COLLAPSIBLE CELL-ENCAPSULATING SCAFFOLDS TO ENHANCE MAGNETICALLY-INDUCED SECRETOME STABILISATION AND POTENCY

FIELD OF THE INVENTION

The present invention generally relates to methods of producing magnetically induced secretomes with enhanced potency and stability, the methods comprise inter alia, employing polyacrylic acid based polymers, preferably a carbomer, to form a collapsible cell-encapsulating scaffold and dispersing donor cells within said scaffold prior to induction via a directionally-specific pulsing electromagnetic field (PEMF), or dispersing said donor cells in a "collapsed" polyacrylic acid based polymer scaffold prior to the PEMF induction. Also provided are the improved conditioned media thereof, suitable for use in medical and commercial applications.

BACKGROUND OF THE INVENTION

Cells communicate by virtue of their secretomes, which act as signaling molecules to facilitate cell-to-cell communication and promote therapeutic benefits like tissue regeneration, anti-inflammatory and immunomodulatory effects. In recent times, secretomes have gained attention and shown promise in therapy, particularly in regenerative medicine, as it can be used in cell-free approaches. However, stabilization of secretome factors from cells has proven to be a bottleneck to industrial exploitation. For example, the inventors' ongoing research has shown that the secretomes released from cells into "clean" saline basal media is labile and degrade within 18-24 hours of secretion. This limitation makes commercial applications requiring long-term storage and shipping unrealizable.

Molecular confinement and macromolecular crowding (MMC) are known to influence various biological processes, including protein folding, functional stability, enzymatic activity, and interactions with other proteins. Confining agent such as agarose hydrogel and crowding agent like the biopolymer dextran have been shown to enhance protein stabilization (Ross ML et al.(2020)). However, commonly used polymers are either not cleared for human consumption, are indigestible to varying degrees, or require combinations of distinct polymers for the distinct roles (Ross ML et al.(2020), Lee MH et al. (2016), Ramalingam R. et al. (2023), Zeugolis DI. (2021)).

Carbomers are a family of high molecular weight, cross-linked polyacrylic acid polymers that swell in water to form a gel-like structure. Carbomers are commonly used as rheology modifiers in semisolid and liquid applications for pharmaceutical and cosmetic applications including lotions, creams, gels, and toothpaste. They are typically used to provide a thicker

consistency to products, increasing viscosity and suspending dispersed components inside an aqueous media. Carbopol (a trademark brand name for a type of carbomer produced by Lubrizol Corporation) are generally considered safe for use in cosmetics and skincare products, and has been approved for use in food as an ingestible. A few scientific studies have examined cell behaviour within carbopol matrices (e.g., Bhattacharjee T et al. (2016)), but not with the objective of recapturing nor improving the viability of cellular products secreted into the carbopol matrix. Given its safety profile and its hydrogel-forming abilities, the use of carbomers like carbopol might hence represent a viable approach in improving cell secretome stability for use in clinical, commercial and other applications.

Accordingly, there is a need to provide methods that improve cell secretome release, stabilisation and/or potency, or at least ameliorate one or more of the disadvantages described above.

SUMMARY OF THE INVENTION

In a first aspect, there is provided a method of producing a conditioned medium (CM) capable of conditioning recipient cells, the method comprises the steps:

(i) providing an agent that is a particulate or a polymer or a combination thereof capable of forming a material-spanning microstructure;

(ii) mixing progenitor and/or stem cells in a media with the agent of step (i), preferably at a pH suitable for cell culturing, to obtain a mixture,

(iii) exposing the mixture to low amplitude pulsed electromagnetic fields (PEMFs),

(iv) incubating the mixture preferably at a temperature suitable for cell culturing, for a time period,

(v) centrifuging the mixture to obtain a supernatant, and

(vi) collecting the supernatant to obtain the conditioned medium (CM), and optionally ultracentrifuging the supernatant to obtain separate components of the CM, consisting of a vesicular fraction separated from the soluble secretome components.

In one preferred embodiment, the agent is provided in a suspension and forms the material-spanning microstructure in the suspension, prior to mixing with said cells in step (ii), and the cells are suspended within the material-spanning microstructure in the mixture in step (ii) upon mixing. Preferably, the method further comprises the steps of: disrupting the material-spanning microstructure prior to centrifuging the mixture in step (v), filtering the supernatant

in step (v) prior to step (vi), and/or the step of ultrasonicating the suspension comprising the material-spanning microstructure in step (i), prior to the mixing in step (ii).

In another preferred embodiment, the agent is provided in a suspension wherein the agent forms the material-spanning microstructure in the suspension. Preferably, the material-spanning microstructure in the suspension in step (i) is disrupted prior to mixing with the cells in step (ii). More preferably, the suspension comprising the material-spanning microstructure of step (i) is ultrasonicated prior to the disruption of the material-spanning microstructure in the suspension, and the method may further comprise the steps of filtering the disrupted suspension prior to mixing with the cells in step (ii).

In a second aspect there is provided a conditioned medium capable of conditioning cells, produced by the methods as disclosed herein.

In a third aspect, there is provided a method of stabilizing cell extracts and secretome, comprising contacting an efficacious amount of the conditioned medium of the second aspect with said cell extracts and secretome.

In a fourth aspect, there is provided a method of pre-conditioning proliferating, differentiating or senescent (oxidatively stressed) progenitor and/or stem cells for use in the production of a PEMF-conditioned media, wherein the method comprises contacting a sample of proliferating, differentiating or senescent (oxidatively stressed) progenitor and/or stem cells with the condition medium of the second aspect. Preferably, the contacting with the CM of the second aspect enhances the secretory response of said proliferating, differentiating or senescent (oxidatively stressed) progenitor and/or stem cells upon later exposure to PEMF.

In a fifth aspect, there is provided a use of the conditioned medium according to the second aspect in conditioning recipient cells.

Advantageously, the methods disclosed herein improve cell secretome release, stabilization, viability and potency. More advantageously, the methods and conditioned media disclosed herein also have minimal GMP (good manufacturing practice) barriers, thus making them suitable for therapeutic, clinical and commercial exploitations. These and other advantages of the present disclosure will become readily apparent to those skilled in this art from the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

The accompanying drawings illustrate disclosed embodiments and serve to explain the principles of the disclosed embodiments. It is to be understood, however, that the drawings

are designed for purposes of illustration only, and not as a definition of the limits of the invention.

FIG. 1 shows a graph depicting the rheological behaviour of Carbopol gel (CPG) (0.6 – 1% CP 974 and 0.5% CP 980) and Collapsed Gel (0.8% CP 974) in the presence of different salts (error bars indicate the standard deviation of three repeat experiments). The dashed horizontal line represents the yield-stress fit from a Herschel-Bulkley model. Error bars from some repeated experiments that are smaller than the data points have been neglected for clarity.

FIG. 2 depicts a graph which shows the rheological behaviour of 0.8% ultrasonicated Carbopol gel (UCPG) (n = 1). Compared to 0.8% CPG, no plateau was observed, indicating more free volume in ultrasonicated materials. Linear polyacrylic acid gel (LPAAG) behaved similarly to UCPG. CP 974 was used in this experiment.

FIG. 3 depicts microscopic images showing CP aggregates in 0.5% CPG 980 and 0.8% CPG 974. The mean size area (black area) for n = 5 and the standard deviation are indicated.

FIGS. 4A-4B are microscopic images illustrating CP aggregates in (**FIG. 4A**) CPG prepared with varying CP concentrations and (**FIG. 4B**) Collapsed Gel 0.8% CP 974 with different salt additions. The mean size area (black area) for n = 5 and the standard deviation are indicated.

FIG. 5 are microscopic images illustrating aggregates in CPG, UCPG and LPAAG. Among all gel samples, the aggregates in CPG exhibited the largest sizes as they are made of cross-linked LPAA chains. LPAAG showed larger particle sizes compared to UCPG at both 0.08% and 0.8%, likely due to aggregation of the linear polymers.

FIGS. 6A – 6B depicts images of tubes after centrifugation, showing (**FIG. 6A**) CPG with different CP 974 concentrations and (**FIG. 6B**) 0.8% CPG 974 collapsed with various salt types.

FIG. 7 shows a graph depicting the recovery percentage and pH of the CPS after the collapse of 0.8% CPG 974 with varying NaCl concentrations (mean ± standard deviation; n = 3). As the NaCl concentration increased, the recovery percentage rose while the pH of the CPS decreased. Despite the NaCl concentration reaching 1500 mM, the recovery percentage remained around 80%.

FIG. 8 shows a graph depicting the recovery percentage and pH of the CPS after the collapse of 0.8% CPG 974 with varying $CaCl_2$ concentrations (mean ± standard deviation; n = 3). The recovery percentage increased while the pH of CPS decreased with increasing $CaCl_2$ concentration, achieving almost full recovery at 50 mM with a pH of ~7.2.

FIG. 9 is a schematic workflow diagram for the experimental conditions utilized in determining the presence of residues in the CPS obtained from CPG 974.

FIG. 10 depicts the DLS spectra of blank DMEM and CPS with and without salt addition (mean ± standard deviation; n = 3). The presence of CP residues in the CPS is confirmed by the overlap of particles size peaks with the CPS without salt addition (at ~20 and ~200 nm) and the lack of these peaks in blank DMEM.

FIG. 11 is a schematic workflow diagram for the experimental conditions utilized in determining the gel sizes in 0.08% UCPG and residue sizes in 0.08% UCPS.

FIG. 12 shows the DLS spectra of 0.08% UCPG and UCPS (with and without filtration through 0.2 µm pore size) recovered from the collapse of 0.08% of UCPG using 25 mM of $CaCl_2$.

FIG. 13 are microscopic images illustrating aggregates in 0.08% UCPG and UCPS (with and without filtration through 0.2 µm pore size) recovered from the collapse of 0.08% of UCPG using 25 mM of $CaCl_2$.

FIG. 14 shows a schematic workflow diagram of the various experimental conditions utilized in cell culture experiments of the present invention.

FIG. 15 depicts a bar chart which shows the relative fold change of recipient C2C12 (24 h proliferation) cells exposed to conditioned media from magnetically-induced recipient myoblast suspension cells that were conditioned for 1 h, with or without Carbopol Gel (CPG) (CP 980 at 0.5% w/v). The data was normalized to the 0 mT condition (-CPG). Data represents the average of n = 2 biological replicates. Error bars represent the standard error of the mean. FIG. 15 shows that CPG improves secretome conditioning post magnetic exposure.

FIGS. 16A - 16B demonstrate that the secretome protective properties of CPG (CP 980 at 0.5% w/v) allow for the conditioning of media for longer to ultimately sustain greater secretome accumulation and collection. **FIG. 16A** (Left 3 bars; -CPG): Bar chart shows the relative fold change of C2C12 recipient cells (24 h proliferation) after the provision with 1.5 mT conditioned media, normalized to the 0 mT condition. Conditioned media were collected from C2C12 suspension cells after 1 h incubation post magnetic exposure. (Right 3 bars; +CPG): Donor suspension C2C12 cells in CPG were exposed to magnetic fields and left to condition the media for either 1 h or 2 h before the collection and provision to recipient C2C12 cells. Collection of conditioned media from donor cells suspended in CPG is associated with enhanced stability and potency of cell secretome that conferred improved cell proliferation responses in recipient C2C12 cells. Data was taken from n = 1 biological replicate. The same would apply for other CP configurations. **FIG. 16B** Bar chart shows the response of recipient

C2C12 cells to exosomes (vesicular fraction) collected from donor suspension C2C12 myoblasts. The optimal conditioning time for the isolation of exosomes (for enhanced proliferation) is 60 min. C2C12 donor myoblasts were exposed to PEMF and incubated for 30 min to 120 min prior to the start of exosome isolation (n = 3 biological replicates with each consisting of 3 technical replicates). Error bars represent the standard error of the mean and data was analyzed using One-Way ANOVA with Sidak's multiple comparisons test.

