(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 April 2024 (18.04.2024)

WIPO | PCT

(10) International Publication Number
WO 2024/080932 A1

(51) International Patent Classification:
A23L 13/00 (2016.01) *C12N 5/077* (2010.01)
A23L 33/185 (2016.01) *A23J 3/34* (2006.01)

(21) International Application Number:
PCT/SG2023/050688

(22) International Filing Date:
12 October 2023 (12.10.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10202251361N 12 October 2022 (12.10.2022) SG

(71) Applicant: **NATIONAL UNIVERSITY OF SINGAPORE** [SG/SG]; 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(72) Inventors: **KONG, Yan**; c/o National University of Singapore, Faculty of Science / Department of Food Science and Technology, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **HUANG, Dejian**; c/o National University of Singapore, Faculty of Science / Department of Food Science and Technology, 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(74) Agent: **DONALDSON & BURKINSHAW LLP**; P.O. Box 3667, Robinson Road Post Office, Singapore 905667 (SG).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST,

(54) Title: A MICROCARRIER, A METHOD OF MAKING A MICROCARRIER, A SYSTEM FOR PRODUCING A CELL CULTURED FOOD PRODUCT AND A METHOD OF PRODUCING A CELL CULTURED FOOD PRODUCT

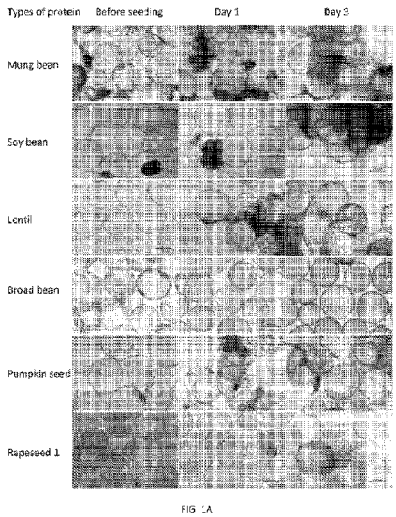


FIG. 1A

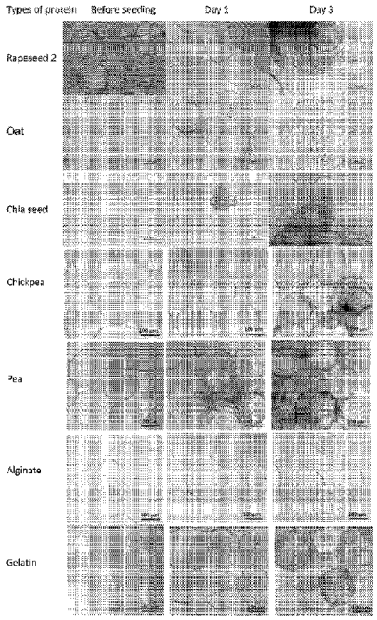


FIG. 1A continued

(57) Abstract: There is provided a microcarrier, a method of making a microcarrier, a system for producing a cell cultured food product and a method of producing a cell cultured food product, the microcarrier comprising, a core particle; and a coating layer disposed on a surface of the core particle; wherein the core particle and coating layer comprise one or more materials derived from plant-based sources.

WO 2024/080932 A1

[Continued on next page]

SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

A MICROCARRIER, A METHOD OF MAKING A MICROCARRIER, A SYSTEM FOR PRODUCING A CELL CULTURED FOOD PRODUCT AND A METHOD OF PRODUCING A CELL CULTURED FOOD PRODUCT

TECHNICAL FIELD

The present disclosure relates broadly to a microcarrier, a method of making a microcarrier, a system for producing a cell cultured food product and a method of producing a cell cultured food product.

BACKGROUND

Conventional animal-based farming practices have raised significant sustainability and global warming concerns due to their carbon footprint, environmental, social, and economic impacts. Some of the concerns associated with conventional animal-based farming practices include animal welfare, greenhouse gas emissions, land use and deforestation, water usage, food security and safety, energy consumption and the associated carbon footprint. To address these sustainability and global warming concerns, there is a growing interest in alternative food production methods.

Cell-based meat (CBM), also termed cultured meat, is an emerging technology which aims to manufacture meat at commercial scale directly from cell culture in a controlled environment. CBM may provide a sustainable and ethical alternative to traditional meat production. It has the potential to address many of the environmental and ethical concerns associated with conventional animal agriculture while providing consumers with a familiar and nutritious source of protein.

However, there are many technological hurdles to overcome before CBM can be economically competitive with conventional meat to make an impact to the food supply.

Two-dimensional (2D) systems have been used to produce cultured meat in laboratories. In 2D systems, tissue culture flasks or petri dishes are used as an adherent mechanical support for cells. However, a major drawback of a 2D culture is the limited space for proliferation. In addition, cells that are randomly grown on a 2D surface hardly communicate with one another and there is barely any cell–matrix interactions, resulting in slower proliferation, limited differentiation, and failure to form cohesive tissue features, such as tubular and cyst structures, typically found in epithelial tissues.

Three-dimensional (3D) systems have also been used to produce cultured meat in laboratories. For example, microcarriers may be used to provide anchorage for cells to adhere to and proliferate in a 3D environment. The large surface to volume ratio in microcarriers may facilitate scaling up for industrial production. However, one of the difficulties faced in designing microcarriers is the lack of functional groups with high cytoaffinity and recognition. Microcarriers have been coated with RGD-containing materials such as gelatin to promote cell attachment and proliferation. However, the use of animal derived proteins for cell-based meat culture is not feasible for industrial scale operation because of their high costs, risk of viral contamination, and batch to batch variations.

Thus, there is a need for a microcarrier, a method of making a microcarrier, a system for producing a cell cultured food product and a method of producing a cell cultured food product, which seek to address or at least ameliorate one of the above problems.

SUMMARY

In one aspect, there is provided a microcarrier comprising, a core particle; and a coating layer disposed on a surface of the core particle; wherein the core particle and coating layer comprise one or more materials derived from plant-based sources.

In one embodiment of the microcarrier as disclosed herein, the core particle is a hydrogel-based particle.

In one embodiment of the microcarrier as disclosed herein, the core particle comprises one or more materials selected from the group consisting of alginate, cellulose, chitosan, gum, hemicellulose, lignin, pectin, soy, starch, and zein.

In one embodiment of the microcarrier as disclosed herein, the coating layer comprises a protein or a protein hydrolysate.

In one embodiment of the microcarrier as disclosed herein, the protein hydrolysate has a degree of hydrolysis of from 1% to 30%.

In one embodiment of the microcarrier as disclosed herein, the coating layer comprises at least 3 amino acids selected from the group consisting of arginine, histidine, aspartic acid, glutamic acid, proline, serine, glycine, and alanine.

In one embodiment of the microcarrier as disclosed herein, the coating layer comprises amino acid sequences selected from the group consisting of RGD (SEQ ID NO: 1), IKVAV (SEQ ID NO: 6), YIGSR (SEQ ID NO: 7), DGEA (SEQ ID NO: 8), PHRSN (SEQ ID NO: 9), PRARI (SEQ ID NO: 10), permutations thereof or sequence order variations thereof.

In one embodiment of the microcarrier as disclosed herein, the coating layer is coupled to the core particle via a cross-linker.

In one embodiment of the microcarrier as disclosed herein, the cross-linker comprises a natural cross-linker selected from the group consisting of flavanones, genipin, quercetin, tannic acid and transglutaminase.

In one embodiment of the microcarrier as disclosed herein, the coating layer exhibits self-fluorescence.

In one embodiment of the microcarrier as disclosed herein, the plant-based sources include one or more selected from the group consisting of broad bean, chickpea, chia seed, corn, lentil, mung bean, oat, pea, pumpkin seed, rapeseed, rice, soybean, wheat, and winter squash.

In another aspect, there is provided a method of making a microcarrier, the method comprising, providing a core particle; and forming a coating layer on a surface of the core particle to obtain the microcarrier, wherein the core particle and coating layer comprise one or more materials derived from plant-based sources.

In one embodiment of the method as disclosed herein, providing the core particle comprises electrospraying a precursor solution to form the core particle in the form of a microbead.

In one embodiment of the method as disclosed herein, forming the coating layer comprises immersing the core particle in a coating solution comprising a protein or a protein hydrolysate.

In one embodiment of the method as disclosed herein, prior to forming the coating layer, the method further comprises preparing the coating solution comprising the protein hydrolysate.

In one embodiment of the method as disclosed herein, the protein hydrolysate has a degree of hydrolysis of from 1% to 30%.

In one embodiment of the method as disclosed herein, preparing the coating solution comprising the protein hydrolysate comprises hydrolyzing a protein using an enzyme-substrate concentration (E/S%) of from 0.1% to 50%.

In one embodiment of the method as disclosed herein, the method further comprises immersing the core particle coated with the coating layer in a cross-linking solution.

In one embodiment of the method as disclosed herein, the cross-linker comprises a natural cross-linker selected from the group consisting of flavanones, genipin, quercetin, tannic acid, and transglutaminase.

In another aspect, there is provided a system for producing a cell cultured food product, the system comprising, a population of cells; and a plurality of microcarriers configured to allow the population of cells to be grown thereon; wherein the microcarrier comprises a core particle and a coating layer disposed on a surface of the core particle; and wherein the core particle and coating layer comprise one or more materials derived from plant-based sources.

DEFINITIONS

The term "microcarrier" as used herein is to be interpreted broadly to refer to a discrete particle with physical dimensions in the order of micrometers, which is suitable for carrying a target agent, e.g., cells. For example, the microcarrier may be suitable for use in culturing cells by providing a substrate or scaffold onto which cells may attach. Microcarriers may be in any suitable shape, such as a sphere, hemisphere, ellipsoid, rod, disk and the like. In various embodiments, the

physical dimensions include but are not limited to length, width, thickness, diameter and the like. In various embodiments, the micrometer order of magnitude is defined as not more than about 1000 μm, not more than about 950 μm, not more than about 900 μm, not more than about 850 μm, not more than about 800 μm, not more than about 750 μm, not more than about 700 μm, not more than about 650 μm, not more than about 600 μm, not more than about 550 μm, not more than about 500 μm, not more than about 450 μm, not more than about 400 μm, not more than about 350 μm, not more than about 300 μm, not more than about 250 μm, not more than about 200 μm, not more than about 150 μm, not more than about 100 μm, not more than about 50 μm, not more than about 40 μm, not more than about 30 μm, not more than about 20 μm, or not more than about 10 μm.

The term "core particle" as used herein is to be interpreted broadly to encompass both solid and semi-solid particles e.g., gel-like particles. The core particle may be in any suitable shape, such as a sphere, hemisphere, ellipsoid, rod, disk and the like.

The term "coating layer" as used herein is to be interpreted broadly to encompass a layer of material that is at least partially covering a target surface e.g., surface of the core particle or part thereof.

The term "hydrogel" as used herein is to be interpreted broadly to refer to three-dimensional cross-linked polymer networks, which can absorb and retain a relatively large amount of water.

The term "microbead" as used herein is to be interpreted broadly to refer to a bead or particle of any material having dimensions of from about 1 μm to about 1000 μm.

The term "substrate" as used herein is to be interpreted broadly to refer to any supporting structure.

The term "layer" when used to describe a first material is to be interpreted broadly to refer to a first depth of the first material that is distinguishable from a second depth of a second material. The first material of the layer may be present as a continuous film, as discontinuous structures or as a mixture of both. The layer may also be of a substantially uniform depth throughout or varying depths. Accordingly, when the layer is formed by individual structures, the dimensions of each of individual structure may be different. The first material and the second material may be same or different and the first depth and second depth may be same or different.

The term "continuous" when used to describe a film or a layer is to be interpreted broadly to refer to a film or a layer that is substantially without gaps or holes or voids across the film or layer. In this regard, a continuous film or a continuous layer is also intended to include a film or a layer that may have trivial gaps or holes or voids that may not appreciably affect the desired properties of the film or the layer.

The term "biocompatible" as used herein is to be interpreted broadly to refer to the ability of a material to perform its intended function without inducing significant inflammatory response, immunogenicity, or cytotoxicity to native cells, tissues, or organs.

The term "biodegradable" as used herein is to be interpreted broadly to refer to a material that is capable of being broken down physically and/or chemically within the body of a subject by the action of living things (such as microorganisms), e.g., by hydrolysis under physiological conditions, by natural biological processes such as the action of enzymes present within the body, etc., to form smaller chemical species which can be metabolized and/or excreted.

The term "cytoaffinity" is to be interpreted broadly as an affinity to cells, e.g., an affinity of a material or structure to cells.

The term "cytotoxic" as used herein is to be interpreted broadly to refer to the ability of a compound or process to disrupt the normal metabolism, function and/or structure of a cell.

The term "food grade" as used herein is to be interpreted broadly to refer to any substance that is approved for human consumption by a relevant authority in a jurisdiction, e.g., the Food and Drug Administration (FDA) in the U.S. and the Singapore Food Agency in Singapore.

The term "micro" as used herein is to be interpreted broadly to include dimensions from about 1 micron to about 1000 microns.

The term "nano" as used herein is to be interpreted broadly to include dimensions less than about 1000 nm.

The term "particle" as used herein broadly refers to a discrete entity or a discrete body. The particle described herein can include an organic, an inorganic or a biological particle. The particle used described herein may also be a macro-particle that is formed by an aggregate of a plurality of sub-particles or a fragment of a small object. The particle of the present disclosure may be spherical, substantially spherical, or non-spherical, such as irregularly shaped particles or ellipsoidally shaped particles. The term "size" when used to refer to the particle broadly refers to the largest dimension of the particle. For example, when the particle is substantially spherical, the term "size" can refer to the diameter of the particle; or when the particle is substantially non-spherical, the term "size" can refer to the largest length of the particle.

The terms "coupled" or "connected" as used in this description are intended to cover both directly connected or connected through one or more intermediate means, unless otherwise stated.

The term "associated with", used herein when referring to two elements refers to a broad relationship between the two elements. The relationship includes, but is not limited to a physical, a chemical or a biological relationship. For example, when element A is associated with element B, elements A and B may be directly or indirectly attached to each other or element A may contain element B or vice versa.

The term "adjacent" used herein when referring to two elements refers to one element being in close proximity to another element and may be but is not limited to the elements contacting each other or may further include the elements being separated by one or more further elements disposed therebetween.

The term "and/or", e.g., "X and/or Y" is understood to mean either "X and Y" or "X or Y" and should be taken to provide explicit support for both meanings or for either meaning.

It will be appreciated that for use of substantially any singular and/or plural terms herein, those skilled in the art will be able to interpret the plural in the singular and/or the singular in the plural as appropriate to the context and/or application. Various singular/plural alterations may be expressly set forth herein for clarity. The singular ("a" or "an") does not exclude the plural.

Further, in the description herein, the word "substantially" whenever used is understood to include, but not restricted to, "entirely" or "completely" and the like. In addition, terms such as "comprising", "comprise", and the like whenever used, are intended to be non-restricting descriptive language in that they broadly include elements/components recited after such terms, in addition to other components not explicitly recited. For example, when "comprising" is used, reference to a "one" feature is also intended to be a reference to "at least one" of that feature. Terms such as "consisting", "consist", and the like, may in the appropriate context, be considered as a subset of terms such as "comprising", "comprise", and the like. Therefore, in embodiments disclosed herein using the

terms such as "comprising", "comprise", and the like, it will be appreciated that these embodiments provide teaching for corresponding embodiments using terms such as "consisting", "consist", and the like. Further, terms such as "about", "approximately" and the like whenever used, typically means a reasonable variation, for example a variation of +/- 5% of the disclosed value, or a variance of 4% of the disclosed value, or a variance of 3% of the disclosed value, a variance of 2% of the disclosed value or a variance of 1% of the disclosed value.

Furthermore, in the description herein, certain values may be disclosed in a range. The values showing the end points of a range are intended to illustrate a preferred range. Whenever a range has been described, it is intended that the range covers and teaches all possible sub-ranges as well as individual numerical values within that range. That is, the end points of a range should not be interpreted as inflexible limitations. For example, a description of a range of 1% to 5% is intended to have specifically disclosed sub-ranges 1% to 2%, 1% to 3%, 1% to 4%, 2% to 3% etc., as well as individually, values within that range such as 1%, 2%, 3%, 4% and 5%. It is to be appreciated that the individual numerical values within the range also include integers, fractions and decimals. Furthermore, whenever a range has been described, it is also intended that the range covers and teaches values of up to 2 additional decimal places or significant figures (where appropriate) from the shown numerical end points. For example, a description of a range of 1% to 5% is intended to have specifically disclosed the ranges 1.00% to 5.00% and also 1.0% to 5.0% and all their intermediate values (such as 1.01%, 1.02% ... 4.98%, 4.99%, 5.00% and 1.1%, 1.2% ... 4.8%, 4.9%, 5.0% etc.,) spanning the ranges. The intention of the above specific disclosure is applicable to any depth/breadth of a range.

Additionally, when describing some embodiments, the disclosure may have disclosed a method and/or process as a particular sequence of steps. However, unless otherwise required, it will be appreciated that the method or process should not be limited to the particular sequence of steps disclosed. Other sequences of steps may be possible. The particular order of the steps disclosed

herein should not be construed as undue limitations. Unless otherwise required, a method and/or process disclosed herein should not be limited to the steps being carried out in the order written. The sequence of steps may be varied and still remain within the scope of the disclosure.

Furthermore, it will be appreciated that while the present disclosure provides embodiments having one or more of the features/characteristics discussed herein, one or more of these features/characteristics may also be disclaimed in other alternative embodiments and the present disclosure provides support for such disclaimers and these associated alternative embodiments.

DESCRIPTION OF EMBODIMENTS

Exemplary, non-limiting embodiments of a microcarrier, a method of making a microcarrier, a system for producing a cell cultured food product and a method of producing a cell cultured food product are disclosed hereinafter.

MICROCARRIER

In various embodiments, there is provided a microcarrier comprising, a core particle; and a coating layer disposed on a surface of the core particle; wherein the core particle and coating layer comprise one or more materials derived from plant-based sources.

In various embodiments, the microcarrier may have a core-shell structure wherein the core particle forms the core, and the coating layer forms the shell. In various embodiments, the core particle and the coating layer may be made from the same or different materials. In various embodiments, the surface of the core particle may be partially or completely coated by the coating layer. In various embodiments, the core particle may be encapsulated/ encompassed/ covered by

the coating layer. In various embodiments, the core particle may be functionalized by the coating layer disposed/ formed/ deposited/coated thereon.

