A bioreactor with an electro-responsive elastomeric membrane for mimicking intestinal peristalsis

Daniele Cei[1], Giulia Gori[1], Joana Costa[1], Gabriele Frediani[2], Claudio Domenici[3], Federico Carpi[2], Arti Ahluwalia[1*]

[1] Research Center "E.Piaggio" and Department of Information Engineering, University of Pisa, Largo L Lazzarino, 56126 Pisa, Italy

[2] Queen Mary University of London, School of Engineering & Materials Science, Mile End Road, London E1 4NS, UK

[3] CNR Institute of Clinical Physiology, Via G. Moruzzi 1, Pisa 56124, Italy

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ACCEPTED VERSION

ABSTRACT

This study describes an actuated bioreactor which mimics the pulsatile contractile motion of the intestinal barrier using electro-responsive elastomers as smart materials that undergo deformation upon electrical stimulation. The device consists of an annular dielectric elastomer actuator working as a radial artificial muscle able to rhythmically contract and relax a central cell culture well. The bioreactor maintained up to 4 hours of actuation at a frequency of 0.15 Hz and a strain of 8-10%, which are analogues to those of the cyclic contraction and relaxation of the small intestine. In-vitro tests demonstrated that the device was biocompatible and cell-adhesive for Caco-2 cells, which formed a confluent monolayer following 21 days of culture in the central well. In addition, cellular adhesion and cohesion were maintained after 4 hours of continuous cyclic strain. These preliminary results encourage further investigations on the use of dielectric elastomer actuation as a versatile technology that might overcome the limitations of commercially available pneumatic driving systems to obtain bioreactors that can cyclically deform cell cultures in a biomimetic fashion.

Keywords: dielectric elastomer actuator, bioreactor, in-vitro model, intestinal contraction, artificial muscle

INTRODUCTION

In-vitro models are replicates of the structure and function of biological tissues which allow for modelling and predicting physiological responses to a variety of stimuli. Although traditional static models and conventional substrates for cell culture (plastic flasks, dishes, plates, wells or cover slips) offer ease of use and have low cost, they are not fully representative of in-vivo responses, as they do not mimic key aspects of the biological environment. Indeed, there is currently great interest in developing biomimetic in-vitro models which better recapitulate the complexity of the biological milieu as alternatives to in-vivo experiments. In particular, significant efforts are focused on attempts to mimic the dynamics of the cellular environment [1–7]. Within the body, cells are continuously exposed to dynamic stimuli, such as flow and motion. Cells sense the mechanical signals and convert them into biochemical responses, through mechano-transduction**.** A dynamic environment is in fact crucial for many processes such as cell growth, alignment, proliferation, remodelling, migration, differentiation, gene expression, as well as for the progression of many diseases [8].

A variety of deformable cell culture substrates with different configurations, length scales and actuation mechanisms have been proposed to mimic the mechanical stimuli present in-vivo. For instance, Akbari & Shea [9,10] described arrays of electroactive polymer micro-actuators for stretching individual cells. Other smart responsive materials have also been used to convey mechanical strains to single cells, such as piezoelectric films [11,12] and shape memory alloys [13]. These micro-actuators represent some of the several micro-scaled devices proposed in literature for cell mechanical stimulation (Fior et al., 2011; Shimizu et al., 2011; Chang et al., 2013; Tremblay et al., 2014; Wang et al., 2014). Most of the commercially available devices work at larger scales and are used to stimulate cell cultures as ensembles. They generally consist of flexible membranes multiaxially or uniaxially stretched either by vacuum pressure (such as the Flexer-Cell strain unit by

Flexcell international Corp.) or mechanical motors (e.g. the Cell Stretching System by Strex Inc.) [17,18].

This work is focused on mimicking the physiological motion of the small intestine, which undergoes both circular segmental and longitudinal peristaltic contractions induced by actuation of the circular and longitudinal muscular layers of the bowel. Segmental contractions aid in mixing and absorption of chyme, while peristaltic contractions allow for the propulsion of digested juices through the intestinal tract [19]. Not only do these contractions play a fundamental role in the digestion and absorption of nutrients, they are also crucial for the proliferation and differentiation of the epithelial interface [20]. Although the pattern of movement is rather complex and still not fully understood [21], a model of wall motion resulting from the combined contraction of circular and longitudinal muscles can be represented by a small element of the curved surface of the intestinal wall, which deforms radially, as illustrated in Figure 1. Typical values for frequency and strain reported for the intestine are 7-20 contractions per minute and 8-10%, respectively [22,23].