FIGS. 17A – 17D depict bar charts which show the relative protein expression (normalized to GAPDH) of (**FIG. 17A**) phosphorylated JNK, (**FIG. 17B**) phosphorylated ERK, (**FIG. 17C**) Cyclin B1 and (**FIG. 17D**) Cyclin D1, from recipient cells. Recipient C2C12 myoblasts were previously seeded in 6-well plates (30,000 cells/well) for 24 h before the provision with secretome from suspension myoblasts exposed to magnetic fields in the presence of CPG (CP 980 0.5% w/v) (1 h post-magnetic conditioning). The myogenic effect of conditioned media in CPG was compared to recipients grown under standard DMEM or DMEM supplemented with FBS (DMEM + FBS). The protein analyses on recipient cells were performed 24 h after the provision of secretome or after media change to DMEM or DMEM + FBS, using standard RIPA lysis for Western Blotting. A reduction of p-JNK and an increase in p-ERK after magnetic exposure in the presence of CPG relative to DMEM + FBS indicated better survival. An increase in both Cyclin B1 and Cyclin D1 after magnetic exposure in the presence of CPG indicated increased cell proliferation. Data represent the average of n = 2 biological replicates. Error bars represent the standard error of the mean. FIGS. 18A - 18D show that secretome from magnetically-stimulated C2C12 myoblasts suspended in CPG improves myogenic survival.

FIG. 18 depicts a bar chart which shows the relative fold change of C2C12 recipient cells (24 h proliferation) exposed to conditioned media (2^{nd} and 3^{rd} bars) or EVs (4^{th} and 5^{th} bars) from donor C2C12 suspension cells after conditioning for 1 h post magnetic exposure in the presence of CPG (CP 980 at 0.5% w/v), and was normalized to the CPG-only condition (no addition of cells). Data represent the average of n = 2 biological replicates. Error bars represent the standard error of the mean. FIG. 18 shows that CPG enhanced the potency of conditioned media as well as extracellular vesicles (EVs; vesicular secretome factors).

FIGS. 19A – 19B show that collapsed CPG supernatant (CPS) exhibited greater secretome protective capacity than adding small amounts of CP to basal media prior to magnetically-induced cell secretome release and conditioning of the media. CPS was generated by collapsing CPG (CP 980 at 0.5% w/v) with NaCl (110 mM) and then using the supernatant as conditioning media for C2C12 myoblasts. (**FIG. 19A**) CPS maintained secretome efficacy after 24 h and 48 h of storage post exposure. (**FIG. 19B**) Prior addition of CP 980 (5%) to

conditioned media collected after magnetic exposure did not improve the efficacy of the secretome after 24 h and 48 h of storage. Bar chart shows the relative fold change of C2C12 recipient cells (24 h proliferation) after the provision with conditioned media. Data represents the average of n = 2 biological replicates. Error bars represent the standard error of the mean.

FIG. 20 depicts a bar chart which shows that collapsed CPG supernatants (CPS) preserved the potency of the PEMF conditioned media following storage at 4°C for 1 week. CPS was generated by collapsing CPG (CP 980 at 0.5% w/v) with NaCl (110 mM) and then using the supernatant as conditioning media for C2C12 myoblasts. Bar charts show the relative fold change of C2C12 recipient cells relative to DMEM + FBS (foetal bovine serum) condition (Bar 2) 24 h after the provisioning of conditioned media. Fresh conditioned media (Bars 3 to 6) was compared to conditioned media stored at 4°C for 1 week. Bar 11 consists of DMEM + FBS condition which was stored in 4°C for 1 week as a control. Data represents the average of n = 2 biological replicates. Magnetic exposure (black bars) enhanced the potency of the conditioned media in all cases except storage without CPS. Error bars represent the standard error of the mean.

FIG. 21 depicts a bar chart that shows that collapsed CPG supernatant (CPS) preserved the potency of the extracellular vesicles (EVs) following storage at 4°C for 24 h. CPS was generated by collapsing CPG (CP 974 at 0.8% w/v) with NaCl (110 mM) and then using the supernatant (CPS) as conditioning media for C2C12 myoblasts before isolation of EVs. EV proliferative efficacy persists after storage for 24 h at 4°C in the presence of CPS. The CPS media performance experiences only a slight decrease, while the media without CPS has significantly degraded (24 h proliferation of C2C12 recipient cells). Magnetic exposure (black bars) enhanced the potency of the EVs in all cases except storage without CPS. FBS refers to Foetal Bovine Serum. Data represents the average of n = 2 biological replicates. Error bars represent the standard error of the mean.

FIG. 22 depicts a bar chart which shows that collapsed CPG supernatant (CPS) (CP 974 at 0.8% w/v) preserved the potency of the PEMF conditioned media after storage at 37°C for 2 or 6 Hours. CPS was generated by collapsing CPG with NaCl (110 mM) and then using the supernatant as conditioning media for C2C12 myoblasts. CPS maintained secretome efficacy and outperformed the efficacy of DMEM + FBS media. cCM and pCM refer to media conditioned without (control, 0 mT) and with (1.5 mT) PEMF exposure. In this instance, pCM matched or surpassed the efficacy of FBS (Foetal Bovine Serum) in all cases. Bar chart shows the relative fold change of C2C12 recipient cells (24 h proliferation) after the provision with conditioned media. Data represents the average of n = 3 biological replicates. Error bars represent the standard error of the mean.

FIG. 23 depicts a bar chart which shows that collapsing cell-impregnated CPG (CP 974 at 0.8% w/v) with calcium chloride ($CaCl_2$) increases the potency of the rendered conditioned media. The potency of the conditioned media generated following $CaCl_2$ (25 mM) collapsing was compared with that following 110 mM sodium chloride (NaCl) and 25 mM magnesium chloride ($MgCl_2$) collapsing. Magnetic exposure (1.5 mT) consistently enhanced the potency of the rendered conditioned media regardless of CPG or collapsing salt. Bar chart shows proliferation of C2C12 recipient cells (24 h proliferation) after the provision with conditioned media. Data represents the average of n = 1 biological replicates. Error bars represent the standard error of the mean.

FIGS. 24A – 24F depict bar charts which show that collapsing cell-impregnated CPG with calcium chloride ($CaCl_2$) rendered a conditioned media that best promoted myogenic survival. The myogenic potency of the conditioned media generated following $CaCl_2$ (25 mM) collapsing was compared with that following 110 mM sodium chloride (NaCl) and 25 mM magnesium chloride ($MgCl_2$) collapsing. Magnetic exposure (1.5 mT) best enhanced the myogenic potency of the rendered conditioned media. The effect of $CaCl_2$ was compared with other salts like 110 mM sodium chloride (NaCl) and 25 mM magnesium chloride ($MgCl_2$). Bar charts show the relative protein expression (normalized to GAPDH) of (FIG. 24A) phosphorylated JNK, (FIG. 24B) phosphorylated ERK, (FIG. 24C) TRPC1, (FIG. 24D) Cyclin B1, (FIG. 24E) Cyclin D1, and (FIG. 24F) P21 from recipient cells. Recipient C2C12 myoblasts were previously seeded in 6-well plates (30,000 cells/well) for 24 h before the provision with secretome from suspension myoblasts exposed to magnetic fields in the presence of CPS (1 h post-magnetic conditioning). The myogenic effect of conditioned media in CPS was compared to recipients grown under standard DMEM or DMEM supplemented with FBS (DMEM + FBS). The protein analyses on recipient cells were performed 24 h after the provision of secretome or after media change to DMEM or DMEM + FBS, using standard RIPA lysis for Western Blotting. A reduction of p-JNK and an increase in p-ERK after magnetic exposure in the presence of CPG relative to DMEM + FBS indicated better survival. An increase in Cyclin D1 (CD1) after magnetic exposure in the presence of $CaCl_2$ indicated increased cell proliferation. Data represents the average of n = 3 biological replicates. Error bars represent the standard error of the mean.

FIG. 25 depicts a bar chart showing that cell-impregnated ultrasonicated CPGs (UCPG) render enhanced conditioned media compared to cell-impregnated Carbopol Gels (CPG) or Carbopol Gel Supernatants (CPS). Bar charts show the relative fold change in C2C12 recipient cell number relative to DMEM alone (Bar 1) 24 hours after the provisioning of the specified conditioned media. C2C12 donor cells were conditioned in either CPG, ultrasonicated CPG (UCPG) or CPS. Ultrasonicated CPG was generated by sonicating CPG in an ice bath for 2.5

minutes at 30% amplitude using a FB-505 sonic dismembrator ultrasonic processor as described in methodology. Data represents the average of n = 1-2 biological replicates. Error bars represent the standard error of the mean.

FIG. 26 depicts a bar chart showing that Ultrasonicated Collapsed Carbopol Gel Supernatant (UCPS) (CP 974 at 0.8% w/v) enhanced the pro-proliferative capacity of isolated extracellular vesicles (EVs) following magnetic exposure. Bar charts showing the proliferation of C2C12 recipient cells 24 hours after the provision of EVs isolated from conditioned media derived from CPS (Bars 3-4), UCPS (Bars 5-6) or linear poly-acrylic acid (LPAA; Bars 7-8). Data represents the average of n = 1 biological replicates. Error bars represent the standard error of the mean. Acrylic acid is the monomer of both CP and poly-acrylic acid. These results indicate that other PAA-based materials are also capable of sustaining proliferation, albeit not as strongly as carbopol.

FIG. 27 shows that the resuspension of extracellular vesicles (EVs) In Ultrasonicated CPG Supernatant (UCPS) (CP 974 at 0.8% w/v) or Collapsed CPG Supernatant (CPS) (CP 974 at 0.8% w/v) following stage one conditioning in collapsed CPG Supernatant (CPS) further enhances EV proliferative efficacy. The C2C12 donor cells were first conditioned in either CPS or fresh DMEM with (0 mT) or without (1.5 mT) magnetic exposure (Stage 1 conditioning). After ultracentrifugation, the EVs were then resuspended in either UCPS or CPS. Bar charts show the proliferation of C2C12 recipient cells 24 hours after the provision of EVs isolated from conditioned media derived from UCPS (Bars 3-4; 7-8) or CPS (Bars 5-6; 9-10). Data represents the average of n = 1 biological replicates. Error bars represent the standard error of the mean.

FIG. 28 shows that ultrasonication enhanced the potency of the extracellular vesicles (EVs) rendered from CP supernatants in a time-dependent manner. Bar charts showing relative fold change in C2C12 recipient cell numbers relative to DMEM alone (Bar 1) 24 hours after the provision of EVs rendered from either UCPS (CP 974 at 0.8% w/v) or CPS (CP 974 at 0.8% w/v) conditioned for one or two hours. Magnetic exposure (10 minutes) and conditioning of C2C12 donor cells was performed within CPS or UCPS. Media conditioning was conducted for one or two hours at 37°C within a standard tissue culture incubator. Data represents the average of n = 1 - 2 biological replicates. Error bars represent the standard error of the mean.

