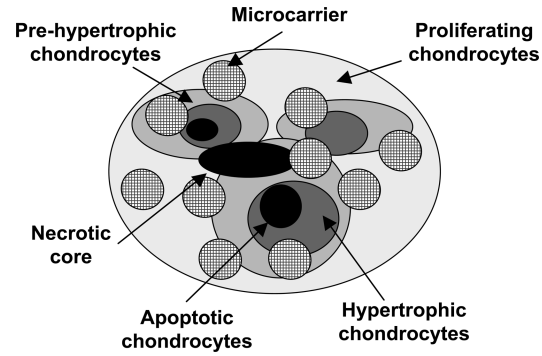


**Figure 7.** If instead of following a biomimetic process composed of distinct successive unit operations for the growth plate development, we follow a one-stage process, according to the paradigm of 3D cell growth and differentiation, placing the cells on the microcarrier surface and leaving the construct size to increase in the same system through microcarrier clustering and cluster fusion, we should not expect any organization to occur. The size of the construct increases gradually, the chondrocyte differentiation progresses. However, no cell organization can occur, because the structure of the growth plate is planar and not spherical. If the constructs will be cultured further in the same system, the cell differentiation will reach the final states of hypertrophy and apoptosis, when the secretion of minerals from the cells start. Such minerals, which are dispersed randomly inside the cluster mass around the cells, "freeze" the construct structure, since they do not allow any further cell movement. However, if cells cannot move inside the cluster, they cannot take the appropriate positions needed for their organization. In other words while the 3D cell growth and differentiation paradigm can achieve the progression of cell differentiation and construct size, if the system of cells on microcarriers in the rotation bioreactor will be used, it is unable to lead to cell organization, imposing the inappropriate conditions at the developing constructs to proceed to cell organization. Therefore, the culture should continue in another unit operation before the mineralization of clusters, as seen in Figure 9 (presented later in this work), where conditions compatible with the geometry of the growth plate, as well as conditions compatible with the operation of the Ihh/PTHrP negative feedback loop (see Figure 4) that is responsible for the columnar organization of growth plate, will be provided.

the culture, a break of the constructs is expected to happen, because of a misbalance of mechanical forces, which increase with the construct size with the collision and assembly of cell-microcarrier clusters and biological factors as the ability of cells to retain and renew their extracellular matrix that keeps them together in the construct.

Several technical modifications of the process can be done; however, this time, they will be rational and well-supported by Developmental Biology, instead of attempts of trial-and-error. If we observe a high differentiation rate in comparison to the rate of the increase of the clusters size, since our goal is to synchronize the evolution of the three variables (cell differentiation, tissue size, and cell organization) and ensure that differentiation will only be completed at the final construct size in the last unit operation, appropriate technical choices can be made. For example, microcarriers with a density less than the medium could be used. In this case, the microcarriers or the cell-microcarrier clusters migrate toward the central region of the bioreactor as they move in circular trajectories.[181] The fast migration toward the center results in the formation of cell-microcarrier clusters stabilized by cell bridges with cells still not having reached their final differentiation state, so that further differentiation will occur in the next unit operations. This means that not all the cells will have started their differentiation and cells still at the proliferation state will be found inside the cell-microcarrier cluster that will start their differentiation in the next unit operations for the reasons explained below. As we see, this example of technical choice is rational from the point of view of developmental phenomena (biomimetic process), instead of being compatible with cell properties only (3D cell growth and differentiation paradigm). In the second case, since only the cell differentiation is considered to be an important factor, a construct with fully differentiated chondrocytes is acceptable, even if their distribution in the construct is random.

**4.2.2.3. Technical Feasibility of the Second Unit Operation.** The technical feasibility of performing the second unit operation to initiate the chondrogenic differentiation of mesenchymal stem cells inside the mass of cell-microcarrier clusters arises from already-performed studies in similar systems (chondrocytes, microcarriers, and rotation bioreactor). The rotation bioreactor with chondrocytes on microcarriers has been used by Baker and Goodwin,[180] as mentioned previously. Those authors observed that the production of extracellular matrix proteins that are specific for chondrocytes, such as fibronectin, collagen II, chondroitin-4-sulfate, chondroitin-6-sulfate, and vimentin, was superior in the rotation bioreactor than when the chondrocytes were cultured in Petri dishes. Duke et al. also used the rotation systems for studies in chondrogenesis with rat embryonic limb cells grown on beads, where they observed cell aggregation and differentiation.[182] However, these studies are under the 3D cell growth and differentiation paradigm and, therefore, do not address the chondrocyte heterogeneity and spatial organization, referring only to construct size and expression of differentiation markers. Microcarriers have been also used with mesenchymal stem cells in the rotation bioreactor for their differentiation to osteoblasts and formation of bone-like aggregates.[183] The feasibility of this process as the second unit operation is further supported by the development of mathematical models that describe the kinetics of aggregation between a single bead and an aggregate, the assembly between two aggregates and the collision between two single beads[184] or the calculation of shear stress on microcarriers.[185]

**4.2.3. Third Unit Operation−Cluster Fusion.** The third unit operation is similar to the second one. However, in this unit operation, the cells are cultured for a longer time, giving larger-sized cell-microcarrier clusters, as the smaller-sized ones of the previous unit operation fuse together via the same mechanism through cell bridges. In such larger clusters, the cells have proceeded further to their differentiation but still not until the final stage of mineralization. Then, from the second and third unit operations, we have two constructs of different size and in different differentiation states (we can use another unit operation to make larger and more-differentiated constructs). Still, no cell organization has started. It is interesting to note that the third unit operation is performed continuously with the second one on the same system. It only requires the removal of some of the cell-aggregate clusters at the end of the second operation and the continuation of the process, with that remaining in the bioreactor forming larger clusters of more-differentiated cells.

**4.2.4. Fourth Unit Operation−Cell Orientation (Cell Organization). 4.2.4.1. Technical Feasibility of Multilayered Hydrogel Bioreactors: Examples of Bioartificial Articular Cartilage.** In this unit operation, we will attempt to provide conditions that will facilitate the cell organization. In the simplest case, we could use hydrogel layers, filling each one with the constructs of the previous operations, and place them on top of each other, with the hydrogel layer having the differentiating cells in an earlier state on the top (see Figure 9, presented later in this paper). Such types of bioreactors already have been used in Tissue Engineering, in nonbiomimetic processes to design the zonal structure of artificial cartilage.[186−189] Articular cartilage is formed by endochondral ossification in the epiphysis (the end of a long bone at its joint with other bones) of long bones (see Figure 8), following developmental events similar to those observed in the elongation of long bones, such as formation of the (secondary) ossification center (Figure 8a), chondrocyte differentiation in it, and hypertrophy of chondrocytes in its center (Figure 8c).

The cartilage of the epiphysis is replaced by bone, leaving only a surface layer of articular cartilage 2−4 mm thick (see Figure 8b). The articular cartilage provides a smooth surface for joint movement and protects the ends of long bones from wear at points of contact with other bones. It also helps to absorb shock and distribute forces. The bioartificial articular cartilage is destined to be used in defects of the articular cartilage of the joints after damage due to trauma or aging-related degeneration, such as that observed in osteoarthritis.[190−192] Although several technical aspects of the in vitro process under the 3D cell growth and differentiation paradigm were examined, such as selection of appropriate mesenchymal cells, scaffold design, the use of growth and differentiation factors to promote the chondrogenic differentiation, the design of bioreactors that impose mechanical loading to promote the condrocyte differentiation, etc., the process of bioartificial articular cartilage still remains under investigation, necessitating further improvement.[193,194] Until recently, no considerations related to the heterogeneity of the differentiating chondrocytes and attempts to design the bioartificial cartilage restoring the chondrocyte interactions have appeared in bioartificial articular cartilage design, with the few exceptions mentioned below. The chondrocytes spread in the scaffold pore network and proceed in the differentiation process without developing spatial relations, and, consequently, no structural organization at the level of the entire scaffold can be established. However, as the growth plate, articular cartilage also is composed of zones with different composition. The special mechanical properties of articular cartilage reflect the different mechanical properties of chondrocytes in its different zones that secrete different extracellular matrix molecules in

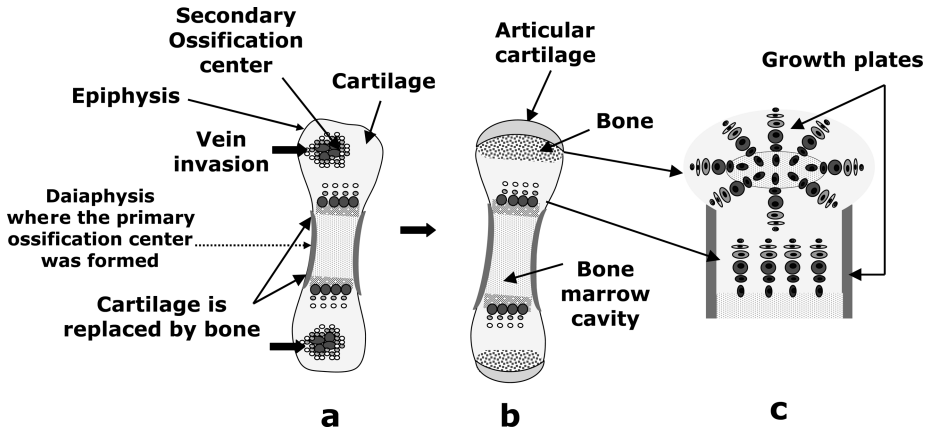


**Figure 8.** The developmental mechanism that is followed for the growth of the bones of the body that participate in joints and bear weight is indirect bone formation through cartilage (endochondral ossification; see Figure 3). The same phenomenon occur in the epiphyses of long bones. They are initially composed of cartilage and, with the vein invasion (panel a), the hypertrophic chondrocytes die and are replaced by bone (panel b), leaving only a surface layer of articular cartilage 2−4 mm thick. The spatial arrangement of chondrocytes at different differentiation stages in the epiphyses is similar to that of the growth pattern formed in the middle of the bone (recall Figures 3 and 4a), as shown in panel c.

each one. Consequently, the tensile strength arising from collagens and compressive stiffness arising from proteoglycal aggrecan are the same as the natural ones only when the zonal structure of the articular cartilage is recapitulated in the bioartificial one. There are four zones of articular cartilage. In the superficial one, the chondocytes produce the protein lubricin; in the zone below that, they produce decorin and type VI collagen; in the deep zone, they produce aggrecan; and the last zone is calcified, containing hypertrophic chondrocytes, producing type X collagen, reminiscent of the pattern of growth plate, since articular cartilage is formed in the secondary ossification centers via the same mechanism of endochondral ossification[195] (see Figure 8). However, Tissue Engineering of articular cartilage, except for the examples mentioned above with the zonal structure,[186−189] is performed in one-stage processes that result in homogeneous tissue that has no resemblance to the native zonal organization of articular cartilage and, therefore, suboptimal mechanical properties, as mentioned in ref 188. Although, in the above study,[188] the authors used only two layers of hydrogels to simulate the superficial and deep zone, this bilayered constructs demonstrated greater shear and compressive strength than homogeneous cell constructs. This study has also showed that interactions between zone-specific chondrocytes affect the biological and mechanical properties of engineered cartilage.[188] The importance of the zonal system that is composed of hydrogels is not the 3D growth and differentiation of the cells, but rather the ability to bring in contact two different cell cultures along the hydrogels interface and the ability to separate the cultures without disturbing the cells. Evidence has been provided that the bilayered hydrogel system for articular cartilage allowed cell signaling along interfaces between cells in different layers (according to Elisseeff in ref 196). Therefore, such a unit operation of multilayered hydrogels is technically feasible, since it has been tried several times with satisfactory results. Especially, the cell signaling that occurs between cells residing in successive layers through the layers interfaces is an important factor for the fourth unit operations, which must restore the exchange of Ihh/PTHrP signals through the hydrogel layers, mimicking the in vivo situation.

