

DISSOLVABLE MICROCARRIERS FOR CULTURING CELLS AND RELATED METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C §120 of U.S. Provisional Application Serial No. 62/861,084 filed on June 13, 2019, the content of which is relied upon and incorporated herein by reference in its entirety.

[0002] This application is related to commonly-assigned U.S. Patent Application Nos. 14/899,394 and 15/579,739, and U.S. Patent Nos. 8,404,85 and 8,426,176, the contents of which are relied upon and incorporated herein by reference in their entirety.

BACKGROUND

Field

[0003] The present disclosure relates generally to digestible substrates, and more specifically to digestible microcarriers that may be used, by way of example, for the isolation of proteins, cells, and viruses and also for diagnostic applications and cell cultivation, as well as methods of using digestible substrates, and more specifically to method of culturing and recovering cells, proteins, and viruses from digestible substrates.

Technical Background

[0004] Microcarriers enable efficient cell scale-up in controlled bioreactors for therapeutic applications. In contrast to cell culture on flat surfaces where adhesive cells can reach high confluence and thus limit cell expansion via cell-to-cell contact inhibition, spherically-shaped microcarriers having a high ratio of surface area/volume present an attractive platform for efficient cell culture scale-up or expansion where either harvested cells or conditioned media can be the desired product.

[0005] Incumbent to cell culture is adequate oxygenation and supply of nutrients to the cells. An associated challenge includes stirring of the microcarriers to provide the required oxygen and nutrients without introducing hydrodynamic stresses sufficient to damage the growing cells. Conventionally the stirring is done using impellers.

[0006] A further challenge involves separating the microcarriers from the cells or conditioned media. Enzymatic treatment may be used to harvest adhesive cells, for example, though the addition of enzymes can damage the cells. Proteolytic enzymes, for example, may non-selectively clear cell surface receptors.

[0007] Effective cell recovery from microcarriers often requires the use of concentrated enzymes, extended treatment times, and continuous centrifugation / filtration cycles, which can negatively affect cell health and recovery yield. Further, there are questions about possible by-products from microcarrier digestion and the effect such by-products may have on cell health and recovery yield.

[0008] In view of the foregoing, it would be advantageous to provide cell growth surfaces including microcarriers having with defined digestion by-products, as well as methods of effectively culturing and harvesting cells from growth surfaces while controlling digestion by-products.

BRIEF SUMMARY

[0009] In accordance with embodiments of the present disclosure, a cell culture article comprises a cell culture article is providing. The article includes a substrate having a polygalacturonic acid compound crosslinked with a divalent cation, the polygalacturonic acid compound selected from at least one of: pectic acid; partially esterified pectic acid, partially amidated pectic acid and salts thereof. The substrate is digestible by digestion reagents into components comprising of galacturonic acid monomers and the divalent cation.

[0010] In other embodiments, a method of harvesting cultured cells from a dissolvable substrate is provided. The method include separating the cultured cells from the substrate by digesting the substrate via exposure of the substrate to (i) a chelating agent, (ii) an enzyme, or (iii) a chelating agent and an enzyme, the separating resulting in a harvest solution; and

performing a series of wash and/or centrifugation cycles of components of a harvest solution following the separating. The substrate comprising a polygalacturonic acid compound selected from at least one of: pectic acid; partially esterified pectic acid, partially amidated pectic acid and salts thereof, and an adhesion polymer on the surface of the polygalacturonic acid compound.

[0011] Additional features and advantages of the subject matter of the present disclosure will be set forth in the detailed description which follows, and in part will be readily apparent to those skilled in the art from that description or recognized by practicing the subject matter of the present disclosure as described herein, including the detailed description which follows, the claims, as well as the appended drawings.

[0012] It is to be understood that both the foregoing general description and the following detailed description present embodiments of the subject matter of the present disclosure, and are intended to provide an overview or framework for understanding the nature and character of the subject matter of the present disclosure as it is claimed. The accompanying drawings are included to provide a further understanding of the subject matter of the present disclosure, and are incorporated into and constitute a part of this specification. The drawings illustrate various embodiments of the subject matter of the present disclosure and together with the description serve to explain the principles and operations of the subject matter of the present disclosure. Additionally, the drawings and descriptions are meant to be merely illustrative, and are not intended to limit the scope of the claims in any manner.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The following detailed description of specific embodiments of the present disclosure can be best understood when read in conjunction with the following drawings, where like structure is indicated with like reference numerals and in which:

[0014] Fig. 1 is a phase contrast image showing hMSC cells on externally crosslinked PGA microcarriers according to an embodiment;

[0015] Fig. 2 is a phase contrast image showing hMSC cells on externally crosslinked PGA microcarriers according to a further embodiment;

- [0016] Fig. 3 is a phase contrast image showing hMSC cells on externally crosslinked PGA microcarriers according to a still further embodiment;
- [0017] Fig. 4 shows phase contrast images showing MRC5 and Vero cells on gelatin-coated PGA microcarriers according to an embodiment;
- [0018] Fig. 5 is a graph of fold expansion for Vero cells on gelatin-coated dextran microcarriers and on gelatin-coated externally crosslinked microcarriers;
- [0019] Fig. 6 is a graph of fold expansion for MRC5 cells on gelatin-coated dextran microcarriers and on gelatin-coated externally crosslinked microcarriers;
- [0020] Fig. 7 is a graph of fold expansion for hMSC cells on both gelatin-coated digestible microcarriers and microcarriers provided with a Corning Incorporated Synthemax® II surface;
- [0021] Fig. 8 is a phase contrast image showing monodisperse PGA beads made according to an embodiment;
- [0022] Fig. 9 is a phase contrast image showing MRC5 cells on externally crosslinked PGA microcarriers according to an embodiment;
- [0023] Fig. 10 is a phase contrast image showing comparative microcarriers illustrating the broad size distribution obtain by emulsification and internal gelation; and
- [0024] Fig. 11 is a phase contrast image of hMSC cells in serum-free media after seeding on VN-grafted PGA microcarriers.
- [0025] Fig. 12 is a graph of the absorbance of PGA and digestion reagents using size-exclusion chromatography (SEC);
- [0026] Fig. 13 is a graph of absorbance of PGA polymer digestion by pectinase and EDTA using SEC;
- [0027] Fig. 14 is a graph mass spectrometry analysis of PGA digestion in water;
- [0028] Fig. 15 is a graph of PGA microcarrier digestion using SEC;
- [0029] Fig. 16 is a flow diagram of a method of washing and centrifuging a cell suspension, according to one or more embodiments;
- [0030] Fig. 17A is a graph of UV-VIS spectra of PGA and digestion components from an undiluted sample;

- [0031] Fig. 17B is a graph of UV-VIS spectra of PGA and digestion components from a diluted sample;
- [0032] Fig. 17C is a graph of the optical densities of PGA and digestion components at different wavelengths;
- [0033] Fig. 17D is a graph of the optical densities of the components from Fig. 17C before and after various washes;
- [0034] Fig. 18 are images of Synthemax II staining of dissolvable microcarriers;
- [0035] Fig. 19 are graphs of the fluorescence intensity of the staining in Fig. 19, as measured using a spectrophotometer;
- [0036] Fig. 20 are images of anti-Synthemax II antibody staining of dissolvable microcarriers;
- [0037] Fig. 21 is an image of a dot blot analysis of soluble Synthemax II in digestion solutions;
- [0038] Fig. 22A is a graph of Vero cell concentrations based on various dilutions of the harvest solution;
- [0039] Fig. 22B is a graph of Vero cell concentrations based on various dilutions of pectinase; and
- [0040] Fig. 22C is a graph of Vero cell concentrations based on various dilutions of EDTA.

DETAILED DESCRIPTION

- [0041] Reference will now be made in greater detail to various embodiments of the subject matter of the present disclosure, some embodiments of which are illustrated in the accompanying drawings. The same reference numerals will be used throughout the drawings to refer to the same or similar parts.
- [0042] Disclosed are cell culture articles that promote cell attachment and growth. According to some embodiments, the disclosed cell culture articles allow for cell harvesting without the use of protease. Example cell culture articles are microcarriers, which are also referred to as beads or microbeads (collectively “microcarriers”).
- [0043] In embodiments, the cell culture article is a smooth and transparent (or translucent) bead comprising a gel that includes pectic acid, partially esterified pectic acid, or salts thereof.

The cell culture articles may be spherical or substantially spherical and are formed by external gelation. The calcium content of the cell culture articles may be adjusted to afford rapid cell harvesting under mild conditions that mitigates damage to the cells. Molecules promoting the attachment of anchorage-dependent cells may be attached to the surface of the cell culture article by chemical coupling or physical adsorption.

[0044] In contrast to the presently-disclosed external gelation route, microcarriers may be formed via emulsification and internal gelation. With this internal gelation process, beads are formed via gelation of a polygalacturonic acid (PGA) aqueous solution containing an insoluble calcium salt dispersed in the aqueous phase, which is emulsified within an oil phase (also called a continuous phase or dispersion medium).

[0045] In the case of internal gelation, crosslinking is initiated by addition of an oil-soluble acid that releases soluble divalent metal ions (e.g., Ca^{2+} or Mg^{2+}) from the salt. With such a method, however, a large volume of the oil phase is required as is a significant amount of surfactant to stabilize the emulsion. While vegetable oils can be used as the continuous phase, beads prepared in this dispersion medium are difficult to rinse.

[0046] A further drawback to the internal gelation process is that a portion of the metal ion source (salt) may remain intact and manifest as heterogeneities in the microcarriers, which may compromise surface roughness and transparency. Further, such retained metal salt may be released over time during use of the microcarriers, which may be detrimental to cell culture or inhibit digestion of the microcarriers during cell harvest.

[0047] The disclosed external gelation methods provide an inexpensive and environmentally-friendly synthetic route for the preparation of highly-transparent PGA microcarriers that are free of undesired inclusions (second phases) and surface defects and which support non-proteolytic cell separation and harvesting. As defined herein, transparent microcarriers exhibit at least 90% transmission over the visible spectrum, i.e., 90, 92, 94, 96, 98, 99 or 100% transmission, including ranges between any of the foregoing values, from 390 to 700 nm.

[0048] In addition, in embodiments, uniform size distribution of the microcarriers can be provided. Uniform size distribution ensures faster and cleaner separation of microcarriers from supernatant during use. This can make medium exchange and final production isolation more

predictable, more reliable, and less expensive. In embodiments, microcarrier size can be precisely tuned to different ranges. This allows the settling speed of the beads to be customized to match different bioprocess needs without changing the material properties of the beads.

[0049] According to some embodiments, a calcium-crosslinked polygalacturonic acid (PGA) microcarrier is provided that can be dissolved using ethylenediaminetetraacetic acid (EDTA) and pectinase. Characterization of such PGA polymers and microcarriers, as well as dissociation agents, and by-products is discussed herein, and the impact of such components on subsequent cell growth is determined. Based on this, efficient PGA microcarriers and methods of culturing and harvesting cells using PGA microcarriers have been determined.

