A new electro-mechanical bioreactor for soft tissue engineering

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ABSTRACT: By enabling the maintenance of controlled chemical and physical environmental conditions, bioreactors proved that electro-mechanical stimulation improves tissue development in vitro, especially in the case of tissues which are subjected to stimuli during embryogenesis and growth (ie skeletal and cardiac muscle tissue). However, most of the bioreactors developed in the last 20 yrs, designed to suit specific applications, lack versatility. With the aim to provide researchers with a yielding, versatile tool, we designed and realized in this study an electro-mechanical stimulator capable of dynamically culturing four biological constructs, delivering assignable stretching and electrical stimulation patterns. The device has been conceived to be easy to handle and customizable for different applications, while ensuring sterility along with stimuli delivery. The gripping equipment, modular and adaptable to scaffolds of different consistencies, is provided with dedicated tools for supporting sample insertion into the culture chamber performed under a laminar flow hood. As to performance, a wide range of electro-mechanical stimulation patterns and their relative occurrence can be accomplished, permitting the adjustment of the dynamic culture parameters both to the specific cell species and to the developmental phase of the cultured cells. (Journal of Applied Biomaterials & Biomechanics 2007; 5:)

KEY WORDS: Bioreactor design, Dynamic culture, Soft tissue development, Mechanical stimulation, Electrical stimulation, Muscle tissue engineering

INTRODUCTION

Bioreactors are generally defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental conditions (eg, pH, temperature, pressure, nutrient supply and waste removal). Such devices have traditionally been used in industrial fermentation processing, in food processing and pharmaceutical production. Due to the high degree of reproducibility, control and automation of bioreactor-based processes, in the last 20 yrs bioreactors have been extensively used in the field of in vitro tissue engineering, where they play a key role in several procedures (1).

Bioreactors have been widely used to optimize cell seeding procedures (2, 3) or to maintain the viability of 3D constructs by perfusion or diffusion systems (4, 5). Moreover, they have been classically employed as mechanical and electrical stimulators of differentiated or differentiating cells previously cultured in static conditions on 3D matrices (scaffolds). Through the use of bioreactors, it was possible to prove that physical factors improve and accelerate tissue development and maturation in vitro, especially in the case of tissues which are subjected to physical (mechanical, electrical, fluid dynamical) stimuli during embryogenesis and growth. An example, pulsatile shear stress was found to increase endothelial cell proliferation (6), while dynamic loading or shear stress of chondrocytes embedded in a 3D environment was proved to stimulate GAG synthesis (7-9) and increase the mechanical properties of the resulting tissue (9, 10). Furthermore, cyclic mechanical stretch was found to increase tissue organization and the expression of elastin by smooth muscle cells (SMCs) seeded in polymeric scaffolds (11). Pulsatile radial stimulation of tubular scaffolds seeded with SMCs improved structural organization and suture retention of engineered blood vessels (12). Regarding electrical stimulation, neural gene expression promotion by the activation of calcium channels was observed as a result of the application of physiological electrical patterns to primary sensory neurons (13).
One of the most promising applications of bioreactors, however, emerged in the field of musculoskeletal and cardiac tissue engineering, with the aim to improve biological construct organization and maturation. Based on the finding that stretching plays a key role in muscle tissue development (14, 15), the effect of mechanical stretching on cultured muscle cells has been investigated in several studies employing bioreactors. Bach et al (16) demonstrated that applied mechanical forces are crucial aspects to the in vitro development of differentiated functional muscle tissue, while direct mechanical tension influenced the organization of myoblasts (muscle cells precursors) into functional aligned myotubes, and provided a stimulus for the expression of mature isoforms of myofibrillar proteins such as myosin heavy chain (MHC) (17). Powell et al (18) also reported that mechanical stimulation improves the structure of the engineered skeletal muscle tissue by increasing the mean myofiber diameter and their elasticity. Moreover, cyclic mechanical stretch was found to enhance proliferation and matrix organization by human heart cells seeded on gelatin-matrix scaffolds (19).

Another approach towards the development of differentiated and functional muscle tissue is the application of electrical stimulation (20-25). Mimicking nerve stimulation during myogenesis and during regeneration of injured muscle, induced contractile activity of mammalian skeletal muscle cells in bioreactors was shown to enhance the development of functional sarcomeric architectures (20). Furthermore, it was observed that chronic electrical stimulation promotes myotube differentiation, while primary myoblast MHC expression is modulated depending on different impulse patterns (22, 25). Promising results of electrical stimulation were also found in the field of cardiac muscle tissue engineering: electrical stimulation of neonatal rat ventricular myocytes, in particular, induced cell alignment and coupling, increased the amplitude of synchronous contractions and resulted in a remarkable level of ultrastructural organization (24).