**4.2.4.2(a). Mechanical Properties of Bioartificial Tissues under the 3D Cell Growth and Differentiation Paradigm.** Since we spoke previously about the mechanical properties of the bioartificial articular cartilage being most important for this construct to be suitable as an implant, we should mention the general aspect of the mechanical properties of the bioartificial tissues. Although mechanical engineers engaged in Tissue Engineering focus on the mechanical properties of a scaffold as a whole, this may give the impression that they integrate cellular functions to the mechanical properties of the cell-bearing scaffold, to achieve the desired global properties, such as the in vivo mechanical properties. However, as we will show, the situation is not different from the biochemical properties of bioartificial tissues that are examined by trial-and-error methods at the cell level without integration from the cell level of heterogeneous cell functions to the functions at tissue level. In the early years of Tissue Engineering, tissue engineers relied largely on the use of existing materials (according to Ingber in ref 33). Scaffolds had to meet several design criteria: their surfaces had to permit cell adhesion and migration and promote cell growth; they had to be biocompatible without provoking inflammation or toxicity in vivo; they had to be biodegradable and eventually eliminated after implantation; they had to have interconnected pores, to increase the cell number and the sufficiency of nutrients; they had to have an optimal porosity with adequate surface area and mechanical strength; etc.[42] These properties had to be different for different bioartificial tissue. For example, for skeletal tissues, the degradation rate should be slow, to maintain the mechanical strength until tissue regeneration; however, for skin tissue, the bioartificial construct should not remain long, because it may later retard the tissue regeneration, rather than promote it.[42] Consequently, the work of material scientists started, and still continues, with the tailoring of the properties of biomaterials for particular applications modifying their composition and the methods of their synthesis. It was found, for example, that the degradation rate of a block copolymer such as PLGA is affected by the ratio of hydrophilic poly(glycolic acid) (PGA) to hydrophobic poly(lactic acid) (PLA).[197] The degradation rate of poly(D,L)-lactide coglycolide was degreased, making a blend of three copolymers with different viscosities, rendering such a copolymer more suitable for bioartificial cartilage, because of the longer-term retention of their mechanical properties after implantation.[198] However, note that the above group[198] in a recent study used a scaffoldless method in co-cultures of meniscal fibrochondrocytes (MFCs) and articular chondrocytes (ACs).[199] It was found that the tensile modulus was proportional to the percentage of MFCs employed. The 100% MFC group yielded the greatest mechanical stiffness, with 432.2 ± 47 kPa tensile modulus and an ultimate tensile strength of 23.7 ± 2.4 kPa, with the 50% MFC constructs being the most similar to an idealized meniscus shape. When these

polymer constructs were compared to PGA scaffold-based constructs, they were 2−4 times stiffer and were also stronger in tension that the PGA constructs. The authors concluded that co-culturing ACs and MFCs without a scaffold is a promising new method for the Tissue Engineering of fibrocartilaginous tissues, because it exhibits a spectrum of mechanical and biomechanical properties closer to the miniscus. This is consistent with what we have previously mentioned for the prevention of cell organization by the inappropriate use of scaffolds in one-stage processes: the suppression of tissue dynamic development[93] and the new method of "*cell sheet engineering*", in which the 3D construct is formed by successive cell layers. We will show this below.

One of the signs of crisis of a paradigm that signifies that the time has come for its replacement, like the 3D cell growth and differentiation paradigm, is the imbalance between complexity and accuracy.[1] It is true that the complexity of scaffold development has increased enormously over the last two decades. However, this did not lead to the expected improvement of the bioartificial tissues that will make them suitable for clinical applications. This is a sign of crisis that also has been observed in the past. The complexity of calculations of the Ptolemaic astronomy for the predictions of the apparent motion of moon, sun, and planets before its replacement by the Copernican heliocentric system, was increasing more rapidly than its accuracy (as quoted by Kuhn in ref[1] and taken from the work of Dreyer[200]). Similar perceptions for the negative role of scaffolds in Tissue Engineering, in addition to those that we have previously mentioned, were concluded in accordance with the newly proposed biomimetic process paradigm. Herring, in ref 196, suggested that it may not be advisible to use a 3D porous scaffolds for intramembranous bone formation (direct differentiation of mesenchymal stem cells to osteoblasts without passing through the stage of chondrocytes, as in the endochondral bone formation of the growth plate). Instead, a two-dimensional vascular network will be the best choice, since, in vivo, a 3D bone is created by a 2D periosteum, which promotes osteogenesis and angiogenesis,[201] providing an extension of the vascular network in three dimensions. The scaffold, in this case, is a natural one, established by the process itself, as the growth plate is the self-established scaffold for endochondral bone formation. This means that the two processes—vascularization and bone formation—are synchronized, facilitating each other and assuring that vasculature is, at each time, optimal for the size of the bone deposited by osteoblasts as in a biomimetic process of bioartificial growth plate formation the time evolution of tissue variables should be synchronized.

Coming back to the start of Tissue Engineering, we could see that the mechanical properties of scaffolds were the first preoccupation, as well as the first experience of material scientists. The results of the work of developing new scaffolds with new properties are numerous, and such studies continue until now. For example, for a scaffold to provide temporary mechanical strength to the reconstructed bone region, a composite biomaterial based on an unsaturated linear polyester mixed with a monomer that cross-links the double bonds of the unsaturated polyester was designed.[202] It was found that the prepolymer molecular weight, the presence of a leachable salt, and the amount of cross-linking monomer had strong effects on the strength and modulus of the composite. In another study, the photopolymerization of poly(hydroxyethylmethacrylate) (HEMA), in the presence of poly(hydroxybutyrate-co-hydroxyvalerate) (PHBA), increased the modulus of elasticity, the failure stress, and the failure strain.[203] In a recent study, mechanical properties, such as relatively high strength and self-hardening of calcium phosphate cement (CPC)-chitosam composites, were tested and found to be superior to CPC.[204] In another recent study, composites prepared from supramolecular polycaprolactone and UPy-grafted hydroxyapatite (HApUPy) showed increased mechanical properties and, according to the authors, can be easily formed into microporous biomaterials.[205] However, we should mention that the biomaterial synthesis and mechanical properties that are checked provide only one of the tools to Tissue Engineering and, therefore, are more Material Science than Tissue Engineering. The same approaches could be used for any application not related to Tissue Engineering. Although material scientists check these properties at the entire scaffold level, this is not the same as we have discussed in this article. Specifically, until now, we have discussed how the cells communicate to establish tissue functions. In most of the biomaterial studies, cells are not used. Modifications are made in the material components of the scaffold, and the overall mechanical properties are examined in vitro or in vivo.

However, this situation is similar to other Tissue Engineering studies. For example, the overall tissue function of the bioartificial liver, which is blood detoxification of a patient with liver failure, is expressed using various indices related to the cell function under the influence of different components of the device that is used. Such examined design parameters include the following examples: the hepatocytes used;[206,207] the medium composition and the growth factors contained within it;[208−210] the scaffolds from various biomaterials that have different physical properties (such as porosity) and are modified with attached biomolecules;[211−214] issues that are the same for any Tissue Engineering application; and several different bioreactor types (such as hollow fiber, flat-plate bioreactors where the hepatocytes are attached on a surface, bioreactors with the hepatocytes attached on polymeric porous beads or scaffolds, and bioreactors where the hepatocyte spheroids are encapsulated in a polymer material).[215,216] Other studies involving mathematical models have tried to design the medium that optimizes the metabolism of the hepatocytes,[217−219] instead of optimizing the bioartificial liver device performance in a direct way, such as, for example, implementing the hepatocyte zonation for the optimization of metabolic functions.

Therefore, we see the work of material scientists is not different from this of tissue engineers under the 3D cell growth and differentiation paradigm that examine other aspects of the in vitro process. All of them may have had the overall tissue function in mind, either mechanical or chemical; however, the indices that were checked referred to the cell functions, because no appropriate methodology to decipher tissue functions from cells functions was available. Therefore, the important issue to be examined here is how the overall tissue functions arise from the cooperation of a heterogeneous cell population, as in the Ihh/PTHrP negative feedback loop of the growth plate. In a recent study, the mechanical properties of hierarchically structured biological materials were studied with the interactions of components at all scales, from the nanoscale to the bulk scale of the entire material.[220] Although this approach is reminiscent of ours, examining the tissue forms that appear during development and their interactions to ensure compatibility of the conditions moving from the one tissue size scale (first unit operation) to the large one of the bioartificial tissue, the cells were missing from the above-mentioned study; therefore, this work also belongs to Material Science and not to Tissue Engineering, even if the intention is for this biomaterial to be used in Tissue Engineering.

Although material scientists have been preoccupied with the methods of synthesis and the mechanical tests of their materials, they moved gradually to the examination of the cell behavior on scaffolds. For example, while agarose hydrogels with chondrocytes can give constructs with mechanical properties comparable to native cartilage, agarose is immunogenic and nondegradable. Silk hydrogels were then used, giving biochemical and mechanical properties similar to constructs based on agarose.[221] In another case, the surface of polycaprolactone/poly(2-hydroxyethyl methacrylate) (PCL/pHEMA) polymer blends has been modified with bone sialoprotein (BSP), which is an extracellular matrix protein, significantly enhancing osteoblastic cell attachment and spreading.[222] Later, the work of material scientists moved to the influence of scaffolds to stem cell growth and differentiation. For example, in a recent study, embryonic stem cells have been used for the formation of embryoid bodies (EBs).[223] The EB cells then were cultured to form a cell sheet. After the cell differentiation, a continuous sheet of cardiomyocytes with cell beating was obtained. The produced sheets were then sandwiched into the sliced porous scaffold. The seeded cells were redistributed uniformly throughout the scaffold, with a significant increase in mechanical strength, and they could be used as cardiac patches. Since this work starts with embryonic stem cells, the addition of several steps in the differentiation process it is unavoidable, as in our case of biomimetic processes, although not fully in agreement with the information from Developmental Biology, contrary to our work here, which uses considerable information about the developmental stages and implements this in process design. In another recent study, human embryonic stem cells were cultured in scaffolds composed of poly(L-lactic acid) (PLLA) and efficiency-differentiated into osteoblasts, as assessed by the expression of osteocalcin.[224] In another similar study, mouse embryonic stem cells after forming EBs were seeded in a biodegradable polymer scaffold composed of PLLA and PGA.[225] Growth factors were added to induce hepatic differentiation. The 3D differentiated hepatocyte-like cells were able to express several liver-specific markers. Many other studies exist in literature for the differentiation of stem cells inside scaffolds. However, all are conducted under the 3D cell growth and differentiation paradigm. Instead of checking the cell differentiation in the 2D dishes of Biology, similar tests at the cell level are performed by Tissue Engineering in three dimensions. Consequently, no tissue structures appear; instead, only some cell functions with cells randomly distributed in the scaffolds appear.