[0050] The byproducts of PGA microcarrier digestion (e.g., galacturonic acid monomers, calcium, EDTA, and pectinase) were characterized using Size Exclusion Chromatography (SEC), Electrospray Ionization Mass Spectrometry (ESI-MS), and Inductively-Coupled Plasma Mass Spectrometry (ICP-MS). UV-Vis Spectroscopy was used to show confirm that these soluble components can be removed through methods disclosed herein, which include a series of wash and/or centrifugation cycles.

[0051] Also as discussed below, by analyzing the location of the Synthemax® II coating on the microcarriers after dissolution using an antibody against the Synthemax® II peptide, it is shown that the majority of Synthemax® II for the PGA microcarriers of this disclosure becomes soluble after bead digestion. In some embodiments, where small residual amounts of Synthemax® II remain associated with released cells, this level can be reduced by adding a protease (e.g., trypsin) to the harvest solution.

[0052] The impact of digestion solution components, pectinase, and EDTA, on cell growth in 2D cultures was analyzed by adding back known concentrations of pectinase and EDTA to cells to identify the concentrations at which there was minimal impact to cell growth. According to some embodiments, methods including steps where the concentration of EDTA is kept at or below 1 mM and pectinase at or below 30 U/mL. In addition, the these components can be removed via wash and/or centrifugation or filtration prior to passaging or cryopreserving cells.

[0053] Thus, according to embodiments disclosed herein, microcarriers are provided that have reduced or removed digestion by-products. Additionally, by knowing or controlling the by-

products according to the articles and methods herein, practitioners can have increased knowledge and confidence in the use of the PGA microcarriers, and any biological and/or therapeutic implications.

[0054] Although some embodiments are directed to microcarriers, the substrates can take various forms and are not limited to microcarriers or beads. In some embodiments, the substrate can take a form suitable for use in a packed-bed bioreactor, and may be a foam scaffold having a cylindrical, rectangular, triangular, or disc shape, for example. Thus, according to embodiments, a dissolvable foam scaffold for cell culture is provided. The dissolvable foam scaffold includes an ionotropically crosslinked polygalacturonic acid compound selected from at least one of: pectic acid; partially esterified pectic acid, partially amidated pectic acid and salts thereof. In some embodiments, the scaffold can include at least one first water-soluble polymer having surface activity.

[0055] PGA Polymers

[0056] Polygalacturonic acid (PGA), or pectic acid, is a water-soluble biopolymer of pectin degradation found in ripe fruits and some vegetables. It is most well known in the food industry as a thickening agent, but can also be used as a matrix or binding agent for drug, protein, and cell delivery in pharmaceutical and biotechnology industries. The viscosity of the polymer is dependent on the molecular weight, degree of esterification, concentration, pH, and presence of counterions in solution. PGA polymers produce gels independent of sugar content, show little sensitivity to changes in pH, and require a defined amount of calcium, or other divalent cation, to control gelation. Microcarriers of PGA polymer can be made, for example, by dropping a solution of PGA into a calcium chloride bath, which results in quick gelation of the droplet as a spherical bead. Excess calcium chloride is removed from the microcarriers through a series of wash cycles before the microcarriers are coated with cell attachment-promoting substrates, such as Synthemax® II or denatured collagen.

[0057] PGA microcarriers can be destabilized by removal of calcium ions using ethylenediaminetetraacetic acid (EDTA). PGA polymers can be further degraded using the enzyme pectinase, which hydrolyzes the linkages between the monomeric units, and hence reduces the molecular weight of the PGA polymer and increases its solubility. In general,

pectinase is associated with fruit ripening, as it promotes the softening of plant cell walls; as such, pectinase has been issued a Generally Regarded As Safe (GRAS) notification by the FDA for use as a direct human food ingredient.

[0058] Microcarriers may be made using at least one ionotropically crosslinked polysaccharide. Examples include pectic acid, also known as polygalacturonic acid (PGA), or salts thereof, or partly esterified pectic acid (PE PGA) known as pectinic acid, or salts thereof.

[0059] Pectic acid can be formed via hydrolysis of certain pectin esters. Pectins are cell wall polysaccharides and in nature have a structural role in plants. Major sources of pectin include citrus peel (e.g., peels from lemons and limes) and apple peel. Pectins are predominantly linear polymers based on a 1,4-linked alpha-D-galacturonate backbone, interrupted randomly by 1,2-linked L-rhamnose. The average molecular weight ranges from about 50,000 to about 200,000 Daltons.

[0060] The polygalacturonic acid chain of pectin may be partly esterified, e.g., with methyl groups and the free acid groups may be partly or fully neutralized with monovalent ions such as sodium, potassium, or ammonium ions. Polygalacturonic acids partly esterified with methanol are called pectinic acids, and salts thereof are called pectinates. The degree of methylation (DM) for high methoxyl (HM) pectins can be, for example, from 60 to 75 mol% and those for low methoxyl (LM) pectins can be from 1 to 40 mol%.

[0061] In embodiments where pectinic acid is selected, the degree of esterification may be 40 mol% or less (e.g., 1, 5, 10, 20, 30 or 40 mol%, including ranges between any of the foregoing values). Higher degrees of esterification make bead formation by ionotropic crosslinking ineffective. Without being bound by theory, it is believed that a minimum amount of free carboxylic acid groups (not esterified) are needed to obtain a desirable degree of ionotropic crosslinking.

[0062] In embodiments, microcarrier beads were formed using LM pectins such as polygalacturonic acid that contains 20 mol% or less of methoxyl groups, e.g., 0, 5, 10, 15 or 20 mol%. Such a polygalacturonic acid may have no or negligible methyl ester content as pectic acids. As used herein, pectinic acid having no or only negligible methyl ester content and low methoxyl (LM) pectins are referred to collectively as PGA.

[0063] In embodiments, microcarrier beads were formed using a mixture of pectic acid and pectinic acid. Pure pectic acid and/or pectinic acid may be used. Blends with compatible polymers may also be used. For example, pectic or pectinic acid may be mixed with polysaccharides such as dextran, substituted cellulose derivatives, alginic acid, starches, glycogen, arabinoxylans, agarose, etc. Glycosaminoglycans like hyaluronic acid and chondroitin sulfate, or various proteins such as elastin, fibrin, silk fibroin, collagen and their derivatives can be also used. Other water soluble synthetic polymers can be also blended with pectic acid and/or pectinic acid. Non-limiting examples include polyalkylene glycol, poly(hydroxyalkyl(meth)acrylates), poly(meth)acrylamide and derivatives, poly(N-vinyl-2-pyrrolidone), polyvinyl alcohol, etc. Compatible polymers may be anionic, neutral or cationic provided that their inclusion does not impair digestion of the microcarriers.

[0064] External ionotropic gelation

[0065] External gelation, also called diffusion setting, involves the introduction of a hydrocolloid (PGA) solution to an ionic solution, with gelation occurring via diffusion of ions into the hydrocolloid solution. In embodiments, an aqueous, negatively-charged polysaccharide solution was dispensed drop-wise into a solution of divalent cations, such as calcium, magnesium or barium, which induces crosslinking of the PGA polymer. The crosslinking is ionic crosslinking, which in contrast to covalent crosslinking allows for subsequent digestion of the crosslinked polymer.

[0066] According to embodiments, the PGA concentration in the hydrocolloid solution ranged from 0.5 to 5 wt.%, e.g., 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 or 5 wt.%, including ranges between any of the foregoing values.

[0067] Example methods for forming droplets of the hydrocolloid (PGA) solution included dripping or extrusion with a syringe; jet breakup or pulverization, for which bead formation is accomplished by a coaxial air stream that pulls droplets from a nozzle; electrostatic bead generation, which uses an electrostatic field to pull droplets from a nozzle into a gelling bath; magnetically driven vibration; jet cutting, for which bead formation is accomplished by a rotating cutting tool that cuts a jet into uniform cylindrical segments; and spinning disk atomization.

[0068] Droplets of the PGA solution may be spherical or substantially spherical and have an average diameter ranging from 10 to 500 micrometers, e.g., 10, 20, 25, 50, 75, 100, 150, 200, 252, 300, 350, 400, 450 or 500 micrometers, including ranges between any of the foregoing values.

[0069] The gelling bath may comprise an aqueous solution of a divalent metal salt. In embodiments, the salt (e.g., calcium chloride) concentration in the gelling bath is at least 1% (w/v), e.g., 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 or 20%, including ranges between any of the foregoing values. If the calcium content is too low, the beads exhibit poor stability due to a too low crosslinking density.

[0070] The aqueous solution may comprise an alcohol such as ethanol. The ratio (v/v) of alcohol to water may range from 0/100 to 80/20, e.g., 0/100, 10/90, 20/80, 30/70, 40/60, 50/50, 60/40, 70/30 and 80/20.

[0071] In embodiments, some covalent crosslinking can occur but the level of such crosslinking, being irreversible, should be sufficiently low, for example, less than about 10 to 20 mol%, so as to maintain the digestibility of the beads.

[0072] The microcarrier beads may be spherical or substantially spherical and have an average diameter ranging from 10 to 500 micrometers, e.g., 10, 20, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450 or 500 micrometers, including ranges between any of the foregoing values. In embodiments, the coefficient of variation (CV) of the microcarrier beads, also referred to as the relative standard deviation, is less than 20%, e.g., 2, 5, 10 or 15%, including ranges between any of the foregoing. In embodiments, the size spread $\Delta d5-d95$ (the difference between $d95$ and $d5$, where $d5$ is the microcarrier diameter that is larger than the diameters of 5% of the microcarrier population and $d95$ is the microcarrier diameter that is larger than the diameters of 95% of the microcarrier population) is less than 25 micrometers, e.g., 10, 15 or 20 micrometers, including ranges between any of the foregoing. In embodiments, the size spread $\Delta d10-d90$ (the difference between $d90$ and $d10$, where $d10$ is the microcarrier diameter that is larger than the diameters of 10% of the microcarrier population and $d90$ is the microcarrier diameter that is larger than the diameters of 90% of the microcarrier population) is less than 20 micrometers, e.g., 5, 10 or 15 micrometers, including ranges between any of the foregoing. In embodiments, the radius of

curvature spread from d_5 to d_{95} (the difference between the radius of curvature of the microcarrier diameter that is larger than the diameters of 5% of the microcarrier population and the radius of curvature of the microcarrier diameter that is larger than the diameters of 95% of the microcarrier population) is less than 10 cm^{-1} , e.g., 2, 5 or 8 cm^{-1} , including ranges between any of the foregoing.

[0073] Control of Bead Size

[0074] In embodiments, microcarrier beads can be manufactured within narrow and specific size ranges. That is, they can be size-controlled. Control of the size of microcarrier beads is important for several reasons. If there is a wide size distribution, ranging from small to large microcarriers, microcarriers with smaller size will be in suspension much longer than larger size microcarriers. Exact settling time in the process would be much longer (because of the presence of smaller beads) or difficult to define. In use, more time will be required to ensure that the supernatant is clear from microcarriers.