In various embodiments, the microcarrier is manufactured using materials that are derived from natural sources. In various embodiments, it will be appreciated that the materials that are derived from natural sources e.g., plant sources, may undergo processing into one or more forms that are suitable for making the microcarrier. In various embodiments, processing may include extraction, purification, solubilization, drying, hydrolysis, and the like. In various embodiments, the microcarrier may be substantially devoid of any synthetic substance(s).

In various embodiments, the microcarrier is manufactured using materials that are derived from plant sources. In various embodiments, the microcarrier may be a plant-based microcarrier. In various embodiments, the microcarrier may comprise plant proteins, e.g., purified plant protein. In various embodiments, the microcarrier may be substantially devoid of any substance(s) derived from animal source(s). In various embodiments, materials that are derived from plant sources are advantageously more cost effective as compared to materials that are derived from animal sources. In various embodiments, materials that are derived from plant sources advantageously obviate the concerns associated with animal derived substances, e.g., animal derived proteins, such as spreading of infectious diseases, growth factor contamination, and batch to batch variations. In various embodiments, the plant-based sources include but are not limited to broad bean, chickpea, chia seed, corn, lentil, mung bean, oat, pea, pumpkin seed, rapeseed, rice, soybean, wheat, and winter squash. In various embodiments, the plant-based sources include one or more selected from the group consisting of broad bean, chickpea, chia seed, corn, lentil, mung bean, oat, pea, pumpkin seed, rapeseed, rice, soybean, wheat, and winter squash.

In various embodiments, the microcarrier is edible. In various embodiments, the microcarrier may be safe for human and/or animal

consumption. In various embodiments, the microcarrier may be manufactured using food-grade materials. In various embodiments, the microcarrier may be manufactured in a food processing facility. In various embodiments therefore, the microcarrier may be used or may be present, e.g., embedded, in a food and/or beverage product without food safety concerns. In various embodiments, the microcarrier is substantially free of pathogens. In various embodiments therefore, there is no need to detach cells from the microcarrier when the microcarrier is used for cell culture of a food product. In various embodiments therefore, there is no need to remove the microcarrier from the cell cultured food product.

In various embodiments, the microcarrier is biocompatible. In various embodiments, the biocompatibility of the microcarrier may depend on its surface properties, such as stiffness, surface charge, chemical functionalities, roughness, and wettability. In various embodiments, the microcarrier is substantially non-cytotoxic to cells. In various embodiments, the microcarrier is biodegradable. In various embodiments, degradation by-products of the microcarrier is substantially non-cytotoxic to cells. In various embodiments, the microcarrier is manufactured using a fabrication process that is non-toxic.

In various embodiments, the microcarrier is sterilizable. In various embodiments, the microcarrier may remain stable under heat. For example, the diameter of the microcarrier may not substantially change before and after the application of heat. In various embodiments, cross-linking of the coating layer may impart stability to the microcarrier. In various embodiments therefore, the microcarrier may be sterilized using the autoclave process. In various embodiments, the microcarrier may be provided in a sterile form.

In various embodiments, the microcarrier or the core particle has a diameter of from about 50 μm to about 1000 μm. In various embodiments, the microcarrier or the core particle has a diameter falling in a range with approximate start and end points selected from the following group of numbers: 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240,

250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, and 1000 μm. In some embodiments, the microcarrier or the core particle has a diameter of from about 150 μm to about 200 μm. In various embodiments, the microcarrier or the core particle has a size (e.g., diameter) which is compatible with the dimensions of a cell to be cultured thereon. A microcarrier or the core particle of an appropriate size would enable the cells to attach and proliferate thereon. In various embodiments, a larger microbead may result in a lower growth surface area to volume ratio. In various embodiments, a larger microbead may require a higher energy input in order to achieve complete microcarrier suspension in culture. In various embodiments, a smaller microbead may increase the difficulty of cell adhesion and harvesting, resulting in a lower final cell yield and higher costs incurred. In various embodiments, the size of the microcarrier or the core particle may be controllable.

In various embodiments, a plurality of microcarriers or core particles have a substantially uniform size or diameter. In various embodiments, the plurality of microcarriers have a coefficient of variation of the diameter of less than about 20%, less than about 19%, less than about 18%, less than about 17%, less than about 16%, less than about 15%, less than about 14%, less than about 13%, less than about 12%, less than about 11%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1%. Advantageously, cells that are cultured on microcarriers having a substantially uniform size or diameter reach confluence at approximately the same time.

In various embodiments, the plurality of microcarriers or core particles may have a particle size distribution calculated by (d90-d10)/d50, where d10 is defined

as the diameter of a microcarrier at which 10% of the microcarriers are smaller than this diameter, d50 is defined as the diameter of a microcarrier at which 50% of the microcarriers are smaller than this diameter, d90 is defined as the diameter of a microcarrier at which 90% of the microcarriers are smaller than this diameter.

In various embodiments, the microcarrier has a density of from about 0.9 g/cm^3 to about 1.3 g/cm^3. In various embodiments, the microcarrier has a density falling in a range with approximate start and end points selected from the following group of numbers: 0.9, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, 0.99, 1, 1.01, 1.02, 1.03, 1.04, 1.05, 1.06, 1.07, 1.08, 1.09, 1.1, 1.11, 1.12, 1.13, 1.14, 1.15, 1.16, 1.17, 1.18, 1,19, 1.20, 1.21, 1.22, 1.23, 1.24, 1.25, 1.25, 1.27, 1.28, 1.29, and 1.3 g/cm^3. In various embodiments, the microcarrier may have a density for allowing suspension of the microcarrier in a suspension cell culture.

CORE PARTICLE

In various embodiments, the core particle is a microbead. In various embodiments, the core particle is a solid or semi-solid particle. In various embodiments, the core particle is a semi-solid particle, e.g., gel particle.

In various embodiments, the core particle is a hydrogel-based particle. In various embodiments, when used in cell culture applications, a hydrogel may advantageously allow the cells to be placed in a more physiological three-dimensional environment as compared to the classical, flat two-dimensional cell culture. In various embodiments, hydrogels may provide a hydrophilic and bioinert microenvironment for cell culture. In various embodiments, hydrogels may be customized to better mimic the natural environment of the cells to be cultured. For example, the stiffness/rigidity of the environment may be adjusted to match with the natural stiffness of the cells/tissue of origin. In various embodiments, a hydrogel-based microcarrier (i.e., a microcarrier comprising a hydrogel-based core particle) may be configured with specific water retention capacity at high temperatures for enhancing juiciness of a cooked food product.

In various embodiments, the core particle may be fabricated using materials derived from natural sources, e.g., natural polymers for fabricating hydrogels. In various embodiments, the core particle may be fabricated using materials derived from plant sources. In various embodiments, the use of materials derived from plant sources may advantageously obviate the drawbacks of using materials derived from animal sources (e.g., gelatin). Such drawbacks include, for example, difficulty in industrial scale operation due to high costs, risk of microbial contamination, and batch to batch variations. In various embodiments, the use of materials derived from plant sources may advantageously obviate the drawbacks of using synthetic materials. Such drawbacks include, for example, the use of enzymatic digestion to separate cells from the microcarrier during cell harvest, cytotoxicity of chemical cross-linking agents, difficulty in removing chemical cross-linking agents, degradation of the microcarrier when exposed to high temperatures required for sterilization, changes in pH during degradation of the microcarrier leading to cell death, low biodegradability, inedibility, and relatively high prices.

In various embodiments, the core particle is a plant-based hydrogel. In various embodiments, the plant-based hydrogel may be a plant lignocellulose materials-based hydrogel (e.g., cellulose-based hydrogel, hemicellulose-based hydrogel, lignin-based hydrogel), a plant polysaccharide-based hydrogel (e.g., starch-based hydrogel, pectin-based hydrogel, plant gum-based hydrogel), a plant protein-based hydrogel (e.g., soy protein-based hydrogel, zein-based hydrogel), or a combination thereof. In various embodiments, the core particle comprises one or more materials selected from the group consisting of alginate, cellulose, chitosan, gum, hemicellulose, lignin, pectin, soy, starch, and zein. For example, polysaccharides such as alginate and pectin may possess ionic gelation capabilities. For example, mushroom carboxymethyl chitosan may be capable of forming porous microspheres for chondrocyte cultures.

In various embodiments, the core particle may be an alginate hydrogel microbead, e.g., sodium alginate hydrogel microbead, calcium alginate hydrogel microbead. In various embodiments, a core particle made of an alginate hydrogel may provide one or more of the following advantages: good mechanical integrity, cell compatibility, simple preparation method, abundance of raw material sources, ease of controlling the properties of the alginate hydrogel within a large range (e.g., physicochemical property, mechanical strength, degradation rate and surface property).

COATING LAYER

In various embodiments, the coating layer is coupled to the core particle. In various embodiments, the coating layer may be coupled to the core particle via physical adsorption. In various embodiments, the coating layer may further be coupled to the core particle via cross-linking. In various embodiments, the surface of the core particle and the coating layer may have opposite electrical charges. The opposite electrical charges may advantageously encourage adsorption of the coating layer on the core particle. For example, where the core particle comprises sodium alginate, the surface of the core particle would have a net negative charge, thereby encouraging the adsorption of a coating layer with a net positive charge, e.g., a coating layer comprising amino acids with a positive charge.

In various embodiments, the microcarrier may comprise more than one coating layer. In various embodiments, the microcarrier may comprise multiple coating layers. Each coating layer may have a composition that is different from the other (e.g., adjacent) coating layer(s). Each coating layer may impart a specific set of one or more properties to the microcarrier.

In various embodiments, the coating layer comprises one or more materials derived from plant source(s). In various embodiments, the coating layer may be partially or completely made of one or more materials derived from plant source(s). In various embodiments, the plant source may comprise seeds, fruits,

vegetables. Examples of materials derived from plant sources include but are not limited to chickpea, mung bean, soybean, lentil e.g., red lentil, broad bean, pumpkin seed, rapeseed, oat, chia seed, pea, garden pea, winter squash, wheat, rice, corn, or combinations thereof. Food grade plant proteins are abundant and can be upcycled from oil seed processing by-products such as chickpea, pumpkin seed meal, rapeseed meal, and sunflower seed meal.

In various embodiments, the coating layer comprising one or more plant-based material may be capable of supporting growth of cells, for example, by promoting better adhesion of cells to, proliferation and/or differentiation of cells on the microcarrier. Without wishing to be bound by theory, it is believed that plant proteins may contain active domains that can be used to replace the animal-based ECM for meat culture applications. In various embodiments, plant proteins may be capable of mimicking ECM proteins. Advantageously, the plant-based material may be used in place of extracellular matrix (ECM) components e.g., ECM proteins. ECM components may include, for example, collagen, fibronectin, laminin, poly-L-lysine, gelatin, keratin, vitronectin, elastin, heparan sulphate, dextran, dextran sulphate, chondroitin sulphate, heparan sulfate proteoglycans, and derivatives or fragment thereof.

In various embodiments, the coating layer comprises one or more types of cyto-adhesive functional molecules. In various embodiments, the coating layer comprises one or more compatible, biologically relevant, or physiologically relevant substance, e.g., cyto-adhesive functional molecules, capable of supporting growth of cells. In various embodiments, the presence of cyto-adhesive functional molecules bonded to the surface of the core particle may advantageously improve cyto-affinity of the microcarrier.

In various embodiments, the coating layer comprises a protein or a protein hydrolysate. In various embodiments, the coating layer is a protein coating layer. In various embodiments, the coating layer is a protein hydrolysate coating layer. In various embodiments, the coating layer comprising a protein, or a protein

hydrolysate may provide improved cytoaffinity to the microcarrier. In various embodiments, the protein may be a plant-based protein. In various embodiments, the protein hydrolysate may be a hydrolysate of the plant-based protein.

In various embodiments, the protein hydrolysate may be obtained by subjecting a protein to enzymatic hydrolysis using a suitable enzyme. In various embodiments, enzymes such as proteinase/protease/proteolytic enzyme may be used to digest the protein to form protein hydrolysate. Examples of enzymes may include but are not limited to trypsin, papain, alcalase, and flavorzyme. In various embodiments, the proteinase/ protease/ proteolytic enzyme has an optimal working pH of more than 7. In various embodiments, the proteinase/protease/proteolytic enzyme is an enzyme found in the gut e.g., or the small intestine of a mammalian subject.

In various embodiments, enzymatic hydrolysis may advantageously generate bioactive peptides. For example, plant proteins may be rich in cell adhesion peptides such as Arg-Gly-Asp (RGD)-containing peptide. In various embodiments therefore, selective enzyme hydrolysis may be used to improve the bioactivity of plant proteins. In various embodiments, the enzyme may be configured to cleave a peptide at sites to expose arginine and/or lysine residues. Enzymatic hydrolysis may be employed in food and pharmaceutical industries because the process is typically conducted under mild controlled conditions (e.g., pH, temperature, substrate concentration, and enzyme activity), provides high specificity and the absence of residual organic solvents and toxic chemicals in the final peptide composition.

In various embodiments, the protein hydrolysate has a degree of hydrolysis of from about 1% to about 30%. In various embodiments, the degree of hydrolysis of the protein hydrolysate may fall in a range with approximate start and end points selected from the following group of numbers: 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5. 21, 21.5,

22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, and 30%. In various embodiments, the degree of hydrolysis (DH) is defined as the proportion of the number of peptide bonds being broken in protein hydrolysis, or the proportion of cleaved peptide bonds in a protein hydrolysate. For example, if a starting protein containing one hundred peptide bonds is hydrolyzed until ten of the peptide bonds are cleaved, then the DH of the resulting hydrolysate is 10%. The degree of hydrolysis may be determined using techniques known in the art such as the trinitrobenzene sulfonic (TNBS) colorimetric method or the ortho-phthaldialdehye (OPA) method.

In various embodiments, it will be appreciated that the higher the degree of hydrolysis, the greater the extent of protein hydrolysis. Typically, as the protein is further hydrolyzed (i.e., the higher the DH), the molecular weight of the peptide fragments decreases, the peptide profile changes accordingly. A lower DH may result in longer peptides which could lead to stronger forces of attraction between the core particles, e.g., beads, and the cells due to the larger molecular weight. An increase in DH may result in a higher number of peptide bonds being cleaved but it may not equate to higher bioavailability. In some embodiments, the degree of hydrolysis of the protein hydrolysate may be from about 2.75% to about 6.0%. In some embodiments, the degree of hydrolysis of the protein hydrolysate may be about 3.5%.

In various embodiments, the coating layer comprises a protein source which has been selectively hydrolyzed. Common hydrolysis by alkaline can break down the cross-link in disulfide of cereal proteins and render proteins more soluble by the ionization of acidic and neutral amino acids. Enzymes such as papain, alcalase, and flavourzyme also have a very broad specificity of cleavage sites. The selective hydrolysis of the enzyme describes the cleavage of specific types of amino acid after which the enzyme can hydrolyze a peptide bond (e.g., Lys and Arg for trypsin). This may include using a suitable enzyme with cleavage sites that specifically expose amino acid residue(s) and/or peptide fragment(s) with bioactive effects, prior to coating the core particles with the protein

hydrolysate. In various embodiments, the enzyme for hydrolyzing the protein may be trypsin. In various embodiments, trypsin hydrolysis may result in desirable peptides that support cell growth and improve cytoaffinity. As trypsin has two cleavage sites – Arginine and Lysine, enzymatic hydrolysis with trypsin may lead to longer chain peptides as compared to an enzyme with broader specificity and more cleavage sites, which may lead to shorter chain peptides. Without wishing to be bound by theory, it is hypothesized that the adhesion of shorter chain peptides on beads may not be as strong as longer chain peptides, leading to the loss of protein during cell growing stages. Thus, even when shorter chains may be favored for cell growth, the inability to adhere to the beads would mitigate the positive effects on cell proliferation.

In various embodiments, a protein hydrolysate coating may advantageously provide better cyto-affinity as compared to a protein coating of the same material. For example, chickpea protein may be selectively hydrolyzed using protease to obtain a chickpea protein hydrolysate for coating and cross-linking to a core particle. By doing so, the cyto-affinity of the chickpea protein hydrolysate may be significantly enhanced relative to the chickpea protein.

In various embodiments, the coating layer comprises amino acids. The amino acids may include alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine or combinations thereof. In various embodiments, the coating layer comprises at least 3 amino acids selected from the group consisting of arginine, histidine, aspartic acid, glutamic acid and alanine. In various embodiments, the coating layer comprises at least 3 amino acids selected from the group consisting of arginine, histidine, aspartic acid, glutamic acid, proline, serine, glycine, and alanine. In various embodiments, the coating layer comprises amino acid sequences selected from the group consisting of RGD (SEQ ID NO: 1), IKVAV (SEQ ID NO: 6), YIGSR (SEQ ID NO: 7), DGEA (SEQ ID NO: 8), PHRSN (SEQ ID NO: 9), PRARI (SEQ ID NO: 10), permutations thereof or sequence order

variations thereof (e.g., GRD (SEQ ID NO: 2), DGR (SEQ ID NO: 3) etc.). In various embodiments, the coating layer comprises amino acid sequences which advantageously have cell adhesion properties.

In various embodiments, the coating layer comprises peptide fragments having an arginine residue and/or peptide fragments having a lysine residue. In various embodiments, the arginine and lysine residues may be found at the amino terminal and/or carboxyl terminal of the peptide/polypeptide fragments. In various embodiments, hydrolysis of a protein may advantageously expose peptide fragments that promote cell adhesion, e.g., peptide fragments having an arginine or lysine residue.

In various embodiments, the coating layer comprises one or more cell adhesion peptides. In various embodiments, the coating layer is configured to bind to a cell receptor responsible for cell adhesion. In various embodiments, the presence of cell adhesion peptides in the coating layer may advantageously improve cytoaffinity. In various embodiments, cell adhesion peptides may include but are not limited to peptides with the following amino acid sequences: RGD (SEQ ID NO: 1), RGN (SEQ ID NO: 4), DGR (SEQ ID NO: 3), NGR (SEQ ID NO: 5), DGEA (SEQ ID NO: 8), IKVAV (SEQ ID NO: 6), YIGSR (SEQ ID NO: 7), PHRSN (SEQ ID NO: 9), and PRARI (SEQ ID NO: 10). In various embodiments, cell adhesion peptides may be found in plants including but not limited to mung bean, soybean, lentil, broad bean, chickpea, garden pea, pumpkin, winter squash and rapeseed. In various embodiments, the coating layer may comprise DGR-containing peptides. In various embodiments, the coating layer may comprise peptides found in chickpea protein with the following amino acid sequences RQSHFANAQP (SEQ ID NO: 11) and GAGAGS (SEQ ID NO: 12).