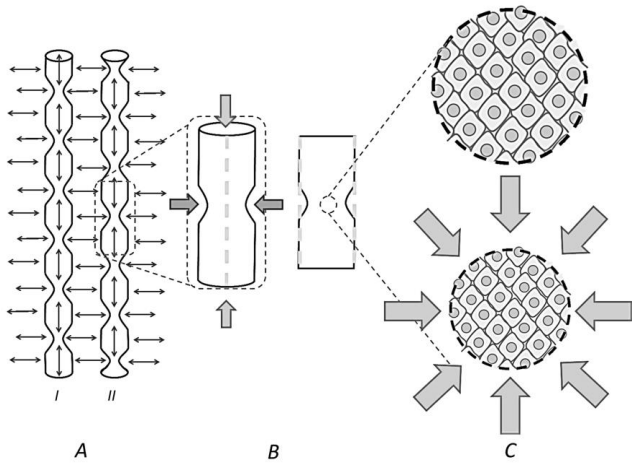

Figure 1: Schematic of the longitudinal and circular contractions of the small intestine (A). The resultant of these rhythmic contractions on a small element of the curved surface of the luminal wall (B) can be approximated as cyclic radial deformations (C).

Classical in-vitro models of the intestine involve the use of the Transwell system [24], a rigid, static culture chamber which cannot recreate the mechanical microenvironment of the intestine. To explore the importance of intestinal mobility, Kim et al [25] developed a human 'Gut-on-a-Chip', a living cell-based model of the intestine that permits fluid flow and cyclic uniaxial strain, similar to some of the mechanical forces experienced by the cells in the intestinal tract. However, as for most micro-scaled devices, this system contains few functional units and thus it is not capable of mimicking long-range interactions between cells [26]. Moreover, micro-scaled systems are difficult to use, requiring significant skills when seeding and culturing cells.

Here, we propose a versatile and easy-to-use bioreactor having the same size of traditional static cell culture-ware, designed to mimic the rhythmic contraction and relaxation of the intestinal wall. The distinctive feature of the device is its ability to mimic the resultant of both the longitudinal and the circular strains experienced by enterocytes on the luminal wall of the small bowel. This was achieved by developing a particular type of a dielectric elastomer actuator (DEA) acting as an artificial muscle.

Dielectric elastomers are a class of electromechanically active polymers, assmart materials capable of reversible transduction from electrical to mechanical energy [27–30]. They are drawing increasing interest as soft actuators, combining simplicity of structure and compact size with large strains (10-100%) and relatively high stresses (up to 1 MPa) in response to an electrical stimulus, low power consumption, low specific gravity and low costs [28]**.** The development of artificial muscle systems is one of the most emblematic uses of these materials as biomimetic actuators [28,29]. The DEA technology shows an attractive high potential to develop dynamic cell culture systems. Indeed, it makes use of the same type of elastomeric materials adopted in the field and, especially, it can add unprecedented value and functionality to those conventional materials, enabling biomimetic motion through electromechanical actuation. Following the first acknowledgements of this potential in 2009 [Bashkin J S, Kornbluh R, Prahlad H and Wong-Foy A.

Chapter 21 - Biomedical Applications of Dielectric Elastomer Actuators. In Biomedical Applications of Electroactive Polymer Actuators, Carpi F and Smela E., Editors, Chichester: Wiley, 2009; Carpi F, Frediani G and De Rossi D 2009. Electromechanically active polymers: new opportunities for biomaterials and tissue engineering. Proc. of Medical Physics and Biomedical Engineering - World Congress 2009, IFMBE Proc. 25/X, O. Dössel, W.C. Schlegel, Editors, pp. 53–56, 2009.] the first micro-devices to mechanically stimulate single cells were described in 2012 [9,10]**. I**n this paper, we present the concept, design, prototype implementation and testing of a bioreactor that exploits the DEA technology to apply cyclic radial strains to epithelial cell cultures, reproducing the physiological motion of the small intestine.

Commento [FC1]: Queste sono 2 reference da aggiungere che avevo già segnalato a Daniele tempo fà.