FIGS. 29A – 29B show that cell-impregnated Carbopol 974 Gel (CP 974 at 0.8% w/v) and Collapsed Carbopol 974 Supernatant increased the potency of the rendered PEMFed conditioned media as compared with conditioned media produced from Carbopol 980 Gel (CP 980 at 0.5% w/v). (**FIG. 28A**) Conditioned media from cell-impregnated Carbopol 974 Gel had

increased potency than conditioned media from cell-impregnated Carbopol 980 Gel (**FIG. 28B**) CPS collected from collapse of Carbopol 974 Gel increased the potency of the PEMFed conditioned media when compared with CPS from Carbopol 980 Gel. Bar charts showing the proliferation of C2C12 recipient cells 24 hours after the provision of Conditioned Media. Data represents the average of n = 1 biological replicates. Error bars represent the standard error of the mean.

DETAILED DESCRIPTION OF THE INVENTION

Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference but their mention in the specification does not imply that they form part of the common general knowledge.

Definitions

For convenience, certain terms employed in the specification, examples and appended claims are collected here.

In general, technical, scientific and medical terminologies used herein has the same meaning as understood by those skilled in the art to which this invention belongs. Further, the following technical comments and definitions are provided. These definitions should in no way limit the scope of the present invention to those terms alone, but are put forth for a better understanding of the following description.

As used herein, "a" or "an" may mean one or more than one unless indicated to the contrary or otherwise evident from the context.

As used herein, the term "comprising" or "including" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term "comprising" or "including" also includes "consisting of". The variations of the word "comprising", such as "comprise" and "comprises", and "including", such as "include" and "includes", have correspondingly varied meanings.

As used herein, the term "conditioned media" refers to a cell culture supernatant enriched with secretomes (preferably secretomes of interest) from cultivated cells. The secretomes may be induced (for example, with magnetic induction) or released constitutively. For example, a cell culture comprising myoblast cells may be magnetically induced to produce secretomes that

promotes proliferation. Accordingly, the resultant conditioned media is enriched with proliferation-enhancing secretomes and thus may promote proliferation.

As used herein, the term "conditioning recipient cells" refers to providing/feeding recipient cells with a conditioned media to influence the recipient cells' biological processes such as proliferation, differentiation and/or senescence. For example, recipient cells may be conditioned with a conditioned media capable of promoting proliferation and thus, the recipient cells will show enhanced proliferation upon conditioning.

As used herein, the term "promoting proliferation", "enhancing proliferation" or any other of equivalent grammatical meaning thereof may be used interchangeably and refer to the improvement of one or more characteristics and/or functions of cellular proliferation (i.e., the process of generating an increased number of cells through cell division) in a treated/recipient cell as compared to a control cell (for example, a cell cultured in the conditioned medium of the present invention compared to a control cell cultured in normal growth medium). Examples of characteristics and/or functions of cell proliferation would be understood by those skilled in the art to include, but not limited to, rate of cell division, rate of cell growth, cell size, upregulation of certain proliferative signalling, downregulation of growth suppressors etc.

As used herein, the term "promoting differentiation", "enhancing differentiation" or any other of equivalent grammatical meaning thereof may be used interchangeably and refer to the improvement of one or more characteristics and/or functions of cellular differentiation (i.e., the process of converting one cell type into another cell type, typically from an immature unspecialized, cell to a mature, specialized form and function) in a treated/recipient cell as compared to a control cell (for example, a cell cultured in the conditioned medium of the present invention compared to a control cell cultured in normal growth medium). Examples of characteristics and/or functions of cell differentiation would be understood by those skilled in the art to include, but not limited to, rate of change, change in morphological structures such as cell shape, cell size, membrane potential, and metabolic activities, upregulation of certain differentiation-related signalling etc.

As used herein, the term "promoting senescence", "enhancing senescence" or any other of equivalent grammatical meaning thereof may be used interchangeably and refer to the improvement of one or more characteristics and/or functions of cellular senescence (i.e., the process by which a cell ages and permanently stops dividing but does not die) in a treated/recipient cell as compared to a control cell (for example, a cell cultured in the conditioned medium of the present invention compared to a control cell cultured in normal growth medium). Examples of characteristics and/or functions of cell senescence would be

understood by those skilled in the art to include, but not limited to, morphological changes such as flattened and enlarged morphology, presence of molecular markers such as senescence-associated heterochromatin foci (SAHF), expression of tumour suppressors and cell cycle inhibitors etc.

A description of exemplary, non-limiting embodiments of the invention follows.

The present invention is based, in part, on the concept of applying molecular confinement and/or macromolecular crowding to improve cell secretome production, stability and/or efficacy. To this end, the inventors have found that impregnating cells in polyacrylic acid polymer-based gel such as carbomer gel, and/or mixing cells with a polyacrylic acid polymer or collapsed carbomer gel prior to applying pulsing electromagnetic fields (PEMFs) to induce secretome production advantageously enhances the stability, viability and/or potency of the secretomes produced. In this regard, the inventors have successfully employed a carbomer gel-based paradigm and developed, inter alia, a conditioned media with enhanced secretome properties.

Accordingly, provided in one aspect is a method of producing a conditioned medium (CM) capable of conditioning recipient cells, the method comprises the steps:

(i) providing an agent that is a particulate or a polymer or a combination thereof capable of forming a material-spanning microstructure such as a gel-like microstructure;

(ii) mixing progenitor and/or stem cells with the agent of step (i), preferably at a pH suitable for cell culturing, to obtain a mixture,

(iii) exposing the mixture to low amplitude pulsed electromagnetic fields (PEMFs),

(iv) incubating the mixture preferably at a temperature suitable for cell culturing for a time period,

(v) centrifuging the mixture to obtain a supernatant, and

(vi) collecting the supernatant to obtain the conditioned medium (CM), and optionally ultracentrifuging the supernatant to obtain separate components of the CM, consisting of a vesicular fraction separated from the soluble secretome components.

In one embodiment, the material-spanning microstructure throughout the mixture and the progenitor and/or stem cells are suspended in the material-spanning microstructure, upon mixing.

In another embodiment, the agent is provided in a suspension and forms the material-spanning microstructure, such as a gel, in the suspension, prior to mixing with said cells in step (ii).

Preferably, the mixing of the cells in step (ii) causes the cells to be suspended within the material-spanning microstructure in the suspension mixture. In this regard, the cells are thus confined within the material-spanning microstructure network, reducing its mobility and affecting its interactions and behaviour. Advantageously, the entrapment and confinement of the cells within the material-spanning structural network enhances the cell secretome bioactive properties, such as increased stability and potency. Additionally, the material-spanning microstructure provides substrate support for cells in three-dimensions during magnetic exposure for improved cell survival.

As would be appreciated by a person skilled in the art, the mixing in step (ii) is preferably carried out at a pH suitable for cell culturing in order to promote optimum cell growth, metabolism, and overall health. In some embodiments, the mixing is carried out at a pH of about 6 to about 8. In some embodiments, the mixing in step (ii) is carried out at a pH of about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, about 7.5, about 7.6, about 7.7, about 7.8, about 7.9, and about 8.0. In some embodiments, the pH is about 7.4.

In some embodiments, the method further comprises the step of disrupting the material-spanning microstructure prior to centrifuging the mixture in step (v). It would be appreciated by a skilled person that disrupting the material-spanning network in this regard breaks the physical entanglements and releases the cells and its secretomes from the confined spaces into a more fluid environment, thus facilitating its recovery.

In some embodiments, the disruption of the material-spanning structure is carried out by the addition of a substance such as a salt, or by a change in conditions, such as temperature or pH. For example, the salt suitable for the disruption may be, but not limited to, NaCl, $MgCl_2$, $CaCl_2$ and/or combinations thereof.

It would be appreciated that the disruption step should not only facilitate the recovery of the secretomes and/or the conditioned media, it should also take into account the safety considerations with respect to human use. For example, the disrupting agent should be non-toxic, in a concentration or amount that is within a pre-determined safe value, and/or does not disrupt electrochemical gradients within the cell. Thus in some embodiments, the disruption is carried out by adding at least 10 mM, at least 15 mM, at least 20 mM, at least 25 mM, at least

30 mM, at least 35 mM, at least 40 mM, at least 50 mM, at least 55 mM, at least 60 mM, at least 70 mM, at least 75 mM, at least 80 mM, at least 85 mM, at least 90 mM, at least 95 mM, at least 100 mM $CaCl_2$. In some embodiments, the amount of $CaCl_2$ added is about 10 mM to about 50 mM, about 10 mM to about 20 mM, about 10 mM to about 30 mM, about 10 mM to about 40 mM, about 10 mM to about 50 mM, about 10 mM to about 60 mM, about 10 mM to about 70 mM, about 10 mM to about 80 mM, about 10 mM to about 90 mM, and about 10 mM to about 100 mM $CaCl_2$. In some embodiments, the amount of $CaCl_2$ added is about 25 mM.

In another embodiment, the disruption is carried out by adding at least 10 mM, at least 15 mM, at least 20 mM, at least 25 mM, at least 30 mM, at least 35 mM, at least 40 mM, at least 50 mM, at least 55 mM, at least 60 mM, at least 70 mM, at least 75 mM, at least 80 mM, at least 85 mM, at least 90 mM, at least 95 mM, at least 100 mM $MgCl_2$. In some embodiments, the amount of $CaCl_2$ added is about 10 mM to about 15 mM, about 10 mM to about 20 mM, about 10 mM to about 30 mM, about 10 mM to about 40 mM, about 10 mM to about 50 mM, about 10 mM to about 60 mM, about 10 mM to about 70 mM, about 10 mM to about 80 mM, about 10 mM to about 90 mM, and about 10 mM to about 100 mM $MgCl_2$. In some embodiments, the amount of $MgCl_2$ added is about 25 mM.

In some embodiments, the disruption is carried out by adding at least 10 mM, at least 20 mM, at least 30 mM, at least 40 mM, at least 50 mM, at least 60 mM, at least 70 mM, at least 80 mM, at least 90 mM, at least 100 mM, at least 110 mM, at least 120 mM, at least 130 mM, at least 140 mM, at least 150 mM, at least 200 mM, at least 300 mM, at least 400 mM, at least 500 mM, at least 600 mM, at least 700 mM, at least 800 mM, at least 900 mM, at least 1000 mM, at least 1100 mM, at least 1200 mM, at least 1300 mM, at least 1400 mM, at least 1500 mM NaCl.

In some embodiments, the amount of NaCl added is about 10 mM to about 50 mM, about 10 mM to about 60 mM, about 10 mM to about 70 mM, about 10 mM to about 80 mM, about 10 mM to about 90 mM, about 10 mM to about 100 mM, about 10 mM to about 150 mM, about 10 mM to about 200 mM, about 10 mM to about 300 mM, about 10 mM to about 500 mM, about 10 mM to about 1000 mM, about 10 mM to about 1500 mM NaCl. In some embodiments, the amount of NaCl added is about 110 mM.

In another embodiment, the method further comprises the step of filtering the supernatant in step (v) prior to step (vi). In some embodiments, the supernatant is filtered through a filter with a pore size of around 0.2 to around 0.45 microns. In some embodiments, the pore size is 0.2 micron, 0.21 micron, 0.22 micron, 0.23 micron, 0.24 micron, 0.25 micron, 0.25 micron, 0.26 micron, 0.27 micron, 0.28 micron, 0.29 micron 0.30 micron, 0.31 micron, 0.32 micron, 0.33

micron, 0.34 micron, 0.35 micron, 0.36 micron, 0.37 micron, 0.38 micron, 0.39 micron, 0.40 micron, 0.41 micron, 0.42 micron, 0.45 micron or 0.45 micron.