In various embodiments, the coating layer is coupled/attached to the core particle via a cross-linker. In various embodiments, the coating layer may be immobilized/fixed to the core particle by a cross-linker/ cross-linking reagent. In various embodiments, the cross-linker may be formaldehyde. In various

embodiments, the cross-linker may be glutaraldehyde. In various embodiments, the cross-linker may be derived from natural sources. In various embodiments, the cross-linker may be a natural food grade compound. In various embodiments, the cross-linker comprises a natural cross-linker including but not limited to flavanones, genipin, quercetin, tannic acid and transglutaminase. In various embodiments, the cross-linker comprises a natural cross-linker selected from the group consisting of flavanones, genipin, quercetin, tannic acid and transglutaminase. In various embodiments, the cross-linker may be transglutaminase, which is an enzyme that can catalyze the acyl-transfer reaction between the γ-carboxyamide group of peptide-bound glutamine residues and various primary amines. The formation of this cross-link does not reduce the nutritional quality of the food as the lysine residue remains available for digestion. In various embodiment, the cross-linker may be genipin, which is a chemical compound found in Genipa americana fruit extract (i.e., a natural protein cross-linking agent isolated from an edible plant). Genipin is an aglycone derived from an iridoid glycoside called geniposide which is also present in fruit of Gardenia jasminoides. Genipin is an excellent natural cross-linker for proteins, collagen, gelatin, and chitosan cross-linking. It has a low acute toxicity, as compared to glutaraldehyde and many other commonly used synthetic cross-linking reagents.

In various embodiments, the coating layer is fluorescent. In various embodiments, the coating layer is configured to exhibit self-fluorescence. Advantageously, the presence of fluorescence in the coating layer may be utilized, for example, to monitor the degree of coating of the coating layer on the core particle, to ensure the uniformity of the coating layer, to monitor the microcarrier during cell culture, by using a microscope e.g., fluorescent microscope. Microcarriers with fluorescence is an indication of successful coating of the protein or protein hydrolysate. Intensity of the fluorescence may be correlated to the amount of proteins or protein hydrolysate being coated on the surface of the core particle. In one example, where the cross-linker is genipin, cross-linking of the protein or protein hydrolysate with genipin produces blue-

colored fluorescent coating layer, due to the chromophore or fluorophore group formed between genipin and the cross-linked proteins.

METHOD OF MAKING A MICROCARRIER

In various embodiments, there is provided a method of making a microcarrier, the method comprising, providing a core particle; and forming a coating layer on a surface of the core particle to obtain the microcarrier, wherein the core particle and coating layer comprise one or more materials derived from plant-based sources. In various embodiments, the method includes forming a plurality of core particles and forming a coating layer on surfaces of the plurality of core particles.

In various embodiments, providing the core particle comprises forming/fabricating the core particle from its raw material. In various embodiments, the raw material may be sodium alginate for forming an alginate core particle. In various embodiments, the raw material may include but is not limited to pectin, chitosan, zein, and cellulose. In various embodiments, the raw material may be selected from the group consisting of pectin, chitosan, zein, cellulose, and combinations thereof.

In various embodiments, providing the core particle comprises electrospraying a precursor solution to form the core particle in the form of a microbead. In various embodiments, the technique of making microbeads may be based on emulsification. In various embodiments, the technique of making microbeads may be based on microfluidics. In various embodiments, the technique of making microbeads may be based on extrusion. In various embodiments, the technique of making microbeads may be based on a phase-inversion method.

In various embodiments, forming the coating layer comprises immersing the core particle in a coating solution. In various embodiments, the coating

solution may comprise a protein or a protein hydrolysate. In various embodiments, the core particles may be immersed into the coating solution at a percent weight/volume (% w/v) of from about 10% w/v to about 50% w/v. In various embodiments, the percent weight/volume (% w/v) of the core particles relative to the coating solution may fall in a range with approximate start and end points selected from the following group of numbers: 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, and 50% w/v. For example, a percent weight/volume of 10% means that 10 grams of the core particles is immersed in every 100 mL of the coating solution.

In various embodiments, the coating solution may have a concentration of from about 0.1% to about 30% by weight of a coating substance, e.g., protein or protein hydrolysate. In various embodiments, the concentration of the coating solution may fall in a range with approximate start and end points selected from the following group of numbers: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, and 30% by weight. For example, a percent weight of 10% means that 10 g of the coating substance is dissolved in 100 g of solution. In various embodiments, the size of the core particle, e.g., bead size, may be altered depending on the concentration of the coating substance e.g., protein. For example, as protein concentration increases, q (%) may decrease, where q (%) refers to the volume at each particle size (i.e., frequency distribution). Without wishing to be bound by theory, it is believed that moisture may have diffused out of the beads due to differences in osmotic pressure between the beads and protein solution.

In various embodiments, the coating process may be carried out at a pH from about 5.0 to about 10.0. In various embodiments, the coating process may be carried out at a pH falling in a range with approximate start and end points selected from the following group of numbers: 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5,

7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, and 10.0.

In various embodiments, the coating process may be carried out at a temperature of from about 4°C to about 60°C, which is below the protein denaturation temperature to prevent the destruction of the functional properties of peptides. In various embodiments, the coating process may be carried out at a temperature falling in a range with approximate start and end points selected from the following group of numbers: 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, and 60°C. In various embodiments, protein adsorption on solid surfaces generally increases with temperature, and the thickness of protein layers is enhanced with elevated temperature.

In various embodiments, prior to forming the coating layer, the method further comprises extracting a protein for making a protein coating solution. In various embodiments, the protein for making the protein coating solution may be extracted using an isoelectric point precipitation method. In various embodiments, extracting the protein may comprise grounding one or more raw materials into powder form. The raw materials may be obtained from plant sources and may include but are not limited to chickpea, mung bean, soybean, lentil e.g., red lentil, broad bean, pumpkin seed, rapeseed, oat, chia seed, pea, garden pea, winter squash, wheat, rice, corn, or combinations thereof.

In various embodiments, extracting the protein may further comprise dispersing the one or more powders of raw materials in a suitable liquid (e.g., deionized water) to obtain a protein mixture/dispersion. In various embodiments, the one or more powders of raw materials may be dispersed at a percent weight/volume (% w/v) of from about 1% w/v to about 50% w/v. In various embodiments, the percent weight/volume (% w/v) of the powder relative to the liquid may fall in a range with approximate start and end points selected from the

following group of numbers: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, and 50% w/v.

In various embodiments, extracting the protein may further comprise solubilizing the protein in the protein mixture. In various embodiments, solubilizing the protein may further comprise adjusting the pH of the protein mixture such that the protein is dissolved. The pH of the protein mixture may be adjusted using a suitable acid or base. In various embodiments, extracting the protein may further comprise removing insoluble non-protein components from the protein mixture. In various embodiments, extracting the protein may further comprise precipitating the dissolved protein by adjusting the pH of the protein mixture to obtain a solid form of the protein. In various embodiments, extracting the protein may further comprise dissolving the solid form of the protein in a suitable solvent by adjusting the pH to obtain a protein solution. The pH of the protein mixture may be adjusted using a suitable acid or base. In various embodiments, extracting the protein may further comprise freezing the protein solution and lyophilizing the frozen protein solution to obtain a lyophilized protein. It will be appreciated that the various steps of extracting the protein may further involve mixing (e.g., using a vortex or shaker machine) and washing steps (e.g., washing with deionized water).

In various embodiments, the step of extracting the protein may have a protein extraction yield of from about 1% to about 20%, depending on the type of raw material. In various embodiments, the step of extracting the protein may have a protein extraction yield falling in a range with approximate start and end points selected from the following group of numbers: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20%.

In various embodiments, prior to forming the coating layer, the method further comprises preparing the coating solution comprising the protein hydrolysate. In various embodiments, the method further comprises hydrolyzing

a protein source with an enzyme to produce protein hydrolysate, prior to coating the core particles with the protein hydrolysate. In various embodiments, the method further comprises selectively hydrolyzing a protein source. This may include using a suitable enzyme with cleavage sites that specifically expose amino acid residue(s) and/or peptide fragment(s) with bioactive effects, prior to coating the core particles with the protein hydrolysate.

In various embodiments, the protein hydrolysate may be obtained by digesting a protein using an enzyme, e.g., proteinase/protease/proteolytic enzyme. In various embodiments, the protein hydrolysate may have a degree of hydrolysis determined by factors such as the concentration of the protein, type of enzyme used, concentration of the enzyme used, and duration of exposure to the enzyme or duration of hydrolysis. In various embodiments, the protein may be subjected to hydrolysis by more than one type of enzyme. For example, the protein may be subjected to sequential hydrolysis, i.e., treating the protein with a first enzyme to obtain a first protein hydrolysate, deactivating the first enzyme, treating the first protein hydrolysate with a second enzyme to obtain a second protein hydrolysate, and deactivating the second enzyme (repeating the steps of treating with other enzymes and deactivating them may also be further carried out).

In various embodiments, the protein hydrolysate solution has a degree of hydrolysis of from about 1% to about 30%. In various embodiments, the degree of hydrolysis of the protein hydrolysate solution may fall in a range with approximate start and end points selected from the following group of numbers: 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, and 30%.

In various embodiments, the hydrolysis of the protein may be carried out over a period of from about 10 minutes to about 240 minutes. In various

embodiments, the hydrolysis of the protein may be carried out over a period falling in a range with approximate start and end points selected from the following group of numbers: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, and 240 minutes.

In various embodiments, preparing the coating solution comprising the protein hydrolysate comprises hydrolyzing a protein using an enzyme-substrate concentration (E/S%) of from about 0.1% to about 50%. In various embodiments, the hydrolysis of the protein may be carried out using an enzyme-substrate concentration (E/S%) falling in a range with approximate start and end points selected from the following group of numbers: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, and 50%.

In various embodiments, the hydrolysis of the protein may be carried out at a protein dispersion of from about 0.1% to about 30% w/v. In various embodiments, the hydrolysis of the protein may be carried out at a protein dispersion falling in a range with approximate start and end points selected from the following group of numbers: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, and 30% w/v.

In various embodiments, forming the coating layer further comprises mixing the core particles immersed in the coating solution. In various embodiments, the core particles immersed in the coating solution may be mixed using mechanical means, such as vortexing or shaking. In various embodiments, mixing may improve contact between the core particles and the coating solution such that the surfaces of the core particles are coated with the coating solution. It will be appreciated that the mixing step is performed at a level of intensity such that the core particles are not substantially damaged.

In various embodiments, forming the coating layer further comprises immersing the core particle coated with the coating layer in a cross-linking solution. In various embodiments, the cross-linker solution may comprise a cross-linker that is derived from natural sources. In various embodiments, the cross-linker solution may comprise a cross-linker that is a natural food grade compound. In various embodiments, the cross-linker comprises a natural cross-linker selected from the group consisting of flavanones, genipin, quercetin, tannic acid and transglutaminase. In various embodiment, the cross-linker solution may comprise genipin. In various embodiment, the cross-linker solution may comprise transglutaminase.

In various embodiments, the core particles may be immersed into the cross-linker solution at a percent weight/volume (% w/v) of from about 5% w/v to about 50% w/v. In various embodiments, the percent weight/volume (% w/v) of the core particles relative to the cross-linker solution may fall in a range with approximate start and end points selected from the following group of numbers: 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, and 50% w/v. For example, a percent weight/volume of 10% means that 10 grams of the core particles is immersed in every 100 mL of the cross-linker solution.

In various embodiments, the cross-linker solution may have a concentration of from about 0.1 mg/mL to about 10 mg/mL. In various embodiments, the concentration of the cross-linker solution may fall in a range with approximate start and end points selected from the following group of numbers: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, and 10 mg/mL.

In various embodiments, the method may comprise washing the coated core particles prior to and after immersing the coated core particles in the cross-

linker solution. In various embodiments, the coated core particles may be washed using a suitable solvent such as deionized water.

SYSTEM AND METHOD OF PRODUCING A CELL CULTURED FOOD PRODUCT

In various embodiments, there is provided a system for producing a cell cultured food product, the system comprising, a population of cells; and a plurality of microcarriers configured to allow the population of cells to be grown thereon; wherein the microcarrier comprises a core particle and a coating layer disposed on a surface of the core particle; and wherein the core particle and coating layer comprise one or more materials derived from plant-based sources.

In various embodiments, there is provided a method of producing a cell cultured food product, the method comprising, seeding a population of cells onto a plurality of microcarriers configured to allow the population of cells to be grown thereon, wherein the microcarrier comprises a core particle and a coating layer disposed on a surface of the core particle; and wherein the core particle and coating layer comprise one or more materials derived from plant-based sources.

In various embodiments, the microcarrier is a microcarrier for cells. In various embodiments, the coating layer of the microcarrier may provide a substrate for cells to attach/adhere thereon. In various embodiments, the coating layer of the microcarrier may advantageously enhance cell attachment, proliferation and differentiation. In various embodiments, the microcarrier may be used to upscale anchorage-dependent cells such as mammalian cells. In various embodiments, a plurality of microcarriers may advantageously provide a relatively large surface area to volume ratio for cell culture as compared to, for example, two-dimensional cell culture.

In various embodiments, the cells may be stem cells or somatic cells. The stem cells may be pluripotent (e.g., embryonic stem cells or induced pluripotent

stem cells) or multipotent (e.g., adult stem cells). The stem cells may be mesenchymal stem cells (MSCs), embryonic stem cells (ESCs) or induced pluripotent stem cells (iPS). In various embodiments, the somatic cells may include but are not limited to muscle cells (e.g., myoblasts) and fat cells (e.g., adipocytes). In various embodiments, the cells may be cells of a non-human organism including but not limited to poultry (e.g., chicken, duck, turkey, goose, fowl), pig, cattle (e.g., cow), sheep, goat, horse, fish and shrimp. In various embodiments, the microcarrier may be used for producing cultured meat (e.g., red meat or white meat or seafood) or cell-based meat (CBM).

In various embodiments, the microcarrier or a plurality of microcarriers may be used in cell culture. In various embodiments, the microcarrier may remain substantially stable in culture media. For example, the diameter of the microcarrier may not substantially change over the duration of the cell culture. The cell culture may include static cell culture or dynamic cell culture. In a static cell culture, the culture or culture media is not actively agitated. Advantageously, aggregates of cells and/or microcarriers may be allowed to form under reduced agitation of the culture or culture media. In a dynamic cell culture, the culture or culture media is actively agitated to promote exchange of nutrients and waste to and from the cells attached on the microcarriers. Dynamic cell culture may include, for example, stirred spinner and bioreactor culture.

In various embodiments, the cells may be seeded onto the microcarriers at a seeding density of from about 10^3 to about 10^4 cells/mL for every 0.01 gram (g) of the microcarriers. In various embodiments, the cells may be seeded onto the microcarriers at a seeding density falling in a range with approximate start and end points selected from the following group of numbers: 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, and 10000 cells/mL for every 0.01 g of the microcarriers.

In various embodiments, the system may further comprise a cell culture device for holding the cells and microcarriers. In various embodiments, the cell

culture device may be a well plate, e.g., 24-well plate. The well plate may be a low attachment well-plate, i.e., the surfaces of the well plate are treated to minimize/prevent cell attachment onto the surface of the well plate. This would promote the attachment of cells onto the microcarriers. In various embodiments, the cell culture device may be a spinner flask or bioreactor. In various embodiments, the system may further comprise one or more equipment for providing a controlled environment for the growth and cultivation of cells. In various embodiments, the equipment is configured to provide a stable and regulated environment in terms of temperature (e.g., 37°C), humidity, carbon dioxide (CO_2) levels (e.g., 5%), oxygen (O_2) concentration to support the optimal growth and proliferation of cells on the microcarriers. For example, the equipment may be a commercially available cell incubator or a custom-built system for industrial scale production.

In various embodiments, a microcarrier-based system and method of cell culture may provide advantages such as ease of downstream processing in many applications. In various embodiments, the use of microcarriers for cell attachment facilitates the use of stirred tank and related reactors for growth of anchorage-dependent cells. The cells attach to the readily suspended microcarriers. The requirement for microcarriers to be suspended may limit the physical parameters of the microcarriers themselves such as the size of the microcarriers. The size of the microcarriers should be large enough to accommodate many anchorage-dependent cells, while small enough to form suspensions with properties suitable for use in bioreactors.

In various embodiments, the cells remain viable when cultured on the microcarrier over a period of time. In various embodiments, the cells may remain viable when cultured on the microcarrier over a period of time from about 1 to about 28 days. In various embodiments, the cells remain viable when cultured on the microcarrier over a period of time falling in a range with approximate start and end points selected from the following group of numbers: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, and 28 days

In various embodiments, the number of cells cultured on the microcarrier may increase by about 1-fold to about 20-fold, about 2-fold to about 19-fold, about 3-fold to about 18-fold, about 4-fold to about 17-fold, about 5-fold to about 16-fold, about 6-fold to about 15-fold, about 7-fold to about 14-fold, about 8-fold to about 13-fold, about 9-fold to about 12-fold, or about 10-fold to about 11-fold from the time when the cells are seeded on the microcarrier to the time the cells are harvested from the microcarrier for cell counting.

In various embodiments, there is provided a cell cultured food product comprising, a population of cells; and a plurality of microcarriers configured to allow the population of cells to be grown thereon; wherein the microcarrier comprises a core particle and a coating layer disposed on a surface of the core particle; and wherein the core particle and coating layer comprise one or more materials derived from plant-based sources. In various embodiments, the cells may be muscle cells (e.g., myoblasts) and fat cells (e.g., adipocytes). In various embodiments, the cells may be cells of a non-human organism including but not limited to poultry (e.g., chicken, duck, turkey, goose, fowl), pig, cattle (e.g., cow), sheep, goat, horse, seafood (e.g., fish and shrimp). In various embodiments, cell cultured food product is cultured meat / cell-based meat. In various embodiments, the culture meat may be white and/or red cultured meat.

BRIEF DESCRIPTION OF FIGURES

FIG. 1A is a set of brightfield microscope images of C2C12 seeding on microbeads with different protein coating during a 3-day culture. Scale bar = 100 μm. FIG. 1B is a set of fluorescent microscope images of microbeads coated with plant proteins. Scale bar = 100 μm.

FIG. 2A to 2C are charts showing the effect of (A) different coating pH, (B) different coating protein concentration (1 to 20%), and (C) different coating

protein concentration (4 to 10%), on the content of coated protein and fluorescence intensity of genipin cross-linked plant protein-alginate microbeads. FIG. 2D to 2F are particle size distribution diagrams of the microbeads formed at (D) different coating pH, (E) different coating protein concentration (1 to 20%), and (F) different coating protein concentration (4 to 10%).