[0075] Narrow size distribution enables settling of beads at a consistent speed which allows for more predictable separation of microcarriers from supernatant during medium exchange or culture produce isolation. Size of microcarriers can be fine-tuned to different ranges to control the settling speed. This enables customization of settling speed to match different process needs. Size controlled microcarriers have uniform surface area, which provides the same area available for cells to seed per microcarrier. This makes calculating the surface area available for cell seeding easier. In addition, cells will reach confluence at the same, or at a similar, time. As used herein, the terms "confluence" or "confluent" are used to indicate when cells have formed a coherent layer on a growth surface where all cells are in contact with other cells, so that virtually all the available growth surface is used. For example, "confluent" has been defined (R.I. Freshney, Culture of Animal Cells-A Manual of Basic Techniques, Second Edition, Wiley-Liss, Inc. New York, N.Y., 1987, p. 363) as the situation where "all cells are in contact all around their periphery with other cells and no available substrate is left uncovered". As is conventional, the amount of a growth surface that is covered by cells is referred to as a proportion of confluence. For example, a situation where approximately half of the growth surface is covered by cells is referred to herein as 50% confluence, or, in the alternative, as half confluence. Size-controlled

microcarriers can be suspended in the same agitation conditions. This enables fine control of shear force to balance good suspension of microcarriers and may allow conditions that cause less damage to cells. Well defined settling times for different groups of size-controlled microcarriers can help easy separation during continuous cell culture to prevent uneven cell growth on beads fed at different times. For example, cells can be seeded on size-controlled microcarriers with 250 μ m size first. After cells have reached half confluence, size-controlled microcarriers with of 350 μ m size can be added in the bioreactor for bead-to-bead transfer. At the time of confluence for 250 μ m microcarriers, microcarriers with this size can be removed by their unique settle speed or by filtration. Only beads with 350 μ m size and half confluent are left in the bioreactor. Then, fresh 250 μ m microcarriers can be added. After 350 μ m microcarriers reach confluence, they can be collected and fresh 350 μ m microcarriers added. This process may ensure that all the beads are removed when they reach confluence. In contrast, where microcarriers of the same size are used to do bead-to-bead transfer and continuous cell culture, cells on the beads from an earlier feeding will stay in bioreactor much longer than those on beads from a later feeding and the quality of cells can be deteriorated as a result of over confluence.

[0076] In embodiments, dissolvable microcarriers were size-controlled during manufacture using a vibration encapsulator. Size-controlled beads were formed by going through a nozzle with defined hole size, flow rate and vibration frequency. The size of obtained beads was controlled to a narrow range with a coefficient of variation of less than 10%.

[0077] Bead digestion

[0078] Non-proteolytic enzymes suitable for digesting the microcarrier, harvesting cells, or both, include pectinolytic enzymes or pectinases, which are a heterogeneous group of related enzymes that hydrolyze the pectic substances.

[0079] Cell harvesting involves contacting cell-laden microcarriers with a solution comprising a mixture of pectinolytic enzyme or pectinase and a divalent cation chelating agent.

[0080] An example method for harvesting cultured cells comprises culturing cells on the surface of a microcarrier as disclosed herein, and contacting the cultured cells with a mixture of pectinase and a chelator to separate the cells from the microcarrier.

[0081] Pectinases (polygalacturonase) are enzymes that break down complex pectin molecules to shorter molecules of galacturonic acid. Pectinases catalyze the liberation of pectic oligosaccharides (POS) from polygalacturonic acid. Pectinases are produced by fungi, yeast, bacteria, protozoa, insects, nematodes and plants. Commercially-available sources of pectinases are generally multi-enzymatic, such as Novozyme Pectinex™ ULTRA SPL, a pectolytic enzyme preparation produced from a selected strain of *Aspergillus aculeatus*. Novozyme Pectinex™ ULTRA SPL contains mainly polygalacturonase, (EC 3.2.1.15) pectintranseliminase (EC 4.2.2.2) and pectinesterase (EC: 3.1.1.11). The EC designation is the Enzyme Commission classification scheme for enzymes based on the chemical reactions the enzymes catalyze. Pectinases are known to hydrolyze pectin. They may attack methyl-esterified pectin or de-esterified pectin.

[0082] The concentration of pectinolytic enzyme in the digestion solution may be 1 to 200 U, e.g., 1, 2, 5, 10, 20, 50, 100, 150 or 200 U, including ranges between any of the foregoing.

[0083] Example chelating agents include ethylenediaminetetraacetic acid (EDTA), cyclohexanediaminetetraacetic (CDTA), ethylene glycol tetraacetic acid (ETGA), citric acid, tartaric acid, etc. The chelating agent concentration in the digestion solution may be 1 to 200 mM, e.g., 10, 20, 50, 100, 150 or 200 mM. To prevent cytotoxic side effects, the concentration of chelating agent in the digestion solution may be 10 mM or less, e.g., 1, 2, 5, or 10 mM, including ranges between any of the foregoing.

[0084] In embodiments, the total volume of the digestion solution comprising the pectinolytic enzyme and the chelating agent is less than 10 times the microcarrier volume, e.g., 1, 2, 4, 5 or 10 times the volume of the microcarriers including ranges between any of the foregoing values.

[0085] Depending of the digestion time, temperature, and amount of pectinolytic enzyme added, the extent of digestion beads can be selected or predetermined. It has been observed that cells detach from the microcarrier surface before the bead is fully digested. It is therefore possible to harvest cells with or without complete digestion of the beads. In embodiments where cells are harvested from partially-digested microcarriers, separation of the cells from remnant microcarriers may be done by one or more of filtration, decantation, centrifugation, and like processing.

[0086] Beads are readily digested when their calcium content is less than 2 g/l of moist beads, e.g., less than 2, 1.5, 1, 0.8 or 0.5 g/l. When the calcium content of the beads at the harvest stage is greater than 1 g/l, a greater volume and/or concentration of pectinolytic enzyme and divalent cation chelating agent can be used. The time for complete digestion may be less than one hour, e.g., 10, 15, 30 or 45 min. As used herein, the term “complete digestion” refers to digestion of microcarriers that results in a microcarrier particle count that complies with the particle count test as described in The United States Pharmacopeia and The National Formulary Section 788 (USP<788>) entitled “Particulate Matter in Injections”. As indicated in USP<788>, a preparation complies with the test if the average number of particles present in the units tested does not exceed 25 particles per mL equal to or greater than 10 µm and does not exceed 3 particles per mL equal to or greater than 25 µm. In embodiments, the microcarrier particle count for particles having a size of greater than or equal to 10 µm after digestion of the microcarriers is less than 10 particles, e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8 or 9, including ranges between any of the foregoing. In embodiments, the microcarrier particle count for particles having a size of greater than or equal to 25 µm after digestion of the microcarriers is less than 1 particle, e.g., 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 or 0.9, including ranges between any of the foregoing.

[0087] As defined herein the “moist bead” volume is the volume of the bed of beads after decantation or centrifugation. The bed comprises swollen beads as well as interstitial water (i.e., water present between the swollen beads). According to measurements, moist beads contain 70 vol.% swollen beads and 30 vol.% interstitial water. The swollen beads contain 99% water for a 1% PGA solution, 98% water for a 2% PGA solution, 97 % water for a 3% PGA solution, etc.

[0088] By way of example, microbeads prepared from a 3% (w/v) PGA sol contain, at equilibrium, about 1.48 g/l calcium ions. Complete digestion of the microbeads in less than 10 minutes results from exposure to at least 10 mM EDTA and at least 50U enzyme using a 5x volume of digestion solution (compared to the volume of beads).

[0089] Cell attachment

[0090] PGA beads, due to their hydro gel nature and negative charge, do not readily support cell attachment without specific treatment. In order to promote attachment of anchorage dependent cells, the microbeads can be provided with a coating or other surface treatment. By

way of example, the PGA beads can be functionalized with moieties promoting cell adhesion, for example, peptides such as those comprising a RGD sequence.

[0091] Further candidate peptides include those containing amino acid sequences potentially recognized by proteins from the integrin family, or leading to an interaction with cellular molecules able to sustain cell adhesion. Examples include BSP, vitronectin, fibronectin, laminin, Type I and IV collagen, denatured collagen (gelatin), and like peptides, and mixtures thereof. Further example peptides are BSP and vitronectin (VN) peptides having the following sequences: Ac-Lys-Gly-Pro-Gln-Val-Thr-Arg-Gly-Asp-Val-Phe-Thr-Met-Pro-NH₂ (seq. ID No. 1), and Ac-Lys-Gly-Gly-Asn-Gly-Glu-Pro-Arg-Gly-Asp-Thr-Tyr-Arg-Ala-Tyr-NH₂ (seq. ID No. 2), respectively.

[0092] In embodiments, the microbeads are surface functionalized with cell adhesion promoting recombinant proteins, which can be grafted or applied as a coating. Example recombinant proteins include fibronectin-like engineered proteins marketed under the trade names ProNectin® and ProNectin® plus, though other recombinant proteins that promote attachment of anchorage dependent cells can be used.

[0093] *Examples*

[0094] Example 1. 1% PGA microbeads crosslinked with 3% calcium.

[0095] Microbeads were prepared from a 1 wt.% solution of polygalacturonic acid (PGA) by dissolving polygalacturonic acid sodium salt (Sigma catalog number # P3850) into water at 80-85°C under constant agitation. The solution was filtered using a 20 micrometer polypropylene filter under vacuum to eliminate particles in suspension.

[0096] A gelling bath was produced in a separate beaker using 400 ml of a 3% w/v calcium chloride water/ethanol (75/25 v/v) solution, which was stirred using a magnetic stirrer.

[0097] Droplets were produced via the addition of 25 ml of the PGA solution to the gelling bath using a syringe equipped with a 30 Gauge needle. A syringe pressure of about 2 bars was applied.

[0098] Beads were hardened in the calcium chloride bath for 120 minutes before being washing four times with water. The calcium content within the beads was determined as

described in example 9. After four rinses, the calcium concentration was about 0.5-0.6 g/l of moist beads.

[0099] The beads were stored in sterile water in sterile containers at 4°C prior to coating. The beads were highly transparent without any observable surface defects.

[00100] When contacted with 5 mM EDTA/50 U pectinase at 25°C, the beads dissolved completely within 5 minutes.

[00101] Example 2. 1% PGA microcarriers crosslinked with 12 % calcium.

[00102] The procedure from example 1 was repeated except that the 3% w/v calcium chloride water/ethanol (75/25 v/v) solution was replaced with a 12% w/v calcium chloride water/ethanol (75/25 v/v) solution. After four rinses, the calcium concentration was about 0.5-0.6 g/l of moist beads. The beads were highly transparent without any observable surface defects.

[00103] When contacted with 5 mM EDTA/50 U pectinase at 25°C, the beads dissolved completely within 5 minutes.

[00104] Example 3. 1.5% PGA microcarriers crosslinked with 3% calcium.

[00105] The procedure from example 1 was repeated except that a 1.5% by weight solution of polygalacturonic acid and a pressure of 4 bars were used.

[00106] After four rinses, the calcium concentration was about 0.7-0.8 g/l of moist beads. The beads were highly transparent without any observable surface defects.

[00107] Example 4. 1.5% PGA microcarriers crosslinked with 12% calcium.

[00108] The procedure from example 3 was repeated except that a 12% w/v calcium chloride water/ethanol (75/25 v/v) solution was used in lieu of the 3% w/v calcium chloride water/ethanol (75/25 v/v) solution.