In some embodiments, the method also comprises the step of ultrasonicating the suspension comprising the material-spanning microstructure in step (i), prior to the mixing in step (ii). In this regard, the ultrasonication may be conducted in an ice bath for 2.5 minutes at 30% amplitude. Advantageously, the ultrasonication paradigm of the present disclosure provides enhanced pro-proliferative capacity of the conditioned media and/or the extracted extracelluar vesicles.

In another embodiment, the material-spanning microstructure in the suspension in step (i) may be disrupted prior to mixing with the cells in step (ii). Similarly, the disruption may be carried out by the addition of a substance such as a salt, or by a change in conditions, such as temperature or pH, as herein described for other embodiments of the present disclosure. For example, the salt suitable for the disruption may be, but not limited to, NaCl, $MgCl_2$ and $CaCl_2$. Accordingly, the amount of salt to be used herein are similarly as earlier described for the other embodiments.

In this alternative paradigm, while the donor cells are not trapped within the material-spanning microstructure, it appears that macromolecular crowding and confinement still take effect, probably due to the large molecular size of the agent. Surprisingly, this "collapsed" paradigm also advantageously enhances, amongst other things, the stability and potency of the cell secretomes.

In some embodiments, the method further comprises the step of filtering the disrupted suspension prior to mixing with the cells in step (ii). In some embodiments, disrupted suspension is filtered through a filter with a pore size of around 0.2 to around 0.45 microns. In some embodiments, the pore size is 0.2 micron, 0.21 micron, 0.22 micron, 0.23 micron, 0.24 micron, 0.25 micron, 0.25 micron, 0.26 micron, 0.27 micron, 0.28 micron, 0.29 micron 0.30 micron, 0.31 micron, 0.32 micron, 0.33 micron, 0.34 micron, 0.35 micron, 0.36 micron, 0.37 micron, 0.38 micron, 0.39 micron, 0.40 micron, 0.41 micron, 0.42 micron, 0.45 micron or 0.45 micron.

In a more preferred embodiment, the suspension comprising the material-spanning microstructure of step (i) may be ultrasonicated prior to the disruption of the material-spanning microstructure in the suspension. Preferably, the ultrasonication may be conducted in an ice bath for 2.5 minutes at 30% amplitude.

The methods described herein may be adapted to produce an enhanced CM comprising a secretome of a specific characteristic for a specific developmental objective (such as proliferation, differentiation or senescence), by modulating the direction of magnetic field exposure used. For example, the application of a downward magnetic field may produce a CM capable of enhancing proliferation. In another example, the application of a downward magnetic field may also produce a CM capable of enhancing differentiation. In another example, the magnetic field direction may be switched from up to down to obtain a CM with an enhanced senescence or proliferative capabilities.

A person skilled in the art would appreciate that in accordance with the mitohormetic principles, a mild PEMF exposure may induce low levels of oxidative stress that are adaptive and that stimulate the cell secretome, while stronger PEMF exposure may produce greater levels of oxidative stress that are instead damaging and detrimental to the cell's survival. Accordingly, the parameters of PEMF exposure may be modulated to optimise secretome production and release. In some embodiments, the progenitor and/or stem cells may be exposed to the PEMFs for a single 10-30-minute duration, a single 10-25-minute duration, a single 10-20-minute duration, a single 10-15-minute duration or a single 10-minute duration. In particular, the progenitor and/or stem cells may be exposed to the PEMFs for a single 10-minute duration. Preferably, the donor progenitor and/or stem cells may be exposed to the PEMFs for no less than 10 min and/or no longer than 30 min. In this regard, it would be appreciated that a shorter duration of magnetic exposure may be insufficient to condition the medium while a longer duration of exposure may result in stress factors being released and thus contaminate said medium. In some embodiments, a minimum of at least 10 min of PEMF exposure may be required to obtain the most efficacious secretome production and release.

In some embodiments, the PEMF exposure in step (iii) is: a) for a single 10 - 30 minute duration, and/or b) at an amplitude of 1 - 3 mT, preferably 1.5 mT, and/or c) in 20 X 150 µs on and off pulses for 6 ms at a repetition frequency of 15 to 75 Hz, preferably at about 50Hz.

In some embodiments, the PEMF is an upward-directed or a downward-directed PEMF. Preferably, the PEMF is a downward-directed PEMF.

In some embodiments, the PEMF exposure is for a single 10 minute duration in a downward direction, at an amplitude of 1.5 mT, and in 20 X 150 µs on and off pulses for 6 ms at a repetition frequency of 50 Hz.

In other embodiments, the incubation in step (iv) may be for at least 30 min, at least 1 hour, at least 1.5 hours, at least 2 hours, at least 2.5 hours, at least 3 hours, at least 3.5 hours, at least

4 hours, at least 4.5 hours or at least 5 hours. In some embodiments, the incubation step in step (iv) is at least 1-2 hours, at least 1-3 hours, at least 1 to 4 hours or at least 1-5 hours.

As would be appreciated by a skilled person, the incubation in step (iv) is preferably carried out at a temperature that is suitable for cell culturing to facilitate cell growth and productivity. Accordingly, the incubation may be carried out at a temperature of about 25°C to 40 °C, or about 30°C to about 40°C. In some embodiments, the incubation is carried out at a temperature of about 25 °C, about 26 °C, about 27 °C, about 28 °C, about 29 °C, about 30 °C, about 31 °C, about 32 °C, about 33 °C, about 34 °C, about 35 °C, about 36 °C, about 37 °C, about 38 °C, about 39 °C, and about 40 °C. Preferably the incubation temperature is about 37 °C.

As would be appreciated by a skilled a person, centrifugation is required to separate cells from supernatant, and that the time required may be dependent on the volume of the medium to be centrifuged. Accordingly in some embodiments, the centrifugation is for a period of about 5 min to about 30 min, or about 10 min to about 60 min. In some embodiments, the centrifugation period is at least 5 min, at least 10 min, at least 15 min, at least 20 min, at least 30 min, at least 40 min, at least 50 min, or at least 60 min.

In the context of the present disclosure, the agent suitable for use is preferably a particulate or a polymer or a combination thereof capable of forming a material-spanning microstructure (such as a gel-like microstructure) in a suspension, and/or also capable of exerting confinement and or MMC effect. Thus in some embodiments, the agent is selected from poly(acrylic acid) polymer such as a linear poly(acrylic) acid polymer (PAA), poly(acrylic acid) homopolymer, poly(acrylic acid) copolymer, poly(acrylic acid) crosslinked polymer such as a carbomer (including carbopol), and polymethacrylates (PMAA), gelatin, sodium alginate, methylcellulose, poly(N-isopropylacrylamide), laponite and structural or chemical variants thereof. In some embodiments, the agent is a carbomer. In some other embodiments, the carbomer is carbopol 980 or carbopol 974.

In some embodiments, the concentration of the carbomer in the suspension in step (i) is about 0.05% w/v to about 5% w/v. In some embodiments, the carbomer concentration is at least 0.05% w/v, at least 0.1% w/v, at least 0.15% w/v, at least 0.2% w/v, at least 0.25% w/v, at least 0.30% w/v, at least 0.35% w/v, at least 0.4% w/v, at least 0.45% w/v, at least 0.5% w/v, at least 0.55% w/v, at least 0.6% w/v, at least 0.65% w/v, at least 0.7% w/v, at least 0.75% w/v, at least 0.8% w/v, at least 0.85% w/v, at least 0.9% w/v, at least 0.95% w/v, or at least 1.0% w/v. Preferably, the carbomer concentration is at least 0.5% w/v or at least 0.8% w/v.

In some embodiments, the progenitor and/or stem cells may be myoblast cells, neuronal stem cells, hematopoietic stem cells, dental pulp stem cells, fibroblast cells or mesenchymal stromal cells. Preferably, the progenitor and/or stem cells are myoblast cells.

In some embodiments, the hematopoietic stem cells may give rise to red blood cells, reticulocytes, and/or platelets. In this regard, the red blood cells, reticulocytes, and/or platelets may also be suitable to function as donor cells and be subjected to the PEMF induction paradigm as disclosed in the present disclosure for the production of a conditioned medium.

In some embodiments, recipient cells may be myoblast cells, neuronal stem cells, hematopoietic stem cells, dental pulp stem cells, mesenchymal stromal cells or fibroblast cells. In some embodiments, the recipient cells may also be red blood cells and other red blood cell types such as reticulocytes and/or platelets.

In some embodiments, the progenitor and/or stem cells may be differentiated, proliferating or senescent cells. Preferably, the progenitor and/or stem cells are differentiated or proliferating.

In some embodiments, the progenitor and/or stem cells have been prior expanded and/or conditioned to be in a proliferating, differentiating or senescent state in growth media or media of defined composition. Preferably, the progenitor and/or stem cells have been prior expanded and/or conditioned by the CM obtained from any of the method disclosed herein.

In a preferred embodiment, the method comprises the steps:

(i) providing a carbomer in a suspension at a pH of about 6 to about 8, preferably adjusted to 7.4, wherein the carbomer forms the material-spanning microstructure in the suspension;

(ii) dispersing myoblast cells within the suspension of step (i) to obtain a mixture, wherein the myoblast cells are dispersed within the material-spanning microstructure in the mixture;

(iii) exposing the mixture to a downward-directed, low amplitude pulsed electromagnetic fields (PEMFs) for about 10 min at an amplitude of 1.5 mT, at a frequency of 50 Hz;

(iv) incubating the mixture at 37°C for at least 1-2 hours,

(v) disrupting the mixture after incubation by adding salt selected from 110 mM NaCl, 25 mM $MgCl_2$ or 25 mM $CaCl_2$;

(vi) centrifuging the disrupted mixture in step (v) for 15 min to obtain a supernatant,

(vii) filtering the supernatant; and

(viii) collecting the filtered supernatant derived in step (vii) to obtain a conditioned medium (CM), and optionally ultracentrifuging the filtered supernatant to obtain a conditioned medium comprising extracellular vesicles (EV).

Preferably, the step (i) further comprises ultrasonicating the carbomer suspension for 2.5 minutes at 30% amplitude.

In another preferred embodiment, the method comprises the steps:

(i) providing a carbomer in a suspension at a pH of about 6 to about 8, preferably adjusted to about 7.4, wherein the carbomer forms the material-spanning microstructure in the suspension;

(ii) disrupting the material-forming microstructure in the carbomer suspension of step (i) by adding salt selected from 100 mM NaCl, 25 mM $MgCl_2$ or 25 mM $CaCl_2$;

(iii) filtering the disrupted suspension;

(iii) dispersing myoblast cells within the filtered disrupted suspension of step (iii) to obtain a mixture;

(iv) exposing the mixture of step (iii) to a downward-directed, low amplitude pulsed electromagnetic fields (PEMFs) for 10 min at an amplitude of 1.5 mT, at a frequency of 50 Hz;

(v) incubating the mixture at 37°C for at least 1-2 hours,

(vi) centrifuging the incubated mixture in step (v) for 15 min to obtain a supernatant, and

(vii) collecting the supernatant derived in step (vi) to obtain the conditioned medium (CM), and optionally ultracentrifuging the supernatant to obtain a conditioned medium comprising extracellular vesicles (EV).

In some embodiments, the step (i) further comprises ultrasonicating the carbomer suspension for 2.5 minutes at 30% amplitude.

In a second aspect, there is provided a conditioned medium capable of conditioning cells, produced by any of the methods disclosed herein.