FIG. 3A is a chart showing fold changes of C2C12 seeding on microbeads with different protein coating during a 3-day culture. FIG. 3B to 3D are graphs showing correlation between (B) the content of soluble protein, (C) the content of arginine in plant protein, and (D) the content of lysine in plant protein, with respect to fold changes in cell number. Different numbers represent different types of plant proteins (1-mung bean, 2-soybean, 3-red lentil, 4-broad bean, 5-chickpea, 6-pea, 7-pumpkin seed, 8-rapeseed 1, 9-rapeseed 2, 10-oat, 11-chia seed, 12-gelatin); different lowercase letters between columns represent significant differences between tested results ($p < 0.05$).

FIG. 4A and 4B are microscope images of (A) C2C12 and (B) 3T3 cells proliferation on plant-based protein coated microbeads during a 13-day culture and graphs showing fold changes in cell number over the incubation period. Cell nuclei and scaffolds were stained by Hoechst 33342 and F-actin staining kit. Scale bar = 200 μm.

FIG. 5A to 5C are bright-field microscope images of chicken muscle satellite cells cultured on (A) pumpkin seed protein coated microbeads (scale bar = 10 μm), (B) pumpkin seed protein coated microbeads (scale bar = 100 μm), and (C) alginate microbeads (scale bar = 100 μm). FIG. 5D to 5F are bright-field microscope images of primary porcine cells cultured on (D) pumpkin seed protein coated microbeads (scale bar = 10 μm), (E) pumpkin seed protein coated microbeads (scale bar = 100 μm), and (F) alginate microbeads (scale bar = 100 μm). Cells were attached on microbeads on day 3 of culture (see arrows).

FIG. 6A to 6D are charts showing gene expression and differentiation of C2C12 cells grown on microbeads and on 2D plates. (A) Relative expression of integrin β1 and M-cadherin on 6-well plate and microbeads cultured C2C12 (before differentiation); (B) relative expression of MyoD1, C) Myf5 and D) Myh7 on 6-well plate and microbeads cultured C2C12 during differentiation. FIG. 6E and 6F are confocal laser scanning microscope (CLSM) images of C2C12 cells on (E) 6-well plate during a 7-day differentiation, and (F) pumpkin seed protein coated microbeads during a 7-day differentiation.

FIG. 7 is a photograph showing SDS-PAGE profiles of 11 types of plant proteins.

FIG. 8A are brightfield microscope images of primary porcine myoblasts seeded on microbeads with different protein coating at day 7 of culture. Scale bar = 100 μm. FIG. 8B is a chart showing fold changes in primary porcine myoblasts seeded on microbeads with different protein coating during a 7-day culture. FIG. 8C is a chart showing fold change in C2C12 cells seeded on microbeads with different protein coating during a 3-day culture.

FIG. 9A is a photograph showing microbeads in PBS solution. FIG. 9B is a set of fluorescent microscope images of 9 types of protein-coated microbeads (1, mung bean protein hydrolysate coated microbeads; 2, soybean protein hydrolysate coated microbeads; 3, red lentil protein hydrolysate coated microbeads; 4, broad bean protein hydrolysate coated microbeads; 5, chickpea protein hydrolysate coated microbeads; 6, pea protein hydrolysate coated microbeads; 7, pumpkin seed protein hydrolysate coated microbeads; 8, rapeseed 1 protein hydrolysate coated microbeads; 9, rapeseed 2 protein hydrolysate coated microbeads; 10, alginate microbeads).

FIG. 10A is a 3D chart showing fold changes in C2C12 seeding on microbeads coated with different protein hydrolysates after a 3-day culture. Each number represents a different type of plant protein (1-mung bean, 2-soybean, 3-

red lentil, 4-broad bean, 5-chickpea. 6-pea, 7-pumpkin seed, 8-rapeseed 1, 9-rapeseed 2). FIG. 10B and 10C are charts showing the degrees of hydrolysis for (C) different E/S% ratio and (D) different hydrolysis duration. FIG. 10D is a chart showing a comparison of C2C12 cell adhesion with different degrees of hydrolysis. FIG. 10E and 10F are charts showing (E) fold changes in C2C12 cell numbers and (F) fold changes in 3T3 cell numbers with different degrees of hydrolysis.

FIG. 11 is a chart showing fold changes in primary porcine myoblasts seeded on microbeads with different protein hydrolysis during a 7-day culture. Left and right bars for each plant protein represent hydrolysis by trypsin and papain, respectively.

FIG. 12 is a set of brightfield microscope images of primary porcine myoblasts seeded on microbeads with different protein hydrolysate coating during a 7-day culture. Scale bar = 100 μm.

FIG. 13A is a chart showing the effect of different E/S concentrations on the degree of hydrolysis and coated protein on microbeads. FIG. 13B is a chart showing the effect of different hydrolysis durations on the degree of hydrolysis and coated protein on microbeads.

FIG. 14 is a chart showing fold changes in primary porcine myoblasts seeded on microbeads with different degree of hydrolysis during a 7-day culture.

FIG. 15 is a set of brightfield microscope images of primary porcine myoblasts seeded on microbeads with different protein hydrolysis at day 1 and day 7 of culture. Scale bar = 100 μm.

FIG. 16A is a chart showing the coated protein content achieved on microbeads under loading conditions at different pH. FIG. 16B is a chart showing the coated protein content achieved on microbeads under loading conditions at

different temperatures. FIG. 16C is a chart showing the coated protein content achieved on microbeads under loading conditions with coating solutions at different protein concentrations. FIG. 16D is a chart showing the frequency distribution of particle sizes achieved under loading conditions with coating solutions at different protein concentrations.

FIG. 17 is a chart showing primary porcine myoblasts proliferation on microbeads coated with different protein hydrolysis.

FIG. 18A is a set of confocal microscope images of C2C12 cells on CPH coated microbeads. FIG. 18B is a set of confocal microscope images of primary porcine myoblasts on CPH coated microbeads. FIG. 18C is a set of confocal microscope images of chicken muscle satellite cells on CPH coated microbeads. FIG. 18D is a chart showing cell proliferation of C2C12 cells, primary porcine myoblasts and chicken muscle satellite cells on microbeads during a 15-day culture. FIG. 18E is a set of confocal microscope images of 3T3 cells on CPH coated microbeads and a chart showing cell proliferation of 3T3 cells on microbeads during a 15-day culture. Cell nucleus and scaffolds were stained by Hoechst 33342 and F-actin staining kit, shown in blue and red color. Scale bar = 200 μm.

FIG. 19A to 19E are confocal microscope images of cells differentiation on microbeads and on 2D plates. (A) C2C12 cultured on 2D plate, (B) C2C12 cultured on gelatin coated microbeads, (C) C2C12 cultured on CPH coated microbeads, (D) primary porcine myoblasts cultured on CPH coated microbeads, (E) chicken muscle satellite cells cultured on CPH coated microbeads. Samples were stained with anti-desmin (green) with the addition of Hoechst 33342 (Blue), and observed under a confocal microscope. FIG. 19F are brightfield microscope images of 3T3 cells cultured on CPH coated microbeads (left, before Oil Red O staining; right, after staining). Scale bar = 200 μm.

FIG. 20A is a chart showing the content of free lysine and arginine in protein solutions with different treatments (CP, 5% chickpea solution; CP+C, 5% chickpea solution treated by carboxypeptidase B; CP+T, 5% chickpea solution treated by trypsin; CP+T+C, 5% chickpea solution treated by sequential hydrolysis (combination of trypsin and carboxypeptidase B treatment). FIG. 20B is a chart showing fold changes of C2C12 on microbeads with different coating materials (Cytodex, cells cultured on Cytodex-1 microcarriers). FIG. 20C is a chart comparing fold changes of C2C12 and 3T3 cells on different microbeads.

FIG. 21A to 21D are charts showing the protein content of pumpkin seed protein-alginate microbeads with cross-linking agents (A) diacteyl, (B) flavonone, (C) quercetin, and (D) Tgase, as determined by BCA assay conducted one day after cross-linking. The X-axis represents the alginate group without any coating (A), the protein coated group without cross-linking (B), and the cross-linked group (C). Significance of differences between results represented by different lowercase letters ($p < 0.05$).

FIG. 22A and 22B are charts showing the effect of varied TGase concentrations on cross-linked pumpkin seed protein-sodium alginate microbeads during four days of storage. FIG. 22A and 22B are comparisons between days and samples, respectively. Significant differences between results from the same day's BCA assay are denoted by distinct lowercase letters among columns ($p < 0.05$).

FIG. 23A to 23C are optical microscope images of sodium alginate microbeads that have been cross-linked by TGase at different concentrations. (A) TGase concentration is 1 U/mL, (B) 3 U/mL, and (C) 10 U/mL.

EXAMPLES

Example embodiments of the disclosure will be better understood and readily apparent to one of ordinary skill in the art from the following discussions and if applicable, in conjunction with the figures. It should be appreciated that other modifications related to structural, chemical and material changes may be made without deviating from the scope of the invention. Example embodiments are not necessarily mutually exclusive as some may be combined with one or more embodiments to form new exemplary embodiments. The example embodiments should not be construed as limiting the scope of the disclosure.

MATERIALS

Sodium alginate was obtained from Shanghai Ruizheng Chemical Technology Co., Ltd. Calcium chloride, hydrochloric acid, and bovine gelatin were obtained from Sigma-Aldrich Pte. Ltd (Singapore). Oil Red O powder was obtained from Sigma-Aldrich for lipid droplets staining. Genipin was obtained from Challenge Bioproducts (Taiwan). Mung bean, soybean, red lentil, broad bean, pumpkin seed, rapeseed, oat, and chia seed were obtained from Origins Healthcare Pte Ltd for protein extraction. Commercial chickpea, mung bean, soybean and pea protein were obtained from Yantai T. Full Biotech Co., Ltd. Cell Counting Kit-8 (CCK8) was obtained from Scientific Resources Pte Ltd. (Singapore). Primary porcine myoblasts, C2C12 myogenic, 3T3 cell line and chicken muscle satellite cells were used.

METHODS

Production of core particles / microbeads

Electrospraying technique was applied to form alginate beads having a narrow size distribution around 200 μm. First, alginate solution was prepared by adding sodium alginate in water and was stirred continuously until fully dissolved.

The alginate solution was transferred to a 20 mL syringe capped with a blunted stainless-steel needle. Then, a high voltage electric field was applied to the polymer solution (i.e., alginate solution), which was fed at a substantially constant flow rate using a syringe pump to draw alginate beads toward a beaker containing calcium chloride solution (as the gelation bath) placed on a magnetic stirrer. To form the electric field, the needle and a ring were connected to positive and negative electrodes of high voltage supplies, respectively. Uniformity in the size of the microbeads was realized by performing electrospraying in the cone-jet mode, and also stabilized by an additional ring-shape electrode placed on the beaker containing the gelation bath. The collected beads were cross-linked and hardened in the gelation bath for a defined period and Ca-alginate beads were then collected by a cell strainer for further analysis.

Extraction of plant proteins

Grains and oil seeds (soybean, mung bean, red lentil, rapeseeds, pea, chickpea, pumpkin seeds, oat, broad bean, and chia seeds) were ground into powder (Rong Tsong Precision Technology Co., China) for protein extraction. During protein extraction, 150 g of the grounded powder/flour was dispersed in 1.5 L deionized water (10% w/v) and mixed for 2 minutes on a shaker (Rotamax 120, Heidolph instruments GmbH & Co. KG, Germany) at a speed of 150 rpm. For solubilizing proteins, pH was adjusted to 9.0 using 0.5 M NaOH before the dispersion was mixed for another 30 minutes at 150 rpm. Insoluble non-protein components were removed via centrifuging at 12857×g at 23 °C for 5 min (Centrifuge 5810R, Eppendorf AG, Germany). To precipitate proteins, 0.5 M HCl was added to adjust pH of the supernatant to 5.2. The precipitated protein was recovered by centrifugation via the same procedure and washed in deionized water via centrifugation at 12857×g at 23 °C for 1 min. Proteins collected from centrifuge tubes were resuspended in 1.5 L of centrifuge tubes' rinses, via mixing by shaker at a speed of 150 rpm for 5 min. The suspension was adjusted to pH 7.4 to resolubilize proteins followed by freezing at -20 °C at least 24 h prior to lyophilization using VirTis benchtop freeze dryer (SP Industries; Warminster, PA,

USA). The lyophilization process took 1 week to completely dry the samples. The protein extraction yield ranged from 1% to 20%, depending on the types of grains. The purities of the proteins were determined by the Dumas method. Chickpea and pea proteins were dissolved in water and used directly. All protein isolate powder obtained was stored at -26 °C until further application.

Determination of soluble protein concentrations

Plant protein was dissolved in water to prepare 4% protein mixture and insoluble components were removed via centrifuging at 12857×g at 23 °C for 5 min (Centrifuge 5810R, Eppendorf AG, Germany). Soluble protein in supernatant was quantified using the PierceTM BCA Protein Assay Kit (Thermo Scientific), according to manufacturer instructions for the microplate procedure. Six replicates were quantified for each sample. Sample concentration was determined by applying a linear fit to the albumin and using the resulting equation to determine each sample concentration from its absorbance measurement.

Determination of protein molecular weights

Molecular weights of plant proteins were determined by SDS-PAGE. Protein was dissolved at 2 mg/mL to 10 mg/mL in distilled water before a five-fold-concentrated loading buffer was added. Protein samples were denatured for 5 min at 100 °C, loaded onto a polyacrylamide gel made of 10% running gel and 4% stacking gel, and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and de-stained with 50% methanol (v/v) and 7.5% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid. Wide range molecular weight markers including 180, 130, 100, 70, 55, 40, 35, 25, 15, and 10 kDa were used for estimation of molecular weight of proteins. Protein bands were imaged with a

ChemiDocTM gel imaging system from Bio-Rad (Bio-Rad Laboratories, Mississauga, ON, Canada).

Preparation of plant protein hydrolysis

Plant proteins were subjected to enzymatic hydrolysis. Hydrolysis using trypsin (T4799, Sigma-Aldrich Pte. Ltd, Singapore) was carried out in a shaking water bath at 50 °C, pH 8, over 120 minutes with 1% enzyme-substrate concentration (E/S%) in 5% protein dispersion. After hydrolysis, the enzymes were deactivated via heating at 95 °C for 15 minutes. The hydrolysates were centrifuged twice at 10000 g for 15 minutes, and the supernatants were collected and stored at 4 °C.

Sequential hydrolysis was conducted on chickpea protein. After trypsin hydrolysis, the deactivated hydrolysate was treated by carboxypeptidase B (C9584, Sigma-Aldrich Pte. Ltd, Singapore) at 37 °C, pH 7.5, over 120 minutes with 0.2% enzyme-substrate concentration (E/S%) in protein dispersion. After hydrolysis, the enzymes were deactivated via heating at 95 °C for 15 minutes.

Determination of degree of hydrolysis

The degree of hydrolysis (DH) of the hydrolysates was determined via formol titration. For every 2 mL of 5% protein hydrolysate, 68 mL of DI water was added. It was then subjected to titration using 0.05 M NaOH to pH 8.2, 10 mL of formaldehyde solution was added subsequently and titrated using 0.05 M NaOH to pH 9.2. The amount of NaOH used to reach pH 9.2 was recorded down to calculate the DH. The procedure was conducted in triplicate for all hydrolysates. Free nitrogen was first calculated based on the formula shown in Equation 1 and DH was calculated based on Equation 2. The total nitrogen content of different proteins was determined using the Kjeldahl method and each protein was conducted in duplicate.

$$\text{Free Nitrogen} = \frac{(V_1 - V_2) \times c \times 0.014}{V} \times 100 \quad (1)$$

where V_1 is volume of NaOH used in titration of sample (mL); V_2 is volume of NaOH used to titrate the blank (mL); c is concentration of NaOH used (0.05 M); 0.014 is the content of nitrogen equivalent to 1 mL of NaOH, and V is the volume of sample (mL).

$$\text{DH (\%)} = \frac{Free\ Nitrogen}{Total\ Nitrogen} \times 20 \times 100 \quad (2)$$

where free nitrogen is obtained from equation 1; total nitrogen content of individual plant protein was determined using the Kjeldahl method, and 20 is the dilution factor.

Determination of amino acids

Chickpea protein solution, chickpea carboxypeptidase B hydrolysate, chickpea trypsin hydrolysate, and chickpea protein hydrolysate (CPH) by sequential hydrolysis (combination of trypsin and carboxypeptidase B treatment) were prepared for free amino acid analysis. For precipitation, sulfosalicylic acid (200 µL) was added to the sample (800 µL) and incubated at 4 °C for 60 minutes. After centrifuging at 14500 rpm for 15 minutes, the supernatant was passed through a 0.22 µm syringe filter and the filtrate was centrifuged at 10000 g for 5 minutes. The supernatant was transferred into an injection vial with a dilution ratio of 1:4 or 1:16. The samples were analysed using an ARACUS amino acid analyzer (Membrane Pure, Germany).

Coating of microbeads with plant proteins and plant protein hydrolysate

Alginate microbeads were coated by plant proteins and plant protein hydrolysates, respectively. For the plant protein coating, freeze dried plant proteins were used to prepare 4% protein solution by dissolving in water. For the plant protein hydrolysate coating, proteins were firstly digested by proteinase

under different conditions to obtain protein hydrolysates with different degree of hydrolysis. The protein hydrolysis was freeze dried and then used to prepare coating solution with 4% dried powder and water.

The alginate microbeads immersed into the coating solution (w/v of 1/3) were immediately vortexed and then agitated using a shaker (Rotamax 120, Heidolph instruments GmbH & Co. KG, Germany) at a speed of 150 rpm. After coating for 2 hours, microbeads were collected by a 70 µm cell strainer (SPL Lifesciences, Korea) followed by 3 rounds of rinsing with water.

The coated alginate microbeads were cross-linked by immersing in a 1 mg/mL genipin solution with a ratio of 1:4 (beads: genipin solution, w/v) and shaking at 150 rpm in a 60 °C water bath (SW22, Julabo USA Inc., Allentown, PA, USA), then collected and washed with DI water 3 times after cross-linking.

After coating the alginate microbeads and cross-linking by genipin, the coated microbeads were stored in 0.1 M $CaCl_2$ solution with a concentration of 0.2 g beads/mL, before being autoclaved and collected by a cell strainer for cell culture. The coated microbeads can be stored for at least about 3 months. The sterilized beads were used for different physical tests and cell seeding.

Determination of sizes and size distribution of microbeads

Particle size was measured by microscopy images of the microbeads (Olympus Corporation, Tokyo, Japan) and cellSens Standard software (Olympus corporation) which can assess the diameter of beads. The mean diameter of microbeads was calculated by the average data of 3 batches of microbeads with 15 randomly selected beads from each batch.