Several bioreactors have been designed and realized to study the effect of electrical and mechanical stimulation on 3D biological constructs. These devices demonstrated the importance of dynamic culture for functional mature soft tissue development, but at the same time were demonstrated to suffer from some important limitations. Since they were purposely designed to suit a specific application, both with reference to the sample holders (26, 27) and to stimulation patterns (20, 25) the lack of versatility of the devices, in terms of suitability to different biological constructs, represents the major flaw of those bioreactors. Most of the systems are not suitable for the contemporaneous delivery of mechanical and electrical stimulation (25) and, even when this is feasible, the traction-contraction temporal occurrence cannot be planned and controlled (20). The set up procedures are often not thought through in order to be fast and easy or planned taking care of good laboratory practice (GLP) rules (26, 27).

With the aim of addressing the concerns raised above, an innovative highly technological device capable of dynamically culturing 3D engineered constructs of different origins, delivering stretching patterns and/or electrical stimuli, has been developed. The bioreactor was designed to be easily used by the operators, and to satisfy all the security requests of a biological laboratory. The sterility maintenance of the culture was specifically taken into consideration during the device conception. In this article, a detailed description of the device, the electro-tensile bioreactor itself and its performances are reported; a typical example of its possible use in musculoskeletal tissue engineering is also described.

**MATERIALS AND METHODS**

**DEVICE REQUIREMENTS**

The general functions of the bioreactor are the control and the delivery of accurate and reproducible stretching and/or electrical stimulation patterns, and the generation of a biochemical environment suitable for growth and differentiation of several cell phenotypes.

In order to achieve this aim, the first design principle is high versatility both of the actuators and of the stimulation patterns, so that the device can be employed with several biological constructs and for different applications. It is also valuable to assure full compatibility with GLP procedures. Deriving from these general design criteria, the most significant constraints and requirements are the following:

- Cytocompatibility of all the materials in contact with culture medium.
- Corrosion-resistance of the materials in contact with culture medium.
- Ease of sterilization and sterility maintenance.
- Ease of use (assembly in sterile conditions under a laminar flow hood, medium exchange, cleaning, use for non-trained staff).
- Small dimensions, suitable for positioning in a cell culture incubator.
- No medium stagnation during exchange operations.
- Housing of an experimentally significant number of specimens.
- Visual inspection possibility.
DEVICE DESCRIPTION

We designed and realized a bioreactor for dynamic culture of engineered soft tissue constructs. This device can deliver an arbitrary electro-mechanical stimulation under closely monitored and tightly controlled environmental conditions.

System overview

Key constitutive elements of the bioreactor are:
- A culture chamber: a sterile area where cells, seeded on the polymeric scaffold, and culture medium will be housed during the entire duration of the experiments.
- A mechanical stimulation subsystem that provides predefined stretching stimuli to the biological constructs.
- An electrical stimulation subsystem that provides predefined electrical stimuli to the biological constructs.
- A system controller that manages the entire experiment.

Figure 1 schematically depicts the system. During the experiment, the culture chamber, housing the gripping system, the electrodes and the stepping motor are positioned in the incubator. The electrical stimulator and the controller are positioned outside to protect them from the high humidity that characterizes the internal atmosphere of the incubator.

Figure 2 shows the control system components and their mutual connections: the controller, based on a Programmable Logic Controller (PLC), is equipped with an external position module. The latter controls a driver that provides current signals to the stepping motor. The PLC also delivers trigger signals to the electrical stimulator that is programmed by a PC using dedicated software.

Culture chamber

The transparent culture chamber (Fig. 3a), which represents the sterile and cytocompatible environment where cell cultures take place, was obtained through material removal by a micrometrical controlled cutter from a Plexiglas® bulk piece. The inner dimensions are approximately 100*90*60 mm. The rounded edges were conceived to avoid stagnation points and no inlet, discontinuities, fissures, interstices, holes, which are preferable targets for microbial contamination, should be present. In the chamber, two groups of four grips, each of them screwed onto a crossbar, are positioned symmetrically to hold four biological constructs. To allow the mechanical stimulation of the samples, one crossbar is
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fastened to the culture chamber inner wall, while the other is screwed to a couple of drive shafts connected to the stepping motor. A couple of dynamic diaphragms (Fig. 3b) enwrap each drive shaft assuring sterility and allowing motion transmission. An AISI 316 rank provides a fastening structure for the stepping motor and for four toggle latches, constituting at the same time two symmetrical handling points. The chamber is equipped with two lids: one used during mechanical stimulation and the other when electro-mechanical stimuli have to be delivered. The electro-mechanical lid is equipped with a rank to house eight electrodes providing the electrical stimulation to the biological constructs. A D type connector (Fig. 3c), protected by a silicon sheath and by an O-ring, assures electrical connections and was specifically designed to guarantee sterility, allowing at the same time electrical stimulus delivery. The lids can be kept in position by toggle latches, while sterile gas exchange is possible through two high efficiency particulate air filters (HEPA) that are positioned on two holes on the lid. These two inlets can also serve as valuable access for medium exchange procedures and for specific growth factor insertion during the experiment.