The above examples clearly show that, although material scientists refer to the overall mechanical properties of the scaffold, these properties are mostly related with the nonviable components of the scaffold: this is something that was the same before Tissue Engineering. When cells are added to scaffolds, the work is restricted to the trial-and-error determination of the effect of scaffolds on the cell properties and not to the integration of cell and biomaterial properties to tissue functions.

**4.2.4.2(b). An Example of Deciphering the Mechanical Properties of the Scaffold as a Whole from the Cell and Scaffold Functions in Combination.** An exceptional work in which the scaffold properties have been connected with the cell properties and these with the tissue properties refers to the closure of adult mammalian skin wounds.[226] This process occurs in vivo via a combination of wound contraction, scar formation, and induced regeneration. Since the body cannot recreate healthy skin or tissue, it assembles new fibers that serve as a protective barrier. When this barrier is completely healed, it is known as a scar. Scar tissue is a connective tissue which is thicker than the surrounding tissue, of limited blood supply and limited functionality, such as movement and sensation. The scar formation requires the wound contraction in a plane tensile stress field in a skin wound. If the wound contraction is prevented by a scaffold, it leads to induced regeneration of the tissue, instead of scar formation. The author found that a relationship exists between the orientation of axis of a myofibroblast, along which mechanical force develops, and the orientation of collagen fibers synthesized by that cell type. During collagen synthesis, the fibers excreted by the cells have their long axes parallel to the long axis of the cell that secretes them. For this reason, in a wound that was contracting, the axes of contractile myofibroblasts, as well as the collagen fibers that they secrete, are oriented in the plane of the wound surface instead of being quasi-randomly oriented, as in physiological dermis. The scar formation then is due to the collagen fiber synthesis in the presence of the tensile stress generated by a wound contraction. However, the orientation of the fibers could be prevented if the tensile mechanical field is canceled in the plane of the wound. The contraction can be blocked by a scaffold. When a scaffold blocks the contraction, the collagen fibers show poor orientation. The contraction is blocked by the reduction of the number of myofibroblasts inside the scaffold placed in the wound and the reduction of the effectiveness of forces generated by myofibroblasts in the wound. In the absence of a scaffold, the myofibroblasts are densely packed in the plane of the wound with their axes in the plane. However, in the presence of a scaffold, myofibroblasts that have migrated inside the scaffold and become attached on the surface of its pores have their long axes oriented randomly, because the attachment of cells to a scaffold is also random in various directions. Myofibroblasts randomly oriented in space, with regard to direction, cannot apply mechanical forces in the plane of the wound, because the assembly of force vectors, which is the sum of forces, is almost zero, leading to the cancellation of wound contraction.

In the above case, the scaffold was designed according to cell functions that are integrated with tissue function, which is the wound regeneration instead of scar tissue formation. The blockage of the contraction by a scaffold is dependent on its ability to bind most of the contractile myofibroblast cells, which requires specific ligands (short amino acid sequences). At a very small pore size, cells are prevented from entering inside the scaffold; at very large pore sizes, the specific surface becomes very low. However, the internal surface of the scaffold determines the density of ligands attached on the scaffold surface, and, therefore, an optimal pore size exists, in the range of 20−120 $\mu$m. Similarly, the degradation rate should be in an optimal value. A high degradation rate prevents the scaffolds from blocking contraction too early, whereas a high rate (beyond termination of the contraction process) interferes with the synthesis of new tissue.

**4.2.4.3. From the Statistical Zonation of Differentiating Chondrocytes to the Pattern of the Growth Plate with the Use of Multilayered Hydrogel Bioreactor.** Returning to the description of the fourth unit operation (Figure 9), whose purpose is to establish the columnar/zonal pattern of growth plate, we could say that the final construct of at least two hydrogel layers filled with the constructs of the second and third unit operations (or more layers in intermediate construct sizes and cell differentiation states, e.g., another one on the top with constructs of the first unit operation, depending on the previous unit operations that provide the cell-microcarrier clusters in different sizes and different cell differentiation states) has

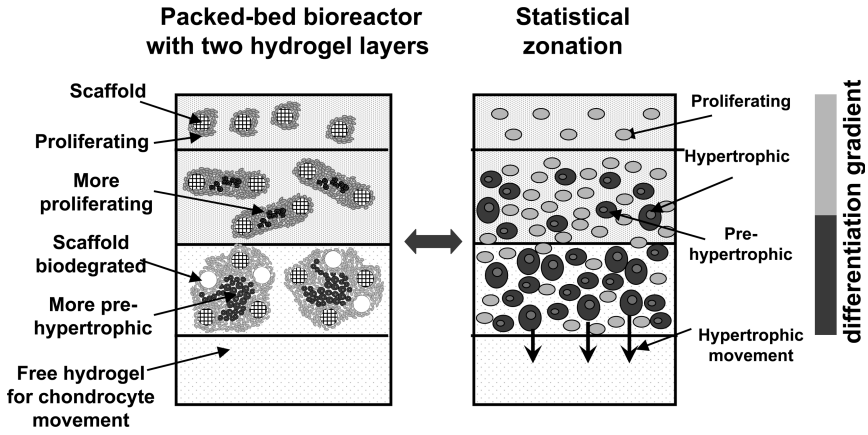


**Figure 9.** Having ensured, from the previous unit operations, constructs with cells in different differentiation states and construct sizes, we can place them successively on top of each other, including them in different hydrogel layers. In this way, we establish a statistical planar organization, since the first layer contains only proliferating cells coming from the first unit operation, the second one more proliferating than prehypertrophic coming from the second unit operation, the third one more prehypertrophic than the second coming from the third unit operation, and the fourth layer is without cells to accommodate the movement of hypertrophic cells. Since the signal exchange of Ihh/PTHrP occurs between these cell types, it will be aligned in one direction along the hydrogels. Therefore, the new proliferating cells that enter their differentiation program will be under the influence of this signaling, aligned, consequently, along the direction of successive hydrogels and forming, in this way, columns. This happens because the construct is dynamic in the sense that it still contains cells that have not yet started their differentiation. In addition, the avoidance of mineralized areas in the third hydrogel layer with the early removal of the constructs from the third unit operation before the chondrocytes start to mineralize their extracellular matrix, as well as the ease of cells to move inside hydrogels designed with the appropriate density, will facilitate them to be aligned along the operating negative feedback loop of Ihh/PTHrP. In this unit operation, a statistical organization of the differentiation states, as well as the facilitation of cell-to-cell communication, can lead to the organization of the construct in columns formed by the new differentiating choondrocytes, as in the real growth plate.

increased the size of the construct. However, and more importantly, it provides a primitive zonal structure (not yet columnar, as in the growth plate) statistically similar to that of the growth plate. The second layer of Figure 9 contains constructs from the second unit operation and has less chondrocytes in an advanced differentiation state (e.g., more proliferating than prehypertrophic or hypertrophic), while the third contains constructs from the third unit operation with more cells in an advanced differentiation state (e.g., more prehypertrophic and hypertrophic cells) than the first layer. We could use another layer at the bottom with less dense hydrogels without cells, to accommodate the increase of the size of hypertrophic chondrocytes and their movement along the zones. Therefore, we could say that the multilayered input of this subprocess is a construct of large size (gradual increase of the tissue size along the process) and with a "statistically organized" structure based on the gradient of differentiation states of the cells from the first hydrogel layer to the second hydrogel layer.

The construct with the four hydrogel layers provides a primitive zonal structure that defines a direction of the cell differentiation progression from one layer to another, instead of a random one in all directions. Exchange of signals will be mostly of Ihh released by the third layer, which contains chondrocytes that are more mature (with regard to their differentiation state), and of PTHrP released from the second one, which has less mature chondrocytes. This certainly does not prevent Ihh and PTHrP from diffusing in various directions inside the layers. However, their sources (mentioned above), as well as their target cells, predominantly reside in different layers: the second one contains more cells that are the target of Ihh (more proliferating chondrocytes), and more cells that are the target of PTHrP (more prehypertrophic cells) reside in the third. Under this statistically one-directional signal exchange, the new chondrocytes that leave the proliferating state (most of them in the first layer), entering their differentiation program, will be under the influence of this signaling and will be aligned along its direction from the first hydrogel layer to the second hydrogel layer, which finally gives a columnar organization of the new differentiating cells. In addition to the not-yet-started-to-differentiate cells of the second and third layers, new cells will enter their differentiation from the first one if the constructs of the first unit operation simply will be packed inside the hydrogel to increase their density between the microcarriers mechanically and reach the density of condensation.

But why has such a statistical organization chances to be transformed to the real one of the growth plate? This is based on the dynamic structure of the growth plate with the continuous cell differentiation, since such dynamic structures are the constructs of the unit operations described above, because they still contain proliferating cells ready to enter their differentiation. Schipani et al., for example, observed a disorganized growth plate in transgenic mice in which a constitutive expression of PTHrP receptors was targeted to the growth plate through a collagen promoter.[227] This means that the receptor of PTHrP was expressed in the differentiating chondrocytes early in their differentiation and, therefore, the signal from Ihh was continuously received, causing the secretion of PTHrP and, consequently, the constant retardation of chondrocyte differentiation. However, when these mice were mated with transgenic mice that did not express PTHrP (contrary to the previously mentioned mice that constitutively express PTH/PTHrP receptors), the resulting animals that had both cell types exhibited an acceleration of chondrocyte differentiation. Most importantly, the generated animals had normal (i.e., organized) growth plates. The critical issue for our discussion is that a mixture of cells with opposite abnormalities—one expressing the receptor constitutively and the other not expressing the ligand, which were randomly distributed in the developing growth plate—cooperated globally in the entire tissue space to balance their effects and cancel each other's deficiencies. This is an example that shows that the partial organization of the growth plate that we have at the beginning of the fourth unit operation can be transformed to a complete one. The new chondrocytes that enter their differentiation, mostly arising from the first and second hydrogel layers, will exhibit different behavior, with respect to their orientation, because they find themselves in a different environment—one of the statistical one-directional Ihh/PTHrP signaling—than that of the cell−microcarrier clusters of the