MATERIALS AND METHODS

Structure and principle of operation

The most elementary configuration for a DEA consists of a dielectric elastomer film sandwiched between two compliant electrodes, so as to form a compliant capacitor. The application of a voltage *V* between the electrodes squeezes the dielectric film (which is incompressible), with a concurrent expansion of its surface (Figure 2A). The effective electromechanical pressure p, also known as Maxwell stress, that compresses the elastomer film is given by the following equation:

$$p = \varepsilon_0 \varepsilon_r E^2 = \varepsilon_0 \varepsilon_r (\frac{V}{d})^2$$

where ε_0 is the permittivity of vacuum, ε_r is the dielectric constant of the elastomer, E is the applied electric field and d is the initial thickness of the elastomer film. In this work, the DEA was shaped as the annular structure shown in Figure 2B. It consists of a dielectric elastomer membrane radially pre-stretched on a circular frame and coated with compliant annular electrodes. By applying a voltage between the electrodes, the annular active region of the elastomer membrane is squeezed in

thickness and, accordingly, it expands in surface area at a constant volume. Hence, the central passive region undergoes a radial compression that tends to release part of the pre-stretch provided during the device assembly. As a result of this, the central region contracts. Figure 2C shows a schematic of the actuating bioreactor concept: the central region of the DE hosts cultured cells within a soft cylindrical chamber, which therefore is radially compressed by the electrically driven actuation of the surrounding DE annulus. When the voltage is turned off, the system returns to the rest state and the central region containing the cells is relaxed.

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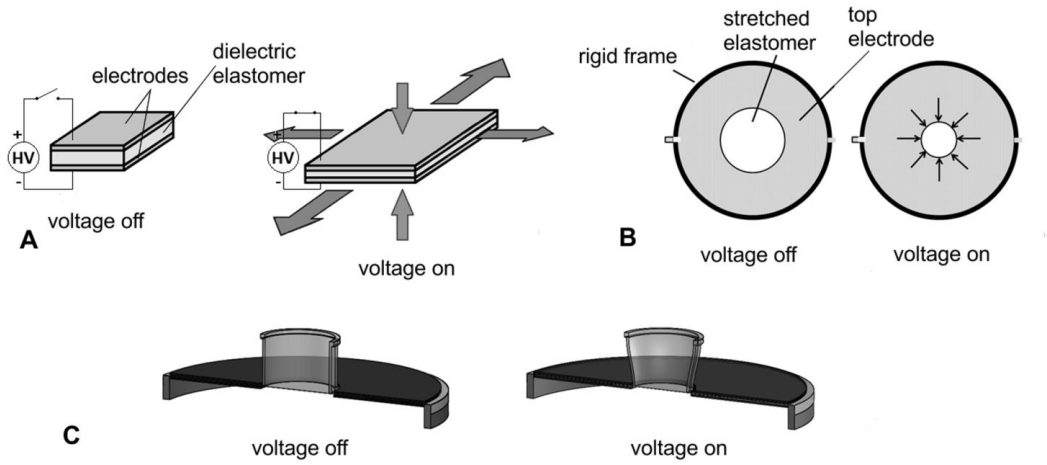

Figure 2: Structure and principle of operation of the DEA-based bioreactor: A) elementary DEA unit; B) annular DEA unit; C) complete actuating bioreactor.

Constitutive materials and manufacturing process

The actuating membrane consisted of a thin acrylic-based co-polymer elastomer film (VHB 4910 by 3M), sandwiched between two compliant electrodes made of carbon grease (846, M.G. Chemicals, Canada). The dielectric membrane was radially pre-stretched by 300% on a rigid circular frame with a diameter of 60 mm. Pre-stretching is necessary for two reasons: firstly, it prevents buckling of the central part when the device is electrically activated; secondly, pre-stretch increases the maximum achievable strain owing to the suppression of electromechanical instabilities

[27,31]. As a result of the pre-stretch, the membrane's thickness changed from 1 mm to approximately 62 µm (calculated value).