**Mechanical stimulation subsystem**

The mechanical stimulation subsystem is composed of a stepping motor driving two drive shafts screwed onto a crossbar that fixes four mobile grips. The dynamic diaphragms that enwrap each drive shaft assuring sterility permit an 8 mm maximum mono-axial dislocation. The micrometrical stepping motor (DRL28PB1G-03D, LIMO series, Oriental Motor, Torrance, Ca, USA), developing a maximum holding force of 30 N, has a 2 µm resolution single step, with a maximum acceleration of 0.2 m/s² and a maximum speed of 24 mm/s.

Four pairs of grips were designed (Fig. 4) to satisfy fully both the holding and the preservation of the integrity of the specimen during the experiment. The grip bodies were obtained from Plexiglas®, while the mobile component was manufactured from an AISI 316 plate. The grasping system is based on a compression spring that pulls the mobile component against the grip body assuring a controlled holding pressure (10⁵ Pa). This pressure is transferred uniformly to the biological constructs, avoiding load concentration points due to two silicon sheets, positioned on the mobile component and on the Plexiglas®. The characteristics of both the spring and the silicon sheet can be customized to adapt the grasping system to the consistency of different biological constructs.

**Electrical stimulation subsystem**

The electrical stimulation subunit is composed of a programmable electrical stimulator (STG 2004 Multi Channel Systems, MCS GmbH, Reutlingen, Germany) and eight transmission lines (four pairs of twisted isolated wires) that deliver the electrical signal to four pairs of platinum electrodes.

The stimulator can be programmed with different patterns by specific software (MC_Stimulus II, Multi Channel Systems, MCS GmbH, Reutlingen, Germany) and then triggered with 5 V Transistor-Transistor Logic (TTL) signals, but it is also supplied with four synchronization outputs that could be set to provide signals to other devices. The electrical stimulation outputs can be both current- or voltage-controlled and a maximum 8 V or a 3.2 mA signal can be delivered with 2 mV or 200 nA amplitude resolution and with 4 V/µs or 100 µA/µs slope time response.
The electrodes (Fig. 5a) were obtained by joining a platinum Hoffman electrode to a copper wire and by protecting the welding both mechanically and chemically with a neutral glass tube. The platinum-glass weld is a perfect medium and air tight. With the aim to simplify the connection-disconnection procedure, an electrical socket was mounted on the other extremity of the copper wire. The electrode insertion in the culture chamber is obtained simply by superimposing the lid (Fig. 5b) to the chamber.

**Controller**

The controller is based on a PLC (226 CPU, SIMATIC S7-200 series, Siemens AG, Munich Germany) that leads the experiment. It controls the mechanical stimulation pattern and the occurrence of trigger events for electrical stimulation based on parameters previously set through a keypad with a panel. The same panel also permits the visualization of a summary of the experiment in terms of duration and step of the stimulation pattern reached.

The control on the electrical stimulator is via four different PLC outputs that are connected by four different BNC cables to the electrical stimulator trigger input. Through single signals, the PLC controls the starting or the stopping of the electrical stimulation patterns.

In addition, the PLC is equipped with a position module (EM 253, SIMATIC S7-200 series, Siemens AG, Munich, Germany) that controls a five-phase driver (CSD5807N-T, Oriental Motor, Torrance, Ca, USA) sending the correct current signal to the stepping motor.
RESULTS