[00109] After four rinses, the calcium concentration was about 0.7-0.8 g/l of moist beads.

[00110] Example 5-a. 2% PGA microcarriers crosslinked with 3% calcium.

[00111] The procedure from example 1 was repeated except that a 2% by weight solution of polygalacturonic acid and a pressure of 5 bars were used. The PGA solution was preheated to 30°C and jetted at 30°C into the gelling bath, which was maintained at 25°C.

[00112] After four rinses, the calcium concentration was about 0.9-1.0 g/l of moist beads. The beads were highly transparent without any observable surface defects.

[00113] Example 5-b. 3% PGA microcarriers crosslinked with 3% calcium.

[00114] The procedure from example 1 was repeated except that a 3% by weight solution of polygalacturonic acid and a pressure of 6 bars were used. The PGA solution was preheated to 40°C and jetted at 40°C into the gelling bath, which is maintained at 25°C.

[00115] After four rinses, the calcium concentration was 1.48 g/l of moist beads. The beads were highly transparent without any observable surface defects.

[00116] When contacted with 5 mM EDTA/50 U pectinase at 25°C, the beads dissolved completely within 10 minutes.

[00117] Example 6. 2% PGA microcarriers crosslinked with 12% calcium.

[00118] The procedure from example 5-a was repeated except that a 12% w/v calcium chloride water/ethanol (75/25 v/v) solution was used.

[00119] After four rinses, the calcium concentration was about 0.9-1.0 g/l of moist beads.

[00120] Example 7. PGA beads coated with 0.1% gelatin crosslinked with glutaraldehyde.

[00121] A 0.1% porcine skin gelatin solution was prepared by first soaking 0.5 g (type A) porcine skin (Sigma #G1890) in 20ml of water and then adding 480 ml heated water (60°C-80°C).

[00122] Ten milliliters PGA beads prepared according to Examples 1-6 were collected by centrifugation to which 10mL of the porcine gelatin solution was added. The resulting mixture was gently shaken and incubated for 60 min at 25°C. The supernatant was removed and the beads were washed one time in water.

[00123] To crosslink the gelatin coating, 100 ml of 0.05% glutaraldehyde solution, prepared from a 25% stock solution (Sigma C5882) was added to the bead bed and incubated for 1 hour at 23°C under gentle shaking. The beads were subsequently rinsed three times with water and stored at 4°C in sterile containers.

[00124] Example 8. PGA beads having a Synthemax® II-SC synthetic copolymer surface

[00125] About 9 ml of swollen beads prepared according to Example 1 were placed in a 50 ml plastic centrifuge tube. Added to the beads was 36 ml of a 0.25 mg/ml aqueous solution of adhesion promoting peptides to form Corning Incorporated Synthemax® II-SC surface. The tube was gently shaken and left undisturbed for 30 min at 40°C allowing the synthetic copolymer

to functionalize the beads. After cooling the functionalized beads were washed three times with DI water. Beads were stored at 4°C water in sterile containers.

[00126] Example 9. Calcium titration

[00127] To quantify the calcium content, 1ml of PGA beads were digested by combining with 10 ml of a 5 mM EDTA/50 U pectinase solution. The suspension was vigorously shaken and left for one hour at 25°C under gentle shaking until digestion was completed.

[00128] The calcium content of the beads was quantified by Inductively Coupled Plasma Optical Emission Spectrometry (Axial ICP-OES, Varian 720 ES tool). The sample to be analyzed was prepared by diluting 100µl of the solution containing the digested beads in 9.9 ml of 1% HNO₃. A calibration curve was built using solutions of known calcium concentrations prepared from an aqueous 1g/l standard solution, which was diluted with HNO₃ as was done for each sample.

[00129] Example 10-a. hMSC static culture with peptide copolymer-coated microcarriers

[00130] Beads prepared according to Example 2 and provided with a Synthemax® II-SC copolymer surface according to Example 8 were sanitized with 70% ethanol/water and twice rinsed with phosphate buffered saline (dPBS) and then with MesenCult™-XF complete medium (MC-XF). Bone marrow-derived mesenchymal stem cell (hMSC) culture was carried out under static conditions in MC-XF in 24 well ULA plates. Cells were seeded at 100k cells/well. Cells were harvested from the microcarriers by treatment with 50U pectinase/ 5 mM EDTA for 5 minutes. Cell morphology 2 days after seeding is shown in **Fig. 1**. Cell morphology 4 days after seeding is shown in **Fig. 2**.

[00131] Example 10-b. hMSC static culture with gelatin-coated microcarriers

[00132] Beads were prepared according to Example 4 and coated with gelatin according to Example 7. **Fig. 3** shows an example phase contrast microscopy image of the adhesion and growth of human bone marrow-derived mesenchymal stem cells (hMSC) 2 days after seeding at 100k cells/well in 24 well plates.

[00133] Example 11. Expansion of Vero cells on gelatin-coated PGA microcarriers

[00134] Vero cells were cultured under continuous stirring on gelatin-coated, externally crosslinked 1% PGA microcarriers prepared according to Example 2 and coated according to Example 7.

[00135] The beads were first sanitized with 70% ethanol/water, twice rinsed with phosphate buffered saline (dPBS), and then rinsed with (IMDM+10% FBS+5ml penicillin streptomycin+5ml Glutamax™ media).

[00136] Cell culture was performed in Corning Incorporated disposable spinner flasks using IMDM supplemented with 10% FBS+5ml penicillin streptomycin+5ml Glutamax™ media as the culture medium. The flasks were seeded with 1M of Vero (p5) without stirring for 2h, followed by continuous agitation. The passage number designation (p#, in this example “p5”) indicates the number (#) of expansion and harvest cycles used to produce the cells (i.e., the number of divisions the cells have had in culture).

[00137] Cell harvesting at day 5 was done with 10 mL of 50U/ml pectinase, 5 mM EDTA.

[00138] **Fig. 4** shows an example phase contrast microscopy image of the adhesion and growth of the Vero cells 4 days after seeding. Fold expansion data is shown in **Fig. 5**.

[00139] Example 12. Expansion of MRC5 cells on gelatin-coated PGA microcarriers

[00140] Human fetal lung fibroblast (MRC5) cells were cultured under intermittent stirring on gelatin-coated, externally crosslinked 1% PGA microcarriers prepared according to Example 2 and coated according to Example 7.

[00141] The beads were first sanitized with 70% ethanol/water, twice rinsed with phosphate buffered saline (dPBS), and then rinsed with (IMDM+10% FBS+5ml penicillin streptomycin+5ml Glutamax™ media).

[00142] Cell culture was performed in Corning Incorporated disposable spinner flasks using IMDM supplemented with 10% FBS+5ml penicillin streptomycin+5ml Glutamax™ media as the culture medium. The flasks were seeded with 1M of MRC5 cells (p4) without stirring overnight, followed by intermittent agitation (1/4h per 2h).

[00143] Cell harvesting at day 5 was done with 10 mL of 50U/ml pectinase, 5 mM EDTA.

[00144] **Fig. 4** shows an example phase contrast microscopy image of the adhesion and growth of the MRC5 cells 4 days after seeding. Fold expansion data is shown in **Fig. 6**.

[00145] The micrographs in **Fig. 4** show that the Vero and MRC5 cells were able to adhere and reach confluence on the PGA microcarriers.

[00146] Example 13. Expansion of hMSC cells on peptide copolymer-coated microcarriers

[00147] hMSC cells were cultured under continuous stirring on externally crosslinked PGA beads prepared as described in Example 1 and provided with a Synthemax® II-SC copolymer surface according to Example 8.

[00148] The beads were first sanitized with 70% ethanol/water, twice rinsed with phosphate buffered saline (dPBS), and then with MesenCult™-XF complete medium (MC-XF). Cells were seeded at 1M cells/flask and cell culture was performed in Corning Incorporated disposable spinner flasks using MC-XF.

[00149] Cells were harvested from the microcarriers at day 7 by treatment with 10 ml 50U pectinase/ 5 mM EDTA for 5 minutes. Fold expansion data is shown in **Fig. 7**.

[00150] Example 14. Expansion of hMSC cells on peptide copolymer-coated microcarriers

[00151] Expansion of hMSC cells under continuous stirring as described in Example 13 was repeated except that externally crosslinked PGA beads prepared from a 2% PGA solution prepared as described in Example 5-a and provided with a Corning Synthemax II synthetic peptide copolymer surface as described in Example 8 were used. Fold expansion data is shown in **Fig. 7**.

[00152] Example 15. Expansion of hMSC cells on gelatin-coated microcarriers

[00153] hMSC cells were cultured under continuous stirring on externally crosslinked PGA beads prepared as described in Example 1 and coated with gelatin as described in Example 7.

[00154] The beads were first sanitized with 70% ethanol/water, twice rinsed with phosphate buffered saline (dPBS), and then with MesenCult™-XF complete medium (MC-XF). Cells were seeded at 1M cells/flask and cell culture was performed in Corning Incorporated disposable spinner flasks using MC-XF.

[00155] Cells were harvested from the microcarriers at day 7 by treatment with 10 ml 50U pectinase/ 5 mM EDTA for 5 minutes. Fold expansion data is shown in **Fig. 7**.

[00156] Example 16. Expansion of hMSC cells on gelatin-coated microcarriers

[00157] Expansion of hMSC cells under continuous stirring conditions as described in Example 15 was repeated except using the externally crosslinked gelatin-coated PGA beads prepared as described in Example 5-a and coated with gelatin as described in Example 7. Fold expansion data is shown in **Fig. 7**.

[00158] Example 17. Chemical stability of PGA microcarriers

[00159] The chemical stability of the microcarrier beads was evaluated by adding 1 ml swollen beads and 5 ml Dulbecco's Phosphate-Buffered Saline (dPBS) (1X) to a plastic centrifuge tube containing. The tube was incubated for 24 hr at 37°C. The volume of the beads after 24 hours was comparable to the initial volume showing that the beads do not dissolve in the phosphate buffer.

[00160] Example 18.

[00161] Monodisperse microcarrier beads were produced from a 1.5 wt.% PGA solution using an electromagnetically-driven laminar jet nozzle system (Nisco Engineering AG, Zurich, Switzerland). The system is equipped with a 100µm nozzle. The frequency was set to 2.5 kHz, and the amplitude to 100%. The solution flow rate, which is generated by applying a pressure of about 3 psi, was about 100 ml/h.

[00162] The nozzle was positioned about 7.5 cm above the surface of a gelling bath (4 wt.% CaCl₂ solution in 50:50 v/v water/ethanol). The bath was continuously stirred (170 rpm).

[00163] The resulting microbeads had an average diameter of $240 \pm 15 \mu\text{m}$, which corresponds to a coefficient of variation (CV) of 6.25%. This narrow size distribution is shown in **Fig. 9** (magnification: 4X).

[00164] The beads were gelatin coated as described in Example 7, except that 50 ml of 0.05% glutaraldehyde solution was used instead of 100 ml to crosslink the gelatin coating.