In a third aspect, there is provided a method of stabilizing cell extracts and secretome, comprising contacting an efficacious amount of the conditioned medium of the third aspect with said cell extracts and secretome.

In a fourth aspect, there is provided a method of pre-conditioning proliferating, differentiating or senescent (oxidatively stressed) progenitor and/or stem cells for use in the production of a PEMF-conditioned media, wherein the method comprises contacting a sample of proliferating, differentiating or senescent (oxidatively stressed) progenitor and/or stem cells with the CM according to the second aspect.

In some embodiments, the contacting with the CM enhances the secretory response of said proliferating, differentiating or senescent (oxidatively stressed) progenitor and/or stem cells upon later exposure to PEMF. Advantageously, exposing donor cells to CM of the present disclosure in their early growth enhances their secretory response upon later exposures to PEMF. In this regard, the cell pre-conditioning paradigm of the present disclosure thus may provide the production of super secretors.

In a fifth aspect, there is provided a use of the conditioned medium disclosed herein in conditioning recipient cells.

Unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in various embodiments, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. "About" in reference to a numerical value generally refers to a range of values that fall within ±10%, in some embodiments ±5%, in some embodiments ±1%, in some embodiments ±0.5% of the value unless otherwise stated or otherwise evident from the context. In any embodiment in which a numerical value is prefaced by "about", an embodiment in which the exact value is recited is provided. Where an embodiment in which a numerical value is not prefaced by "about" is provided, an embodiment in which the value is prefaced by "about" is also provided. Where a range is preceded by "about", embodiments are provided in which "about" applies to the lower limit and to the upper limit of the range or to either the lower or the upper limit, unless the context clearly dictates otherwise. Where a phrase such as "at least", "up to", "no more than", or similar phrases, precedes a series of numbers, it is to be understood that the phrase applies to each number in the list in various embodiments (it being understood that, depending on the

context, 100% of a value, e.g., a value expressed as a percentage, may be an upper limit), unless the context clearly dictates otherwise. For example, "at least 1, 2, or 3" should be understood to mean "at least 1, at least 2, or at least 3" in various embodiments. It will also be understood that any and all reasonable lower limits and upper limits are expressly contemplated.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Green and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2012).

Methods and Materials

Carbopol Gel Preparation

Suspensions of Carbopol gel (CPG) were prepared by slowly adding the as-received Carbopol® 974P (CP 974) or Carbopol® 980 (CP 980) powder (Lubrizol Corporation, Wickliffe, Ohio, United States) to High glucose Dulbecco's Modified Eagle Medium (DMEM; Cytiva SH30243.01; Cytiva, Marlborough, United States) at varying concentrations while stirring. After one hour of stirring, the pH of the CPG suspension was adjusted to 7.4 by adding 1M sodium hydroxide (NaOH) solution prepared from NaOH pellets (Sigma-Aldrich, St. Louis, Missouri, United States) under continuous stirring.

For preparing ultrasonicated CPG (UCPG), the previously described CPG using CP 974 was sonicated in an ice bath for 2.5 minutes at 30% amplitude using a FB-505 sonic dismembrator ultrasonic processor (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a 0.5-inch diameter probe. When necessary, the 0.8% UCPG was diluted with DMEM to a concentration of 0.08%.

Linear polyacrylic acid gel (LPAAG) was prepared at a concentration of 0.8% following the previously described CPG preparation steps, substituting the polymer powder with L-PAA powder (CAS number 9003-01-4, Sigma-Aldrich, St. Louis, Missouri, USA). Dilution with DMEM was done when necessary.

Rheological Characterisation

The presence or absence of the ability to indefinitely suspend cells was confirmed via rheological characterisation, where the presence of a stress plateau at low shear rates indicates a yield stress (σ_Y^{HB} from fitting the Herschel-Bulkley constitutive model) and that cells will remain suspended indefinitely for σ_Y^{HB} greater than approximately 0.01 Pa.

All samples were degassed by using a vacuum chamber before rheological studies. The shear stress was recorded as a function of applied shear rate using a Discovery Hybrid Rheometer HR-30 (TA Instruments, New Castle, DE, USA) equipped with a Peltier temperature controller set to 37°C to simulate cell culture condition. All the measurements were conducted using a concentric cylinder fixture with (546011.941 HA Aluminum Conical DIN Rotor). A stress plateau was observed at low shear rates which is indicative of an apparent yield stress as shown in FIG. 1.

Cell Secretome Conditioning

The conditioning of Carbopol gel (CPG) consists of the following steps:

1. Suspend C2C12 myoblasts in CPG (75,000 cells/ml)
2. Expose the CPG with cells to pulsed electromagnetic fields (PEMF) at 0 mT or 1.5 mT in the downwards direction
3. Incubate the gels in a 37°C incubator for 1 – 2 hours

Ultrasonication

Where applicable, the carbopol gel is ultrasonicated in an ice bath for 2.5 minutes at 30% amplitude using a FB-505 sonic dismembrator ultrasonic processor.

Collapse of Carbopol Gel and Secretome Recovery

The collapse of Carbopol gel (CPG), ultrasonicated Carbopol gel (UCPG) and linear poly-acrylic acid gel (LPAAG) was induced by adding magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), or sodium chloride (NaCl) (Sigma-Aldrich, St. Louis, Missouri, United States) salt. Salt concentrations were chosen to balance cell viability, optimum pH conditions, and recovery of conditioned media. To determine the media recovery efficiency after collapsing, the gels (without conditioning) were centrifuged at 6000 rpm for 15 minutes. The resulting supernatant (CPS) was then recovered via pipetting.

After conditioning, recovered supernatant was provisioned to pre-plated C2C12 recipient cells for cell response analysis. Each well of the recipient cells received 2 ml of supernatant.

Isolation of Extracellular Vesicles

For the isolation of extracellular vesicles, ultracentrifugation was performed on the supernatant to obtain the EV fraction. The EV fractions were reconstituted in DMEM and given pre-plated recipient cells as previously described in Wong CJK et al. (2022), incorporated herein by reference. In this regard, the supernatant was subsequently ultra-centrifuged at 120,000 g for 2 h using Quick-Seal Round-Top ultracentrifuge tubes on Optima XPN-100 Ultracentrifuge (Beckman Coulter, USA) at 4 °C to isolate EVs. EVs were resuspended in 300 µl of basal DMEM; 100 µl of the exosome suspension was given to each of three technical replicates of pre-plated C2C12 myoblasts in 2 ml of fresh growth media. Recipient cells were allowed to grow for 24 h before cell enumeration using the Trypan Blue assay, unless otherwise indicated.

Filtration

Where applicable, filtration is carried out using a syringe filter with 0.22 micron pore sizes (Millex™ PVDF syringe filter)

Pulsed Electromagnetic Fields (PEMFs) Exposure

The PEMF signal used in this study has been described and myogenically characterized in previous studies (Yap, J. L. Y. et al. (2019); Tai, Y. K. et al. (2020), incorporated herein by reference). Briefly, the employed PEMF devices produce spatially homogeneous, time-varying magnetic fields, consisting of barrages of 20 X 150 µs on and off pulses for 6 ms at a repetition frequency of 15 or 50 Hz. The magnetic flux density plateaued at a predetermined amplitude of 1.5 mT within ~50 ms (~17 T/s). As previously described (Crocetti, S. et al., (2013)), all tissue culture flasks, dishes or tubes were placed within a region of greatest magnetic field uniformity within which the entirety of the vessel is exposed evenly to the traversing magnetic field lines. All PEMF-treated samples were compared with time-matched control samples (0 mT) that were manipulated in the same way as the experimental samples, including placement into the PEMF-generating apparatus for the designated time, except that the apparatus was not set to generate a magnetic field. All magnetically-stimulated samples in this study were exposed once for a duration of 10 min to 1.5 mT amplitude PEMFs of different field line directionalities.

Field Directionality

Employed in this study were an *in vitro* coil system (Coil 1) (Yap, J. L. Y. et al. (2019)), animal coil system (Coil 2) (Fields at Work, Zürich), human leg coil system (Coil 3) (FLEX LTD. Singapore), human arm coil system (Coil 4) (HOPE Technik PTE. LTD. Singapore) and human breast cancer coil (Coil 5) (Tai, Y. K. et al. (2021)). All coil systems were designed to generate

analogous magnetic fields as described above. PEMF Coil systems 1 and 2 were designed with an in-built capability to electronically switch field directionality, upwards or downwards. Briefly, the directionality of field exposure is a function of the direction of the current flowing through the field generating coil sub-assemblies. By changing the direction of the current flow, the field direction can be inverted. A polarity-switching H-bridge was implemented to allow for the switching of field directionality by reversing the signal current applied to the coil system. Changes in magnetic field directionality with PEMF coil systems 3, 4 and 5 were accomplished manually by changing the orientation of the coil system relative to the culture flask placed within the lumen of the coils. Field uniformity produced by the distinct coil systems was routinely validated using an ExpoM-ELF (Fields at Work, Zürich) low frequency magnetic fields exposure meter. Magnetically-induced C2C12 murine muscle cell proliferation enhancement was similar in magnitude with all PEMF devices.

Myoblast cultures were exposed to one or more of the following conditions: **1**) unexposed 0 mT; and **2**) 1.5 mT exposure in the downward direction. Culture dishes were placed within the indicated coil system in the intended horizontal (flat) position with the magnetic field lines oriented either parallel or perpendicular to the long axis of the plate, unless otherwise explicitly stated.

The cultures were incubated in a 37°C incubator for 24 h before enumeration using a Trypan Blue assay, unless otherwise stated.

C2C12 Recipient Cell Response

Assessment of secretome response was performed on C2C12 recipient cells seeded 24 h prior to the provision of conditioned media. C2C12 cells were seeded at 30,000 cells per well in a 6-well format and provisioned with 2 ml conditioned media for 24 h (as indicated), and growth of recipient cells was enumerated using Trypan Blue Exclusion Assay. For western analysis, recipient cells were subjected to standard RIPA lysis protocol and analyzed using SDS-PAGE gel electrophoresis.

Western Blot Analysis of Whole Cell Lysates

Protein extraction was performed using RIPA lysis buffer containing 50 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 1% Triton X-100, 0.05% SDS, EDTA-free 1X protease inhibitor cocktail (Nacalai Tesque Inc., Japan), 1X PhosSTOP™ phosphatase inhibitor (Merck, Germany) and 0.1% sodium deoxycholate (Merck, Germany). Whole cell lysates were collected using a cell scraper and incubated at 4 °C for 30 min before being spun at 12,000 rpm for 15 min at 4 °C. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher

Scientific, USA). Whole cell lysates were prepared in 4X Laemmli buffer with added β-mercaptoethanol (Bio-Rad Laboratories and Sigma Life Science, USA, respectively) and were boiled at 95 °C for 5 min. 20 – 25 µg of proteins from the whole-cell lysates were resolved using denaturing and reducing SDS-PAGE and transferred to PVDF membrane (Thermo Fisher Scientific, USA). The antibodies and dilution factors used are listed in Table 1.

Table 1. List of antibodies, vendors and employed dilution factors.