DEVICE PERFORMANCES

Our electro-mechanical stimulator is a versatile system that could be easily used with many soft tissues to induce growth and development of tendons, ligaments, skeletal and cardiac muscles in all the biological laboratories in accordance with GLPs. All the materials are highly cytocompatible and corrosion resistant, and the different components can be sterilized with an ethylene-oxide-based process before setting up the experiment. During the operations preceding sterilization, the culture chamber, the grips and some support tools, consisting of a Teflon® mounting table and a couple of crossbars fixing the distance between the grips (Fig. 6a), are assembled and arranged in order to simplify and minimize the number of set up operations under a laminar flow hood after sterilization. The set up procedure can subsequently be performed under a laminar flow hood in accordance with the GLP rules and maintaining the sterility of the culture chamber. Due to the support tools and to the preliminary device organization, the operations that need to be conducted under laminar flow are just two: (a) specimens grasping on the grips (Fig. 6b); and (b) their insertion with a single motion in the culture chamber (Fig. 6c-f); both of them can be easily performed maintaining the sterility of the culture chamber and of the biological constructs. As is evident from Figure 6, the specimen gripping procedure is conducted in an upside-down configuration as compared with the holding position in the culture chamber. The sterility of the culture chamber is guaranteed during the entire experiment and all the holes (air inlets, mechanical and electrical stimulation passages) are protected with highly efficient air tight systems.

The controller leads the experiment based on the settings of an actual program that manages both the stretching and the electrical stimulation (Fig. 7). It is also possible to set and save 10 different mechanical stimulation patterns and 10 different trigger settings that can be recalled and loaded as an actual program in several mixed culture recipes. The controller settings, both of the actual and of the preset programs, are also simple and easy to use for non-trained staff and can be managed to have a contemporaneous or alternative mechanical and electrical stimulation.

On the mechanical side, the device provides the possibility to set (a) unidirectional traction with different tensile rates, (b) cyclic traction and relaxation with arbitrary amplitudes (never exceeding the 8 mm maximum dislocation imposed by the dynamic diaphragms) and frequencies, and (c) resting periods.
On the electrical side, the electrical stimulator delivers composed patterns using simple (sine, square, ramp) or complex waveforms that can be set due to user friendly software. It is also possible to reproduce physiological waveforms using clinical data collected. As the electrical stimulator is triggered by the controller, in the case of a contemporaneous delivery of stretching and electrical stimulation, it is possible to plan the relative occurrence of traction and contraction, determining the exact loading pattern for the biological constructs. The synchronization outputs on the electrical stimulator could be used to control secondary devices such as chemical sensors or image detectors that should be introduced during future updates of the device.

Due to the absence of inlets, sharp edges or contact junctions that could act as medium stagnation points, medium exchange under a laminar flow hood is extremely easy. After stopping the controller, the culture chamber is transferred from the incubator to the laminar flow hood and the exchange is performed as follows: (a) culture chamber lid opening, (b) medium removal with a pipette-aid, (c) fresh medium insertion, and (d) lid superimposition. After replacing the culture chamber in the incubator, the tissue-conditioning program is restarted.

DISCUSSION

As previously reported, skeletal and cardiac muscle progenitor cells react to specific physical stimuli. It is proved that stretching and contraction play a key role in muscle tissue development in vivo (14, 15) and, miming the embryogenetic process, mechanical force and induced active contraction seem to be an important aspect to the in vitro development of differentiated functional muscle tissue (16, 20).

By enabling reproducible and controlled mechanical and electrical conditioning of engineered constructs, bioreactors contribute to improve cell spatial organization and extra cellular matrix (ECM) deposition (1, 19) and to enhance the development of functional architectures (20, 24); therefore, allowing the development of structurally and functionally optimized engineered tissues.

In our opinion, the devices designed previously have some important drawbacks: in suitability to different biological constructs (26, 27), in electrical and mechanical pattern design versatility (20) and in accordance with GLP rules (26, 27).

Our bioreactor was designed with the aim to overcome these limitations in order to provide a leading edge powerful device capable of mechanically and/or electrically conditioning 3D engineered constructs of different origins. The main target of our work was to realize a bioreactor that could be used with several biological constructs testing their response to different stimulation patterns. In order to reach this goal, our device was designed to be customizable to the specific application. It can be used in tendon and ligament in vitro tissue engineering by using only mechanical functionalities, and in cardiac and skeletal muscle development by making use of electro-mechanical functionalities. In particular, the grasping pressure can be easily regulated by changing the compression spring in the grip while the load distribution is regulated using silicon sheets. We could assert that the grip design permits the matching of the grasping system to the specific biological construct permitting a proper holding pressure both on several synthetic polymers (classical degradable polyesters, polyetherurethanes etc) and on biologic polymers (derived from hyaluronic acid, collagen or fibrin, for example). Once the specimens are housed in the culture chamber, the operator can freely deliver mechanical and electrical stimulation patterns in a contemporaneous or alternative mode mixing different mechanical patterns with several electrical stimuli. The mechanical pattern is created choosing among or composing (a) unidirectional traction at different rates, (b) cyclic traction and relaxation with tunable amplitudes and frequencies, and (c) rest periods to let the cells recover from the previously induced stress. The electrical pattern can be designed selecting in a wide range of simple (sine, square, ramp) or complex waveforms and mixing them arbitrarily. Reproducing physiological waveforms using clinical collected data is also feasible due to common text files. In the case of a contemporaneous delivery of mechanical and electrical stimulation, the relative occurrence of traction and contraction can be easily