Particle size and distribution of microbeads were also measured by LA-960 laser particle size analyzer (Horiba, Ltd., Tokyo, Japan). The characteristic diameters were obtained along with the diagram: mean diameter, median

diameter, d_{10} (10% of the particles smaller than this diameter), d_{50} (50% of the particles smaller than this diameter), and d_{90} (90% of the particles smaller than this diameter). The particle size distribution was calculated by $(d_{90}-d_{10})/d_{50}$.

Microscope imaging and fluorescence analysis of the microbeads

The appearance of microbeads and degree of cross-linking were investigated by an Olympus BX51 fluorescence microscope (Olympus corporation, Tokyo, Japan). A total of 3 batches of microbeads were analyzed on 15 randomly selected beads from each batch.

Determination of coated proteins

The coated proteins on microbeads were quantified using the PierceTM BCA Protein Assay Kit (Thermo Scientific), according to manufacturer instructions. Microbeads were quantified after a 20x dilution. Six replicates were quantified for each sample. Sample concentration was determined by applying a linear fit to albumin and using the resulting equation to determine each sample concentration from its absorbance measurement.

Cell seeding on microbeads

C2C12 cells were cultured in Growth Medium (GM) containing Dulbecco's Modified Eagle's Medium (DMEM)-High Glucose (Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin in 75 cm^2 flasks to get the required amount of cells.

For protein screening, 10^4 cells/mL cells were seeded onto 0.01 g of microbeads in low attachment 24-well plate on day 0. CCK-8 assay and microscope images were performed on day 1 and 3 (with cells being seeded at day 0) to measure cell metabolic activity.

C2C12 proliferation on pumpkin protein coated microbeads and chickpea hydrolysate coated microbeads were determined by seeding the cells onto the 0.01 g microbeads at 3×10^3 cells/mL in each well of low attachment 24-well plate. The number of cells seeded were estimated by CCK-8 assay on day 1, 3, 5, 7, 9, 11, and 13 (with cells being seeded at day 0). Half of the cell culture medium was changed every two days. After being incubated at 37 °C and 5.0% CO_2 for 1 to 13 days, cells were taken out every 2 days and stained by Hoechst 33342 and F-actin. Morphology of stained cells were observed by a Zeiss LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

C2C12 differentiation was induced to 80% confluence by replacing the GM with differentiation medium (DM) containing DMEM-High Glucose supplemented with 2% horse serum (Hyclone Laboratories, Inc., Logan, UT, USA) and 1% antibiotic–antimycotic. Day 0 (D0) indicates the day on which the cells were switched from GM to DM.

The protocol of 3T3 cells proliferation was same with C2C12 cells. Primary porcine myoblasts were cultured according to a procedure described in "Ding, S., Wang, F., Liu, Y. et al. Characterization and isolation of highly purified porcine satellite cells. Cell Death Discov. 3, 17003 (2017)." to obtain the required amounts. Chicken muscle satellite cells were cultured according to the AcceGen protocol.

Immunofluorescence staining of muscle cells

After differentiation, muscle cells were fixed by adding 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4 for 10 minutes at room temperature followed by incubation for 10 minutes in PBS containing 0.1% Triton X-100 for permeabilization. For blocking, cells were incubated with 1% BSA, 22.52 mg/mL glycine in PBST (PBS + 0.1% Tween 20) for 30 minutes to block unspecific binding of antibodies. After blocking, cells were incubated in the diluted antibody in 1% BSA in PBST in a humidified chamber for 1 hour at room

temperature, followed by a second incubation with the secondary antibody in 1% BSA for 1 hour at room temperature in the dark. Finally, cells were incubated in 0.1 µg/mL Hoechst for 1 minute for DNA staining. After each step, cells were washed 3 times in cold PBS, for 5 minutes per wash.

Oil Red O staining of 3T3

Oil Red O stock solution was prepared by dissolving Oil Red O (0.1 g) in isopropanol (200 mL) to give stock solution, which was diluted to working solution by mixing 3 parts of Oil Red O stock solution with 2 parts of water. The Oil Red O Working Solution is stable for 2 hours and were prepared 15 minutes before use.

3T3 differentiation was induced by differentiation media (GM) containing 0.5 mM IBMX (Isobutylmethylxanthine, 1-Methyl-3-Isobutylxanthine), 0.25 µM dexamethasone, 1 µg/mL insulin, and 2 µm rosiglitazone) for 2 days post-confluence, followed by 2 days of maintenance media containing 1 µg/mL insulin, and finally, back to GM with media replacement every 2 days.

After removing the spent media from the culture plates, cells ready to be stained were washed twice with PBS and fixed with 10% formalin to the cells and incubated for 30 minutes at room temperature. The formalin was discarded and the cells were washed twice with water. To the cells, isopropanol (60%) was added and incubated for 5 minutes. The solvent was removed, and the cells covered evenly with Oil Red O working solution. The plates were rotated and incubated for 10 to 20 minutes. The Oil Red O solution was removed, and the cells washed t 2 to 5 times with water until no residual stain was observed under a microscope.

RNA extraction and RT-qPCR

Total RNA was isolated from 80 % confluent cells grown in 6-well tissue culture plate or on microbeads using a PureNA biospin total RNA extraction kit (Research instruments Pte Ltd), quantified using the Nano Drop (BioDrop μLITE, BioDrop co., UK). The mRNA was converted to complementary DNA (cDNA) with an RNA PCR Kit. A single stranded cDNA equivalent was then subjected to PCR using primers β-actin, integrin β1, M-cadherin, MyoD1, Myf5 and Myh7 which were derived from previously published sequences. The PCR mixture, a total volume of 20μl, contained 1×Go Tag PCR Master Mix, and 0.2 μM of forward and reverse primer and 250 ng/mL cDNA.

Mass spectrometric determination of protein amino sequences

Proteins separated by SDS-PAGE were analyzed by TripleTOF 5600, and identified using ProteinPilot, searching against a combined database of cRAP protein sequences and UniProt Cucurbita maxima reference proteome. False discovery rate (FDR) analysis was performed on all searches, applying an FDR cut-off of 1% to remove low quality measurements.

Statistical analysis

Data processing was performed by the statistical package SPSS 25 (SPSS Inc., Chicago, IL) for one-way ANOVA. The Duncan test was used for the comparison of mean values among treatments, and to identify significant differences ($p < 0.05$) among treatments. Data were expressed as means ± standard deviations of triplicate determinations.

RESULTS AND DISCUSSION

Example 1: Characterization of alginate microbeads with plant protein coating

Tissue engineering approaches typically involve the transplantation of cells or the induction of host cell migration, with synthetic or natural matrices such as type I collagen, ceramics, and poly(lactide-co-glycolide). However, these materials frequently do not adhere cells in a specific and defined manner and may be incapable of tightly regulating the phenotype of contacting cells during tissue development. Cell adhesion to ligands in the surrounding extracellular matrix (ECM) plays an important role in tissue morphogenesis and cell fusion and thus are often used to promote myoblasts adhesion through their interaction with cell-surface receptors of the integrin family.

Many animal gelatins, including those isolated from porcine, bovine, goat, and rat, have been used as tissue sources as ECM. ECM also have been made from human issues such as cadavers. The most common source of ECM is porcine gelatin. However, animal derived proteins suffer from drawbacks such as high costs for industrial scale operation, risk of viral contamination, and batch to batch variations.

Alginate-based microbeads may be used as microcarriers in tissue engineering. Alginate-based microbeads are hydrophilic and have been proven to be effective in encapsulating cells. However, alginate-based microbeads alone may not be suitable for culturing a food product, e.g., cultured meat, due to its poor cytoaffinity arising from a lack of functional groups for interaction with cells, e.g., functional groups that can serve as specific cues for cell adhesion, or functional groups possessed by connective tissues with specific peptides sequences.

Without wishing to be bound by theory, it is believed that plant proteins have structural and composition diversity and may contain functional groups to enhance cytoaffinity, e.g., peptide sequences for attachment of cells. Plant proteins may possess bioactive molecules similar to ECM proteins and may function as ECM material to support cell culture. Hence, alginate microbeads were functionalized by coating with plant proteins.

In the present disclosure, plant proteins were extracted by isoelectric point precipitation methods and their purities and amino acid profiles were characterized (Table 1). The plant proteins studied include soybean, mung bean, red lentil, rapeseeds, pea, chickpea, pumpkin seeds, oat, broad bean, and chia seed. The proteins were extracted such that they are not denatured and have good water solubility needed for coating of the microbeads. The alginate microbeads made by electrospraying were functionalized by coating with plant proteins by physical absorption and cross-linked by genipin, a natural protein cross-linking agent isolated from an edible plant. The coated beads were evaluated for their performance for muscle cell cultures.

Table 1: Protein content of 11 types of plant protein.

	N%	Nitrogen-to-protein conversion factor	Protein content (%)	Soluble protein (mg/mL)
Soybean	14.08	5.66	79.69	352.87±46.99 ^{a}
Mung bean	15.00	6.25	93.75	335.07±42.63 ^{a}
Red lentil	13.78	6.25	86.13	330.92±21.04 ^{a}
Rapeseed 1	9.39	5.53	51.93	121.80±5.61 ^{cde}
Rapeseed 2	11.00	5.53	60.83	113.61±0.94 ^{de}
Pea	12.73	6.25	79.56	151.65±23.92 ^{cd}
Chickpea	11.73	6.25	73.31	162.36±10.16 ^{c}
Pumpkin seed	15.24	5.30	80.77	105.53±7.36 ^{e}
Oat	7.91	5.83	46.12	98.22±14.71 ^{e}
Broad bean	11.62	6.25	72.63	320.39±26.53 ^{a}
Chia seed	8.74	5.71	49.91	264.00±15.52 ^{b}

Alginate microbeads used for coating had clean and smooth surfaces and a diameter of about 200 μm. As shown in FIG. 1A, plant protein mung bean and

soybean coating do not appear even because some black blocks were observed among the beads. Other plant protein coated beads were clearer on their surfaces, suggesting that proteins were coated more evenly on the microbeads.

The coatings of proteins were accomplished by impregnating the microbeads in respective solutions of the proteins and fixed by a cross-linking reagent. As a result, the successfully coated microbeads have self-fluorescence due to the fluorophore group formed between genipin and the cross-linked proteins.

As shown from the fluorescent microscope images of the coated beads (FIG. 1B), the beads with fluorescence are an indication of successful coating of the proteins and the intensity of the fluorescence is correlated to the amount of protein being coated on the surface of the microbeads. The self-fluorescence of protein cross-linking by genipin allows the microbeads to be easily distinguished from cells under the fluorescent microscope.

It is apparent that pea, oat, chia seed protein coated microbeads have lower amounts of proteins coated. Some protein such as chickpea protein, soy protein and rapeseed protein 2 are evenly distributed on the whole microbeads, while other proteins (mung bean, lentil, rapeseed 1, pumpkin seed, and broad bean) can only be coated on the surface of the microbeads. Uneven surfaces were observed on mung bean, broad bean and pumpkin protein coated microbeads (FIG. 1B), and some black spots were found on the microbeads (FIG. 1A). Chickpea, rapeseed, and lentil coated microbeads were similar to that of gelatin coated ones (FIG. 1B), which have smooth surfaces as non-coated alginate microbeads. This suggests that the roughness of surface on beads may not be dependent on the content of loaded protein and may instead be related to protein origin. After coating of the proteins, the microbeads were treated with cell culture media and no significant difference in size and shape was observed, suggesting that the microbeads are physically stable in cell culture media for meat culture applications.

Example 2: Effect of loading conditions on protein loading

Pumpkin protein coated on alginate microbeads was determined by BCA kit. The degree of protein loading was reflected by the fluorescent intensity due to the self-fluorescence of genipin derivatives formed in the cross-linking reaction. The BCA results along with the fluorescent intensity can conveniently reflect the amount of coated protein.

For pumpkin seed protein, the original pH during the coating process was 5.7. The value was adjusted to 5.0, 6.0 and 7.0 to investigate the effect of coating pH on gelatin loading (FIG. 2A and 2D). Coating at the original pH showed the highest protein concentration and fluorescence intensity with little change in size distribution compared with other pH conditions.

The naked alginate microbeads had a mean size of 188.7 ± 74.0 μm. Coating led to a smaller size of 159.5 ± 39.8 μm. For the pumpkin seed protein solution used for coating (FIG. 2B), 10% (w/v) protein led to the highest amount of coating at 293.2 ± 38.4 mg/g, while protein concentrations higher than 10% (w/v) did not lead to any increase of coated protein. However, a higher coating concentration of 20% (w/v) can lead to a larger size of microbeads and a size distribution width of 0.74 (alginate microbeads is 0.60) (FIG. 2E).

In addition, 10% (w/v) protein led to a significantly high fluorescent intensity of 721.7 ± 10.8 mg/g. Lowering the coating protein solution concentration to 4% (w/v) led to a decrease of coated protein and fluorescent intensity. In addition, 1% (w/v) coating protein can result in a much lower coated protein, protein fluorescent intensity and a smaller beads size of 148.2 ± 37.2 μm.

The coating solution was adjusted to 8% (w/v) protein, and it was found that the amount of coated protein, protein fluorescent intensity and beads size can be kept constant with a coating solution of 10% (w/v) protein (FIG. 2C and

2F). However, when the concentration of coating protein solution was lower than 6% (w/v), the coated protein decreased significantly. Therefore, this suggests that the sizes of the beads and the coated protein were mainly due to the amount of protein used.

Example 3: Cytoaffinity of plant protein coated microbeads

Mice-derived C2C12 is a good model to evaluate the performance of the microbeads. C2C12 cells were seeded on plant protein-coated microbeads to investigate the cytoaffinity of coated plant proteins.

C2C12 cells were seeded on microbeads to investigate the cyto-affinities of different plant protein coatings. Cells were grown inside a sterile, ultra-low attachment 24-well plate. DMEM complete medium containing serum-free DMEM with 10% fetal bovine serum, and 1% penicillin-streptomycin was used. Each well contained approximately 0.03 g of coated beads that has a measured density of approximately 0.87 g/cm^3. The beads are added together with 0.5 mL of medium. 0.5 mL of medium with a myoblast cell concentration of 2 × 10^4 /mL was inoculated into each well which resulted in a final count of 10^4 /well. The plate was left to incubate at 37 °C in 5% CO_2. The concentration of cells was prepared after determining the number of cells in the stock solution via the hemocytometer. Cells were stained with trypan blue for cell counting. For routine maintenance, media were changed every two days by resuspension in fresh medium. Cell morphology was observed using the ECLIPSE Ts2 inverted microscope (Nikon, Tokyo, Japan), at 10 times magnification.

The morphologies of cells on plant-based microbeads were shown in FIG. 1A. After cell seeding, C2C12 cells were attached on mung bean, red lentil, and pumpkin seed protein coated beads and formed clusters on day 1 and grew rapidly and formed larger clusters with beads on day 3. Less C2C12 cells were attached on soybean protein and oat protein coated beads and only several individual cells on microbeads were observed on day 1, and cell clusters were

found on day 3. Nearly no cells were attached on protein broad bean, rapeseed 1, rapeseed 2, chia seed coated beads on day 1, and the status was the same for these 4 protein coated beads on day 3.

Cell counting was conducted a day after cell seeding after the cells have attached themselves to the microbeads and over the entire duration of its growth at appropriate time intervals. Cell growth and cell counting analysis during the 3-day incubation were done using the CCK-8 in triplicates. Wells are first drained off the remaining medium before the addition of 10 µL of CCK-8 solution and 100 µL of the medium. The wells are then incubated at 37°C for an hour before transferring the CCK-8 solution into a 96 well plate for absorbance reading at 450 nm.

Fold changes of cells during the 3-day culture on microbeads was shown in FIG. 3A. The change was the highest on pumpkin seed protein coated microbeads, followed by red lentil protein, which was similar to the morphology of cells in the brightfield image. The number of cells grown on pumpkin seed protein and gelatin coated microbeads was significantly higher than on other plant protein coated beads (FIG. 3A and 1A). Cells on pumpkin seed protein coated microbeads increased 6.8 folds within 3 days, which is comparable to gelatin coated microbeads (6.6 folds). The beads coated with broad bean, mung bean, lentil, rapeseed 1 and 2 only led to 4-5 folds cell increase, while the beads coated with soybean and pea protein performed the worse with only about 2-fold increase. Chickpea, oat, and chia seed protein coating led to no observable cytoaffinity.

The results of the cell seeding test demonstrated that pumpkin seed protein coated microbeads having the most concentrated size distribution of 160 µm had comparable cytoaffinity as gelatin coated microbeads.

Example 4: Correlation between characterization of coated protein and cytoaffinity

Proteins extracted from 11 different plant seeds had different protein composition and property (Table 1). The protein content was the highest in mung bean protein (93.75%), followed by red lentil, pumpkin seed, soybean, pea, chickpea, broad bean protein. Protein content in rapeseed, oat and chia seed protein were low, ranging from 46.12 to 60.83%. Soluble protein in mung bean, soybean, red lentil, and broad bean protein were significantly higher, more than 320 mg/mL in 4% (w/v) protein solution. Other soluble proteins were in small variation, ranged from 98.22 ± 14.71 to 162.36 ± 10.16 mg/mL, except for chia seed soluble protein which was 264.00 ± 15.52 mg/mL. However, it was found that the solubility of protein and cytoaffinity has no relationship from FIG. 3B.

The content of positively charged amino acid arginine and lysine having a high cell-penetrating ability is shown in Table 2. The content of arginine varies with different plant proteins, and number having a strong relationship with the increase of cell number (FIG. 3C). Lysine contents in all plant protein were almost same, except for oat and chia seed proteins (FIG. 3D). The content of lysine had a weak correlation with cytoaffinity of the beads.

Table 2: Content of positive charged amino acid in plant proteins

	Lysine (g/100g protein)	Arginine (g/100g protein)
Soybean	6.40	7.40
Mung bean	6.24	6.40
Red lentil	6.70	7.80
Rapeseed	4.70	9.70
Pea	7.60	8.20
Chickpea	6.50	9.00
Pumpkin seed	4.66	14.00
Oat	2.85	5.79
Broad bean	6.40	10.20

Chia seed	2.99	4.23

The BCA and fluorescence intensity results showed that the pumpkin seed protein was successfully coated on alginate microbeads, and when the coating protein solution was 8% (w/v), a significantly high amount of coated protein content was observed.