Protein target and catalogue number	**Vendor, Country**	**Dilution Factor**
Cyclin-dependent kinase inhibitor (P21) (sc-6246)	Santa Cruz Biotechnology, Texas, USA	1:300
Cyclin B1 (CB1) (CST 4138)	Cell Signaling Technology, Massachusetts, USA	1:1,000
Cyclin D1 (CD1) (sc-8396)	Santa Cruz Biotechnology, Texas, USA	1:300
Transient receptor potential canonical 1 (TRPC1) (sc-133076)	Santa Cruz Biotechnology, Texas, USA	1:300
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (60004-1-Ig)	Proteintech, Chicago, USA	1:10,000
Phospho-JNK (sc-6254) and Total JNK (sc-7345)	Santa Cruz Biotechnology, Texas, USA	1:300
Phospho-ERK (sc-7383) and Total ERK (s-514302)	Santa Cruz Biotechnology, Texas, USA	1:300

Results

Rheological Characterisation

Rheological behaviour of CPG (0.6 – 1% CP 974 and 0.5% CP 980) and Collapsed Gel 0.8% CP 974 in the presence of different salts were studied (FIG. 1). The 0.5% CPG 980 exhibited a minimal yield stress of 0.01 Pa. Meanwhile, both 0.8% and 1% CPG 974 showed a yield stress at least ~10 times higher than the minimum yield stress required to suspend cells. CPS

materials show no yield stress due to the addition of salt disrupting the material structure, with divalent salts (i.e. calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) having a more significant effect in decreasing the shear viscosity (values determined from shear stress divided by shear rate) than the monovalent salt (i.e. sodium chloride (NaCl)). This suggests that divalent salts are more effective in inducing the ionic-sensitive gel to de-swell, creating more free space within the gel and increasing media recovery efficiency.

Rheological behaviour of 0.8% UCPG was also studied (FIG. 2). Compared to 0.8% CPG, no plateau was observed, indicating more free volume in ultrasonicated materials.

LPAAG behaved similarly to UCPG.

Microscopy

The microstructural morphology of CPG and CPS were observed using Axiovert 5 Inverted Microscope with Phase Contrast coupled to an Axiocam 208 color digital camera (Carl Zeiss, Germany). The images were saved using Zeiss Labscope 4.0 software. Images in FIG. 3 and FIG. 4) were thresholded for improved visibility.

Comparison between 0.5% CPG 980 and 0.8% CPG 974

Microscopic images showing CP aggregates in 0.5% CPG 980 and 0.8% CPG 974 are shown in FIG. 3. There was no significant difference in the CP aggregate sizes between these two CPGs, yet more free space (white area) was observed in CPG 980 compared to CPG 974. This observation aligns with the higher yield stress observed in 0.8% CPG 974 as shown in the rheological study results.

Comparison between CPG 974 prepared with varying CP concentrations and Collapsed Gel CP 974 due to salt additions

The microscopic images illustrating CP aggregates in CPG prepared with varying CP concentrations and Collapsed Gel 0.8% CP 974 with different salt additions are shown in FIG. 4.

CP aggregates in 0.6% CPG exhibited larger sizes compared to those in 0.08% CPG, likely due to aggregation. However, further increasing the CP concentration to 0.8% and 1% resulted in decreased CP aggregate sizes due to increased compression in the jammed microstructure (confirmed through the emergence of a yield stress in rheological results in FIG. 1). The size of CP aggregates in Collapsed Gel 0.8% CP 974 under different salt conditions follows the order: NaCl < control < $MgCl_2$ < $CaCl_2$. The addition of NaCl salt is believed to shield the repulsion between carboxyl groups on CP chains, reducing CP particle/aggregate swelling. In

contrast, divalent salts not only shield repulsion but also form bridges between the COO- groups on CP chains, resulting in larger CP aggregates and more hydrophobic CP particles. The volume fraction of CP aggregates in images of Collapsed Gels appear high due to sedimentation of aggregates at the imaging plane.

Comparison between CPG 974, UCPG 974 and LPAAG concentrations at 0.08% and 0.8%

Microscopic images illustrating aggregates in CPG, UCPG and LPAAG at 0.08% and 0.8% concentrations are shown in FIG. 5. Among all gel samples, the aggregates in CPG exhibited the largest sizes as they are made of crosslinked LPAA chains. LPAAG showed larger particle sizes compared to UCPG at both 0.08% and 0.8%, likely due to aggregation of the linear polymers.

CPS Recovery

Comparison of supernatant obtained at different CP and salt concentrations

The supernatant obtained at different CP and salt concentrations were compared (FIGs. 6A-6B). As CP concentrations increased, the volume of free CPS recovered decreased. The volume of CPS recovered after adding divalent salts was higher than that with the monovalent salt, with the pH remaining within an acceptable range. The free space within the samples can be estimated from the volume of CPS recovered. These volume recovery results correlate with the rheological study, where a higher volume of CPS recovery was associated with lower shear viscosity, indicating more free space within the material structure. The amount of supernatant recovered after centrifugation is relevant for the media yield in the final stage of the CPG workflow. The recovered percentage is also indicative of the free volume during PEMF conditioning and incubation in both the CPG and CPS cases.

CPS recovery from 0.8% CPG 974 using varying NaCl concentrations

The amount of CPS recovered from 0.8% CPG 974 using varying NaCl concentrations were studied (FIG. 7). As the NaCl concentration increased, the recovery percentage rose while the pH of the CPS decreased. Despite the NaCl concentration reaching 1500 mM, the recovery percentage remained around 80%.

CPS recovery from 0.8% CPG 974 using varying $CaCl_2$ concentrations

The amount of CPS recovered from 0.8% CPG 974 using varying $CaCl_2$ concentrations were studied (FIG. 8). The recovery percentage increased while the pH of CPS decreased with increasing $CaCl_2$ concentration, achieving almost full recovery at 50 mM with a pH of ~7.2.

Size Characterization

Size distributions of the particles in the samples were determined by a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., GB) by the Dynamic Light Scattering (DLS) technique.

Presence of residues in the CPS obtained from CPG 974

FIG. 9 shows the schematic workflow diagram of the steps in the experiment to determine the presence of residues in the CPS obtained from CPG 974. These results confirm the presence of residual particles in the CPS. After collapsing the CPG with salt, the recovered CPS was centrifuged and filtered through a 0.2 µm pore size. The filtered CPS was characterised using DLS and compared to both filtered blank DMEM and CPS recovered without salt (FIG. 10).

Determination of gel sizes in 0.08% UCPG and residue sizes in 0.08% UCPS

FIG. 11 shows the schematic workflow diagram for the experimental conditions utilized in determining the gel sizes in 0.08% UCPG and residue sizes in 0.08% UCPS.

Although the size distribution of UCPG was similar to the CPS obtained with and without salt (FIG. 10), the intensity of larger aggregates in UCPG was higher. The residue size of UCPS was larger than that of UCPG, indicating aggregation in the residues (FIG. 12). After filtration, the residue size distribution became more homogeneous and reduced in size. These DLS results were also supported by microscopic images (FIG. 13).

Example 2: CPG Improves Secretome Conditioning Post Magnetic Exposure

FIG. 14 shows a schematic workflow diagram of the various experimental conditions utilized in cell culture experiments of the present invention (for example, in Examples 2 – 16).

In this regard, the effect of impregnating cells in CPG prior to PEMF exposure is investigated. Recipient C2C12 cells were exposed to conditioned media from magnetically-induced recipient myoblast suspension cells that were incubated for 1 h, with or without Carbopol Gel (CPG) (CP 980 at 0.5% w/v). Significant increase in the cell growth of recipient cells receiving conditioned media with CPG shows that CPG improves secretome conditioning post magnetic exposure (FIG. 15)

Example 3: CPG enhanced C2C12 cell proliferation by increasing potency of magnetically induced conditioned media.

The effect on growth rate of C2C12 recipient cells (24 h proliferation) after the provision with 1.5 mT conditioned media was investigated.

Conditioned media were collected from C2C12 suspension cells after 1 h incubation post magnetic exposure. Donor suspension C2C12 cells in CPG (CP 980 at 0.5% w/v) were exposed to magnetic fields and left to condition the media for either 1 h or 2 h before the collection and provision to recipient C2C12 cells. Collection of conditioned media from donor cells suspended in CPG is associated with enhanced stability and potency of cell secretome that conferred improved cell proliferation responses in recipient C2C12 cells. (FIG. 16A).

The response of recipient C2C12 cells to exosomes (vesicular fraction) collected from donor suspension C2C12 myoblasts was also investigated. (FIG. 16B). The optimal conditioning time for the isolation of exosomes (for enhanced proliferation) is 60 min. C2C12 donor myoblasts were exposed to PEMF and incubated for 30 min to 120 min prior to the start of exosome isolation. The results of FIG. 16 shows that CPG enhanced C2C12 cell proliferation by increasing potency of magnetically induced conditioned media.

Example 4: Secretome from magnetically-stimulated C2C12 myoblasts suspended in CPG improves myogenic survival.

The effect of conditioned media from magnetically-stimulated C2C12 myoblasts suspended in CPG on the expression of A) phosphorylated JNK, B) phosphorylated ERK, C) Cyclin B1 and D) Cyclin D1, in recipient cells were investigated.

Recipient C2C12 myoblasts were previously seeded in 6-well plates (30,000 cells/well) for 24 h before the provision with secretome from suspension myoblasts exposed to magnetic fields in the presence of CPG (CP 980 at 0.5% w/v) (1 h post-magnetic conditioning). The myogenic effect of conditioned media in CPG was compared to recipients grown under standard DMEM or DMEM supplemented with FBS (DMEM + FBS). The protein analyses on recipient cells were performed 24 h after the provision of secretome or after media change to DMEM or DMEM + FBS, using standard RIPA lysis for Western Blotting. A reduction of p-JNK and an increase in p-ERK after magnetic exposure in the presence of CPG relative to DMEM + FBS indicated better survival. An increase in both Cyclin B1 and Cyclin D1 after magnetic exposure in the presence of CPG indicated increased cell proliferation.

The results of FIGs. 17A - 17D shows that secretome from magnetically-stimulated C2C12 myoblasts suspended in CPG improves myogenic survival.

Example 5: CPG enhanced the potency of conditioned media as well as extracellular vesicles (EVs; vesicular secretome factors).

The effects of C2C12 recipient cells' growth rate after exposure to conditioned media or EVs from donor C2C12 suspension cells after conditioning for 1 h post magnetic exposure were investigated (FIG. 18).

Fig 18 shows that the CPG (CP 980 at 0.5% w/v) enhanced the potency of conditioned media as well as extracellular vesicles.

Example 6: Collapsed CPG supernatant (CPS) exhibited greater secretome protective capacity than adding small amounts of CP to basal media prior to magnetically-induced cell secretome release and conditioning of the media.

FIGs. 19A – 19B show that CPS (CP 980 at 0.5% w/v) maintained secretome efficacy after 24 h and 48 h of storage post exposure.

CPS was generated by collapsing CPG (CP 980 at 0.5% w/v) with NaCl (110 mM) and then using the supernatant as conditioning media for C2C12 myoblasts (24 h proliferation). Prior addition of CP 980 (5%) to conditioned media collected after magnetic exposure did not improve the efficacy of the secretome after 24 h and 48 h of storage.

Example 7: Carbopol Gel Supernatants (CPS) Preserved the Potency of the PEMF Conditioned Media Following Storage at 4°C for 1 Week.

CPS was generated by collapsing CPG (CP 980 at 0.5% w/v) with NaCl (110 mM) and then using the supernatant as conditioning media for C2C12 myoblasts. FIG. 20 shows the relative fold change of C2C12 recipient cells relative to DMEM + FBS (foetal bovine serum) condition 24 h after the provisioning of conditioned media. Fresh conditioned media was compared to conditioned media stored at 4°C for 1 week. DMEM + FBS condition which was stored in 4°C for 1 week was used as a control. FIG. 20 shows that magnetic exposure (black bars) enhanced the potency of the conditioned media in all cases except storage without CPS.