Fig. 7 - An example of electro-mechanical stimulation program. The actual program is composed of a stretching stimulation pattern and of some trigger events that control the electrical stimulator. Both of them are widely adjustable.
planned to induce contractile cells into a concentric, isometric or eccentric training. All these features provide the possibility of generating a wide range of pattern combinations permitting the smooth adjustment of the dynamic culture parameters to the specific cell species, to the specific tissue but also to the specific developmental phase of cultured cells. This opportunity is crucial, especially considering that murine and human skeletal muscle are not subjected to the same traction rate during bone growth, and that skeletal muscle does not experience the same contraction pattern as heart muscles do and a fetal muscle cannot face the same loads as an adult muscle.

Once all tissue-conditioning parameters are chosen and the start signal is delivered, the bioreactor is completely autonomous and highly reliable. Typical stimulation programs of 4 or 5 weeks could be executed simply without any external intervention with the only exception of medium exchange. Regarding the compatibility with common cell culture procedures, several expedients have been used to assure sterility inside the culture chamber during the experiment. Once the device is sterilized, a very simple and fast procedure under a laminar flow hood, consisting of only two operations (mounting and insertion), permits a sterile and GLP insertion of the specimens. During cell culture, gas exchange and stimuli delivery are protected by a simple and useful mechanical tight system preventing chamber contamination.

With the aim to verify the potential and performance of the device, the bioreactor was tested conducting a preliminary experiment of dynamic culture in the field of skeletal muscle tissue engineering. Based on previous studies (28) regarding a promising polyesterurethane scaffold (DegraPol®), line myoblasts (C2C12, murine precursors of skeletal muscle cells) were seeded on DegraPol® elastic microfibrous membranes and statically cultured for 4 days in complete growth medium (10% FBS supplemented DMEM). The samples, four identical strips 50*5*0.2 mm in dimensions, were subsequently transferred to the bioreactor and cultured further for 10 days experiencing a stretching pattern adapted from the literature (29). Medium exchange took place every third day. Samples were withdrawn at days 7, 10 and 14 after seeding and evaluated for the degree of cell differentiation via myosin immunostaining, using statically cultured specimens as controls. Throughout the duration of the experiment, the bioreactor was revealed to be a reliable, autonomous and safe device; in particular, the grasping equipment being fully compatible with common laboratory procedures, sample insertion was quickly and easily performed in sterile conditions under a laminar flow hood. The culture chamber, which proved to be extremely comfortable in handling including during medium exchange operations, showed no sign of microbial contamination; air inlets provided the necessary oxygen supply to cultured cells and none of the components was shown to release ethylene oxide after sterilization. During stimulation, the device was capable of applying a precise and repeatable predefined stretching pattern to the samples, allowing a preliminary investigation of the effect of cyclic mechanical load on skeletal muscle cell differentiation. As to this aspect, the applied pattern was demonstrated to allow fusion and differentiation processes of murine myoblasts on DegraPol®, already at day 7.

![Micrograph of C2C12 differentiating on DegraPol microfibrous membranes after 3 days of dynamic culture. Following 4 days of culture in static conditions, the constructs were transferred into the bioreactor and subjected to a stimulation pattern adapted from the literature. Green staining (a-b) represents MHC, a marker of myogenic terminal differentiation, while blue (b) indicates cell nuclei stained with DAPI. The presence of long, multinucleated, unbranched differentiated myotubes is detectable.](image)
after seeding, mechanically stimulated constructs showed parallel arrays of multinucleated unbranched myotubes expressing MHC (a marker of skeletal terminal differentiation). Of particular interest proved to be the myotube length, which reached and even exceeded 700 µm (Fig. 8).

Since our bioreactor still suffers from some limitations, future updates are now under consideration. A force transducer system to measure the applied load and the active forces expressed by the biological constructs is the first target. Moreover, a wide range of control of the chemical parameters and an imaging system will be considered in future developments of the device. The data collected would permit the characterization of the constructs and the supervision of the environment enabling more controlled and reproducible cell cultures. In order to optimize the culture to the specific cell response, the data collected would also be used to implement a closed loop control of the experimental parameters. In addition, an automatic medium conditioning and exchange system will be considered with the aim of reducing contamination risks and preserving the homeostasis. Finally, a device housing a larger number of specimens divided into two different medium chambers is under consideration to study the effect of biochemical factors in a physically identical environment.

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