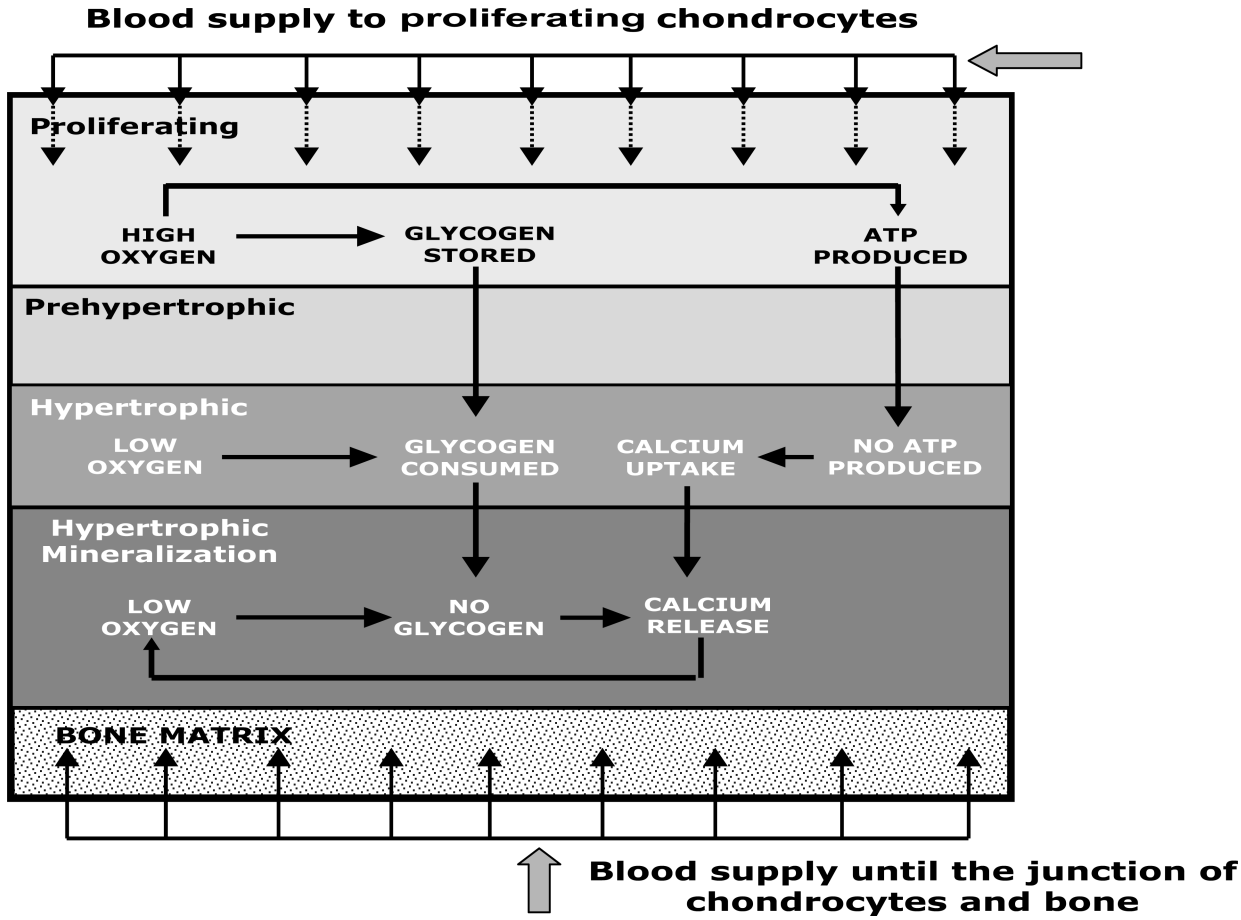


**Figure 10.** Another functional characteristic arising from the structure of the growth plate which at the same time participates in the formation of this structure (these two cannot be distinguished during the development) is the metabolic heterogeneity of chondrocytes of different zones and their spatial cooperation. In the proliferating zone, the oxygen concentration is high, to serve the energy requirements of high cell proliferation. In the hypertrophic zone, the oxygen concentration is low, which results in anaerobic metabolism. The glycogen that the cells had stored in the high oxygen zone of proliferating cells is consumed and, until near the middle of the zone, is depleted. Because of the lack of oxygen, the mitochondria switch from forming ATP to accumulating calcium. However, with the calcium retention, they have lost their energy and there is no way to replenish it in the last part of the hypertrophic zone, because of the lack of oxygen. The mitochondria then release calcium, to save energy. The release of calcium at the end of the hypertrophic zone is an important event that triggers the blood vein invasion and the start of endochondral ossification, which replaces the dying chondrocytes with osteoblasts that produce a bone matrix. Here, we see that the heterogeneity in the microenvironments where the cells reside determines their metabolic properties. These properties are well-integrated in a network that leads to the calcium release at the end of hypertrophic zone. Such a localized release of calcium arising from the metabolic cooperation of the different chondrocyte types in the zones of the growth plate is not a cell but a developing tissue property, as such, is the control of chondrocyte differentiation and growth plate elongation through the negative feedback loop of IHH/PTHrP operating between different chondrocyte zones.

second and third unit operations. The multilayered hydrogel bioreactor of the fourth unit operation provides a direction along which the gradient of PTHrP/Ihh negative feedback loop is established, facilitating the differentiating chondrocytes orientation in columns along this gradient, which finally gives the cell organization.

We could get further information from Developmental Biology to design the fourth unit operation more precisely. For example, the proliferative zone is well-supplied with blood, but none of the branches of these arteries penetrate the cartilage of the growth plate beyond the uppermost part of the proliferative zone[228,229] (see Figure 10) The bone−cartilage junction in the zone of dying hypertrophic chondrocytes is richly supplied by blood but not the upper part of the zone of hypertrophic chondrocytes, which has a low blood supply (see Figure 10). This indicates that tangential flow in the upper part of the first layer and the lower part of the second layer (recall Figure 9) seems to be the most in-vivo-like medium supply.

**4.2.4.4. Spatial Organization of the Metabolic Activities of the Chonrocytes of Growth Plate.** The blood supply mostly at the ends of the growth plate is related to the global metabolic activities of the growth plate. Another cooperative metabolic heterogeneity of chondrocytes of different zones, besides that of the negative feedback loop of Ihh/PTHrP, that we encounter is the case of oxygenation. In the proliferating zone, the oxygen concentration is high, and, as a result of aerobic metabolism, glycogen is stored and mitochondria form ATP, which serves the energy requirements of high cell proliferation.[230] At the hypertrophic zone, the oxygen concentration is low, which results in anaerobic metabolism. Glycogen is consumed and, until near the middle of the zone, is depleted. In the first half of the hypertophic zone, the mitochondria switch from forming ATP to accumulating calcium.[231] However, the retention of calcium is an active process that requires energy[232] that, however, is not available in the last part of the hypertrophic zone, because of the lack of oxygen. Because of the lack of energy when the glycogen supply is exhausted, the mitochondria release calcium to save energy. The release of calcium at the end of the hypertrophic zone is an important event that triggers the blood vein invasion and the start of endochondral ossification, which replaces the dying chondrocytes with osteoblasts that produce a bone matrix.

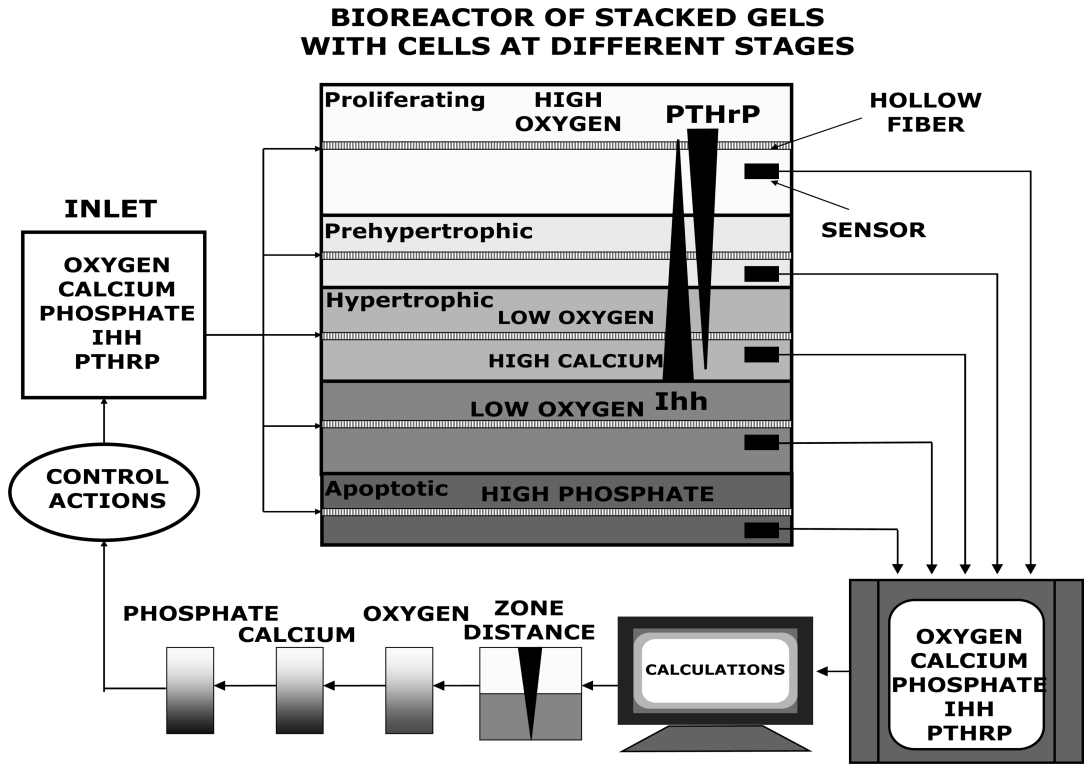


**Figure 11.** Dynamic control strategy for the maintenance of a growth plate structure and function based on online, in situ detection of metabolites and proteins for the estimation of relative position of the zones that directs separate external actions in each one, to ensure that the cells in each zone are provided with the appropriate microenvironment.

Here, we see that the heterogeneity in the microenvironments where the cells reside determines their metabolic properties and their metabolic cooperation but, on the other hand, their metabolic properties determine these microenvironments. These properties are well-integrated in a network of interactions that leads to calcium release at the end of the hypertrophic zone. If the oxygen concentration was not low in the hypertrophic zone, the cells could not release calcium. However, they had accumulated calcium in the upper part of the hypertrophic zone, where they had energy stored in glycogen. Moreover, this energy was stored because of the high oxygen concentration in the zone of proliferating cells. The release of calcium is a cell property, but its release at the right time and position where the ossification occurs is a property of the growth plate that emerges from the metabolic cooperation of chondrocytes along its structure.

**4.2.4.5. A More-Advanced Design of Multilayered Hydrogel Bioreactor for Growth Plate Formation. 4.2.4.5(a). Online Measurements for a Dynamic Control of Growth Plate.** According to the above information for the oxygen gradient in the growth plate, besides using different numbers of biodegradable microcarriers in each layer, so that the tangential flow at the top of the first layer will be appropriately distributed along the layers not reaching the last one washing out the growth factors, we could use other methods. One of these is the use of hollow fibers that penetrate horizontally the different zones. Bioreactors with hollow fibers have been used for a long time in Biochemical Engineering,[233−235] in studies of oxygen or mass-transfer phenomena,[236,237] as well as in Tissue Engineering, especially for bioartificial liver devices,[238] either with cells[239] or cell aggregates.[240] For example, a high oxygen concentration is required in the proliferating zone, where the chondrocytes grow before they start their differentiation, and a low oxygen concentration is required at the end of the hypertrophic zone, which can induce the release of calcium. In a more-advanced design, since the thickness of the hypertrophic zone changes dynamically as new cells divide and differentiate, elongating the columns, the hollow fibers can supply different media at different layers, according to the differentiation stages of the chondrocytes, following the changes of the zones along the vertical direction of the bioreactor. This can occur only if there is information concerning the components of each zone that are related with their movement in the vertical direction. Therefore, biosensors that can provide information about the oxygen and calcium concentration could be placed in each hydrogel layer, with the first one being expected to decrease and the second one being expected to increase when the cells start to become hypertrophic at the last zone. We do not know in detail how the two tissue properties—the control of differentiation of the chondrocytes of the growth plate with the negative feedback loop of Ihh/PTHrP, and the controlled release of calcium in the hypertrophic zone—are connected. If such information would be known, we could use only the online measurements of metabolites to infer for the zonation, instead of measuring the proteins Ihh and PTHrP.