Example 8: Carbopol Gel Supernatants (CPS) Preserved the Potency of the Extracellular Vesicles (EVs) Following Storage at 4°C for 24 h.

CPS was generated by collapsing CPG (CP 974 at 0.8% w/v) with NaCl (110 mM) and then using the supernatant (CPS) as conditioning media for C2C12 myoblasts before isolation of EVs. EV proliferative efficacy persists after storage for 24 h at 4°C in the presence of collapsed Carbopol supernatant. Collapsed Carbopol supernatant media performance experiences only a slight decrease, while the media without collapsed Carbopol supernatant has significantly degraded (24 h proliferation of C2C12 recipient cells). FIG. 21 shows the relative fold change of C2C12 recipient cells (24 h proliferation) after the provision with conditioned media.

Magnetic exposure (black bars) enhanced the potency of the EVs in all cases except storage without CPS (FIG. 21).

Example 9: Carbopol Gel Supernatants (CPS) Preserved the Potency of the PEMF Conditioned Media After Storage at 37°C for 2 or 6 Hours.

CPS was generated by collapsing CPG (CP 974 at 0.8% w/v) with NaCl (110 mM) and then using the supernatant as conditioning media for C2C12 myoblasts (24 h proliferation). Collapsed Carbopol gel supernatant (CPS) maintained secretome efficacy and outperformed the efficacy of DMEM + FBS media. cCM and pCM refer to media conditioned without and with 1.5 mT PEMF exposure. In this instance, pCM matched or surpassed the efficacy of FBS in all cases (FIG. 22).

Example 10: Collapsing Cell-Impregnated Carbopol Gels with Calcium Chloride ($CaCl_2$) Increases the Potency of the Rendered Conditioned Media.

The potency of the conditioned media generated from CPG (CP 974 at 0.8% w/v) following $CaCl_2$ (25 mM) collapsing was compared with that following 110 mM sodium chloride (NaCl) and 25 mM magnesium chloride ($MgCl_2$) collapsing. Bar chart shows proliferation of C2C12 recipient cells (24 h proliferation) after the provision with conditioned media. Magnetic exposure (1.5 mT) consistently enhanced the potency of the rendered conditioned media regardless of CPG or collapsing salt (FIG. 23).

Example 11: Collapsing Cell-Impregnated Carbopol Gels with Calcium Chloride ($CaCl_2$) Rendered a Conditioned Media That Best Promoted Myogenic Survival.

The myogenic potency of the conditioned media generated following $CaCl_2$ (25 mM) collapsing was compared with that following 110 mM sodium chloride (NaCl) and 25 mM magnesium chloride ($MgCl_2$) collapsing. Magnetic exposure (1.5 mT) best enhanced the myogenic potency of the rendered conditioned media. The effect of $CaCl_2$ was compared with other salts like 110 mM sodium chloride (NaCl) and 25 mM magnesium chloride ($MgCl_2$).

FIGs. 24A – 24F shows the relative protein expression (normalized to GAPDH) of A) phosphorylated JNK, B) phosphorylated ERK, C) TRPC1, D) Cyclin B1 and E) Cyclin D1, F) P21 from recipient cells. Recipient C2C12 myoblasts were previously seeded in 6-well plates (30,000 cells/well) for 24 h before the provision with secretome from suspension myoblasts exposed to magnetic fields in the presence of CPS (1 h post-magnetic conditioning). The myogenic effect of conditioned media in CPS (CP 974 at 0.8% w/v) was compared to recipients grown under standard DMEM or DMEM supplemented with FBS (DMEM + FBS). The protein analyses on recipient cells were performed 24 h after the provision of secretome

or after media change to DMEM or DMEM + FBS, using standard RIPA lysis for Western Blotting. A reduction of p-JNK and an increase in p-ERK after magnetic exposure in the presence of CPG relative to DMEM + FBS indicated better survival. An increase in Cyclin D1 (CD1) after magnetic exposure in the presence of $CaCl_2$ indicated increased cell proliferation.

Example 12: Cell-Impregnated Ultrasonicated CPGs (UCPG) Render Enhanced Conditioned Media Compared to Cell-Impregnated Carbopol Gels (CPG) or Carbopol Gel Supernatants (CPS).

FIG. 25 shows the relative fold change in C2C12 recipient cell number relative to DMEM alone (Bar 1) 24 hours after the provisioning of the specified conditioned media. C2C12 donor cells were conditioned in either CPG (CP 974 at 0.8% w/v), ultrasonicated CPG (UCPG) or CPS. Ultrasonicated CPG was generated by sonicating CPG in an ice bath for 2.5 minutes at 30% amplitude using a FB-505 sonic dismembrator ultrasonic processor as described in the methodology.

Example 13: Ultrasonicated Collapsed Carbopol Gel Supernatant (UCPS) Enhanced the Pro-Proliferative Capacity of isolated Extracellular Vesicles (EVs) Following Magnetic Exposure.

FIG. 26 showing the proliferation of C2C12 recipient cells 24 hours after the provision of EVs isolated from conditioned media derived from CPS (CP 974 at 0.8% w/v) (Bars 3-4), UCPS (Bars 5-6) or linear poly-acrylic acid (LPAA; Bars 7-8). Acrylic acid is the monomer of both CP and poly-acrylic acid. These results indicate that other PAA-based materials are also capable of sustaining proliferation, albeit not as strongly as CP.

Example 14: Resuspension of Extracellular Vesicles (EVs) In Ultrasonicated Collapsed Carbopol Gel Supernatant (UCPS) or Collapsed Carbopol Gel Supernatant (CPS) Following Stage One Conditioning in Collapsed Carbopol Gel Supernatant (CPS) Further Enhances EV Proliferative Efficacy.

FIG. 27 shows the proliferation of C2C12 recipient cells 24 hours after the provision of EVs isolated from conditioned media derived from UCPS (CP 974 at 0.8% w/v) (Bars 3-4; 7-8) or CPS (CP 974 at 0.8% w/v) (Bars 5-6; 9-10). The C2C12 donor cells were first conditioned in either CPS or fresh DMEM with (0 mT) or without (1.5 mT) magnetic exposure (Stage 1 conditioning). After ultracentrifugation, the EVs were then resuspended in either UCPS or CPS.

Example 15: Ultrasonication Enhanced the Potency of the Extracellular Vesicles (EVs) Rendered From CP Supernatants in a Time-Dependent Manner.

FIG. 28 shows the relative fold change in C2C12 recipient cell numbers relative to DMEM alone (Bar 1) 24 hours after the provision of EVs rendered from either UCPS (CP 974 at 0.8% w/v) or CPS (CP 974 at 0.8% w/v) conditioned for one or two hours. Magnetic exposure (10 minutes) and conditioning of C2C12 donor cells was performed within CPS or UCPS. Media conditioning was conducted for one or two hours at 37°C within a standard tissue culture incubator.

Example 16: Cell-Impregnated Carbopol 974 Gel and Collapsed Carbopol 974 Supernatant Increased the Potency of the Rendered PEMFed Conditioned Media as compared with conditioned media produced from Carbopol 980 Gel.

FIG. 29A shows that conditioned media from cell-impregnated Carbopol 974 Gel (CP 974 at 0.8% w/v) had increased potency than conditioned media from cell-impregnated Carbopol 980 Gel (CP 980 at 0.5% w/v). FIG. 29B shows that CPS collected from collapse of Carbopol 974 Gel increased the potency of the PEMFed conditioned media when compared with CPS from Carbopol 980 Gel. Bar charts show the proliferation of C2C12 recipient cells 24 h after provision with conditioned media.

Summary

We commence by dispersing and swelling the CP polymer particles within a basal cell culture media to produce a swollen CP particle gel (CPG). Cells are then dispersed within the CPG, a CPG containing evenly distributed cells is the result. The CPG creates an environment providing molecular confinement as well as macromolecular crowding that have been shown to facilitate the secretion of trophic factors and extracellular matrix proteins that slow protein degradation (Ross ML et al. (2020)). This aspect of the invention is amenable to the industrial environment where 3D bioreactors are the state of the art. In order for confinement and crowding to occur, the additive must be present at a sufficiently high concentration and volume fraction. If CP polymer particles are too dilute they will have negligible mechanical interaction with each other or distributed cells.

Secretome can then be further induced by brief (for example, 10 min) exposure to low energy (1.5 mT @ 50 Hz) and safe directionally-specified magnetic exposure (see PCT/SG2023/050453, incorporated herein fully by reference) or any other intervention amenable to the platform.

Alternatively, the structure of the CP polymer particles within the basal culture media may be disrupted or modified via ultrasonication to produce an Ultrasonicated Carbopol Gel (UCPG) prior to the dispersal of cells.

We next actively trigger the disruption of the polymer configuration by de-swelling the particles by increasing the salt concentration to a pre-determined safe value, facilitating recovery of the conditioned media. $CaCl_2$ (25 mM) or NaCl (110 mM) is not toxic to the cells and does not disrupt electrochemical gradients within the cell, unlike KCl, etc. Distinct from other applications where the collapse of the material structure is used to allow for recovery of embedded solid components (see for example, Nelson AZ et al. (2020)), in the present disclosure, the recovery target is the fluid media containing the cell secretome agents, residual collapsed additive, and dissolved salt without causing disruption of the cells which may contaminate the supernatant (conditioned media) with damaging intracellular agents such as proteases, ribonucleases, deoxyribonucleases, etc.

It has been previously shown that one hour of conditioning generated the greatest level of secretome from cells suspended in "clean" basal media after magnetic exposure, whereas the secretomes collected from cells in suspension for longer than one hour were less potent; unexposed cells showed low levels of secretome release that was time invariant ((see for example, Nelson AZ et al. (2020)).

In this disclosure, it is shown that the presence of poly(acrylic acid) in the form of therapeutic grade CP during cell secretome conditioning within CPGs is protective of the secretome opening numerous avenues to commercial exploitation. Allowing cells to condition the CPG environment results in greater levels of secretome recovery at a given time point compared to the cell conditioning of "clean" basal media alone as well as allows for longer conditioning periods of the CPG environment compared to conditioning of basal media alone.

In this regard, CP polymers act simultaneously to create an environment of molecular confinement as well as provide macromolecular crowding (MMC) agents that have been shown to promote cell survival, increase secretion and improve protein stability (resistance to degradation), i.e., CPGs provide both MMC agents and molecular confinement. MMC and molecular confinement are individually known to improve cell regeneration by facilitating secretion. Their combination gives synergistic effects.

Further, CPG provides substrate support for cells in three-dimensions during magnetic exposure for improved cell survival (i.e. improved cell health). In contrast, cells in suspension (without substrate support) experience stress after a few hours. This will be reflected in an inflammatory secretome being produced. Our experiments have shown that cells within CPGs are healthier and produce more beneficial secretomes.

Furthermore, the present paradigm also has minimal GMP barriers and low toxicity, considering CP is already being used for human use as a topical and ingestible additive and NaCl and $CaCl_2$ are safe and allowed for human consumption.

In our second paradigm, it is shown that the supernatant *per se* released from collapsed CPGs or UCPGs holds secretome protective capacity. Collapsed CPG supernatant (CPS) or collapsed ultrasonicated CPG supernatant (UCPS) that had been used as a conditioning media for magnetically exposed cells parallels the secretome stabilization factor of fetal bovine serum.