Prior to application of the electrodes, a soft cylindrical well-shaped chamber was fabricated and glued to the elastomer membrane (Figs. 3A, 3B). The chamber was fabricated from PDMS (Sylgard 184 Dow Corning, USA) using the millimolding method described by Mazzei et al [32]. To reduce the stiffness of the structure, we used a 30:1 monomer-curing agent ratio rather than the 10:1 ratio recommended by the manufacturer. Higher ratios were also tested but then discarded because of defective curing. To fabricate the well-shaped chamber, a degassed solution of premixed monomer and curing agent was poured into a custom-made aluminium mould and cured at 70°C for 8 hours. The PDMS well (inner diameter 15 mm, outer diameter 17 mm and height 22 mm) was glued to the central passive area of the DEA membrane using a thin layer of PDMS (30:1 monomer-curing agent ratio) and then polymerized *in situ* at room temperature for 24 hours. The well dimensions were based on the size of a 12-well tissue culture plate, so as to facilitate its integration with standard cell culture practices and subsequent sample analysis.

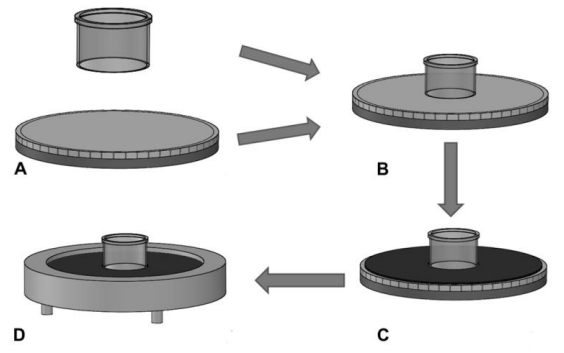

Figure 3: Schematic of the fabrication process of the DEA-based bioreactor. A) The membrane is stretched on a frame while the soft chamber is fabricated by milli-molding; B) The chamber is glued to the membrane, creating a central cell culture well; C) Carbon grease is then deposited on both sides of the membrane, obtaining the two annular compliant electrodes; D) The membrane is lodged in a ring-shaped case for protection and ease of handling.

Commento [FC3]: Bisogna aggiungere uno step intermedio tra B e C, come suggerito da vecchio revisore:
"The fabrication steps presented in this figure do not correspond to the fabrication protocol explained in the text. Applying carbon grease on the membrane should appear as the final step, after cells are cultured and growth medium is added in the culture well. This is an important aspect of the fabrication protocol that should be visible in this figure."

Compliant electrodes were prepared by smearing conductive carbon grease (590-846-80G, MG Chemicals, USA) on both sides of the membrane (Fig. 3C). The amount of deposited grease was controlled by weighting the membrane before and after deposition. The electrodes were created only after the period of incubation, just before the actuation performance measurements. This choice was motivated by the fact that preliminary investigations had shown that samples fully equipped with electrodes didn't survive the incubation, showing electrical failure as soon as they were tested afterwards. Furthermore, the carbon electrodes are difficult to sterilise and so they could easily contaminate the environment. To overcome these limitations (whose management requires further detailed studies) we opted for creating the electrodes only after the incubation.

Commento [FC4]: Chidere a Daniele se è vero esattamente.

Thin strips of aluminium foil were used as electrical contacts for the cables of the voltage generator used to drive the unit (as described in the following Section). The membrane with the coupled well was finally arranged within a plastic case fabricated by 3D printing (Fig. 3D).

Electromechanical characterization

One of our main interests was to determine the robustness and electromechanical performance of the DEA bioreactor after storage within a cell culture environment for 3 weeks, which is the differentiation period of the cell line employed (as described in the section on cell culture). The electromechanical performance was assessed by measuring the electrically achievable radial strain of the soft central well when the device was maintained in different conditions. The bioreactor was characterized using both static and dynamic tests; in all cases the experiments were performed with 1 mL of culture medium in the central well.

The static tests consisted in the application of square wave voltages (50% duty cycle) of increasing amplitude (with voltage steps of 0.5 kV), up to electrical breakdown. The deformation was measured one minute after the onset of the voltage step. After each measurement, the voltage signal

was turned off for one minute to allow the elastomer to relax before applying the next voltage step. These tests were repeated both at room temperature and humidity soon after fabrication and after placing the bioreactor in incubator-like conditions (37°C, 95% humidity) for 1, 6 and 21 days, so as to assess the effect of the environment on performance . The dynamic tests consisted in the application of a sinusoidal waveform with a peak-to-peak amplitude of 4.5 kV (2.25 kV D.C. offset) and a frequency of 0.15 Hz for up to 4 hours.