[00165] Example 10-c. MRC5 static culture with gelatin-coated microcarriers

[00166] Human fetal lung fibroblast (MRC5) cells were cultured in static conditions on microbeads prepared and coated with gelatin according to Example 18. Cells were seeded at 100k cells/well in 24 well ULA plates using IMDM supplemented with 10% FBS as the culture medium. Cell morphology 1 day after seeding is shown in the phase contrast microscopy image of **Fig. 10**.

[00167] Example 19. Grafting of vitronectin peptide to PGA microcarriers

[00168] About 3 ml of swollen beads prepared according to Example 18 were placed in a 15 ml plastic centrifuge tube. Twelve milliliters of an aqueous solution comprising 200 mM N-ethyl-N'-(3-(dimethylamino)propyl)carbodiimide (EDC) and 50 mM N-hydroxysuccinimide (NHS) was added. The tube was gently agitated for 30 min at 23°C allowing the activation of carboxylic acid groups within the PGA beads. The activated microcarriers were collected by centrifugation and rinsed three times with 10 ml deionized water.

[00169] The rinsed microcarriers were re-suspended in 12 ml borate buffer (pH 9.2) containing 49 mg of vitronectin peptide (Ac-Lys-Gly-Pro-Gln-Val-Thr-Arg-Gly-Asp-Val-Phe-Thr-Met-Pro-NH₂; catalog number: 341587 available from American peptide). The suspended microcarriers were left to react for 30 minutes under gentle agitation.

[00170] The peptide conjugated-microcarriers were collected by centrifugation and washed three times with 10 ml PBS buffer, pH 7.4. Excess activated ester was deactivated by blocking with 12 ml 1M ethanolamine (pH 8.4) for 60 minutes.

[00171] The peptide grafted and blocked microcarriers were collected and rinsed three times with PBS. After removing excess PBS, the microcarriers were rinsed 2 times with ethanol/water (70/30 v/v) and stored prior to cell culture at 4°C in sterile containers.

[00172] Example 10-d. hMSC static culture using VN-grafted microcarriers

[00173] Human mesenchymal stem cells (hMSC 2637, p3) were cultured in static conditions on VN-grafted microcarrier beads prepared according to Example 19. Cells were seeded at 50k cells/well in 24 well ULA plates. **Fig. 12** is a phase contrast image showing the hMSC cells in serum free medium (Mesencult XF) 24h after seeding.

[00174] Data from Examples 10a-10d demonstrate that wholly synthetic microcarriers and gelatin-coated microcarrier each support hMSC attachment and growth.

[00175] Example 20. Generation of Dissolvable Microcarriers Having Different Sizes Using External Gelation

[00176] Polygalacturonic acid sodium salt was dissolved 2% in water. External gelation was processed using NISCO single nozzle vibration encapsulator system. The beads were dropped

into 4% CaCl₂ dissolved in 1:1 (v/v) mixing of ethanol and water. For targeted size ranges, different sized nozzles, flow rates and vibration frequencies were used according to Table I.

Table I

Targeted Size	Nozzle Size	Flow Rate	Frequency	Obtained Size	CV
250µm	100µm	120ml/hr	3.00 kHz	243±15µm	0.062
350µm	100µm	120ml/hr	1.15 kHz	346±11µm	0.032
450µm	250µm	400ml/hr	1.15 kHz	485±17µm	0.035

[00177] Example 21. Settling Time Measurement and Analysis

[00178] To evaluate settling speed, embodiments of microcarriers of different sizes were compared with three commercially available microcarriers: Cytodex®-1 (cross-linked dextran-based microcarriers commercially available from GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania), SoloHill® P102-1521 (plastic cross-linked polystyrene microcarriers commercially available from Pall Corporation, Port Washington, New York) and Hillex® II (modified polystyrene microcarriers commercially available from Pall Corporation, Port Washington, New York). These three types of commercially available microcarriers range from hydrogel to solid plastic and have densities ranging from 1.02 to 1.09. Optical Density (OD) was measured, as described in more detail below, and used to determine the concentration of beads in suspension. Microcarriers are able to block visible light due to obscuration. Generally, a lower OD correlates to a lower concentration of microcarriers in suspension.

A method to measure OD involves letting beads settle in cuvettes at different stages. A light path used may be close the bottom of the cuvette. There can be a change in OD over a period of time during which the microcarrier beads settle out of solution. Generally, at the beginning of the measurement, the microcarrier beads are completely suspended in solution and light is blocked at the highest level. As the microcarrier beads start to settle, the top part of the suspension begins to clear because the microcarrier beads move in the same direction, although

the concentration of microcarrier beads in the path of the light remains relatively unchanged. As the microcarrier beads begin to settle below the upper limit of the path of the light, OD decreases. When the microcarrier beads reach approximately the middle of the path of the light, OD is reduced by half. The period of time for the microcarrier beads to reach approximately the middle of the path of the light is represented by t_m . When the height of total suspension and the position of the path of the light are fixed, the faster the beads settle, the smaller will be. Settling speed, represented herein by v_m , can be estimated by the time, represented herein by t_m , for the microcarriers to travel the distance, represented herein by l_m , from the top of the suspension to the middle of the path of the light.

This can be shown in Formula (1):

$$v_m = \frac{l_m}{t_m} \quad (1)$$

[00179] For a population of microcarrier beads with non-uniform size, different settling speeds are expected for different sized microcarrier beads. As such, settling speed v_m represents a medium settling speed of the population.

[00180] The path of the light has a width, represented herein by l_w , and there is a time, represented herein by t_w , for the microcarrier beads to travel the width l_w . When the microcarrier bead population has a uniform settling speed (i.e.: the microcarrier beads have a uniform size distribution), t_w can be estimated using the width of the path of the light l_w and settling speed v_m as shown in Formula (2):

$$t_w = \frac{l_w}{v_m} \quad (2)$$

[00181] When the microcarrier bead population has a distribution of different settling speeds (i.e.: the microcarrier beads have a non-uniform size distribution), the fastest settling microcarrier beads will reach the path of the light sooner than the slowest settling microcarrier beads. As the fastest settling microcarrier beads pass through the path of the light, a reduction of OD is observed, however, not until the slowest settling microcarrier beads pass through the path of the light is a complete reduction of OD observed. A microcarrier bead population having a

distribution of different settling speeds will exhibit a longer t_w than a microcarrier bead population having a uniform settling speed. As such, the shorter the t_w , the more uniform the settling speed of the population of the microcarrier beads and the more uniform the size distribution of the population of the microcarrier. While the above assumes that a starting point and an ending point of a change in OD can be determined, it should be understood that such starting and ending points may be difficult to define. As such, the slope of the change of OD can be used to represent the magnitude of the variation of the settling speeds in a microcarrier bead population.

[00182] In practice, it is preferable to wait for all the beads to completely separate from the supernatant in order to conclude the settling process. Therefore the time, represented herein by t , to observe a complete reduction of OD is more relevant to estimate final settling time. Final settling time may be determined using both the average settling time of a microcarrier bead population and the variation is of the the settling times of the microcarrier bead population. For quantitative measurement, final settling time may be determined by using t_m and t_w or using the slope of the change of OD.

[00183] In the present experiment, the three types of commercially available microcarriers were rehydrated and suspended in DPBS solution. Dissolvable microcarriers (DMCs) with three different sizes (250 μm , 350 μm and 450 μm) were formed as described in Experiment 1. About 0.5 ml of packed microcarriers was added into each cuvette. Then, DPBS was filled into the cuvettes to a total volume of 3.5 ml. Immediately before measurement, the microcarriers were suspended by pipetting up and down 10 times. OD was measured in accordance with the method described above and was measured at a wavelength of 400 nm. Other visible wavelengths can be chosen as well. OD measurements were performed every 2.0 seconds. Because the various types of microcarriers are formed from different materials, having different optical indexes, are different sizes and have different optical clarities, Initial OD was used to normalize the measurement of each sample so that the different samples could be compared.

[00184] The three sizes of DMCs were compared with the three commercially available microcarriers. The results showed that DMCs of 350 μm diameter settled 2 times as fast as the DMCs of 250 μm , and DMCs of 450 μm diameter settled 3 times as fast as the DMCs of 250 μm .

By changing the size of the DMCs, the settling speeds were able to match the medium settling speeds of the three commercial beads made of different materials and with different densities. As compared with the Cytodex®-1 and SoloHill® P102-1521 microcarriers, the DMCs having sizes of 250 µm and 350 µm demonstrated comparable medium settling speeds, but demonstrated much shorter t_w and steeper slopes of change of OD. As will be discussed in more detail in Example 21, the shorter t_w and steeper slopes of change of OD is the result of a smaller size distribution than the commercially available microcarriers which provides a more consistent settling speed as compared with the commercially available microcarriers. Comparing settling time t , DMCs having sizes of 250 µm and 350 µm settle quicker than Cytodex®-1 and SoloHill® P102-1521 microcarriers, which suggests that DMCs will need much shorter time to complete settling compared to the commercially available microcarriers.

[00185] Example 21. Microcarrier Size Distribution and Radius of Curvature Analysis

[00186] The size distribution and radius of curvature of dissolvable microcarriers (DMCs) formed in accordance with the microcarriers having a targeted size of 250 µm in Example 20 were compared to three commercially available microcarriers: Cytodex®-1, SoloHill® P102-1521 and Cytodex®-3 (cross-linked dextran-based microcarriers commercially available from GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania). Microcarrier size was determined using known optical microscopy techniques and software to analyze images captured with an optical microscope. Radius of curvature was then calculated from the determined microcarrier size using Formula (3):

$$|K| = \frac{1}{R} \quad (3)$$

where K is microcarrier radius of curvature and R is microcarrier radius. For each type of microcarrier, Table II shows size range d5-d95 (where d5 is the microcarrier diameter that is larger than the diameters of 5% of the microcarrier population and d95 is the microcarrier diameter that is larger than the diameters of 95% of the microcarrier population), size range d10-d90 (where d10 is the microcarrier diameter that is larger than the diameters of 10% of the microcarrier population and d90 is the microcarrier diameter that is larger than the diameters of 95% of the microcarrier population), size spread $\Delta d5-d95$ (the difference between d95 and d5),

the coefficient of variation for d5-d95, size spread Δd_{10-d90} (the difference between d90 and d10) and the coefficient of variation for d10-d90.

Table II

	Cytodex®-1	Cytodex®-3	SoloHill®	DMC
d5-d95	202-290 μm	165-236 μm	134-193 μm	233-256 μm
d10-d90	210-279 μm	174-230 μm	138-188 μm	234-252 μm
Δd_{5-d95}	88 μm	71 μm	59 μm	23 μm
d5-d95 CV	0.179	0.177	0.180	0.047
Δd_{10-d90}	69 μm	56 μm	50 μm	18 μm
d10-d90 CV	0.141	0.139	0.153	0.037

[00187] For each type of microcarrier, Table III shows average microcarrier diameter (d), average radius of curvature (K), radius of curvature at d5, radius of curvature at d95, and radius of curvature spread from d5 to d95 (the difference between d5 radius of curvature and d95 radius of curvature).