Importantly, collapsed CPG supernatant and collapsed UCPG supernatant had greater secretome protective capacity than adding small amounts of CP to basal media prior to magnetically-induced cell secretome release and conditioning of the media. This result indicates that the de-swelling step of CPGs produces PAA-based remnants of high biological efficacy.

Furthermore, employing the collapsed CPG supernatant as a conditioning media effectively improves secretome protection as well as reduces the level of CP in contact with the cells as well as within the secretome. Moreover, employing CPS as a conditioning media effectively reduces the level of CP remnants in the secretome. Thus, this provides secretome protection with decreased CP contamination (i.e. secretome protection with a lower level of CP remnants). This would serve to ease consumer acceptance and lower (already nominal) GMP barriers.

Other polyacrylic acid (PAA) -based materials are also capable of sustaining proliferation and improving secretome protection.

In view of the above, the platforms of the present disclosure may serve as a basis for the testing of similar agents which are capable of forming a material-spanning microstructure with distinct microstructure-disrupting or collapsing agents and/or agents with similar chemical properties that are cell and human safe. For example, polymeric agents such as gelatin, sodium alginate, methylcellulose, poly(N-isopropylacrylamide), and poly(acrylic acid) with differently engineered microstructure (e.g. crosslinking density, molecular weight, etc.) may be used as dilute suspensions, bulk gels or engineered into microgels, similar to CP. Particulate agents that may provide the necessary microstructure qualities may include laponite, silica, and bentonite. These can also have their chemical or microstructure disrupted/de-swelled/disintegrated with innocuous agents/stimuli such as temperature drop, calcium chelation, pH, ion concentration etc.

References

Bhattacharjee T, Gil CJ, Marshall SL, Urueña JM, O'Bryan CS, Carstens M, Keselowsky B, Palmer GD, Ghivizzani S, Gibbs CP, Sawyer WG, Angelini TE. Liquid-like Solids Support Cells in 3D. ACS Biomater Sci Eng. 2016 Oct 10;2(10):1787-1795. doi: 10.1021/acsbiomaterials.6b00218. Epub 2016 Jun 20. PMID: 33440476.

Crocetti, S., Beyer, C., Schade, G., Egli, M., Fröhlich, J., and Franco-Obregón, A. (2013) Low intensity and frequency pulsed electromagnetic fields selectively impair breast cancer cell viability. *PLoS One* 8, e72944.

Lee MH, Goralczyk AG, Kriszt R, Ang XM, Badowski C, Li Y, Summers SA, Toh SA, Yassin MS, Shabbir A, Sheppard A, Raghunath M. ECM microenvironment unlocks brown adipogenic potential of adult human bone marrow-derived MSCs. Sci Rep. 2016 Feb 17;6:21173. doi: 10.1038/srep21173. PMID: 26883894; PMCID: PMC4756694.

Nelson AZ, Kundukad B, Wong WK, Khan SA, Doyle PS. Embedded droplet printing in yield-stress fluids. Proc Natl Acad Sci U S A. 2020 Mar 17;117(11):5671-5679. doi: 10.1073/pnas.1919363117. Epub 2020 Mar 3. PMID: 32127482; PMCID: PMC7084155.

Ramalingam R, Jiang G, Larjava H, Häkkinen L. Macromolecular crowding regulates matrix composition and gene expression in human gingival fibroblast cultures. Sci Rep. 2023 Feb 4;13(1):2047. doi: 10.1038/s41598-023-29252-1. PMID: 36739306; PMCID: PMC9899282.

Ross ML, Kunkel J, Long S, Asuri P. Combined Effects of Confinement and Macromolecular Crowding on Protein Stability. Int J Mol Sci. 2020 Nov 12;21(22):8516. doi: 10.3390/ijms21228516. PMID: 33198190; PMCID: PMC7697604.

Tai, Y. K., Ng, C., Purnamawati, K., Yap, J. L. Y., Yin, J. N., Wong, C., Patel, B. K., Soong, P. L., Pelczar, P., Fröhlich, J., Beyer, C., Fong, C. H. H., Ramanan, S., Casarosa, M., Cerrato, C. P., Foo, Z. L., Pannir Selvan, R. M., Grishina, E., Degirmenci, U., Toh, S. J., Richards, P. J., Mirsaidi, A., Wuertz-Kozak, K., Chong, S. Y., Ferguson, S. J., Aguzzi, A., Monici, M., Sun, L., Drum, C. L., Wang, J. W., and Franco-Obregón, A. (2020) Magnetic fields modulate metabolism and gut microbiome in correlation with Pgc-1α expression: Follow-up to an in vitro magnetic mitohormetic study. *Faseb j* 34, 11143-11167.

Wong CJK, Tai YK, Yap JLY, Fong CHH, Loo LSW, Kukumberg M, Fröhlich J, Zhang S, Li JZ, Wang JW, Rufaihah AJ, Franco-Obregón A. Brief exposure to directionally-specific pulsed electromagnetic fields stimulates extracellular vesicle release and is antagonized by streptomycin: A potential regenerative medicine and food industry paradigm. Biomaterials.

2022 Aug;287:121658. doi: 10.1016/j.biomaterials.2022.121658. Epub 2022 Jul 6. PMID: 35841726.

Yap, J. L. Y., Tai, Y. K., Fröhlich, J., Fong, C. H. H., Yin, J. N., Foo, Z. L., Ramanan, S., Beyer, C., Toh, S. J., Casarosa, M., Bharathy, N., Kala, M. P., Egli, M., Taneja, R., Lee, C. N., and Franco-Obregón, A. (2019) Ambient and supplemental magnetic fields promote myogenesis via a TRPC1-mitochondrial axis: evidence of a magnetic mitohormetic mechanism. *Faseb j* 33, 12853-12872.

Zeugolis DI. Bioinspired in vitro microenvironments to control cell fate: focus on macromolecular crowding. Am J Physiol Cell Physiol. 2021 May 1;320(5):C842-C849. doi: 10.1152/ajpcell.00380.2020. Epub 2021 Mar 3. PMID: 33656930.

CLAIMS

1. A method of producing a conditioned medium (CM) capable of conditioning recipient cells, the method comprises the steps:

(i) providing an agent that is a particulate or a polymer or a combination thereof capable of forming a material-spanning microstructure;

(ii) mixing progenitor and/or stem cells in a media with the agent of step (i), preferably at a pH suitable for cell culturing, to obtain a mixture,

(iii) exposing the mixture to low amplitude pulsed electromagnetic fields (PEMFs),

(iv) incubating the mixture at a temperature suitable for cell culturing, for a time period,

(v) centrifuging the mixture to obtain a supernatant, and

(vi) collecting the supernatant to obtain the conditioned medium (CM), and optionally ultracentrifuging the supernatant to obtain separate components of the CM, consisting of a vesicular fraction separated from the soluble secretome components.

2. The method of claim 1, wherein the agent forms the material-spanning microstructure throughout the mixture and the progenitor and/or stem cells are suspended in the material-spanning microstructure.

3. The method of claim 1, wherein the agent in step (i) is provided in a suspension and forms the material-spanning microstructure in the suspension, prior to mixing with said cells in step (ii).

4. The method of claim 3, further comprising the step of ultrasonicating the suspension comprising the material-spanning microstructure in step (i), prior to the mixing in step (ii).

5. The method of any one of claims 2 to 4, the method further comprises the step of disrupting the material-spanning microstructure prior to centrifuging the mixture in step (v).

6. The method of any one of claim 2 to 5, further comprising the step of filtering the supernatant in step (v) prior to step (vi).

7. The method of claim 3, wherein the material-spanning microstructure in the suspension in step (i) is disrupted prior to mixing with the cells in step (ii).

8. The method of claim 7, further comprising the step of filtering the disrupted suspension prior to mixing with the cells in step (ii).

9. The method of claims 7 or 8, further comprising ultrasonicating the suspension comprising the material-spanning microstructure of step (i) prior to the disruption of the material-spanning microstructure in the suspension.

10. The method of any one of claims 1 to 9, wherein the PEMF exposure in step (iii) is:

a) for a single 10 - 30 minute duration, and/or

b) at an amplitude of 1 - 3 mT, preferably 1.5 mT, and/or

c) in 20 X 150 µs on and off pulses for 6 ms at a repetition frequency of 15 to 75 Hz, preferably at about 50Hz.

11. The method of claim 10, wherein the PEMF is a downward-directed PEMF.

12. The method of claim 11, wherein the PEMF exposure is for a single 10 minute duration, at an amplitude of 1.5 mT, and in 20 X 150 µs on and off pulses for 6 ms at a repetition frequency of 50 Hz.

13. The method of any one of claims 1 to 12, wherein the incubation in step (iv) is for at least 1-5 hours.

14. The method of any one of claims 1 to 13, wherein the incubation in step (iv) is carried out at a temperature of about 30 °C to about 40 °C, preferably at about 37 °C.

15. The method of claims 5 or 7, wherein the material-spanning microstructure is disrupted by the addition of a substance such as a salt, or a change in conditions, such as temperature or pH.

16. The method of claim 15, wherein the substance is a salt selected from the group consisting of NaCl, $MgCl_2$, $CaCl_2$ and combinations thereof.

17. The method of claim 16, wherein the material-spanning microstructure is disrupted by the addition of 10 to 200 mM NaCl, 10 – 50 mM $MgCl_2$ or 10 – 50 mM $CaCl_2$.

18. The method of any one of claims 1 to 17, wherein the centrifugation is for a period of 15 min.

19. The method of any one of claims 1 to 18, wherein the agent is selected from poly(acrylic acid) polymer such as a carbomer, a linear poly(acrylic acid) polymer, a poly(acrylic acid) homopolymer, poly(acrylic acid) copolymer, poly(acrylic acid) crosslinked polymer and/or a derivative thereof, gelatin, sodium alginate, methylcellulose, poly(N-isopropylacrylamide), laponite and structural or chemical variants thereof, wherein the agent is preferably carbomer.

20. The method of any one of claims 1 to 19, wherein the agent is a carbomer, preferably carbopol.

21. The method of claim 20, wherein the concentration of the carbomer in the suspension in step (i) is about 0.05% w/v to about 5.0% w/v.

22. The method of claim 21, wherein the concentration of the carbomer in the suspension in step (i) is about 0.5% w/v or about 0.8% w/v.

23. The method of any one of claims 1 to 22, wherein the progenitor and/or stem cells are myoblast cells, neuronal stem cells, hematopoietic stem cells, dental pulp stem cells, fibroblast cells or mesenchymal stromal cells.

24. The method of claim 23, wherein the progenitor and/or stem cells are myoblast cells.

25. The method of claims 23 or 24, wherein the progenitor and/or stem cells have been prior expanded and/or conditioned to be in a proliferating, or differentiating state, optionally wherein the progenitor and/or stem cells have been prior expanded and/or conditioned by the CM obtained from the method of any one of claims 1 to 24.

26. A conditioned medium capable of conditioning cells, produced by the method of any one of claims 1 to 25.

27. A method of stabilizing cell extracts and secretome, comprising contacting an efficacious amount of the conditioned medium of claim 26 with said cell extracts and secretome.

28. A method of pre-conditioning proliferating, differentiating or senescent (oxidatively stressed) progenitor and/or stem cells for use in the production of a PEMF-conditioned media, wherein the method comprises contacting a sample of proliferating,

differentiating or senescent (oxidatively stressed) progenitor and/or stem cells with the conditioned medium according to claim 26.

29. Use of the conditioned medium (CM) according to claim 26 in conditioning recipient cells.