All tests were performed by driving the actuator with a high-voltage amplifier (615-10, Trek, USA), interfaced to a PC via an A/D board (USB- 6251 DAQ, National Instruments, USA) controlled with a LabView® interface to generate the desired voltage signals. The electrically induced deformation of the membrane was measured via automatic post-processing of image frames extracted from video recordings. In particular, the circular edge of the electrode-free area of the bioreactor was patterned with four black reference markers, whose position was tracked with a high definition camera at a frame rate of 30 fps (Trust WB-1400T Webcam, Italy). A dedicated image processing and speckle tracing software developed with Matlab (The Mathworks Inc) was used to automatically estimate the radial strain, using the pre-stretched rest state as a reference.

Cell culture and actuation

The commonly accepted model of the intestinal epithelium consists of a monolayer of Caco-2 cells, derived from the human colon adenocarcinoma [33,34]. Caco-2 cells exhibit morphological and functional features typical of intestinal epithelia after about 3 weeks in culture, when they spontaneously differentiate into enterocyte-like cells and are characterized by a paracellular permeability comparable to that of the human colon. The cells (kindly supplied by Dr. Isabella De Angelis, Istituto Superiore di Sanità, Italy) were cultured in Dulbecco's modified medium (high glucose) supplemented with 1% non-essential amino acids and containing 10% foetal bovine serum (FBS), 4 mM glutamine, 100 U.I./mL penicillin and 100 µg/mL streptomycin (all reagents from

Sigma-Aldrich, Italy). Subculture was performed at 50% confluence and all experiments were performed between passages 103 and 108.

Before cell seeding, the bioreactor structure was sterilized with 70% ethanol followed by 15 minutes of exposure to UVC light (254.6 nm) under a laminar flow hood, on either side. The central seeding area was then pre-conditioned with 1 mL cell culture medium overnight in an incubator. The conditioning medium was replaced with 500 µL gelatin (0.1% w/v) and incubated for 2h. Once the excess gelatin was removed, cells were seeded at a density of 3×10^5 cells/cm^2 and covered with 1 mL of medium. Gelatin coated glass coverslips were used as controls. The cells were maintained in culture for 21 days, changing the medium every two or three days. The cell culture was observed with a microscope at regular intervals.

After 21 days of culture, the annular carbon grease electrodes were created on the elastomeric membrane and the so-completed bioreactor was actuated with a sinusoidal driving voltage (4.5 kV peak, 0.15 Hz) in an incubator at 37°C, 95% humidity, for 4 hours. Beyond 4 hours some samples showed electrical failure and so for the sake of this study the investigations were limited to that length of time. That length, by the way, was sufficient for an effectivemechanical stimulation of Caco-2 cells, as a few hours of cyclic strain have been reported to be sufficient to increase proliferation significantly for that type of cells [36].

At the end of the mechanical stimulation experiments the cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature, permeabilized for 5 minutes in 0.1 % Triton and labelled with Rhodamine-Phalloidin (Thermo Fischer, Italy), diluted 1:400 in 1% BSA followed by DAPI (4' 6-diamidino-2-phenylindole; 1µg/mL in 1% BSA) for 20 minutes. The samples were imaged using a confocal microscope (A1 Confocal Microscope System, Nikon Italy).

Data Analysis

At least 3 samples were analysed for each electromechanical test performed. The strain data were expressed as means ± standard deviation. Statistical differences between the different conditions were analysed using analysis of covariance (ANOCOVA Matlab, Statistical Toolbox), setting significance at $p<0.05$.

RESULTS AND DISCUSSION

Electromechanical characterization

Photographs of the DEA-based bioreactor in different stages of fabrication are shown in Figure 4.

Figure 4: The bioreactor at various stages of fabrication: A) the membrane, B) the flexible central well, C) the complete bioreactor mounted within its case, containing the cultured cells and1 mL of medium within the well and equipped with the carbon grease electrodes.

Figure 5A reports the radial strain of the central passive area of the bioreactor as a function of the applied voltage for different experimental conditions: soon after fabrication (0 days) and after 1, 6 and 21 days in a humidified incubator. In all cases the central well contained 1 mL of culture medium and the electrodes were applied just before the actuation tests. The strain data are not significantly different (p-value = 0.12, ANOCOVA), demonstrating that the actuator performance was not affected by storage in a humid environment for up to 21 days.