Table III

	Cytodex®-1	Cytodex®-3	SoloHill®	DMC
Avg. diameter (μm)	244	201	161	240
Avg. K (cm^{-1})	82.0	99.5	124.2	83.3
K at d5 (cm^{-1})	99.0	121.2	149.3	85.8
K at d95 (cm^{-1})	69.0	84.7	103.6	78.1
$\Delta K_{d5} - K_{d95}$ (cm^{-1})	30.0	36.5	45.6	7.7

[00188] The data in Tables II and III shows that DMCs as disclosed herein have a more uniform size distribution and a more uniform radius of curvature than the three commercially

available microcarriers. As previously described, a uniform size distribution enables settling of beads at a consistent speed which allows for more predictable separation of microcarriers from supernatant during medium exchange or culture produce isolation. A uniform size distribution and a uniform radius of curvature also provides the same surface area available for cells to seed per microcarrier which enables cells to reach confluence at the same, or at a similar, time.

[00189] Example 22. Microcarrier Particle Count Analysis

[00190] Debris particle count resulting from dissolvable microcarriers (DMCs) formed in accordance with microcarriers described in Example 20 was compared to two commercially available microcarriers: SoloHill® P102-1521 and Cytodex®-3. Multiple steps of washing each type of microcarrier were performed prior to stirring to reduce the number of particles to less than 2 particles per mL. For each type of microcarrier, a volume of microcarriers of about 1000cm² was placed in separate Corning® 125mL Disposable Spinner Flasks (commercially available from Corning, Inc., Corning, New York) and suspended in about 100 mL Dulbecco's phosphate-buffered saline (DPBS). Two Disposable Spinner Flasks were filled with DMCs. The microcarriers were continuously stirred at a speed of about 60 rpm at room temperature for a total of 6 days. The DMCs in one of the Disposable Spinner Flasks were dissolved in accordance with methods described herein, and the DMCs in the other of the Disposable Spinner Flasks were not dissolved. After stirring was complete, the microcarriers were separated from the DPBS and the particle count in the DPBS was measured with an HIAC 9703+ Particle Counter (commercially available from Beckman Coulter Life Sciences, Indianapolis, Indiana) using the light obscuration particle count test as described in The United States Pharmacopeia and The National Formulary Section 788 (USP<788>) entitled "Particulate Matter in Injections". As indicated in USP<788> a preparation complies with the test if the average number of particles present in the units tested does not exceed 25 particles per mL equal to or greater than 10 µm and does not exceed 3 particles per mL equal to or greater than 25 µm. Table IV shows the number of particles remaining having a size of greater than or equal to 25 µm per mL of DPBS for each of the microcarriers, including non-dissolved DMC and dissolved DMC. Table V shows the remaining number of particles having a size of greater than or equal to 10 µm per mL of DPBS for each of the microcarriers, including non-dissolved DMC and dissolved DMC.

Table IV

	# of Particles ≥ 25 μm per mL
Cytodex®-3	0.4
SoloHill®	1.0
DMC (non-dissolved)	0.3
DMC (dissolved)	0.1

Table V

	# of Particles ≥ 10 μm per mL
Cytodex®-3	26
SoloHill®	17
DMC (non-dissolved)	7
DMC (dissolved)	9

[00191] As shown in Tables IV and V, where the DMCs were both dissolved and non-dissolved, fewer DMC particles remained after stirring in the Disposable Spinner Flasks than remained after stirring the other two commercially available microcarriers. This was true for particles having a size of greater than 10 μm and for particles having a size of greater than 25 μm .

[00192] Comparative example 1

[00193] Vero cell culture was repeated as described in Example 11 except that non-digestible Cytodex®-3, substrates area used instead of the PGA microcarriers. Trypsin was needed to detach the cells from the surface of the Cytodex®-3 beads. Fold expansion data is summarized in **Fig. 5**. The expansion obtained with the digestible microcarriers is comparable to the expansion on Cytodex®-3.

[00194] Comparative example 2

[00195] MRC5 cell culture was repeated as described in Example 12 except that non-digestible Cytodex®-3 substrates are used instead of the PGA microcarriers. Trypsin was needed to detach the cells from the surface of the Cytodex® beads. Fold expansion data is summarized in **Fig. 6**. The expansion obtained with the digestible microcarriers is comparable to the expansion on Cytodex®-3.

[00196] Comparative example 3

[00197] **Fig. 11** is a phase contrast microscopy image of beads formed via internal gelation according to Example 1 of WO2014/209865. The beads have an average diameter of 231 ± 54 μm , which corresponds to a coefficient of variation (CV) of 23%.

[00198] The disclosed methods provide an inexpensive and environmentally-friendly route for the preparation of highly-transparent PGA microcarriers that are free of undesired inclusions and surface defects and which support non- proteolytic cell separation and harvesting.

[00199] Characterization of By-Products from PGA Digestion

[00200] To show the composition of the dissolvable microcarrier digestion, size-exclusion chromatography (SEC) was used to separate PGA polymer digestion byproducts by their molecular weight. Figure 12 shows the results of each individual reagent (left), as well as a close-up of the data to better resolve the peaks in PGA and salt (right). As shown in Figure 12, each individual reagent was run as a control to determine the relative elution profile and retention time. From this, strong pectinase and EDTA peaks were observed due to high absorbance at 214 nm. The PGA peak had the shortest retention time of 2.1 minutes, which suggests that it has the highest molecular weight, and pectinase showed multiple peaks between 2.2 and 3.0 minutes, consistent with a mixture of multiple enzyme components. There is some overlap between the low molecular weight PGA peaks and pectinase peaks, which could interfere with detection of larger digestion fragments from PGA. Similarly, EDTA had a peak at 3.91 minutes due to a relatively low molecular weight of 292.17 Daltons; this could also mask viewing of PGA monomer and dimer peaks with expected molecular weights of 194.14 Daltons and 370.26 Daltons, respectively.

[00201] To better show the small-molecule digestion byproducts of PGA, analysis of the digestion of PGA polymer by pectinase was first performed. A solution of PGA polymer and

pectinase were mixed together without EDTA. As shown in Figure 13, new periodic peaks were generated at low molecular weight, most likely a result of different lengths of digested galacturonic acid oligomers each separated by one residue. As digestion time increased, the new low molecular weight peaks became stronger, suggesting that PGA oligomers were continually digested to lower molecular weight oligomers. After extended time, the periodic peaks disappeared, resulting in a strong low molecular weight peak at 4.17 minutes. The retention time suggests that its molecular weight is lower than that of EDTA and is most likely the galacturonic acid monomer. Since the mobile phase for SEC (e.g., Dulbecco's phosphate-buffered saline, or DPBS) prevented the use of mass spectrometry to determine the molecular weight and identity of each peak, a separate digestion solution was analyzed using Electrospray Ionization Mass Spectrometry, the results of which are shown in Figure 14. A strengthening peak ($m/z = 193$) with an increase in digestion time was observed (Figure 14), supporting the SEC data that the PGA polymer will completely digest into monomers. The Mass Spectrometry (MS) data supports a rapid increase in monomer peak in less than 15 minutes, but changed much less from 15-60 minutes. The SEC analysis, in contrast, showed the most increase in monomer peak after 10 minutes. Without wishing to be bound by theory, this suggests two potential explanations: (1) the digestion occurs rapidly, and the different sample introduction methods used in different analyses, MS versus SEC, could introduce some shifting in actual time, and (2) MS peak intensity may not respond as quantitatively to sample concentration as absorbance in SEC.

[00202] Next, the degradation byproducts of the PGA polymer were compared to that of PGA microcarriers. When only EDTA was added, the microcarriers were dissociated into PGA polymer. As shown in Figure 15, a PGA peak around 2.10 minutes and EDTA peaks at 3.91 and 4.03 minutes. When pectinase and EDTA both were added, microcarrier dissociation and PGA polymer digestion occurred at the same time. Additional pectinase peaks are shown between 2.2 and 3.0 minutes, multiple periodic peaks after 3.5 minutes which weakened with increased digestion time, and single strong peak at 4.17 minutes corresponding to the PGA monomer (Figure 15). Unlike the SEC data from the PGA polymer, PGA oligomer peaks could not be resolved between 3.85 and 4.10 minutes due to masking from the EDTA peaks.

[00203] Calcium and EDTA Concentration

[00204] To facilitate the dissolution of PGA microcarriers, EDTA can be added to sequester and capture calcium ions, thereby destabilizing the polymer network and bead structure. Thus, Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) in combination with SEC to better understand the expected amount of calcium ion released upon bead dissolution and the amount of EDTA necessary to sequester released calcium ions. Samples were prepared similarly for both analyses as described in the “Methods” section below, and the resulting calcium content measured by ICP-MS was 90-110 mg/L, or approximately 2.25-2.75 mM calcium ions in the digested solution (data not shown). SEC data showed an approximately 60% reduction in the EDTA peak at 3.91 minutes in a digestion solution containing 4 mM EDTA (data not shown), which agrees well with the ICP-MS results of the amount of calcium released. This data suggests that most of the calcium released from dissolved microcarriers binds to EDTA, and EDTA is in excess at 4mM. It is noted that existing digestion protocols may recommend 10 mM EDTA to ensure rapid bead digestion. However, according to embodiments of this disclosure, as evidenced by the above data, much lower EDTA concentrations can be used for bead dissolution.

[00205] Removal of Soluble Digestion Components from Recovered Cells

[00206] Dissolvable microcarrier digestion products include: small PGA oligomers (monomers), EDTA, calcium, and pectinase. To illustrate how these components can be separated from cells, post-recovery, a series of wash and centrifugation cycles were used, in accordance with some embodiments of methods of this disclosure. As described in the Methods section, and shown in Figure 16, cells were recovered from dissolved microcarriers by centrifugation, and 75% of the supernatant from each wash cycle was analyzed via UV-VIS spectroscopy. The UV-VIS spectra of individual digestion solution reagents are shown in Figure 17A. The optical density (OD) at four different wavelengths was used to represent the characteristic spectrum of different components: 215 nm (typically used for general organics); 260 nm (typically used for DNA); 280 nm (typically used for protein); and 400 nm for visible color. The OD at 215 nm was inaccurate in undiluted samples due to over saturation; therefore, a 1:10 dilution was used for quantitative comparison (Figure 17B). As shown in Figure 17C, each reagent had a unique combination of OD at the four selected wavelengths; ratios between these OD were used to identify each reagent in the digestion solution and further quantify their

concentrations, as shown in Figure 17D. The OD values at each wavelength for the experimental samples agreed with the additive ODs of the individual digestion reagents and PGA.

[00207] Further, the reduction in OD with each wash / centrifugation cycle agreed with a theoretical 75% medium reduction model, demonstrating that removal of the digestion reagents and microcarrier byproducts followed a similar dilution scheme and can be reduced through a series of wash cycles, according to embodiments. Based on these results and considering the byproducts are proteins (pectinase) and small water-soluble molecules (PGA oligomers, EDTA), tangential flow filtration or other commonly-used cell purification methods will be able to remove these byproducts.