In the example of the artificial structure of stacked gels in the packed-bed bioreactor, we can see how we can ensure the appropriate conditions in each zone and how we can develop a dynamic method to restructure the initial imposed structure to the in vivo patterned one. In Figure 11, we see a future experimental setting for the online and in situ detection of the important metabolites and proteins that could be used for an accurate control of the process, although, as we already stated, the control of the growth plate pattern is internal and, for this, its structure is robust. However, in the case that some information from Developmental Biology is missing in the process design, a combination of internal and external control seems to be suitable. However, we believe that no critical information is missing, because the Developmental Biology of cartilage is well-advanced. Since partial organization to a growth plate pattern

has been even observed in very simple micromass and pellet cultures of mesenchymal stem cells, as we have mentioned,[109] the process presented above composed of four unit operations that have been designed in accordance to the information for the natural growth plate development has chances to achieve the columnar organization of the growth plate, especially when the appropriate advanced tools, bioreactors and biomaterials, are used. The existing tools of Tissue Engineering have been already proposed for studies of Developmental Biology, especially in biomimetic process with co-cultures in multilayered hydrogel bioreactor systems (according to Elisseeff in ref 196).

Although we know that low oxygen is required in the hypertrophic zone and we can supply this through the hollow fiber in this zone, we do not know exactly where the hypertrophic zone is situated, since the thickness of the zones changes along the process. According to the simple version of the negative feedback loop, the expression of Ihh is restricted in the prehypertrophic cells and the protein diffuses up to the zone of proliferating cells where it induces the expression of PTHrP that diffuses downward, preventing the cells from becoming hypertrophic. Therefore, the peak of Ihh marks the prehypertrophic cells and close to this is the lower concentration of PTHrP. The peak of PTHrP marks the proliferating zone. If immunosensors will be used for the detection of these proteins, the relative distance between the zones will be known and the supply of oxygen and calcium can be at the appropriate positions. The detection of proteins from immunosensors has been already used, as for example for the detection of hepatocyte growth factor/scatter factor (HGF/SF) in the serum-containing medium of a bioreactor with hepatocytes.[241] This biosensor consists of antibodies against HGF (anti-HGF) that were immobilized covalently on the biosensor surface. Immunosensors have been also developed for other applications, such as the detection of troponin T secreted by cardiomyocytes during exposure to toxic substances,[242] as well as for the detection of cardiac troponin T and fatty acid binding protein 3 as toxicity markers in cardiomyocytes derived from human embryonic stem cells,[242] for the detection of cortisol,[243] for detection of interleukine 6,[244] etc. We expect that the immunosensors will be soon available to assist in the establishment and maintenance of the negative feedback loop of a bioartificial growth plate process. In the meantime, the determination of the position of different zones can be done indirectly through the online measurements of metabolites such as oxygen. From the oxygen measurements, the oxygen consumption can be calculated, giving an indication for the proliferating activity in different positions along the layers. Off-line examination of Ihh and PTHrP expression could be correlated with the oxygen consumption, and the relative position of zones could be then determined.

Other considerations could be taken into account, according to information provided by Developmental Biology. If, for example, a fast increase of the hypertrophic zone will be detected, although, in vivo, it could be retarded with the negative signal of PTHrP, the timing is different in vitro, because the proteins diffuse into an artificial material and we can make use of the system design, introducing an amount of PTHrP. Other proteins that interfere with the negative feedback loop also could be introduced. Since it is easier to introduce proteins in the bioreactor instead of removing them, if a removal of PTHrP is required, such as in case the differentiation is slow, another protein, such as BMP-2, that has opposite effect of PTHrP and even is dominant can be introduced.[245]

**4.2.4.5(b). The Robustness of Developmental Tissue Forms is Reflected in the Robustness of Their Mathematical Models.** All the available measurements can be introduced in a mathematical/computational model that can reconstruct the zonal pattern of the developing growth plate and indicate control actions for its restoration to physiological limits, as in the case mentioned in the previous section (see Figure 11). Such "control software" already has been used in Biochemical Engineering in bioreactors for mammalian cell cultures. For example, a mathematical model describing the dynamics of hybridoma growth and monoclonal antibody production in suspension cultures was developed, using the oxygen consumption rate of the cell culture as input.[246] Oxygen was monitored online and was used as the only measurement information to provide estimates for living and dead cell densities and the medium composition. Another control software was used in perfusion cultures of hybridoma.[247] Using the measurement of a single component in the culture medium (in this case, glucose), the software provided an evaluation of the time variation of the concentrations of living and dead cells, of glutamine and antibodies. (For a review on software control, see the comments in ref 248.) Generally, the use of software control is due to the limited hardware sensor measurements of the variables that describe the process dynamics. Specifically, for our subject of growth plate control, mathematical models have also appeared in the literature and can be further developed and modified for the particular experimental settings. Such a model for the interaction of two key signaling molecules, Indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP), has been developed.[249] The model is a steady-state linear approximation to a reaction-diffusion system where only diffusion and absorption mechanisms are considered. The authors predicted the robustness of the growth plate. For example, the size of the proliferative zone in the growth plate is rather insensitive to variations in the flux coefficients for Ihh and PTHrP. The model also showed that its solution is also insensitive—and therefore robust—to large changes in the value of the PTHrP concentration, which marks the transition from proliferative zones to hypertrophic zones, irrespective of the particular diffusivities of Ihh and PTHrP. In another model, the authors modeled the differentiation and growth of chondrocytes from the proliferation zone to the hypertrophic zone with a reaction−diffusion regulatory loop between Ihh and PTHrP.[250] The proliferation and hypertrophy of the chondrocytes are considered to be directionally dependent, modeling, in this way, the column formation.

Additional data related to known physical and kinetic properties of the growth plate in vivo and additional aspects of chondrocyte biology can simplify the process by restricting the mathematical analysis to a reasonable physiological range for the parameter values of the model, even if we generally know its robustness to parameter changes. Examples of such data include the following: diffusion coefficients;[251] solute transport rates;[252] volume increase of hypertrophic chondrocytes;[98] cell proliferation rates;[98] cell growth by cellular division, matrix synthesis throughout the growth plate, and chondrocytic enlargement during hypertrophy;[253,254] and other relevant information from the literature. In addition, the expression of many genes along the zones of the growth plate has been already been determined accurately.[255,256] Thus, we have sufficient data from in vivo Developmental Biology to construct a mathematical model for online prediction of the thickness of the zones, based on the measurements of biosensors.

However, because of the robustness of the growth plate, as any other developmental module, it is expected that the development of a mathematical model for the negative feedback loop and the metabolic functions should not be difficult in the sense of the amount of information that should be included. A particularly instructive example of a developmental module is the segmentation appearing during the development of the fruit fly, *Drosophila*. Modularity, as a global property of the spatially extended biological system, arises from the way a limited number of genes influences the expression of another limited number of genes, forming a complex network of strong mutual interactions that is only weakly connected to the rest of the genes. The gene expression network of one cell extends its action to other cells, influencing their gene expression and activating various signaling pathways through secreted proteins. In turn, these cells respond to the first, activating its signaling pathways in a specific way, so that finally the gene expression is stabilized. Just as the developmental module is robust macroscopically, the same robustness is exhibited by the gene and protein interaction network. It operates autonomously without interference from other genes or proteins (as the Ihh/PTHrP negative feedback loop also is autonomous) and keeps the interactions of its components continuously active, stabilizing the spatially differentiated gene expression pattern. In this example, we can see the development of insects. The various parts of the body of insects develop on particular segments, i.e., layers of cells that appear during embryogenesis. The genes expressed in each segment specify the correct number of body parts and the correct polarity of each one. In *Drosophila*, a complex network of gene interactions converts a single-celled *Drosophila* egg to a multicellular embryo with 14 segments, forming a spatial pattern of parallel stripes with each segment bounded by a stripe of cells, expressing different genes. Von Dassow et al. have taken the step of modeling the activated signaling pathways and relevant genes of the interaction between cells of the different segments,[257] which can be considered as the different zones of the growth plate. The authors developed a mathematical model of 136 ordinary differential equations (ODEs) for the core network, comprised of five genes, (en, wg, ptc (patched), cid; (*cubitus interruptus*) and hh), and their proteins. The equations described the time evolution of the concentration of mRNA, protein, or protein complexes, and have terms for synthesis, decay, transformation, and transport. The model was surprisingly robust and able to predict the correct pattern of segments over a large range of parameters and initial conditions. It was primarily the topology of the organization of the gene interacting network (which gene interacts with what else) that provided the stability of the model and not the details of molecular interactions. Von Dassow and Odell characterized this network as a module, "a device unto itself", because it can accomplish the task of maintaining the spatial gene expression pattern "without any persistent, extrinsic spatial or temporal biases on any of its components".[258]

In conclusion, we could say that the above system, with online, in situ measurements of metabolites and proteins, together with the mathematical/computational estimation of the zones of the growth plate, establishes a coordinated communication of the cells, well-informed from Developmental Biology, with a dynamic in vitro environment facilitating the establishment of cellular interactions that lead to cell organization and finally to tissue properties. Therefore, we could speak for dynamic organization of cell interactions during the culture, instead of only expecting the organization to occur, ensuring the appropriate initial conditions. In this system, if something is not appropriate from the developmental point of view (e.g., very slow differentiation), it can be corrected with external actions without the need to restart the experiment. This does not mean "forcing" the system but rather changing slightly accordingly and differently each time, and, in small steps, so that the system will be brought in the best conditions to follow its inherent time evolution toward a mature organized tissue. Therefore, it is a pseudo-dimension that is introduced with the online, in situ measurements and control of bioreactor that facilitates the steps of the natural and inherent tissue development. The introduction of a pseudo-dimension makes it clear that this is not a magic pot system but a system whose critical part is the design of this pseudo-dimension (i.e., the external interference to the internal dynamics of the construct that must be designed and applied online through a sophisticated control algorithm that respects the cell communication). This system has the ability for the introduction of other proteins in later stages to interfere with the basic negative feedback loop and, with the help of a mathematical model, the protein measurements can be coupled with the diffusion rates and the control actions become feed-forward, anticipating the predicted outcomes. This is a sophisticated culture system that, although not compared with the sophistication of the human body, where the process of growth plate formation occurs, it can be successfully used for in vitro growth plate formation, because it includes sufficient critical information from Developmental Biology and the possibility to interfere with the tissue development in a way to correct and facilitate, instead to direct, any inappropriate (in terms of in vivo development) conditions that arise because of the use of artificial tools, such as scaffolds and bioreactors.