Commento [FC5]: Non credo che sia possibile in questo journal

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Figure 5B shows the typical radial strain of the central passive region of the bioreactor in response to a 0.15 Hz, 4.5 kV sinusoidal driving voltage after the device had been stored for 21 days at 37°C, 95% humidity, maintaining 1 mL of culture medium in the well. The maximum strain achieved was 8.92 ± 0.11% for four samples analysed, mimicking the amplitude and frequency of intestinal contractions. The evident drift of the cyclic strain response during the first few minutes of actuation was due to viscoelastic creep of the elastomeric membrane. After about 100 seconds of cyclic deformation the electromechanical response of the actuator reached a steady state, with pretty stable oscillations for at least 4 hours of continuous actuation.

It is worth noting that this drift is more important than a simple transient occurring over the first 100 seconds. Indeed, in the steady state the cells are exposed to a compressive strain that is about 4-9%, meaning that they are constantly under at least a 4% compressive strain. While the maximum value is consitent with those reported by the literature for the intestine *in vivo* (as remarked above), as of today it is not clear whether the non-null minimum strain might have contraindications. While this aspect should be addressed by future studies, clearly the use of less viscous elastomers would reduce that problem. To this end, silicones are expected to offer a promising solution, as it has already been shown for different types of DEA-based systems [37].

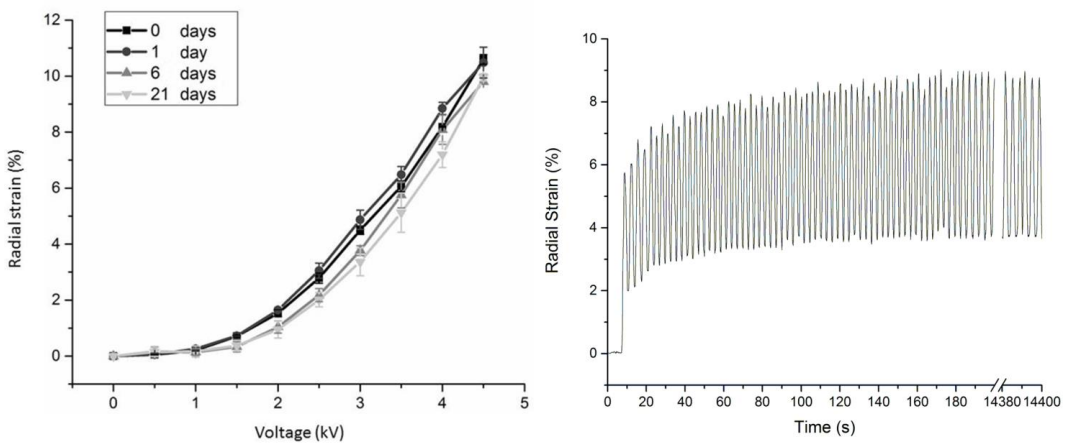

Figure 5: A) Voltage induced radial strain of the central passive area of the actuated bioreactor containing 1 mL of culture medium in the central well, stored for different times in an incubator at 37°C, 95% humidity. Error bars represent the standard deviation n=3 samples. B) Typical cyclic electromechanical response of the device after storage in an incubator for 21 days with 1 mL of medium in the well.

Cell culture

Caco-2 cells cultured on the DEA membrane were imaged with bright-field microscopy after 14 and 21 days of culture. As shown in Figure 6, the images demonstrate that the cells were able to proliferate on the membrane retaining their typical morphology. After 21 days, small domes (Fig. 6B), indicative of cell differentiation towards an epithelial phenotype were observed.

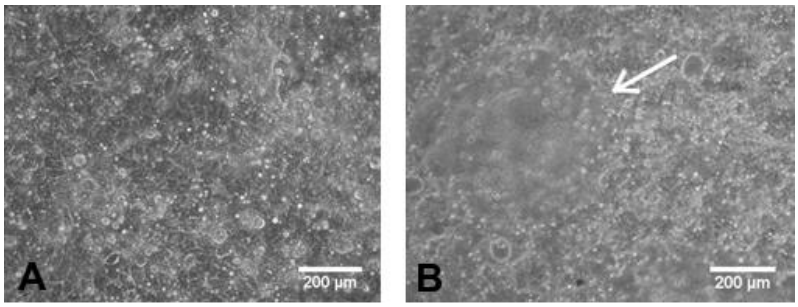

Figure 6: Bright-field micrographs of Caco-2 cells cultured on the DEA membrane: A) after 14 days; B) after 21 days. The white arrow points to a "dome", characteristic of differentiated Caco-2 cells.