Example 5: Cell proliferation on microbeads coated with pumpkin proteins

Pumpkin seed protein was selected for further study and the amount of coated protein was maximized to 8% (w/v). The resulting microbead was used for evaluation of their performance on cell culture. To investigate the proliferation of C2C12 cells in longer incubation period, 3×10^3/mL C2C12 cell were seeded on the coated beads and the cell growth during the 13-day incubation were monitored by CCK8 and confocal microscope. The confocal microscope image showed a few cells attached on the self-fluorescent microbeads on day 1 (FIG. 4A). On day 3, more cells were found evenly distributed on the surface of coated microbeads. The cells on pumpkin seed protein coated microbeads were able to fully cover the beads and the cells connected the adjacent beads forming a cluster on day 5. From day 7 onwards, there were larger cell and microbeads clusters observed. The cell numbers experienced a slightly slower increase from day 5 and reached the max value of 17.2 folds increase on day 7. The cessation of cell growth was observed from day 7 and the cell numbers gradually decreased and stabilized until day 13. The reason for growth arrest is unclear. One possible reason could be that the cells might need some stimulation to keep growing.

To expand the scope of the pumpkin seed protein coated microbeads on other cells, 3×10^3/mL 3T3 cell were seeded on the microbeads. The cell growth during the 15-day incubation was monitored by CCK8 and confocal imaging which showed a few cells attached on the self-fluorescent microbeads on day 1 (FIG. 4B). On day 3, more cells were found on the surface of some coated microbeads. The cells on the microbeads were able to fully cover the beads and

the cells connected the adjacent beads together forming a cluster since day 5. A sharp increase in cell numbers was observed from day 3 to day 5, consistent with the confocal images. Cell numbers experienced a slightly slower increase from day 5 and reached the maximum value of 8.7 folds on day 7. The cessation of cell growth was observed from day 7. Cell numbers gradually reduced from day 7 and stabilized from day 9 to 13. However, a decrease of cell number was observed from day 13. The results showed that besides muscle cells C2C12, adipocyte 3T3 cells can also attach on pumpkin seed protein coated microbeads and achieve 8.7-fold increase in cell number, making pumpkin protein coating comparable with gelatin (9-fold increase of 3T3) for culturing adipose tissue.

To further explore the utility of the microbeads for meat culture, primary porcine myoblasts and chicken muscle satellite cells were seeded on pumpkin seed protein-coated microbeads and alginate microbeads. The cell growth was monitored by bright-field microscope. For alginate beads, no chicken and porcine cells attached on the microbeads, and the suspended cells formed clusters by themselves because of the low cyto-affinity of alginate (FIG. 5C and 5F). In contrast, it was observed that cells attached on the protein coated microbeads. Some clusters can be observed in FIG. 5A and 5D, and the magnified images (FIG. 5B and 5E) showed these clusters were formed by cells connected to adjacent microbeads. The morphology of chicken and porcine cells were different when they attached on microbeads. As shown by the arrows, the chicken muscle satellite cells appeared round shape which is a typical characteristic of most satellite cells during the duplicative phase, and the porcine myoblasts typically appeared spindle-shaped, and can cross the adjacent microbeads and fuse into adjacent fibers.

Taken together, the results showed that microbeads with pumpkin seed protein coating have good cytoaffinity and could be used as a microcarrier for different cell types. Although tissues grown from C2C12 and 3T3 cells may not be accepted by consumers because of its origin from mice, they are good model cells to evaluate the performance of microbeads due to its fast-growing rates. In

contrast, chicken and porcine cell-based meat may have better acceptance. The results showed that primary porcine myoblasts and chicken muscle satellite cells both had better attachment on the coated microbeads as compared with the cells seeded on naked alginate microbeads. These results further proved that the pumpkin seed protein coating have high cytoaffinity for different cell types.

Example 6: C2C12 cell differentiation on pumpkin seed protein coated microbeads

The expression levels of integrin β1, M-cadherin, MyoD1, Myf5 and Myh7 in C2C12 were determined by RT-qPCR (FIG. 6A-D). Relative expression levels of integrin β1 and M-cadherin were both significantly higher in C2C12 cells seeded on pumpkin seed protein coated microbeads than the cells grown on a 2D plate. After differentiation, the expressions of MyoD1 and Myf5 in C2C12 cells seeded on pumpkin seed protein coated microbeads were the highest on day 4. The expressions of MyoD1 and Myf5 on microbeads were also significantly higher on day 4 of differentiation than that on a 2D cultured C2C12 cells. The expression level of Myh7 on microbeads was the highest on day 7 and exceeded that of the cells grown on a 2D plate. The confocal images (FIG. 6E) showed that C2C12 cells on 2D plates can fuse during differentiation and some myotubes containing more than one cell nuclei can be observed on day 4 and day 7 of differentiation. On the other hand, C2C12 cells on microbeads can fuse and form fibers during 7-day differentiation (FIG. 6F).

Example 7: Characterization of pumpkin seed proteins

An analysis of the proteins was conducted in order to understand the high cytoaffinity and activity for cell proliferation of pumpkin seed coated proteins. Pumpkin seed protein has 4 bands in 20, 37, 40, and 55 kDa, of which the first two bands (P1 20 kDa and P2, 37 kDa) have much higher concentrations (FIG. 7). These two bands and one broad bean band from SDS-page named B1 were subjected to tryptic digestion for proteomic analysis (LC-MS), which detected

18406 fragments from P1, 19672 spectra for P2, and 26449 for B1 hydrolysates. After a strict cut-off for unused protein score > 2.0 (99% confidence), 21 proteins (1634 peptides) for P1, 28 proteins (1825 peptides) for P2, and 13 proteins for B1 (131 peptides) were identified from mass spectra by sequence by similarity searching.

The identified pumpkin proteins were derived from 11S globulin, vicilin-like seed storage protein, superoxide dismutase and aspartic proteinase. Most of the peptides were from 11S globulin with minor variation in sequences. It was suggested that cell adhesion peptides RGD (SEQ ID NO: 1) and DGEA (SEQ ID NO: 8) may be responsible for the high cytoaffinity of proteins. Therefore, a search was conducted for peptide fragments in pumpkin seed protein and it was found that a few peptides containing cell adhesion peptides were identified from P1 (nine) and P2 (ten), respectively. For example, the four peptide sequences containing such units:

RGDMIVVPAGTVQWCHNDGGQDLIAIAFLDLNNEDNQLDLR (SEQ ID NO: 13),

RGDMIVVPA (SEQ ID NO: 14),

RGDMIVVPAGTVK (SEQ ID NO: 15),

LVYVV**DGEA**NFQISDDYGNQVFNER (SEQ ID NO: 16)

are found in both P1 and P2.

Other peptides presented in Table 3 have similar sequence with these 4 peptides, except for GGQRGDEQQWEEEQEEEQER (SEQ ID NO: 17) in P1.

The identified broad bean proteins were derived from elongation factor 1-alpha, ATP synthase subunit alpha, glucose-1-phosphate adenylyltransferase, convicilin and others. Two peptides containing cell adhesion peptides RGD were from ATP synthase subunit alpha. The two peptides VVDALGVPIDGR (SEQ ID NO: 18) and ELIIGDR (SEQ ID NO: 19), may have contributed to the cyto-affinity

of broad bean protein as well because both RGD (SEQ ID NO: 1)- and DGR (SEQ ID NO: 3)-containing peptides have been demonstrated to compete with adhesive proteins for integrin interaction. In contrast, most identified peptides from broad bean containing arginine (Table 4) were located in the C-terminal of peptides.

Table 3. Pumpkin seed protein containing cell adhesion peptides.

Origin	%Cov(95)	Accessions	Name	Conf	Sequence
P1	66.7	tr|A0A6J1HXD8_CUCMA	11S globulin subunit beta	99.0	GGQ**RGD**EQQWEEEQEEEQER (SEQ ID NO: 17)
P1	56.9	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	99.0	**RGD**MIVVPAGTVQWCHNDGGQDLIAIAFLDLNNEDNQLDLR (SEQ ID NO: 13)
P1	56.9	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	99.0	**RGD**MIVVPAGTVQ (SEQ ID NO: 20)
P1	56.9	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	70.0	**RGD**MIVVPA (SEQ ID NO: 14)
P1	56.9	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	99.0	**RGD**MIVVPAGTVQWCHNDGGQDLIAIAFLDLNNEDNQLDLR (SEQ ID NO: 13)
P1	53.0	tr|A0A6J1K8H1_CUCMA	11S globulin seed storage protein	99.0	**RGD**MIVVPAGTVK (SEQ ID NO: 15)
P1	53.0	tr|A0A6J1K8H1_CUCMA	11S globulin seed storage protein	99.0	**RGD**MIVVPAGTVKWCHNDGGQDLVIVSFLDLNNEDNQLDLR (SEQ ID NO: 21)
P1	53.0	tr|A0A6J1K8H1_CUCMA	11S globulin seed storage protein	70.0	**RGD**MIVVPA (SEQ ID NO: 14)
P1	56.9	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	99.0	LVYVV**DGEA**NFQISDDYGNQVFNER (SEQ ID NO: 16)
P2	73.5	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	99.0	**RGD**MIVVPAGTVQWCHNDGGQDLIAIAFLDLNNEDNQLDLR (SEQ ID NO: 13)
P2	73.5	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	99.0	**RGD**MIVVPAGTVQW (SEQ ID NO: 22)
P2	73.5	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	72.3	**RGD**MIVVPA (SEQ ID NO: 14)

P2	67.1	tr|A0A6J1K8H1_CUCMA	11S globulin seed storage protein	99.0	**RGD**MIVVPAGTVK (SEQ ID NO: 15)
P2	67.1	tr|A0A6J1K8H1_CUCMA	11S globulin seed storage protein	72.3	**RGD**MIVVPA (SEQ ID NO: 14)
P2	73.5	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	99.0	LVYVV**DGEA**NFQIS (SEQ ID NO: 23)
P2	73.5	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	99.0	LVYVV**DGEA**NFQISD (SEQ ID NO: 24)
P2	73.5	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	99.0	LVYVV**DGEA**NFQISDDYG NQVFNER (SEQ ID NO: 16)
P2	73.5	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	99.0	VV**DGEA**NFQISDDYGNQV FNER (SEQ ID NO: 25)
P2	73.5	tr|A0A6J1HV45_CUCMA	11S globulin seed storage protein	59.5	LVYVV**DGEA**NFQ (SEQ ID NO: 26)
B1	2.0	tr|R4IV40_VICFA	ATP synthase subunit alpha	74.7	VVDALGVPI**DGR** (SEQ ID NO: 18)
B1	2.0	tr|R4IV40_VICFA	ATP synthase subunit alpha	99.0	ELII**GDR** (SEQ ID NO: 19)

Table 4: Broad bean protein containing arginine.

Origin	%Cov(95)	Accessions	Name	Conf	Sequence
B1	17.7	sp|O24534|EF1A_VICFA	Elongation factor 1-alpha	99.0	**RG**FVASNSKDDPAK (SEQ ID NO: 27)
B1	2.0	tr|R4IV40_VICFA	ATP synthase subunit alpha	74.7	VVDALGVPI**DGR** (SEQ ID NO: 18)
B1	2.0	tr|R4IV40_VICFA	ATP synthase subunit alpha	99.0	ELII**GDR** (SEQ ID NO: 19)
B1	17.7	sp|O24534|EF1A_VICFA	Elongation factor 1-alpha	44.0	LGGIDK**R** (SEQ ID NO: 28)
B1	17.7	sp|O24534|EF1A_VICFA	Elongation factor 1-alpha	98.1	FEKEAAEMNK**R** (SEQ ID NO: 29)
B1	17.7	sp|O24534|EF1A_VICFA	Elongation factor 1-alpha	49.4	EAAEMNK**R** (SEQ ID NO: 30)
B1	17.7	sp|O24534|EF1A_VICFA	Elongation factor 1-alpha	99.0	YYCTVIDAPGH**R** (SEQ ID NO: 31)

B1	8.2	sp|P52417|GLGS2_VICFA	Glucose-1-phosphate adenylyltransferase	97.8	AKPAVPLGANY**R** (SEQ ID NO: 32)
B1	8.2	sp|P52417|GLGS2_VICFA	Glucose-1-phosphate adenylyltransferase	36.2	SSPIYTQP**R** (SEQ ID NO: 33)
B1	8.2	sp|P52417|GLGS2_VICFA	Glucose-1-phosphate adenylyltransferase	99.0	IINSDNVQEAA**R** (SEQ ID NO: 34)
B1	2.0	sp|P53536|PHSL_VICFA	Alpha-1,4 glucan phosphorylase L isozyme	99.0	DAWNITQ**R** (SEQ ID NO: 35)
B1	2.0	sp|P53536|PHSL_VICFA	Alpha-1,4 glucan phosphorylase L isozyme	85.0	AREIVGL**R** (SEQ ID NO: 36)
B1	2.7	tr|A0A059PAV5_VICFA	Actin	99.0	AGFAGDDAP**R** (SEQ ID NO: 37)
B1	2.7	tr|A0A059PAV5_VICFA	Actin	55.7	DAYVGDEAQSK**R** (SEQ ID NO: 38)
B1	2.7	tr|A0A059PAV5_VICFA	Actin	71.1	VVAPPE**R** (SEQ ID NO: 39)
B1	2.5	tr|O04919_VICFA	Lipoxygenase	99.0	DTMNINALA**R** (SEQ ID NO: 40)
B1	2.3	tr|B0BCL7_VICFA	Convicilin	65.4	NKNGYI**R** (SEQ ID NO: 41)
B1	2.3	tr|B0BCL7_VICFA	Convicilin	69.9	AILTVLSPN**DR** (SEQ ID NO: 42)
B1	2.3	tr|B0BCL7_VICFA	Convicilin	99.0	NSYNLE**R** (SEQ ID NO: 43)
B1	2.3	tr|B0BCL7_VICFA	Convicilin	23.7	EDEQQERNKQVQ**R** (SEQ ID NO: 44)
B1	2.3	tr|B0BCL7_VICFA	Convicilin	29.9	NKQVQ**R** (SEQ ID NO: 45)
B1	0.7	tr|A0A023I2W9_VICFA	DNA-directed RNA polymerase subunit	99.0	MLSLSPS**R** (SEQ ID NO: 46)
B1	1.2	tr|Q43855_VICFA	Beta-fructofuranosidase; cell wall invertase I	72.8	GIQAIP**R** (SEQ ID NO: 47)

B1	2.0	sp|P53536|PHSL_VICFA	Alpha-1,4 glucan phosphorylase L isozyme	99.0	EMNAAE**R** (SEQ ID NO: 48)
B1	2.7	tr|R4IUB4_VICFA	Uncharacterized protein	99.0	IGLSIG**GR** (SEQ ID NO: 49)
B1	10.2	tr|R4ITZ7_VICFA	NADH-ubiquinone oxidoreductase	99.0	GPRYTVLICVGP**R** (SEQ ID NO: 50)
B1	6.6	tr|B6RPT3_VICFA	Phosphogluconate dehydrogenase	99.0	LPANLVQAQ**R** (SEQ ID NO: 51)
B1	7.3	RRRRRtr|R4IV20_VICFA	REVERSED Uncharacterized protein	99.0	TSGIMIN**R** (SEQ ID NO: 52)
B1	2.1	sp|Q06215|PPO_VICFA	Polyphenol oxidase	95.7	DPIFYSHHSNV**DR** (SEQ ID NO: 53)
B1	7.3	RRRRRtr|R4IU44_VICFA	REVERSED ATP synthase	91.4	IGLSLA**R** (SEQ ID NO: 54)
B1	5.4	tr|B0BCL8_VICFA	Convicilin	72.8	NENGHI**R** (SEQ ID NO: 55)
B1	1.4	tr|A0A023I2L2_VICFA	ATP synthase subunit alpha	65.4	LSADLFNAGI**R** (SEQ ID NO: 56)

Although pumpkin seed proteins have relatively poor solubility as compared to other proteins, it is sufficient to achieve coating of microbeads that exhibited the best performance as ECM materials for cell culture. From the SDS-PAGE bands, the isolated pumpkin seed proteins showed two major bands in 20 and 37 kDa, which are characteristic 11S proteins consisting of an acidic polypeptide (27-37 kDa) and a basic polypeptide (20-24 kDa) linked by a disulfide bond. Some fibroblast growth factors such as ECGF, aFGF, and (HBGF)-1 are acidic polypeptide, the band around 37 kDa might be an important part for cell growth. Moreover, some peptide sequences known as transduction domains or membrane translocation signals contain positively charged amino acid residues such as arginine and lysine, which may have a high cell-penetrating ability. Lysine content in pumpkin seed protein was close to that of other plant proteins, the high arginine content is a prerequisite for its superior cytoaffinity.

Arginine is a positively charged amino acid, and is the initial amino acid of cell adhesion tripeptide RGD (SEQ ID NO: 1). Other peptides exhibiting cell adhesion property include RGD, IKVAV (SEQ ID NO: 6), YIGSR (SEQ ID NO: 7), DGEA (SEQ ID NO: 8), PHRSN (SEQ ID NO: 9), and PRARI (SEQ ID NO: 10). The RGD and DGEA sequences were found in pumpkin seed proteins. These cell-binding peptide sequences could couple with integrin receptors and activate on osteoblast gene expression. The higher cell attachment ability of pumpkin seed protein might be caused by higher peptide sequences containing RGD units. In agreement, gelatin also contains good amount of RGD peptides. In contrast, broad bean protein coated microbeads had poor cytoaffinity. Although there are many R-residues in broad bean peptides, there are only two DGR (SEQ ID NO: 3)-containing peptides identified from broad bean proteins.

Example 8: Characterization of alginate microbeads with commercial protein coating

Commercial plant proteins (chickpea, mung bean, soybean, and pea protein) powders were purchased from Yantai T. Full Biotech Co., Ltd. These plant proteins were used to prepared 4% (w/v) protein solution by dissolving in water as coating solution using the protocol described in the Methods section.

As shown in FIG. 8A, pea protein coating was not even because some black blocks were observed among the microbeads. Other plant protein coated beads were much clearer on the surface, suggesting that proteins were coated more evenly on the microbeads.

Primary porcine myoblasts were seeded on microbeads with a density of 3×10^4 /mL, grown inside a sterile, ultra-low attachment 24-well plate. Every 500 mL of complete medium contained 420 mL of F10 medium, 15% fetal bovine serum, 1% penicillin-streptomycin, and 10 ng/mL FGF2. Each well contained approximately 0.03 g of coated beads that has a measured density of approximately 0.87 g/cm^3. The beads were added together with 0.5 mL of

medium. 0.5 mL of medium with a myoblast cell concentration of 6×10^4 cells/mL was then inoculated into each well which then resulted in a final count of 3×10^4 /well. The plate was then left to incubate at 37 °C in 5% CO_2. The concentration of cells was prepared after determining the number of cells in the stock solution via the hemocytometer. Cells were stained with trypan blue to facilitate cell counting. For routine maintenance, media were changed every two days by resuspension in fresh medium. The morphology of cells on plant-based microbeads at day 7 is shown in FIG. 4. There was no cell growth or little growth displayed for non-hydrolysed plant proteins at day 7. The fold change in cell number based on Cell Counting Kit-8 (CCK8) was around 2.5 on plant protein chickpea, mung bean and pea protein coated microbeads (FIG. 8B). The cells number was nearly not changed after 7-day culture on soy protein coated microbeads. Similar to the result in the brightfield microscope image, the cells displayed little growth after seven days.