**4.2.5. The Importance of the Growth Plate Developmental Module as a Building Block of Other Bioartificial Tissue and the Feasibility of Such Processes.** Although the above approach may seem overly complex, we should consider that the modular intermediate tissue forms are particularly important in Tissue Engineering, because they are the building blocks of complex tissues. In the case of the growth plate, after establishment of the columnar pattern, we could combine the fourth unit operation with other unit operations that they generate osteoblasts. Osteoblasts can now be generated from embryonic stem cells. For example, a suspension bioreactor seems to be an efficient way to generate osteoblasts from embryonic stem cells.[259] They also can be generated with the formation of embryonic cell bodies that subsequently are cultured with osteoinductive media.[260] In another way, they can be generated from mesenchymal stem cells, as was the situation before the use of embryonic stem cells, such as mesencymal stem cells generated for human adipose tissue in the rotation bioreactor attached to scaffolds that was proven as a superior method, compared to static cultures in 2D dishes,[261] or from mesenchymal stem cells isolated from adult human bone marrow in gelatin-hyaluronic acid scaffolds in a spinner or rotating bioreactor,[262] or from human mesenchymal stem cells attached in nonwoven poly(ethylene terepthalate) (PET) fibrous scaffolds in a perfusion bioreactor,[263] or with bone mesenchymal stem cells attached on gelatin microcarriers (Cultispher S).[264,265] The objective of using scaffolds in the above studies is the generation of bioartificial bone. For our purpose of establishing a biomimetic process for bioartificial bone formation, the co-culture of the bioartificial growth plate with osteoblasts, one method that can be used to multiply the osteoblasts after the differentiation of stem cells, is to culture them either on

microcarriers such as collagen microcarriers in spinner culture systems, in which the expanded osteoblasts isolated from human trabecular bone can be recovered by collagenase treatment of the aggregates of microcarriers,[266] as in the second and third unit operations previously mentioned for the culture of chondrocytes, or in nonporous microcarriers in a rotation bioreactor, where the cells are recovered by trypsine or collagenase.[267] Depending on the specific designed process, we could get bioartificial bone or bioartificial osteochondral tissue (as we will see below) that is used for deep defects of articular cartilage that penetrate the subchondral layer, reaching the cancellous bone.[268] We could also use the bioartificial growth plate as the natural scaffold for bone fracture repairs that, in some cases, follows the mechanism of endochondral ossification.[269−271] By introducing in the bioartificial bone generated from the growth plate osteoclasts generated by another unit operation, we could restore the function of bone remodelling (osteoclasts and osteoblasts work closely together in time and space to remove old bone locally and deposit new bone, respectively), to test for bone resorption before the bone is implanted.[272] It was also suggested that the introduction of osteoclasts in the bioartificial bone can enhance its mechanical strength, because only osteoblasts are unable to form lamellar bone-like structures and deposit the characteristic mineral of native bone.[273]

**4.2.6. Other Methods of Gradual Increase of Bioartificial Tissue Size.** Conclusively, we can say that the above-described simplified process of four unit operations has been designed considering, as the most important factor, the concerted evolution of the three variables along the process, trying to adjust the rate of chondrocyte differentiation to construct size, which is the only way to achieve the cell organization, as it happens in vivo preserving these cell-to-cell communications that are responsible for cell organization. For this, we have selected the appropriate information from the studies of Developmental Biology to optimize each unit operation, as well as optimize and match the needs of developmental processes with the technical factors of biomaterial and bioreactor systems.

The gradual increase of bioartificial tissue size has been already introduced in Tissue Engineering methods, in the context of restoring the cell-to-cell communication rather than to imitate natural developmental processes explicitly. These methods provide evidence of feasibility of the example of growth plate process that we presented and also provide initial examples of further technical approaches that could be used to implement gradual increase of tissue size in biomimetic processes. One of these methods that shows divergence from the methods of one-stage processes dictated by the 3D cell growth and differentiation paradigm are methods-based 2D constructs in multiple stages, such as the "cell sheet engineering", which is 3D construction from 2D constructs. This method even has been used for tissues that do not have a planar geometry, such as liver[274] or bone.[275] The cell sheet engineering method uses cell culture surfaces coated with a temperature-responsive polymer. When cells reach confluence, they can be detached as a cell sheet by reducing the temperature. The temperature reduction makes the culture surface hydrophilic and, therefore, non-cell-adhesive. By layering the cell sheets, 3D cellular constructs can be formed. This method has been used to fabricate 3D bioartificial liver constructs that preserve the cell-to-cell contacts and thus promote persistent survivability, as the authors mentioned.[274] It also has been used with layered cardiomyocyte sheets for the reconstruction of myocardial tissue.[275] As the authors claimed, their method allowed the formation of gap junctions between cells in the different layers, which is indispensable for the properly organized intercellular electrical communication that, with their method, lead to synchronized pulsation.[276] This approach, which is conceptually similar to that which we have previously discussed for the gradual increase of the tissue size, ensuring the basic biological phenomena in each unit operation, serves the same goal: a functionally organized 3D bioartificial construct that exhibits global properties at the tissue level (e.g., synchronized pulsation) or control of chondrocyte differentiation in the growth plate (which also is a global property). Another recently developed method of cell sheet engineering, "Magnetic Force-Based Tissue Engineering" (Mag-TE), enhances cell-to-cell adhesion in the vertical direction.[277] This method decreases cell adhesion on the surface of plates through the use of ultralow-attachment plates. At the same time, it increases the adhesion between cells bearing magnetic nanoparticles by application of a magnetic field. The cell sheets can be detached and delivered with the help of a magnet placed above the sheet. This method has been used to provide sheets of several tissues as well as heterotypic layers composed of different cell types.[278−280] As the authors mentioned,[280] a bilayered co-culture system composed of one layer of hepatocytes and another layer of endothelial cells enhanced the albumin secretion by hepatocytes, compared to monotypic cultures of hepatocytes. It can also provide tubular structures when the cells are attracted around a cylindrical magnet.[281] When a cylindrical magnet was rolled onto the cell sheet, the cell sheet that was attracted to the magnet formed a tube around it. When the magnet was removed, a tubular structure was formed. The authors used urinary tissue, which consisted of a monotypic urothelial cell layer, and vascular tissue, which consisted of heterotypic layers of endothelial cells, smooth muscle cells, and fibroblasts.

The controlled formation of folded cell sheets offers the possibility of recapitulating in vitro basic mechanism of folding and refolding happening in the early stages of in vivo development, such as, for example, the formation of germ layers during gastrulation (the early phase of development during which the embryo is reorganized to form the three germ layers: ectoderm, mesoderm, and endoderm, from which individual organs develop) germ layer transformation (e.g., tube formation in endoderm, formation of the neural folds and neural tube) and establishment of interactions between the germ layers (e.g., organ-specific bud formation in the endodermal tube). Furthermore, the issue of tissue, if taken with the mathematical meaning, shows that the concept of 3D cell growth and differentiation is an oversimplification, because the dimension of the tissues, if they are examined as fractals (structures composed of units and these, in turn, of subunits and so on, with its sub...unit resembling the entire structure), as they are, is less than three, because they are composed on folded sheets (i.e., 2D objects that are folded in the 3D space)[282] and, therefore, the method of cell sheet engineering is more appropriate for tissue formation. With the sheets and tubular structures, we have the basic topological operations "stretching" and "folding" that are responsible for the creation of chaotic dynamics and fractal structures of chaotic attractors (see the "Smale horseshoe map" in ref 283). Therefore, it is expected that further advancement of this method would be critical in implementing the fractal geometry in bioartificial tissue.

**4.2.7. Cell Self-Organization to Tissue Structures in a Biomimetic Process Has a Chance To Happen Only in the Proper Environments That Do Not Restrict the Cells from Operating In Vivo.** Examples of in vitro experiments

from the literature have already shown how such an interdependence of biological and physical factors influence the cell-to-cell signaling that controls morphogenetic processes, so that it could be able to either (i) establish a morphogenetic patterns if cells are allowed to define, by themselves, the space where they are distributed or (ii) prevent it from happening if the cells are restricted in a noncompatible space, as is the case of a scaffold of fixed size. Quintana et al. have worked on studying the in vitro conditions that can lead to an early embryonic pattern of mouse embryonic fibroblasts inside scaffolds.[284] The authors showed that, when the cells were embedded in a soft scaffold, such as the self-assembling peptide RAD16-I, which had the form of a disk, they were able to contract. The cell contraction was facilitated by the ability of the scaffold to contract. The resulting short distances between cells allowed the exchange of signals that lead to a morphogenetic process (as in the case of condensation of mesenchymal stem cells that induces the chondrogenic differentiation) that transformed the shape of the scaffold, which followed the cell contraction, from the form of a disk to a symmetrically bilateral structure with a distinct central axis such as that obtained in vivo. According to the authors' observations, when cell proliferation was prevented with staurosporine treatment, no such bilateral structure was formed, because the number of cells was not sufficient for the initial size of the scaffold to produce the required signals in sufficient quantity for the induction of the cell contraction process. In addition, the cells acquired chondrogenic potential in that scaffold while this did not happen in an agarose scaffold, which has poor contracting capacity, to allow the cell contraction so that distances among cells remained large causing the dissipation of their signaling that induces chondrogenesis.

What we actually see in these experiments is that a cell condensation could initiate either a morphogenetic process or chondrogenic differentiation without any externally imposed conditions. As the authors have mentioned, the scaffolds were noninstructive (i.e., they did not contain any specific peptide to induce the expression of any signal by the cells). The self-initiated developmental phenomena were induced and came to completion only if cell activities were not prevented, either by preventing cell proliferation with staurosporine or by preventing cell condensation in a scaffold with no contracting ability. This is another example that shows that the perception for the role of the scaffold in the biomimetic process paradigm is different from that of the previous paradigm; it is not that much to guide the cells than to allow the cells to follow their natural program, as we have also seen in the suggestion of Herring in ref 196 for the avoidance of scaffolds in intramembraneous bone formation in a biomimetic process, since the scaffold could be self-made progressively from the interaction between a 2D vascular network and bone in a way to optimally serve the nutrition and oxygenation of the bone cells as the vascular network grows together with the bone growth.

The work of Quintana et al.[284] is a simple and elegant example that provides evidence that it is only when the cells are allowed to define, by themselves, their proper distances, either with their growth in a scaffold or with their contraction (or both), the cell distances are gradually adjusted synchronized with their signaling capacity that also changes at the single cell and cell population level, (cell proliferation). The authors called this phenomenon "self-organization", with this cell self-organization however occurring only in the proper environment that permits the cells to operate normally, according to their natural abilities.