The device was then actuated, subjecting the cells to cyclic compression and relaxation by applying a sinusoidal voltage for 4 hours in the incubator, in order to evaluate morphological changes in response to the mechanical stimuli. Figure 7shows fluorescence image of Caco-2 cells after 21 days of culture on the membrane, taken right after the incubation (Fig. 7A) and right after 4 hours of cyclic actuation (Fig. 7B) . ~~Unactuated controls are reported in Figure 7B~~.

Commento [FC6]: Non so se sia vero. Daniele puoi verificare?

In realtà direi che per un fare un vero controllo l'immagine avrebbe dovuto essere ripresa dopo 4 ore dalla incubazione, ossia facendo trascorrere un tempo equivalente a quello trascorso per il campione soggetto ad attuazione, per verificare che una eventuale differenziazione in realtà non sia in grado di avvenire anche in condizione statiche

Commento [FC7]: Nella caption della figura A e B erano invertiti e quindi qui ho cambiato, ma non so se è corretto. Quale è la versione giusta?

After the exposure to cyclic strain the cell monolayer was uniform and intact, and there were no apparent changes in actin filament distribution or cell integrity. This was observed in all of the three tested samples.

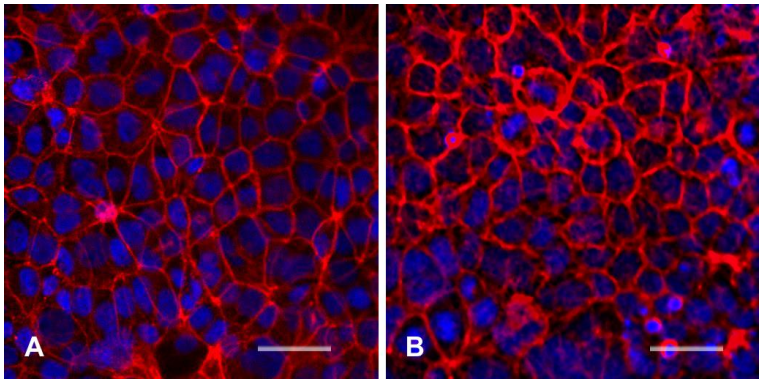

Figure 7: Confocal images of Caco-2 cells stained with DAPI (blue nuclear stain) and phalloidin (red, actin fibers) after 21 days of culture on the DEA membrane: A) not actuated sample; B) actuated sample after 4 hours of cyclic actuation at 0.15 Hz in an incubator at 37°C and 95% humidity. Scale bar=50 μm.

Commento [FC8]: Dobbiamo dire di più qui, in relazione a quanto scritto sopra: "a few hours of cyclic strain have been reported to be sufficient to increase proliferation significantly for that type of cells [36]". Si riscontra un simile increment della proliferazione a seguito della stimolazione per 4 ore, rispetto al controllo? Dalle figure non mi sembra (da inesperto) che si noti alcuna differenza. Se così fosse, non abbiamo dimostrato nulla in termini di efficacia della stimolazione. Avremmo solo dimostrato che si può stimolare e che questo non fa danni, almeno apparenti visivamente, alle cellule. Questo va detto.

Inoltre, se diciamo questo, allora dobbiamo eliminare il pezzo sopra in cui diciamo che poche ore sono sufficienti a indurre proliferazione.

Inoltre, qui potremmo commentare che è necessari aumentare durability del device ad un numero di ore maggiore di 4, per verificare se quel tipo di stimolazione può indurre differenziazione.

Commento [FC9]: Nel testo A e B erano invertiti. Quale è la versione giusta?

CONCLUSIONS AND FUTURE DEVELOPMENTS

An innovative bioreactor with muscle-like actuation for the study of physiological phenomena and prediction of in-vivo responses was described. The bioreactor is based on electro-responsive elastomers, smart materials that undergo deformation upon electrical stimulation. Working as a radial artificial muscle, the elastomer is able to cyclically compress and relax a central culture well, mimicking the peristaltic and segmental motions of the intestinal wall. The integrated device combines several advantages such as compactness, versatility of design, ease of use, low cost and capability of mimicking the dynamic micromechanical milieu of the small bowel on a scale compatible with the standard cell culture ware, so conserving consolidated cell biology protocols.