[00208] Soluble byproducts of PGA microcarrier dissolution should be reduced or removed through washing. However, the fate of the attachment substrates, Synthemax® II (a synthetic peptide from the extracellular matrix protein (ECM), vitronectin) or denatured collagen, were previously unclear. Thus, residual peptide on cells was measured using an anti-Synthemax® II antibody as a function of cell density. Briefly, human mesenchymal stem cells (hMSC) were seeded on Synthemax® II (S2) dissolvable microcarriers (DMC) at increasing cell concentrations: 15×10^3 , 30×10^3 , and 60×10^3 cells per well. Denatured collagen (DC) DMC were seeded with 60×10^3 cells per well as a negative control. Cells were expanded for 5 days and then harvested with a digestion solution of pectinase and EDTA. Digested cell suspensions were immunostained with anti-Synthemax® II antibody as described in Methods. Unseeded DMC were stained using the same method.

[00209] As shown in Figure 18, Synthemax® II staining was evident on unseeded Synthemax® II DMC (NSM) when compared to negative control denatured collagen beads (NDC). Similarly, low background fluorescence was observed on cells harvested from DC DMC (DC 60). In contrast, fluorescence corresponding to Synthemax® II immunodetection was observed on cells harvested from Synthemax® II DMC (S2 15, 30, and 60). The overall fluorescence signal increased with cell density; however, the amount of Synthemax® II detected per cell was similar across the different conditions (Figure 19) based on quantitative fluorescence intensity as measured using a spectrophotometer. Further, when fluorescence intensity was normalized to the total fluorescence of Synthemax® II DMC (lower graph of Figure 19), the amount of

Synthemax® II associated with harvested cells was 1-6% of the total Synthemax® II present on the microcarriers prior to digestion.

[00210] According to some embodiments, a method of digesting microcarriers can include a standard cell culture protease during microcarrier digestion to facilitate breakdown of cell-ECM networks and promote a single cell suspension. To determine whether the addition of trypsin to the pectinase/EDTA digestion solution would decrease the amount of Synthemax® II associated with harvested cells, several harvest conditions were compared: pectinase/EDTA (NSM PE), trypsin alone (NSM trypsin), trypsin/pectinase/EDTA (NSM TPE), and a trypsin first, until cells begin to detach from the beads, followed by pectinase/EDTA (NSM T+PE). Conditions were then immuno-stained with the anti-Synthemax® II antibody as done previously, and samples were visualized using fluorescence microscopy as illustrated in Figure 20. When trypsin was used during the cell harvest process, the fluorescence intensity corresponding to Synthemax® II was significantly reduced. Further, a pre-treatment with trypsin followed by pectinase/EDTA solution resulted in less Synthemax® II signal compared to a parallel treatment of trypsin/pectinase/EDTA.

[00211] Further, a mock digestion solution was exposed to a series of centrifugation and wash cycles, as described in Figure 16, to determine if soluble Synthemax II peptides could be detected in DMC digestion solutions. Based upon anti-Synthemax® II antibody dot blot analysis of samples from each wash cycle, Synthemax® II was detected in the original digestion solution, wash 1, and wash 2 (Figure 21). Traces of Synthemax® II were detected in wash 3, which was considered at the limit of detection ~100 pg (5 uL blot of 20 ng/mL) based on the Synthemax® II serial dilutions. These results confirm that the soluble byproducts of microcarrier dilution can be removed or reduced through washing and only small amounts of Synthemax® II (<6%) remain associated with recovered cells.

[00212] Digestion Components Impact on Cell Growth

[00213] To demonstrate the impact of digestion byproducts and reagents on subsequent cell growth, Vero cells on denatured collagen DMC were harvested and pelleted to completely remove the digestion solution and then reseeded into T75 flasks in fresh culture media containing a dilution of the collected digestion solution (1:5 to 1:100). Cell attachment and growth was

monitored for several days. As shown in Figure 22A, Vero cell growth was significantly impacted by the presence of the harvest solution at low dilutions (1:5-1:10), and modest inhibition of growth was observed up to 1:40 dilution. These results suggest that there are components in the digestion solution that will inhibit cell growth. To further investigate the impact of pectinase and EDTA on reseeding Vero cells on DMC, pectinase (10-100 U/mL final concentration) or EDTA (1 to 5 mM final concentration) were spiked into disposable spinner flasks containing dissolvable microcarriers. Vero cells were then added to each spinner flask at 10,000 cells/cm². Cells were harvested and quantified on day 3.

[00214] As shown in Figure 22B, the presence of pectinase in the cell culture medium at concentrations ≥ 40 U/mL resulted in decreased cell yield, and the cell concentration was minimally impacted by pectinase concentrations up to 30 U/mL (less than 10% reduction). Thus, the digestion solution can be diluted greater than 1:3 to minimize inhibition of cell growth due to pectinase, according to some embodiments. Similarly, EDTA had a significant impact on cell growth, and concentrations ≥ 2 mM had an impact on DMC integrity which resulted in DMC dissolution (Figure 22C). Thus, to minimize the impact of EDTA, the final concentration can be less than 1 mM, according to some embodiments.

[00215] Further, the addition of calcium chloride during cell reseeding was unable to neutralize EDTA and pre-equilibrating calcium chloride with EDTA-containing cell suspensions prior to cell seeding had a negligible effect (data not shown). Based upon these results, we would recommend removal of the microcarrier digestion solution from cells via centrifugation, filtration, or perfusion prior to reseeding or optimize microcarrier dissolution at lower concentrations of pectinase and EDTA (e.g. 30 U/mL pectinase, 1 mM EDTA).

[00216] Thus, according to embodiments of this disclosure, and as demonstrated by the above, soluble components resulting from microcarrier dissolution include: PGA monomers/oligomers, calcium, EDTA, pectinase, and surface coatings, and these can be reduced or removed from recovered cells through a series of wash / centrifugation cycles. Also, a small amount (1-6%) of residual Synthemax II coating remain associated with recovered cells, and this can be further reduced with use of a protease during bead digestion (e.g., trypsin). Further, because residual pectinase and EDTA used for microcarrier digestion can have a negative impact on subsequent

cell growth, methods can remove or significantly reduce these components via centrifugation, filtration, or perfusion prior to cell passage or long-term storage.

[00217] Methods

[00218] For Size Exclusion Chromatography, dissolved microcarriers were prepared as follows: 1 mL of concentrated digestion solution (400 U/mL pectinase $\pm$ 40 mM EDTA) was added to 1 mL packed volume of hydrated DMC beads (or to 1 mL of 1.75% PGA solution, which contained a comparable amount of PGA material) that was diluted in 8 mL of Dulbecco's Modified Buffered Saline (DPBS). A UPLC-PDA Waters Acquity H-class instrument was used with the following settings: column = 4.6X150 mm, BEH 125 SEC (1.7 μ m); Column Temperature = 30°C; flow rate = 0.4ml/min; Mobil Phase = 20mM phosphate (pH5); and injection = 10 μ l.

[00219] For Mass Spectrometry, samples were prepared as done for SEC and diluted in acetonitrile/water mixture (1:9 v/v) to obtain a final dilution of 1:10,000. HPLC grade acetonitrile and water were purchased from Fisher Scientific. Electrospray Ionization (ESI) - Mass Spectrometry (MS) experiments were conducted using Agilent 6560 ion mobility quadrupole time-of-flight system. The Agilent ion mobility system consists of a front funnel, trapping funnel, drift tube, and a rear funnel that couples via a hexapole to the Q-TOF mass analyzer. A sample was introduced to Dual AJS (Agilent jet stream) ionization source at the flow rate of 5 μ L/min by direct infusion for all ESI-MS experiments. Operating conditions were as follows: Gas Temperature: 300 °C, Drying Gas: 7 L/min, Nebulizer Pressure: 35 psi, Sheath Gas Temperature: 275 °C, Sheath Gas Flow: 12 L min⁻¹, VCap: 3500 V and Nozzle Voltage: 2000 V. Data acquisition was collected at the mass range of 50-1700 m/z. All the mass spectra obtained were accumulated and processed via the MassHunter Workstation Software, B.08.00 version.

[00220] For Inductively Coupled Plasma Mass Spectrometry, samples were prepared as done for SEC and analyzed using Agilent 7700s quadrupole Inductively Coupled Plasma Mass Spectrometer (Q-ICP-MS) located in a Class 1,000 Clean Room. Samples were diluted with high purity 1% HNO₃ solution plus addition of Indium, which was used as an internal standard. Calcium was measured only in reaction mode (Hydrogen). Sodium was measured in no gas

mode (Argon only) and in collision mode (Helium). Indium was measured in all three modes. When needed, additional dilutions were made to keep the measured concentrations within the linear range of the calibration curves. The average concentrations of two measurements were reported.

[00221] For Dissolvable microcarrier byproduct clearance study, human mesenchymal stem cells (hMSC) (RoosterBio, Cat. No. MSC-001, 1 million cells) were cultured on 200 cm² Synthemax® II dissolvable microcarriers (Corning Cat. No. 4988) in 125 mL disposable spinner flasks (Corning Cat. No. 3152) containing 50 mL DMEM (Corning Cat. No. 15-018-CM) supplemented with 10% FBS (Corning Cat. No. 35-074-CV) and 2mM L-glutamine (Corning Cat. No. 25-005). A duplicate spinner flask without cells was used as a mock control. Cells attached to microcarriers under intermittent agitation (3 cycles of 30 rpm for 5 minutes, 0 rpm for 6 hours; Wheaton Micro-Stir Platform Cat. No. W900701-A); continuous agitation at 35 rpm and half-volume media exchanges on days 3 and 5 were used to promote cell expansion through day 7.

[00222] To harvest cells from dissolved microcarriers, the contents of each spinner flask were transferred into 50 mL conical tubes and the beads were allowed to settle. Supernatant was removed and the culture was washed twice with 20 mL DPBS. A digestion solution consisting of 50 U/mL pectinase (Sigma, Cat. No. P2611) / 5 mM EDTA (Corning, Cat. No. 46-034-CI) in DPBS mixed 1:1 with 1X TrypLE was added to a final concentration of 10 cm²/mL. Microcarrier digestion was confirmed by microscopy after 10 minutes. Tubes were then centrifuged at 259 x g for 5 minutes. Following centrifugation, 75% of the supernatant was removed and measured on a UV-Vis spectrophotometer (Laxco UV-vis, model No: Alpha-1106). Sample absorbance was measured without dilution or diluted by adding 100 ul (30x dilution) or 300 ul (10x dilution) in 3000ul DPBS to ensure accurate measurements within the limit of detection. DPBS was used both as dilution medium and baseline. All samples were scanned from 200 nm to 500 nm. Next, the same volume of DPBS was used to resuspended the remaining liquid (mock sample) or cell pellet and centrifuged again. This process was repeated for a total of 4 DPBS washes / centrifugation steps.

[00223] For immunostaining of hMSC after DMC dissolution, human mesenchymal stem cells were seeded on 10 mg dissolvable microcarriers per well of a ULA 6-well plate (Corning Cat. No. 3471), and cells were allowed to attach and expand on microcarriers until a desired cell density was achieved (15,000-60,000 cells/well). For cell harvest, microcarriers were transferred to a 15 mL tube to remove spent culture media and wash with DPBS. The wash was discarded and harvest solution containing 50 U/mL pectinase and 5 mM EDTA was added to each suspension. Complete bead digestion was observed after 10 minutes. For cell harvest using trypsin, pectinase, and EDTA, 2 mL of a mix made of 0.125% trypsin, 5 mM EDTA, 50U/mL pectinase was added; whereas, for an incubation with trypsin followed by a treatment with pectinase and EDTA, 1 mL of trypsin 0.25% was added, incubated for 5 minutes, then 1 mL of 5 mM EDTA and 50 U/mL pectinase was added to the reaction for an additional 5 minutes. Released cells were pelleted at 300 x g for 2 minutes and resuspended in 1 mL DPBS.