**4.2.8. Vascularization with Intermediates Assembly.** There are further examples of recent literature of Tissue Engineering that show that the developed methods, until now, are consistent with the needs of biomimetic processes, such as the division of the entire process in subprocess, the gradual increase of the tissue size, or the use of intermediates; therefore, their design should make use of them. Although vascularization is not a problem for the bioartificial growth plate, which is an avascular tissue, except at the ends (see Figure 10), it is a critical problem for other bioartificial tissue. Although not related to developmental processes, such difficult problems have been recently addressed with new methods combining different cell types-intermediates in multistage processes. Kelm et al.[285] produced primary human myofibroblasts (HMF) in spheroids in the first subprocess and coated them in a second subprocess with human umbilical vein endothelial (HUVEC) cells. In a third subprocess they encapsulated the coated HMF spheroids in an agarose mold. The authors observed that the microtissues were assembled into a coherent macrotissue inside the agarose, developing a dense network of endothelial cells throughout the entire macrotissue mass. The implantation of the prevascularized macrotissue into chicken embryos showed the development of a vascular system across the macrotissue/embryo interface, while nonvascularized macrotissue implants were rejected. Similarly, McGuigan et al.[286] have coated collagen cylinders that contain HepG2 cells with HUVEC cells and assembled the cylinders in a larger tube, which developed interconnected channels through which blood or medium could be perfused. It is interesting to mention that, here, we also have a gradual increase of the tissue size by assembling intermediates, which leads to an organization of the tissue structure, with regard to vascularization.

The following works present further examples of assembling intermediates in multistage in vitro processes. Tsuda et al., using the method of cell sheet engineering, fabricated prevascularized bioartificial tissues using multilayered cell constructs with layers of micropatterned endothelial cells between layers of fibroblasts produced in different subprocesses.[287] Kaihara et al. used micromachining technologies on silicon and Pyrex surfaces to generate vascular systems with branched architecture of vascular and capillary networks.[288] The authors cultured hepatocytes and endothelial cells as single-cell monolayers on these 2D molds and they folded them into compact 3D vascularized bioartificial tissue in another subsequent subprocess.

**4.2.9. Considerations in Assembling Biomimetic Processes from Unit Operations.** As we see in Figure 12, we can explore several alternative routes to make a bioartificial growth plate from the one indicated with arrows that has been described previously. In all such routes, we assemble the process from unit operations that involve different culture systems for the different developmental stages, and we must examine the advantages and disadvantages of each one. An important factor besides the suitability of a culture system for a particular stage is the ability to be easily connected with the next developmental stage−unit operation. For example, we could observe cell aggregation−condensation and the onset of chondrogenic differentiation in the packed-bed bioreactor with cells on microcarriers that can form clusters. However, the isolation of clusters of uniform properties is not possible in such cases, because there is just one large cluster that we must break mechanically to smaller ones. In such cases, we lose the uniform differentiation state of small clusters. If these randomly heterogeneous after-the-break clusters will be used in the next unit operation, the chondrocyte differentiation will continue in each cluster from a different point (the one that had at the end of the previous

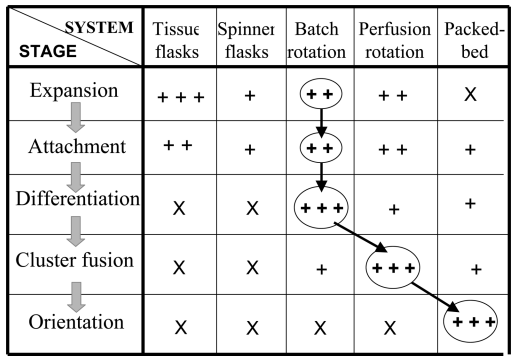


**Figure 12.** The design of a biomimetic process starts with the correspondence between the successive stages of the development and the available bioreactor-biometerial systems. Although several systems could be suitable for particular developmental stages, they may provide constructs that could cause problems in choosing the system for the subsequent unit operation. For example, a packed-bed bioreactor, although it could not be used for cell expansion that requires that the cells are in the same environment that could be better provided in a cell suspension system, can be used for the initiation of chondrocyte differentiation and cluster fusion. However, it will provide a large cluster that must be broken mechanically, therefore losing the homogeneity of the construct properties such as differentiation state and construct size. For the successful continuation of the biomimetic process for growth plate development, we followed the batch, perfusion rotation bioreactor, and, for the last unit operation, the multilayered hydrogel bioreactor. In this way, we ensure uniform properties of the constructs in the rotation bioreactor without the need to treat them mechanically but directly introduce them as inputs to the subsequent unit operation. For the final stage, which requires planar geometry and directional cell-to-cell communication, we must use the multilayered hydrogel bioreactor. However, no further unit operations are needed and, therefore, the construct will remain in this system until its final cell organization. The last unit operation offers also the advantage that, with the achieved organization of the growth plate, the hypertrophic and apoptotic zones appear at one end of the growth plate (or the last hydrogel layer). We then can continue, in the same system, another operation, adding osteoblasts that come from another unit operation for bone formation to occur, replacing the dying chondrocytes.

unit operation) and the final clusters after the fusion of smaller ones will have heterogeneous areas, in terms of differentiation and sizes that would not be related with the differentiation such as those observed in Figure 7. However, in the fourth unit operation, we must embed, in each hydrogel layer, clusters similar in differentiation and size and clusters in less or more advanced differentiation and size in different layers, so that we could restore a statistical one-directional signaling of Ihh/PTHrP. Therefore, it seems that a packed-bed bioreactor can be used for the first, second, and third unit operations but the clusters after that cannot be used in any subsequent unit operation for the statistical zonation.

Other considerations may involve the ease in operations in the scale of size of bioartificial tissue suitable for implantation and even in the scale of large-scale production of growth plates that are needed for bone formation in an industrial scale. For example, cell expansion (cell multiplication with retention of the mesenchymal stem cell ability to differentiate to chondrocytes) can be achieved in tissue flasks, as it normally occurs in biological experiments. However, the high number of flasks and the manipulations required to separate the cells from the surface of flasks, combined with an unavoidable cell damage from the separation procedure, to proceed to the next unit operation, renders this culture system unreliable. An expansion, however, in the rotation bioreactor with cells attached to microcarriers does not require the separation of cells from the microcarriers for the continuation of the process. The constructs of each unit operation are just transferred to the next one, simulating the continuous and undisturbed transformation of tissues during the in vivo developmental process.

## 5. Practical Steps in the Design of a Biomimetic Process

The biomimetic methodology in Tissue Engineering that has been clarified in this article has, as a purpose, the concerted during the tissue development time evolution of cell differentiation, construct size increase, and cell organization (see Figure 13) with the appropriate choices of unit operations that best serve each developmental stage (as described in Developmental Biology) but also make the appropriate choice for the succession of unit operations, to ensure that the three tissue variables previously mentioned advance gradually and simultaneously and reach their final state at the last unit operation.

Several steps should be followed in the design of biomimetic process, with the most important of them described below.

**(1) Define what bioartificial tissue you want to make in scientific terms (i.e., what tissue properties you want your constructs to exhibit).** For example, bioartificial cartilage does not mean anything unless you specify the tissue (and not cell) properties that your constructs must have. In the example presented above, we wanted the bioartificial cartilage to exhibit the function of the growth plate, which is the autonomous, dynamic, continuous, and self-controlled differentiation of chondrocytes in columns.

**(2) Define why you need the bioartificial construct to have these properties.** A bioartificial growth plate with the aforementioned property constitutes the basic unit or "natural scaffold" for endochondral ossification, in the sense that it creates the appropriate environment for the differentiation of osteoblasts and the deposition of bone matrix. Therefore, it could be used in vivo for the formation of osteochondral tissue or bone.

**(3) Check the literature to determine, if these properties are known, how they arise at the molecular level.** If it is not at least qualitatively known how the desired properties arise, you have no criteria to check to determine if the process of in vitro formation is appropriate but only at the end that does not give any information on how you could modify it to take what you have defined as bioartificial construct with tissue properties. For the growth plate, the property of autonomous, dynamic, continuous, and self-controlled differentiation of chondrocytes, to a first approximation, can be attributed to the negative feedback loop of Ihh/PTHrP that determines the rate of chondrocyte differentiation and the size of the growth plate. The appropriate spatial distribution of these proteins then constitutes a measurable criterion for the evaluation of the bioartificial growth plate and the process modification.

**(4) Find evidence that the in vitro process of this tissue formation is feasible.** If this evidence does not exist, you take a great risk, because the system will not exhibit the robustness that is required for the use of engineering systems for the purpose of the simultaneous advancement of cell differentiation and organization and construct size. You will be able to work only with in vitro systems that biologists, after repeated efforts, have found suitable; but they consider usually only one variable, differentiation, or rarely two, including initial stages of cell organization, and never three including size, which is the critical variable that distinguishes Tissue Engineering from Biology, because it is only at the appropriate size that the tissue properties appear, besides the fact that it has a size that is appropriate for implantation. This evidence ranges from scientific (e.g., autonomy of the tissue to maintain its structure) to technical (compatibility of

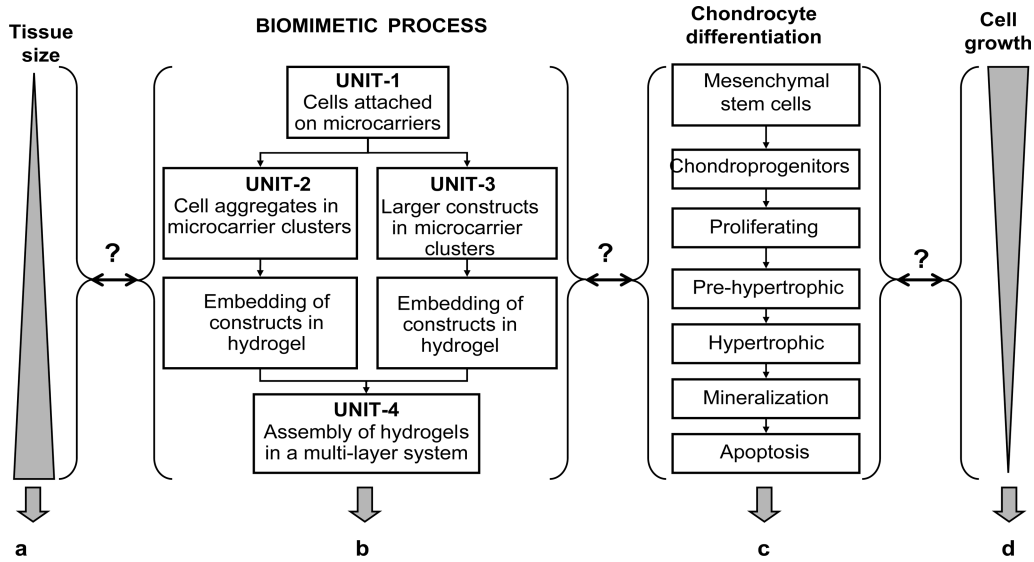


**Figure 13.** The purpose of a biomimetic process design is to synchronize the advancement of the three variable of developing tissue (cell differentiation, construct size, and cell organization), following the developmental stages as described in Developmental Biology. This requires specific choices to be made in the design of unit operations and the bioreactor−biomaterial systems used. In the described example of a biomimetic process for the formation of growth plate, the chosen unit operations correspond to increasingly advanced cell differentiation states as well as construct sizes. For example, mineralization and apoptosis that "freeze" the construct structure are performed in the last unit operation under conditions that facilitate the one-directional Ihh/PTHrP signal exchange between successively placed hydrogel layers that have cells in different differentiation states and are able to either release Ihh or PTHrP that, because of the lack of mineralized areas, are free to move inside the hydrogel and align their direction along the one-directional signal exchange, thereby forming columns. No such consideration of compatibility of biological (signal exchange) and spatial and mechanical factors occurs in the nonbiomimetic process of the 3D cell growth and differentiation paradigm that does not take information from Developmental Biology in the design of one-stage processes into account. Such processes are irrelevant to the developmental stages, and they do not provide the appropriate conditions for the cell organization to occur. Therefore, it seems that Tissue Engineering and Developmental Biology must be combined in one field, "Developmental Engineering", following the methodology of the synchronized progression of the three variables of developing tissue, and establish conditions in each unit operations that do not contradict those of the in vivo tissue development, as Chemical Engineering has done to avoid the incompatibility of inter-related mechanical and chemical factors in its unit operations, making use of the appropriate information from Physics, Physical Chemistry, and Chemistry.

technological tools with the conditions needed in developmental stages). If you want bioartificial bone with tissue functions, you should know that bone and bone marrow constitute the functional unit, and, therefore, any effort to make bioartificial bone only with osteoblasts is destined to end in a mass of semiosteocytes expressing some differentiation markers but unable to exhibit the bone properties. However, if osteoclasts are also used, the bioartificial bone has the remodelling properties and similar mechanical strength as real bone.