As Caco-2 cells typically require 21 days before differentiating to an enterocytic phenotype, one of our main concerns was verifying actuator performance after exposure to cell culture conditions for extended periods of time. Actuator performance in terms of electromechanical response to a series

of incremental voltage steps was not compromised by storage in a humid cell culture environment, even after 21 days. Caco-2 cells were maintained in culture in the central well for 21 days, forming a compact monolayer, confirming that the elastomeric membrane is biocompatible and cell-adhesive. Following storage in such demanding conditions for a polymer-based electrical system, the bioreactor was also able to maintain electromechanical function, with a cyclic strain of about 8%, 0.15 Hz for up to 4 hours. We did not observe any adverse effects on cell morphology and adhesion afterthat cyclic strain.

These results show the suitability of the DEA technology for biomimetic muscle-like actuation of intestinal epithelia and confirms the great potential of that technology for the development of new types of biomimetic bioreactors in general.

Nevertheless, as the technology is still fairly young, a large number of issues still need further study and development. One of the main challenges is the need for high driving electric fields, and, therefore, high voltages. This is due, in general, to the use of off-the-shelf elastomers, developed for other purposes and available/processed as thick films, typically of the order of 100µm, which require voltage typically of some kV. While the generation of voltages so high is not problematic from a technical standpoint, considering that there's no need for high driving powers (typically 10-100 mW) as the loads are capacitive, the implications on the cost of the required driving system and the electrical safety of the operators are certainly a limitation. Further, efforts are required to quantify the amplitude of the fringe electric field (although at very low frequency) impacting the well and assess its possible effects on the cells.

To overcome these problems, ongoing developments within the whole field of the DEA technologies are currently addressing the need for lowering the voltages down to 100-200 V, which is the standard for the low-cost and low-size drives of commercial piezoelectric transducers available in a huge diversity of products today. To this end, efforts are being focused on using dielectric elastomer materials made of silicones, and customizing manufacturing processes to obtain

very thin membranes, lowering their thickness down to the order of 10 μm. While this is challenging for highly stretchable materials, preliminary evidences suggest feasibility [38, 39]. So, future DEA-based systems are expected to be made of silicones, which will offer much greater versatility for design and manufacturing of the actuator, as well as lower creep, thus reducing or even avoiding drifts in the achievable deformation.

Other challenges more specific for the application described here include the development of adequate compliant electrodes. In this work, carbon grease was used a test material for a straightforward approach to simplify the manufacturing of the device and obtain a proof-of-concept demonstration of its functionality. Clearly, in improved versions of the system the electrodes should be solid-state, easy to sterilise and compliant with the wet environments of incubators. Soft protective coatings of electrodes made of silicone-carbon black composites or ionogels, for instance, might be a sensible approach, although the limitations introduced by them in terms of reduction of the actuation strain (owing to a higher stiffness) should be evaluated.

These issues dealing with the identification of the most appropriate materials both for the actuating membrane and its electrodes, as well as their processing techniques, will also have implications on the reliability and lifetime of the devices, which of course have to be improved to enable mechanical stimulations sufficiently accurate and long to be adequately effective.

Commento [FC10]: Suggerisco di eliminare questa frase perché non tiene di conto dei lavori di shea, come giaà fatto notare dal revisore vecchio (forse lui stesso!).

Commento [FC11]: Suggerisco di levare anche questa perché suona come un claim eccessivo.

Acknowledgements

The study was partly funded by the Italian Ministry of Education, University & Research (MIUR) (Project PRIN 2010, MIND, 2010J8RYS7), while JC is recipient of a EU H20202 Marie Curie ITN Grant (MICACT). The authors would also like to thank Isabella De Angelis for her assistance with cell cultures. The software for automatic measurements of radial strain was developed in the framework of an Erasmus mobility grant between the University of Pisa and Queen Mary

University of London, awarded to Cristina Curreli, and it can be downloaded at www.centropiaggio.unipi.it/software.

Codice campo modificato

Author Contributions

DC& GG designed, fabricated and characterised the device and prepared the figures. JC performed cyclic strain and cell culture experiments. GF and FC conceived the annular DEA and provided expertise on EAP. CD supervised the cell culture experiments, AA and FC conceived the bioreactor. AA procured funding. GG, FC && AA wrote the paper.

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