[00224] For cell fixation and immunostaining, cell pellets were resuspended in 500 µL of 4% formaldehyde and incubated at room temperature for 10 minutes. Cells were pelleted, washed with DPBS, and resuspended in 250 µL of primary antibody solution (anti-Synthemax II polyclonal antibody in DPBS, 1:1000 dilution). Primary antibody was incubated for 45 minutes at room temperature. Cells were washed with 1 mL of DPBS and resuspended in 250 µL of secondary antibody solution (anti-rabbit Alexa 488 1:500 in DPBS) for 45 minutes at room temperature in the dark. Cells were washed twice with 1 mL of DPBS and then resuspended in 200 µL DPBS for fluorescence microscopy at 488 nm. Alternatively, 100 µL of stained cells (or beads) were transferred to a 96-well plate for quantitation of fluorescence intensity using a spectrophotometer. Unseeded Synthemax II and denatured collagen beads were stained using the same protocol for microscopy and then the remaining beads were dissolved in 2 mL of pectinase/EDTA harvest solution for fluorescence measurements.

[00225] For dot blot analysis for detection of soluble Synthemax® II, Synthemax® II dissolvable microcarriers (250 mg) were hydrated in water according to standard protocols. Water was removed and replaced by 30 mL of digestion solution containing 100 U/mL pectinase and 10 mM EDTA in DPBS. After 10 minutes, the dissolved bead solution was spun at 125 x g for 10 minutes. The supernatant was discarded, leaving only 2 mL. This remaining solution was

diluted and mixed with 12 mL DPBS (wash 1). After a second centrifugation cycle, the supernatant was removed leaving only 1 mL and 13 mL of DPBS was added (wash 2). This sequence was repeated twice more.

[00226] A sample of each wash was collected and mixed with an equal volume of SDS-PAGE running buffer, and 5 uL were dotted onto a nitrocellulose membrane. Similarly, serial dilutions of Synthemax® II peptide were blotted as controls and for concentration comparison. After blotted samples were dry, the membrane was saturated 30 minutes with 5% dried milk in TBS-Tween buffer, washed, incubated 2 hours with the anti-Synthemax® II antibody (1:1000 dilution in TBS Tween), washed, incubated 1 hour with an anti-rabbit secondary antibody coupled with peroxidase. After a final washing, Synthemax® II peptide was visualized using ECL substrate.

[00227] For the Vero cell growth in the presence of digestion solution reagents, Vero cells were seeded on denatured collagen DMCs at 10,000 cells per cm² for 3-5 days in a 125mL disposable spinner flasks. Cells were harvested, and the culture was centrifuged to completely remove the harvest solution. Cells were reseeded into T-75 flasks in culture medium containing a dilution of the harvest solution (1:2 up to 1:100 dilution). Cells were cultured in the T-flasks for 5-6 days. Images of the cells were captured daily. The attached cells were quantified at the end of the culture period using a ViCell Automated Cell Counter.

[00228] To determine the effect of pectinase and EDTA on cell growth on microcarriers, pectinase (10-100 U/mL final concentration) or EDTA (1-5mM final concentration) was spiked into each spinner flask prior to cell addition (10,000 cells per cm²). Cells were mixed continuously during the cell attachment and cell expansion phases. Images of the DMCs were captured daily. Cells were harvested and quantified on Day 3 or 5.

[00229] Illustrative Implementations

[00230] The following is a description of various aspects of implementations of the disclosed subject matter. Each aspect may include one or more of the various features, characteristics, or advantages of the disclosed subject matter. The implementations are intended to illustrate a few aspects of the disclosed subject matter and should not be considered a comprehensive or exhaustive description of all possible implementations.

[00231] Aspect 1 pertains to a cell culture article, comprising a substrate comprising a polygalacturonic acid compound crosslinked with a divalent cation, the polygalacturonic acid compound selected from at least one of: pectic acid; partially esterified pectic acid, partially amidated pectic acid and salts thereof; wherein the substrate is digestible by digestion reagents into components comprising of galacturonic acid monomers and the divalent cation.

[00232] Aspect 2 pertains to the article of Aspect 1, wherein the substrate is spherical or substantially spherical.

[00233]

[00234] Aspect 3 pertains to the article of Aspect 2, wherein the substrate comprises a diameter of 10 to 500 micrometers.

[00235] Aspect 4 pertains to the article of any of the preceding Aspects 1-3, wherein the divalent cation concentration ranges from 0.5 to 2 g/l of the substrate

[00236] Aspect 5 pertains to the article of Aspect 4, wherein the divalent cation is selected from the group consisting of calcium, magnesium and barium.

[00237] Aspect 6 pertains to the article of any of the preceding Aspects 1-5, further comprising an adhesion polymer on the surface of the substrate.

[00238] Aspect 7 pertains to the article of Aspect 6, wherein the adhesion polymer comprises a polypeptide.

[00239] Aspect 8 pertains to the article of any of Aspects 6-7, wherein the adhesion polymer is grafted to or coated on the surface of the substrate.

[00240] Aspect 9 pertains to the article of any one of the preceding Aspects 1-8, wherein the polygalacturonic acid compound is ionotropically crosslinked.

[00241] Aspect 10 pertains to the article of any one of the preceding Aspects 1-9, wherein the digestion reagents comprise at least one of EDTA and an enzyme.

[00242] Aspect 11 pertains to the article of Aspect 10, wherein the enzyme is pectinase.

[00243] Aspect 12 pertains to the article of any one of Aspects 6-8, wherein at least a portion of the adhesion polymer becomes soluble when the substrate is digested.

[00244] Aspect 13 pertains to the article of Aspect 12, wherein the portion of the adhesion polymer that becomes soluble is at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 95%.

[00245] Aspect 14 pertains to a method for culturing cells, the method comprising contacting cells with a cell culture medium having the cell culture article according to any of Aspects 1-13, and culturing the cells in the medium.

[00246] Aspect 15 pertains to a method for harvesting cultured cells, the method comprising: culturing cells on the surface of the cell culture article of any of Aspects 1-13; and contacting the cultured cells with a mixture of pectinase and a chelator to separate the cells from the cell culture article.

[00247] Aspect 16 pertains to the method of Aspect 15, wherein the chelator comprises EDTA.

[00248] Aspect 17 pertains to a method of harvesting cultured cells from a dissolvable substrate, the method comprising: separating the cultured cells from the substrate by digesting the substrate via exposure of the substrate to (i) a chelating agent, (ii) an enzyme, or (iii) a chelating agent and an enzyme, the separating resulting in a harvest solution; and performing a series of wash and/or centrifugation cycles of components of a harvest solution following the separating, wherein the substrate comprising a polygalacturonic acid compound selected from at least one of: pectic acid; partially esterified pectic acid, partially amidated pectic acid and salts thereof, and an adhesion polymer on the surface of the polygalacturonic acid compound.

[00249] Aspect 18 pertains to the method of Aspect 17, wherein the enzyme comprises a non-proteolytic enzyme.

[00250] Aspect 19 pertains to the method of Aspect 18, wherein the non-proteolytic enzyme is selected from the group consisting of pectinolytic enzymes and pectinases.

[00251] Aspect 20 pertains to the method of any of Aspects 17-19, wherein digesting the dissolvable substrate comprises exposing the dissolvable substrate to between about 1 U/mL and about 200 U/mL of the enzyme, or between about 1 U/mL and about 50 U/mL, or between about 1 U/mL and about 30 U/mL, or less than about 30 U/mL.

[00252] Aspect 21 pertains to the method of any of Aspects 17-20 comprising exposing the dissolvable foam scaffold to the chelating agent at a concentration of less than about 10 mM, less

than about 9 mM, less than about 8 mM, less than about 7 mM, less than about 6 mM, less than about 5 mM, less than about 4 mM, less than about 3 mM, less than about 2 mM, or equal to or less than about 1 mM.

[00253] Aspect 22 pertains to the method according to any of Aspects 17-21, wherein the chelating agent is EDTA.

[00254] Aspect 23 pertains to the method of any one of Aspects 17-22, wherein, after digesting, at least a portion of the adhesion polymer becomes soluble.

[00255] Aspect 24 pertains to the method of Aspect 23, wherein the portion of the adhesion polymer that becomes soluble is at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 95%.

[00256] Aspect 25 pertains to the method of Aspect 23 or Aspect 24, further comprising removing a non-soluble portion of the adhesion polymer by adding a protease to the harvest solution.

[00257] Aspect 26 pertains to the method of Aspect 25, wherein the protease is trypsin.

[00258] As used herein, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a “divalent cation” includes examples having two or more such “divalent cations” unless the context clearly indicates otherwise.

[00259] The term “include” or “includes” means encompassing but not limited to, that is, inclusive and not exclusive.

[00260] “Optional” or “optionally” means that the subsequently described event, circumstance, or component, can or cannot occur, and that the description includes instances where the event, circumstance, or component, occurs and instances where it does not.

[00261] Ranges can be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, examples include from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value

forms another aspect. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

[00262] Unless otherwise expressly stated, it is in no way intended that any method set forth herein be construed as requiring that its steps be performed in a specific order. Accordingly, where a method claim does not actually recite an order to be followed by its steps or it is not otherwise specifically stated in the claims or descriptions that the steps are to be limited to a specific order, it is no way intended that any particular order be inferred. Any recited single or multiple feature or aspect in any one claim can be combined or permuted with any other recited feature or aspect in any other claim or claims.

[00263] It is also noted that recitations herein refer to a component being “configured” or “adapted to” function in a particular way. In this respect, such a component is “configured” or “adapted to” embody a particular property, or function in a particular manner, where such recitations are structural recitations as opposed to recitations of intended use. More specifically, the references herein to the manner in which a component is “configured” or “adapted to” denotes an existing physical condition of the component and, as such, is to be taken as a definite recitation of the structural characteristics of the component.

[00264] While various features, elements or steps of particular embodiments may be disclosed using the transitional phrase “comprising,” it is to be understood that alternative embodiments, including those that may be described using the transitional phrases “consisting” or “consisting essentially of,” are implied. Thus, for example, implied alternative embodiments to a hydrocolloid solution comprising pectic acid and water include embodiments where a hydrocolloid solution consists of pectic acid and water and embodiments where a hydrocolloid solution consists essentially of pectic acid and water.

[00265] It will be apparent to those skilled in the art that various modifications and variations can be made to the present inventive technology without departing from the spirit and scope of the disclosure. Since modifications, combinations, sub-combinations and variations of the disclosed embodiments incorporating the spirit and substance of the inventive technology may occur to persons skilled in the art, the inventive technology should be construed to include everything within the scope of the appended claims and their equivalents.