**(5) Find technological alternatives where it seems that there would be a problem of feasibility.** No tissue is completely autonomous in its development in vivo, but its formation in vitro requires that the complete autonomy is restored with external actions. If, for example, the negative feedback loop requires a signal coming from another tissue that exists in vivo close to the tissue you want to make in vitro, this signal must be provided technically (such as, for example, with the use of microparticles that are releasing the missing factor at the right time).

**(6) Define what cells you need to start to have a bioartificial construct with the specified properties.** For example, you cannot have bioartificial bone with remodelling capacity, which is a tissue property, starting only with mesenchymal stem cells, you need osteoclasts from bone marrow (i.e., you must restore in vitro the functional unit bone−bone marrow). However, you can have a functional bioartificial growth plate, with all its different chondrocytes starting only with mesenchymal stem cells, because the mesenchymal stem cells chondrogenic differentiation can give all the chondrocytes of the growth plate (resting, proliferating, prehypertrophic, hypertrophic, apopototic) and it is only the interaction among these chondrocytes that results in the growth plate properties.

**(7) Define the series of in vitro processes that can lead from the tissue with the higher semiautonomy to the more-complex tissue of less semiautonomy and the properties of constructs in the intermediate steps.** The process complexity (bioreactors−biomaterials−cells−growth factors etc.) is increasing gradually from the bioartificial growth plate to the bioartificial osteochondral tissue as the tissue self-control of its structure is decreasing. The way the process advancement must be performed is determined by the sequence of events during the in vivo tissue formation that can ensure that the first in vitro process not only makes the first bioartificial construct of high autonomy but also provides the base for the next process for the formation of the bioartificial construct of less autonomy that will occur in the tissue microenvironment provided by the first. If this order is not followed and you go directly to make the tissue with the less autonomy (e.g., bone instead of cartilage) in vitro, you must recreate the microenvironment required in an artificial way, by adding or removing growth factors by trial-and-error, because the complete list of factors required is not yet provided from Biology; however, it could be provided by the hypertrophic chondrocytes of the bioartificial growth plate.

**(8) Define the series of subprocesses, unit operations, in which construct transformations (cell growth, cell differentiation, cell, organization, increase of the construct size) can be performed gradually and synchronously, according to Developmental Biology and technological capabilities (bioreactors−biomaterials).** You must examine the development of cartilage in vivo, define the stages, and then take the bioreactor−biomaterial system that you need to simulate this process in vitro. For example, the two stages that can clearly be distinguished—the mesenchymal cell condensation (which induces their chondrogenic differentia-

tion) and the invasion of blood vessels (which separates the central part of the condensation into two growth plates moving horizontally in opposite directions)—require a bioreactor−biomaterial system that supports spherical geometry (rotation systems) and one that supports planar geometry (packed-bed bioreactors). However, there are no technological systems able to provide, with no further considerations, the optimum condensation size or the optimum chondrocyte zonation in the growth plate. It is here that you must consider the technological limitations, as well as opportunities, and define the stages of the in vitro cartilage development that are "versions" of the in vivo stages, according to the technology available. Therefore, you end up studying the cell aggregation, the aggregate fusion, the nodules fusion, the cell orientation, etc. This is actually what makes the in vitro Developmental Biology Developmental Engineering, not the straightforward transfer of in vivo phenomena to in vitro systems, and this is what requires process design instead of "magic ingredients".

**(9) Implement the maximum possible (according to the available technology) unit operations for the series of subprocesses, as have been defined in the previous step (8), and do this at different levels of sophistication or complexity, to ensure that you can have a bioartificial construct already from the first level that you can further improve.** To be certain that you will be able to incorporate the concepts of Developmental Biology in the technology of bioreactors−biomaterials, you must do it in a gradual way, starting with the simplest concepts, such as differentiation stages, then move to the space distribution of the differentiated cells and then to the biological characterization of various entities that appear during the process (e.g., cell-microcarrier clusters) and determine from their space distribution their functional relations exerted on the spatial distributed cells. For example, is the Ihh/PTHrP negative feedback loop operating with the spatial distribution of two types of cell−microcaarrier clusters in two hydrogels (Figure 9)? Or do these clusters have cells in very similar differentiation stages, so that no communication can occur between them to restore the negative feedback loop? There are numerous technological requirements that must be fulfilled to secure the appearance of in vivo phenomena in vitro, and this can be only ensured if there is a broad base of unit operations that can be evaluated and modified each time you move to more-detailed phenomena, as described previously. The more restricted the technological base, the sooner you meet an end point of technical and biological incompatibility. For example, a rotation bioreactor with microcarriers is able to provide, in the third unit operation, constructs of gradually advanced differentiation stage and size, so that the most suitable differentiation stage for which the clusters can communicate with those of the second unit operation (less differentiated chondrocytes) can be found. With a stirred vessel, on the other hand, only small-sized constructs can be taken, and this contradicts the concept of the gradual and synchronized advancement of the developing tissue variables in a biomimetic process.

**(10) Synthesize the entire process from the unit operations.**

**(11) Design tests to check if the bioartificial tissue functionality has been established.** If the tissue functionality is not established at the end of the process, you must distinguish of this is due to scientific or technical problems (e.g., is the tissue structure not autonomous or are the hydrogel properties, alone or together with other technical factors, not suitable for the cell-to-cell communication to occur). In such cases, an external control will help to restore the tissue autonomy (see Figure 11) referring either to factors inside the tissue that are not distributed well (e.g., oxygen in Figure 11) or in growth and differentiation factors that the developing tissue receive from the surrounding co-developing tissues. In the case that the factors that are needed are unknown in Developmental Biology, another biomimetic way should be searched. For example, if the purpose of the process is to produce beta-pancreatic cells, a co-culture of mesodermal cells with endodermal ones allows for the induction of pancreatic development through instructive signals from mesodermal to endodermal cells[289] circumventing the fact that these signals have not been yet identified. Until recently, signals from the mesoderm were considered permissive rather than instructive (i.e., the endodermal cells of the gut were already prepatterned to become pancreatic). Kumar et al.[289] provided evidence that the signals of the mesoderm for the initiation of the development of the pancreas are instructive and able to induce the expression of the pancreatic genes, even in endodermal positions that normally give rise to other organs. This type of information from Developmental Biology greatly facilitates a biomimetic process design. The difference between permissive and instructive signals is critical in process design, because instructive signals offer direct control over the process from the initial stage of the differentiation onward. In other words, making use of instructive signals would ensure correct control over the entire process. After induction is complete, the hydrogel layer with the mesodermal cells must be removed, because the bone morphogenetic proteins (BMPs) that are produced by these cells, although needed in the beginning, later lead to differentiation toward the liver.[290] The co-culture could continue in another unit operation by replacing the mesodermal cell layer with another layer of endothelial cells, which provides a different set of distinct signals to specify a pancreatic fate.[291] Even the case where two different developing tissues must provide signals to another one simultaneously can be accommodated in a multilayered bioreactor system in a sandwich configuration, where the recipient tissue is placed in the middle layer, such as, for example, that observed with a layer of endodermal cells between a layer of mesodermal and a layer of endothelial cells if the timing of signaling by these two tissues is not known. The above information for the restoration of the cell-to-cell communication between mesodermal and endodermal cells for the induction of the pancreatic differentiation argues in favor of feasibility in designing such a biomimetic process. In addition, feasibility in generating mesodermal and endodermal cells also exists.[120]

## 6. Conclusion

Tissue Engineering has been initiated as a distinct field with the three-dimensional (3D) cell growth and differentiation paradigm. The research practice under this paradigm has never unified the involved fields to develop a rational methodology for the design of in vitro processes. Instead, an Edisonian method was followed, with each field solving, in a case-by-case way, practical problems inside its domain of research. Such a method, however, despite the efforts, failed to optimize the bioartificial tissues to make them suitable for clinical applications, because no process design to make physical and biological factors compatible as in vivo was involved, one-stage processes that made it difficult to handle the inter-relation factors were used and optimization criteria were used at the cell instead at the tissue level. As is

expected with all paradigms, at some point after the rapid accumulation of knowledge on specific phenomena dictated by them, they contradict their basic concepts with the data that are generated. The Tissue Engineering research community has detected the aspects in which this paradigm failed, as mentioned above, and it has proposed a new one: that of process biomimetics (i.e., recapitulating in vitro the in vivo process of tissue development). We presented some practical elements of the biomimetic process paradigm to clearify how in vivo developmental processes could be transferred to biomimetic in vitro processes and how information from Developmental Biology should be used in the biomimetic process design. We roughly described a methodology for in vitro process design from sequential unit operations that correspond to in vivo developmental stages that follow a gradual and concerted progression of tissue growth and cell differentiation, leading to the organization of cells into tissue structures, making the physical (space) and biological (cell-to-cell-communication) factors compatible in each unit operation. Despite the fact that this design does not rely on principles such as those provided by Physics or Chemistry, the empirical concepts of Developmental Biology, such as the existence of successive stages with gradual transformation of intermediate tissue forms the dependence of any developmental stage from the previous one, which provides optimal conditions for the process continuation (e.g., osteoblast recruitment and differentiation are dependent on the hypertrophic chondrocytes of the previous developmental stage of growth plate formation), the robustness of final tissue structures that is due to intrinsic factors and, therefore, do not need external control (the negative feedback loop of Ihh/PThrP, which is intrinsic to growth plate), etc., can substitute for this luck. The resulting biomimetic processes are completely different from those designed under the 3D cell growth and differentiation paradigm and certainly rational in terms of developmental events occurring in the in vitro tissue development. The work presented here methodologically substantiates the paradigm of biomimetic processes and shows the importance of the intellectual core of Chemical Engineering in biomimetic process design in Tissue Engineering. We thereby hope that we have narrowed the "deep chasm" that Ingber mentioned in ref 33 for understanding the principles of tissue development in engineering terms.

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