C2C12 cells with a density of 10^4 /mL were seeded on microbeads to investigate the cytoaffinity of different plant protein coating by referring to the above method. The fold change of cells during 3-day culture on microbeads is shown in FIG. 8C. Similar to the primary porcine myoblasts seeding results, there was little change in the cell number.

It was concluded that these commercial plant proteins may not be suitable for primary porcine myoblasts and C2C12 cell growth as the cells displayed little growth.

Example 9: Characterization of alginate microbeads with plant protein hydrolysate coating

Alginate microbeads have clean and smooth surfaces without any fluorescence. Coating was accomplished by impregnating the microbeads in the respective protein hydrolysates and fixed by a cross-linking reagent genipin. Well

coated microbeads will display a dark blue color due to the chromophore formed by the cross-linking agent and amino acid residue of proteins.

As shown from the images of microbeads (FIG. 9A and 9B), the blue color indicates that proteins were successfully coated onto the microbeads. From the microscope images, beads were shown to be coated with evenly distributed proteins coatings on the outer layer. However, broad bean protein hydrolysate not only coated onto the beads but also formed big protein aggregates by themselves. The morphology of beads may differ according to the variety of protein hydrolysate coated.

Before coating, alginate microbeads were about 200 µm in diameter. After coating with the protein hydrolysates, the diameter of microbeads decreased to 160 to 170 µm (Table 5). The autoclaved microbeads were incubated in cell culture media at 60 °C for 3 days to determine the stability of the beads. Uncoated alginate microbeads swelled a little after immersing into media initially, but they shrunk significantly after 3 days. Other microbeads with unstable or thin coating exhibited the same behavior as well. As shown in Table 5, soybean, chickpea, pumpkin, rapeseed protein hydrolysis coated microbeads have no significant difference in size after the 3-day incubation in culture media, indicating that the beads are physically stable.

Table 5: Size of plant protein hydrolysate coated microbeads with different treatment

Coated hydrolysate	Mean diameter (µm)		
	No treatment	D0	D3
Mung bean	164.91 ± 5.04 [a]	178.45 ± 10.10 [b]	169.90 ± 14.99 [a]
Soybean	167.12 ± 6.60	172.86 ± 15.19	169.04 ± 11.85
Lentil	165.53 ± 4.26 [a]	181.69 ± 17.93 [b]	173.04 ± 9.40 [a]
Broad bean	166.22 ± 7.37 [a]	188.48 ± 14.92 [b]	171.21 ± 13.13 [a]
chickpea	167.53 ± 6.84 [a]	191.88 ± 9.49 [b]	186.05 ± 11.74 [b]

Pea	168.41 ± 9.01[a]	188.70 ± 20.88 [b]	169.10 ± 10.79 [a]
Pumpkin	169.02 ± 7.96	175.14 ± 12.99	168.89 ± 12.23
Rapeseed 1	166.81 ± 12.53 [a]	193.99 ± 16.68 [b]	184.34 ± 12.83 [b]
Rapeseed 2	162.41 ± 8.87 [a]	185.17 ± 9.30 [b]	181.32 ± 13.70 [b]
Alginate	201.70 ± 11.11 [b]	204.17 ± 7.24 [b]	183.10 ± 11.69 [a]

No treatment means microbeads kept in storage solution; D0 means microbeads immersed in media at room temperature within 1h; D3 means microbeads immersed in media at 60°C for 3 days. Data are expressed as means ± standard deviation of samples. Different lowercase letters in same row represent significant differences ($p < 0.05$).

Example 10: Cytoaffinity of functionalized microbeads coated with plant protein hydrolysate

C2C12 cells were seeded on plant protein coated microbeads to investigate the cytoaffinity of 9 types of plant proteins. Cell growth during a 3-day incubation was monitored by CCK8. After the 3-day culture, the number of cells grown on chickpea hydrolysate was significantly higher than on other hydrolysates coated beads (FIG. 10A-F). Before hydrolysis, cells on pumpkin seed protein coated microbeads used to have the highest increase of 6.8 folds within 3 days. After hydrolysis, cells on chickpea protein hydrolysate coated microbeads have the highest increase of 8.5 folds, followed by 7.4 folds on red lentil hydrolysate, both were higher than that of pumpkin seed protein coated microbeads (6.6 folds). Among the other 7 types of plant protein hydrolysate, soybean and rapeseed 1 protein hydrolysates displayed only slight improvements in cytoaffinity after hydrolysis, while the hydrolysis of the other proteins led to lower cell proliferation. The results demonstrate that chickpea protein hydrolysate outperformed the rest and therefore was selected to carry out in-depth study to understand the reason behind its superior cytoaffinity.

The result of FIG. 10A showed that the cytoaffinity of chickpea protein was improved as CPH coating has helped to enhance cell proliferation to a considerable extent. Hydrolysis helps to expose arginine to cells and, therefore,

enhanced the cytoaffinity of chickpea protein coating on microbeads. Positively charged lysine is also exposed during trypsin hydrolysis. However, the lysine content only has a weak correlation with cytoaffinity. In addition, the cytoaffinity of other protein hydrolysis did observe the same correlation after trypsin treatment. Therefore, the enhanced cytoaffinity observed may not only be attributed to the increase in positively charged amino acids, but also other factors as well.

Peptides produced by trypsin hydrolysis may also contribute to improved cytoaffinity. RGD-containing peptides having cell adhesion motif can be exposed during trypsin treatment. Additionally, arginine is the initial amino acid of cell adhesion peptide RGD. A search on UniProt was conducted to find RGD-containing peptides in the 9 plant proteins (Table 6), respectively. It was found that rapeseed has the maximum number of RGD-containing peptides of 7066 in types, although most are hypothetical peptides. Soybean has 4122 RGD-containing peptides, of which 17 having been reported. A peptide from soybean, named lunasin, contains the sequence of RGDDDDDDDDD (SEQ ID NO: 57) and was reported to possess cell adhesion effects. Mung bean and pumpkin have relatively high amounts of RGD-containing peptides (2213 and 2051 peptides), followed by chickpea with 1706 peptides. These RGD-containing peptides may contribute cytoaffinity of these plant proteins with muscle cells. In addition, three (CPe)-III (RQSHFANAQP) containing peptides were also identified only from chickpea protein (Table 6).

Table 6. RGD containing bioactive peptides found in plant proteins.

Organism	Containing sequence	Matching entries	
		Unreviewed[1]	Reviewed[2]
Vigna radiata var. radiata (Mung bean)	RGD (SEQ ID NO: 1)	2051	0
Glycine max (Soybean)	RGD	4105	17

Lens culinaris (Lentil) (*Cicer lens*)	RGD	9	2
Vicia faba (Broad bean) (*Faba vulgaris*)	RGD	17	3
Cicer arietinum (Chickpea) (*Garbanzo*)	RGD	1704	2
Pisum sativum (Garden pea)	RGD	68	23
Cucurbita maxima (Pumpkin) (Winter squash)	RGD	2213	0
Brassica napus (Rape seed)	RGD	7060	6
Cicer arietinum (Chickpea) (*Garbanzo*)	RQSHFANAQP (SEQ ID NO: 11)	3	
Cicer arietinum (Chickpea) (*Garbanzo*)	GAGAGS (SEQ ID NO: 12)	13	

[1]Unreviewed means records that await full manual annotation. [2]Reviewed means records with information extracted from literature and curator-evaluated computational analysis.

Example 11: Correlation between degree of hydrolysis and cytoaffinity

The results in Example 10 suggest that enzymatic hydrolysis of chickpea protein could release its potential for cytoaffinity. It is believed that the degree of hydrolysis could affect the performance of the resulting protein hydrolysates. To verify this, hydrolysis of the proteins was conducted with different trypsin and protein ratios to achieve different DH. The hydrolysis was firstly carried out with varying E/S% of 0.1%, 0.5%, 1%, 3%, and 5% with the duration being fixed at 120 minutes. DH, which is defined as the proportion of the number of peptide bonds being broken in protein hydrolysis was determined after the completion of hydrolysis.

Based on the result of DH analysis (FIG. 10B), it was observed that when E/S% increased from 0.1% to 5%, DH increased from 3.5 ± 0.1 to 7.0 ± 0.2 %. However, it did not significantly increase further when E/S% is higher than 3%. To study the DH across varying durations, the hydrolysis was conducted with an

E/S% of 0.5% and the rest of the conditions were held constant. At the 5-, 15-, 30-, 60-, and 120-min mark, the hydrolysate was withdrawn to deactivate the enzymes and to determine the DH. It was established that DH only increased from 1.5 ± 0.2 for 5 minutes hydrolysis to 4.5 ± 0.1% for a 60-minute hydrolysis and entered a stationary phase beyond 60 minutes as the DH did not vary significantly with longer durations of hydrolysis (FIG. 10C), indicating the trypsin has completed the hydrolysis with selected peptide bonds that contain positive amino acid residues (Arg and Lys).

Cell seeding was conducted to establish the correlation between degree of hydrolysis and cell growth. Protein hydrolysates, with five different DHs, formed via various hydrolysis conditions were used to conduct cell seeding for three days. As shown in FIG. 10D, the C2C12 cell adhesion at day 1 was significantly higher in DH 3.5% and 4.5% samples but did not increase with beads coated with protein hydrolysates with higher DH. After the 3-day cell culture, C2C12 proliferation increment rose from 2.1-folds to 12.6-folds when DH is increased from 1.5 to 3.5%. However, when DH is increased to 4.5% and above, cell proliferation remained at 8.1-folds, and did not increase further (FIG. 10E). Besides muscle cells C2C12, adipose cells 3T3-L1 were seeded on microbeads with hydrolysates of differing DH as well. As shown in FIG. 10F, 3T3 cell proliferation increment rose from 2.3-folds to 7.1-folds when DH is increased from 1.5 to 3.5%. However, when DH is increased to 4.5 % and above, cell proliferation reduced to around 3.4-folds, and did not increase further. These results show that there is an optimal degree of hydrolysis for cytoaffinity at around 3.5%.

Trypsin treatment under different conditions leads to various degrees of hydrolysis. Chickpea hydrolysates with different DH has different impacts on cell behavior on the coated microbeads. Lower DH resulted in longer peptides, which could lead to stronger forces of attraction between the beads and the cells due to the larger molecular weight. It has also been reported that peptide fraction of 3.5–7 kDa showed the highest fibroblast growth-stimulating activity. It has also been reported that an increase in DH may result in a higher number of peptide bonds

being cleaved but it does not equate to higher bioavailability. This can be observed in the example as well, partial hydrolysis is favorable, and it was determined that 3.5% is the optimal DH of CPH for cell growth.

Example 12: Alginate microbeads with plant protein hydrolysate coating

Commercial plant proteins were subjected to enzymatic hydrolysis. The hydrolysis using both enzymes was carried out over 120 minutes with 1% E/S% in 5% protein dispersion. After hydrolysis, the enzymes were deactivated via heating at 95 °C for 15 minutes. The hydrolysates were centrifuged twice at 10000 g for 15 min, and the supernatant was collected and stored at 4 °C. Hydrolysis using papain was carried out in a shaking water bath at 60 °C at pH 7 while for trypsin the temperature was set at 50 °C at pH 8.

The resultant hydrolysates were centrifuged to remove the insoluble peptides and the supernatant was used for coating. The alginate beads immersed into the coating solution (w/v of 1/3) were immediately vortexed and then agitated using a shaker (Rotamax 120, Heidolph instruments GmbH & Co. KG, Germany) at a speed of 150 rpm. After coating for 2 h, microbeads were collected by a cell strainer then washed with water for 3 times. The coated alginate microbeads were cross-linked by immersing in a 1 mg/mL genipin solution with a ratio of 1:4 (beads: genipin solution, w/v) and shaken at 150 rpm in a 60 °C water bath (SW22, Julabo USA Inc., Allentown, PA, USA) for 3 h, then collected and washed with DI water for 3 times after cross-linking. After coating alginate microbeads and cross-linking by genipin, the modified microbeads were stored in 0.1 M $CaCl_2$ solution at a concentration of 0.2 g beads/mL, before being autoclaved and collected by the cell strainer for cell culture.

Primary porcine myoblasts were grown on the respective coated beads by referring to the previous method described in Example 8. Fold changes of cells during a 7-day culture are as shown in FIG. 11. It is evident that trypsin hydrolysis for all plant proteins, except for pea protein, resulted in more desirable peptides

that support myoblast cell growth, of which chickpea hydrolysates led to the highest growth with a 3.15 ± 0.60 -fold increase. There is negligible growth for hydrolysates produced via hydrolysis with papain. As papain has a very broad specificity as compared to trypsin which has only two cleavage sites- Arg and Lys, it possibly could have led to the production of shorter chain peptides as compared to the hydrolysis by trypsin. It is hypothesised that the adhesion of shorter chain peptides on beads may not be as strong as longer chain peptides, leading to a loss of protein during cell growing stages. Additionally, as peptides produced via papain hydrolysis tend to be hydrophobic and precipitate as solids, they could have been lost after centrifugation. Thus, even when shorter chains are favoured for cell growth, the inability to adhere to the beads would mitigate the positive effects on cell proliferation.

Similar to the cell growth results shown above, the cell morphology observations in FIG. 12 also showed that protein hydrolysates produced using papain has no effect on cell growth while hydrolysates from trypsin were able to supplement better cell growth. Chickpea which is hydrolysed by trypsin has the highest cell growth on day seven under an inverted microscope followed by pea. No cell growth was observed on soy and mung bean protein coated beads even after hydrolysis by both enzymes, which meant that these two proteins are unsuitable to be used in this study. Chickpea hydrolyzed by trypsin was considered a coating material with better cytoaffinity.

Hydrolysis of chickpea was firstly carried out with varying E/S% of 1%, 3%, 5%, 15%, 20%, 30%, and 50% with the duration being fixed at 120 minutes. DH, which is defined as the proportion of the number of peptide bonds being broken in protein hydrolysis, was determined after the completion of hydrolysis. Based on FIG. 13A, an E/S% of 1% gave the lowest DH of 2.75 ± 0.18 % and it gradually increased to 5.61 ± 0.18 % at an E/S% of 15%. When E/S% continued to increase from 15% to 50%, the DH remained stagnant and did not increase further. Thus, the maximum DH attainable only required a E/S% of 15% for a 2-hour hydrolysis of 5% protein dispersion.

Hydrolysis was conducted with an E/S% of 15% and the rest of the conditions were held constant over a period of 18 hours. At the 2, 4, 8, 13-hour mark, 60 mL of the hydrolysate was withdrawn to deactivate the enzymes and to determine DH. It was established that DH only increased from 5.61 ± 0.18 % for a two-hour hydrolysis to 6.88 ± 0.18 % for a four-hour hydrolysis, and entered a stationary phase beyond four hours as the DHs did not vary significantly with further hydrolysis. This shows that with an E/S% of 15% and 4-hours hydrolysis, it has reached the complete hydrolysis of lysine and arginine of the 5% protein dispersion. BCA protein assay was also conducted for microbeads coated with different hydrolysis duration. Based on FIG. 13B, the protein adhered to the microbeads was the highest for 2 hours of hydrolysis which gave a value of 17.52 ± 1.25 mg/g. However, it decreased significantly to 4.22 ± 1.00 mg/g when hydrolysis was beyond 8 hours. This shows that the optimum DH to achieve the highest amount of protein coated on beads could be 5.61 ± 0.18 %, any DH above this may have a negative correlation with protein concentration coated on the beads.

Based on the results, when DHs increased from 2.75 ± 0.18% to 5.61 ± 0.18% and cell proliferation increment rose from 3.01 ± 0.41-fold to 4.07 ± 0.42 -fold. However, when DH is further increased to 6.88 ± 0.18% (4h hydrolysis), cell proliferation increment decreases to 1.44 ± 0.30-fold. Longer peptides produced by shorter hydrolysis duration are likely to have better adhesion on beads as compared to the shorter peptides or even amino acids that have been produced from longer hydrolysis duration.

Microscope images of cell morphology correspond with the results shown in FIG. 14 as cell growth was the highest on microbeads coated by protein hydrolysis with DH of 5.61%. DH beyond 6%, led to a negative correlation with cell growth. This can be observed in FIG. 15 at day seven, where cells are completely detached from the beads. This shows that the desorption of protein from the beads discourages cell adhesion.

Example 11: Optimization of coating process

Coating was first done across pH values of 5 to 10, over 16 hours, and at room temperature. The isoelectric points (pI) of Arg and Lys are 10.76 and 9.47 respectively. Thus, the pH values below the pI of these amino acids were favoured, since the sodium alginate surfaces have a net negative charge, hence, at pH below the pI values, Arg and Lys would be positively charged. This would encourage the adsorption of these amino acids or peptides containing these amino acids onto the surface of the beads. However, BCA analysis showed that the amount of protein coated onto the beads did not vary significantly with changes in pH (FIG. 16A). Thus, it was determined that the pH of the protein solution for coating does not necessarily need to be adjusted.

Protein adsorption on solid surfaces generally increases with temperature, and the thickness of protein layers is enhanced with elevated temperature. Hence, the coating was determined to take place over the temperature range of 4 °C to 60 °C which is below the protein denaturation temperature to prevent the destruction of the functional properties of peptides. At 4 °C, the amount of protein (2.53 ± 0.74 mg/g) coated on the beads is significantly lower when compared to coating at 60 °C (16.23 ± 0.36 mg/g). Therefore, the BCA results were in line with the study and 60 °C was found to encourage protein adsorption on the alginate beads (FIG. 16B).

After hydrolysis, the hydrolysates were centrifuged, and the supernatant retained for freeze-drying. The freeze-dried powders allow for varying protein concentrations to be used for coating. Protein solutions of 5%, 10%, 15%, 20%, 30% and 40% were prepared using the freeze-dried protein. The resultant solutions are used for coating at 60 °C. BCA analysis was not conducted for beads coated with 40% protein solution as the beads have dissolved during the coating process. Similarly, for beads that were coated with 30% protein, the particle size has decreased to 54.5 ± 2.30 µm which is undesirable to be used in

cell seeding (FIG. 16C). Upon BCA analysis, when protein concentration used is increased from 5% to 20%, the amount of protein coated on beads also increased from 9.25 ± 2.16 to 16.83 ± 1.56 mg/g (FIG. 16C). Even though 20% protein concentration led to the highest protein coated, it is not significantly different from 15%. Thus, 15% protein concentration was chosen as the final protein concentration to be used for coating.

Based on visual inspection under the microscope, beads size was altered depending on the concentration of protein, thus, particle size analysis was conducted. FIG. 16D shows that as protein concentration increases, q(%) decreases. Since q% refers to the volume at each particle size (frequency distribution), this reduction means that there will be fewer beads in the desired particle size intervals for cell seeding, which is not optimal. The median diameter ranged from 124.5 ± 0.95 µm to 111.5 ± 0.4 µm for beads coated with 5% to 20% protein. However, it decreased to 54.5 ± 2.30 µm for beads coated with 30% protein. A possible explanation would be that moisture has diffused out of the beads due to the difference in osmotic pressure between the beads and protein solution.

Cell seeding was conducted with beads coated via improved coating conditions that have been established earlier (original pH, 60 °C coating temperature, and 15% protein concentration). It was also observed that the dark blue colouration of beads dissipates after cell seeding; protein desorption has occurred even though the initial BCA value is the highest after coating. As such, coating was repeated with 5% and 10% protein concentration to further investigate if the high protein concentration has an adverse effect on cell growth. However, only 10% protein concentration displayed a 3.59 ± 0.04 -fold increment on day 10 but cells start to die beyond that, little cell growth was observed for beads coated with 5% protein (FIG. 17).

Biomass calculation was conducted on cells coated with protein hydrolysate as it has the highest primary porcine myoblasts proliferation of 4.44-

fold increment. Based on theoretical calculation, every bead would generate approximately 3.78 cells. A few assumptions were made for this calculation: I) all inoculated cells have attached to the beads and all beads were retained in the well when the medium was removed before the addition of CCK-8 solution; II) negligible cell growth from day zero to day one as fold increment was calculated from day one to the last day of the growth; III) cell growth is evenly distributed on every bead.

However, some cells might have been washed away while withdrawing the medium for CCK-8 analysis, not all cells have attached themselves to the beads and are still suspended in the medium. Additionally, there is some growth from day zero to day one when observed under an inverted microscope and cells tend to grow in clusters instead of individually on every bead.

Example 12: Cell proliferation on microbeads with chickpea hydrolysate (CPH)

A density of 3×10^3/mL C2C12 cell were seeded on CPH with DH 3.5% coated microbeads. The cell growth during a 15-day incubation was monitored by CCK8 and confocal imaging. The confocal images showed that there were only a few cells attached on the microbeads on day 1 (FIG. 18A). Furthermore, the self-fluorescent microbeads were hard to observe due to the weak fluorescence. On day 3, more cells were found on the surface of the coated microbeads, with some connected to the adjacent microbeads together forming small clusters. From day 5 onwards, it was observed that there were larger clusters formed by cells and microbeads. However, the center of the clusters has less F-actin stain though many blue colored nuclei were observed. This may be due to the limited space in the center of the clusters for the cells to expand. The cell numbers experienced a sharp increase in the first 3 days, followed by a period of slower growth from day 3 to day 7 before a rapid increase to reach the maximum value of 15.3-folds on day 9 (FIG. 18D). A decrease in cell number was observed from day 9 until day 15.

Primary porcine myoblasts and chicken muscle satellite cells were seeded on CPH coated microbeads as well. The porcine cell numbers were almost constant during the first 3 days before experiencing a rapid increase (FIG. 18D). Similar to the C2C12 cell proliferation result, the highest fold change (13.8-fold) was observed on day 9 for the primary porcine myoblasts. A decrease in cell number was observed from day 9 to day 15. Clusters formed by adjacent beads and cells can be observed when the maximal cell number was reached on day 9 (FIG. 18B). A 11.7-fold change in chicken muscle satellite cell number was achieved during the 15-day incubation (FIG. 18D). The highest number of chicken cells was observed on day 7. The confocal image in FIG. 18C showed that cell clusters formed on day 7. It was noted that porcine and chicken cells were unable to reach the high density achieved by C2C12 cells. Considering all cell proliferation data across the 3 types of cells, CPH could potentially be used as a coating material on microcarriers for different muscle cell types.

To expand the scope of the CPH coated microbeads on other types of cells, 3×10^3/mL 3T3 cells were seeded on the beads. Cell growth during a 15-day incubation was monitored by CCK8 and confocal imaging. Few 3T3 cells were observed on day 1 and day 3. On day 5, more cells were found on the surface of the coated microbeads (FIG. 18E). The cell growth was observed on the entire surface of the microbeads, which also extended to the adjacent beads, forming clusters since day 5. A sharp increase in cell number was observed from day 3 to day 7, consistent with the cell status in confocal images (FIG. 18E). Cessation of cell growth was observed from day 7 when the cell number increased to the maximal value of 15.7 folds. Therefore, CPH coated microbeads not only can be used in the culture of muscle cells but also adipocyte cells and may potentially be applied in cell-based meat containing muscle and fat tissues.

Example 13: Cell differentiation on chickpea protein hydrolysate coated microbeads

Desmin is a subunit protein of intermediate filaments in muscle tissue known to be at low concentration within replicating myoblasts and satellite cells while high in differentiated myotubes. Expression of desmin in cells on microbeads was detected by immunostaining to determine cell differentiation. The confocal images in FIG. 19A showed that during cell differentiation on a 2D plate, C2C12 cells can fuse together to form myotubes. The green fluorescence of stained desmin was the highest on day 4, and some myotubes containing more than one cell nucleus can be observed on day 7 of differentiation.

Similarly, C2C12 cells on microbeads can fuse and form fibers during a 7-day differentiation period as well. According to the inventors' previous studies, gelatin coated microbeads are promising microcarriers for muscle cells proliferation and differentiation, as gelatin coated material retains cell-binding motifs such as RGD and MMP-sensitive degradation sites, which are important in successful cell adhesion. C2C12 cells on gelatin coated microbeads can start differentiating even on day 1 and are concentrated in the outer layers of the clusters (FIG. 19B). On the other hand, C2C12 cells on CPH coated microbeads achieved the greatest degree of differentiation on day 4 (FIG. 19C). Primary porcine myoblasts (FIG. 19D) and chicken muscle satellite cells (FIG. 19E) also started to differentiate on day 4 as confirmed by the presence of desmin.

For 3T3 cells seeded on CPH coated microbeads, the differentiated cell morphology under bright-field microscope was shown in the left picture of FIG. 19F. After Oil Red O staining, many red oil droplets can be observed in 3T3 cells.

The above results demonstrated that CPH coated microbeads could potentially be used as a substrate in supporting several types of cell differentiation. CPH is edible and biodegradable, thus CPH-based microcarriers or scaffolds can be embedded in the final product or dissolved during the bioprocess. Using CPH to replace animal derived gelatin can eliminate concerns of zoonotic infections, growth factor contamination as well as batch-to-batch variation.

Example 14: Amino acid analysis of chickpea protein hydrolysates

Chickpea proteins and three treated hydrolysates were subjected to free amino acid analysis. For chickpea proteins (CP), chickpea proteins treated with carboxypeptidase B (CP + C) and chickpea protein treated with trypsin (CP+T), only a few amino acids, histidine, lysine, and arginine were detected at low concentrations for lysine and arginine ranging from 56.58 to 226.48 μM. After carboxypeptidase B treatment, lysin and arginine dramatically increased to 2040 and 3502 μM, respectively (FIG. 20A).

The four protein solutions were subsequently used for microbeads coating, after which, the coated microbeads were seeded with C2C12 cells to compare the cytoaffinity with commercial microcarriers Cytodex-1. Among these protein solutions, CP+T showed significantly higher cell fold change than other coatings and Cytodex-1, indicating the high cytoaffinity of CPH and is able to outweigh Cytodex-1. The results showed that commercial microcarriers and scaffolds may have limited cytoaffinity for muscle cells (FIG. 20B). The results demonstrated the importance of peptides containing lysine/arginine/histidine terminal units for cytoaffinity of the chickpea protein hydrolysates.

To verify that the enhanced cytoaffinity of CPH coated microbeads is related to the peptides bonded on the microbeads, carboxypeptidase B was used to further hydrolyse the trypsin hydrolysates. Carboxypeptidase B can release C-terminal lysine and arginine which are exposed by trypsin treatment. The results of the amino acid analysis proved that the trypsin hydrolysis released plenty of C-terminal lysine and arginine. Meanwhile, the cell proliferation result showed that the high proliferation of C2C12 is due to the increased in cytoaffinity of CPH treated by trypsin alone which is related to the exposure of lysine or arginine.

Besides muscle cells, 3T3 cells were able to attach on CPH coated microbeads and achieved a 15.6-fold increase in cell number, which is higher

than cell growth achieved by the gelatin coating (9-fold increase of 3T3). However, CP+T+C and the positively charged Cytodex-1 both had the comparable cell proliferation as well as CP+T (FIG. 20C). Thus, the cytoaffinity of adipocyte cells may not be solely related to the C-terminal lysine and arginine released by trypsin. It has been reported that a higher 3T3 cell proliferation rate can be achieved after treatment with silk fibroin hexapeptide GAGAGS (SEQ ID NO: 12). A search via UniProt found that 13 types of peptides containing the GAGAGS sequence existed in chickpea protein. Therefore, unlike C2C12, the 3T3 proliferation may rely more on the surface charge of microcarriers or other hydrolyzed peptides which have the functionality as growth factor.

Example 15: Enzymatic hydrolysis of protein with trypsin and papain

Some selected plant proteins were subjected to enzymatic hydrolysis. The resultant hydrolysates were centrifuged to remove the insoluble peptides and the supernatant was used for coating. The resultant cell growths using the respective coated beads are as shown in FIG. 11.

It is evident that trypsin hydrolysis for all plant proteins, except for pea protein, resulted in more desirable peptides that support primary porcine myoblast growth, of which chickpea hydrolysates led to the highest growth with a 3.15 ± 0.60-fold increase.

There is negligible growth for hydrolysates produced via hydrolysis with papain. As papain has a very broad specificity as compared to trypsin which has only two cleavage sites- Arg and Lys, it possibly could have led to the production of shorter chain peptides as compared to the hydrolysis by trypsin. It is hypothesised that adhesion of shorter chain peptides on beads may not be as strong as longer chain peptides, leading to a loss of protein during cell growing stages. Additionally, as peptides produced via papain hydrolysis tend to be hydrophobic and precipitate as solids, they could have been lost after centrifugation. Thus, even when shorter chains are favoured for cell growth, the

inability to adhere to the beads would mitigate the positive effects on cell proliferation. Trypsin, which cleaves only next to the positively charged amino acids Lys and Arg, would be better suited for this, as it does not disrupt patches of hydrophobic amino acids.

Similar to the cell growth results shown in FIG. 11, the cell morphology observations in FIG. 12 also showed that protein hydrolysate produced using papain has no effect on cell growth while hydrolysate from trypsin were able to supplement better cell growth. Chickpea cystatin (CPC) which is hydrolysed by trypsin has the highest cell growth on day seven under an inverted microscope followed by pea protein isolate (PPI). No cell growth was observed on soy protein isolate (SPI) and mung bean protein isolate (MBPI) even after hydrolysis by both enzymes, which meant that these two proteins are unsuitable to be used in this example. Therefore, optimisation will be conducted with CPC and trypsin.

Although plant proteins contain RGD units, they may not be accessible to interact with cells on the microbeads. To generate bioactive peptides, enzymatic hydrolysis is practised in food and pharmaceutical industries because it can be conducted under mild controlled conditions (pH, temperature, substrate concentration, and enzyme activity), high specificity and the absence of residual organic solvents and toxic chemicals in the final peptide composition. Some common enzymes such as papain, alcalase, and flavorzyme, have a broad specificity, hence they could have led to the production of shorter chain peptides and free amino acids (Table 7).

Table 7: Optimum reaction condition of enzymes

Enzyme	**Cleavage Sites**	**Optimum Temperature**	**Optimum pH**
Trypsin	Arg, Lys	50	8
Papain	Ala, Leu, Ile, Phe, Trp, Tyr, Arg, Lys, His, Gly, Glu, Val,	60	7

	Asp		
Alcalase	Glu, Met, Leu, Tyr, Lys, Gln, Ile, Gly, Val, Ala, Phe, Trp	50	7.5
Flavourzyme	Ala, Ile, Glu, Lys, Leu, Pro	50	7

On the other hand, trypsin has only two cleavage sites- Arg and Lys. The inventors hypothesized that using trypsin can release RGD-containing and other positively charged peptides, while preserving most of the protein backbone for coating on alginate. The results in FIG. 11 and 12 showed that trypsin hydrolysis led to a higher growth of primary porcine cells. In contrast, there is negligible growth for microbeads coated with hydrolysates produced via hydrolysis with papain (FIG. 11). Therefore, the results showed that selective hydrolysis with trypsin could release the RGD unit due to its high selectivity.

Example 16: Effect of cross-linking agents on the coating of pumpkin seed proteins

Protein coating on microbeads was achieved through immersion in protein solutions followed by immobilization using cross-linking agents. Commonly used cross-linking agents such as glutaraldehyde and diacetyl, which are not derived from plants, possess two aldehyde groups capable of forming Schiff's bases with amino groups of proteins, resulting in protein cross-linking through carbon atoms in the middle. Plant-based cross-linker genipin, which is a commonly used biomaterial, could bring unpleasant blue color in products, and may not be desirable in cultured meat application.

In this example, several natural cross-linkers of food-grade quality, including quercetin, flavanones, and TGase, were tested and compared with these conventional cross-linking agents. The remaining protein content was determined using the BCA method after cross-linking with various cross-linking

agents, and the dosages used were adopted from relevant references (the cross-linking agents used were Diacetyl 9.8%, flavanone 10mg/mL, quercetin 10mg/mL, and TGase 10U/mL).

Duration and temperature of the coating exhibited minimal influence on protein loading. Higher amounts of coated protein were achieved at elevated temperatures ranging from 50 °C to 60 °C. Hence, a coating temperature of 50 °C was chosen, which also corresponds to the optimal temperature for TGase enzyme activity.

As anticipated, the cross-linking of diacetyl led to a higher protein retention rate (2.87 ± 0.65 mg/mL) in comparison to the non-cross-linked group (1.42 ± 0.14 mg/mL) (FIG. 21A). The cross-linking effects of flavanone and quercetin in an aqueous solution were not significant, exhibiting retention amounts of 1.89 ± 0.34 mg/mL and 1.57 ± 0.33 mg/mL, respectively, which did not differ significantly from the non-cross-linked group (FIG. 21B and 21C). Among the food-grade cross-linking agents examined, TGase displayed a relatively high protein retention rate of 1.67 ± 0.16 mg/mL in contrast to 1.18 ± 0.2 mg/mL without cross-linking (FIG. 21D).

The results obtained from the BCA assay provide a convenient means to determine the amount of coated pumpkin seed protein. TGase, when utilized as a protein cross-linking agent, demonstrates the ability to effectively cross-link proteins on microspheres, resulting in a relatively high preservation rate, according to the result.

Example 17: Effect of TGase concentration on protein retention

Although protein attachment to microbeads can be achieved without cross-linkers, the use of cross-linkers such as TGase is advantageous for the long-term storage of pumpkin seed protein-alginate microbeads. However, excessive use of cross-linkers can result in over-crosslinking, leading to protein

modification or deformation of the beads. Therefore, the impact of varying TGase concentrations on the cross-linking ability of the microbeads was investigated (FIG. 22A), and subsequent BCA assays were performed to assess protein retention rates during different storage durations (FIG. 22B).

After a 4-day storage period, the protein content on the surface of microbeads in the uncross-linked sample decreased by nearly a half, from 1.04 ± 0.03 mg/mL to 0.72 ± 0.08 mg/mL. In samples cross-linked with TGase at a concentration of 1 U/mL, the protein retention rate of microbeads did not decrease significantly, remaining at 1.42 ± 0.06 mg/mL and 1.41 ± 0.03 mg/mL. Increasing the concentration of TGase did not significantly increase the amount of protein retention. At a concentration of 20 U/mL, the protein content was still 1.29 ± 0.05 mg/mL, which was not significantly different from the values obtained at 10 U/mL (1.28 ± 0.22 mg/mL) and 3 U/mL (1.37 ± 0.06 mg/mL).

From the results, it is evident that reducing the amount of TGase used in cross-linking of pumpkin seed protein-alginate microbeads did not lead to a significant difference between the protein content of the microbeads and the control group without cross-linking, if the BCA test is performed in a relatively shorter storage time. However, for a longer period of preservation, the content of uncross-linked proteins significantly decreased, while the protein content after cross-linking remained relatively stable, indicating effective retention on the microbeads. Moreover, it was observed that increasing the concentration of TGase does not have a noticeable effect on the protein retention rate after preservation. Therefore, in practical applications, an appropriate concentration of TGase can be used to optimize cost-effectiveness and prevent excessive cross-linking.

Example 18: Effect of concentration of cross-linker on the size and morphology of microbeads

Upon microscopic examination, it was observed that the pumpkin seed protein has undergone successful cross-linking with the sodium alginate microspheres, as manifested by a darker and opaque appearance (FIG. 23B) compared to the uncross-linked microbeads (FIG. 23A), due to the attachment of proteins to its surface. It was observed that as the amount of cross-linking agent increases, some microspheres exhibited an excessive accumulation of cross-linked proteins, and are not in a uniform distribution (FIG. 23C). This uneven protein distribution may potentially exert unfavorable effects on cell culture and growth in these microspheres. A smaller amount of cross-linker may facilitate the cross-linking of an optimal amount of protein to the microspheres, sufficient to support cell growth while avoiding the overloading of proteins on the microspheres.

Alginate microbeads used for coating had clean and smooth surfaces and diameter and an initial diameter of about 200 µm. Intuitively, the microbeads are expected to become larger after the pumpkin seed protein coating. However, in this example, it was observed that the mean diameters of pumpkin seed protein-coated beads became smaller using different concentrations of TGase as cross-linker.

Table 8: Mean diameter and distribution width of coated microbeads

Concentration	1 U/mL	3 U/mL	10 U/mL
Mean diameter (µm)	159.81±7.21	156.23±7.76	156.17±6.00
Distribution width (µm)	0.124	0.123	0.123

Most of the uncoated microbeads had a diameter of around 200 µm. As shown in Table 8, coated microbeads all experienced some shrinkage, and their range of diameter was all below 200 µm. The result indicated that the diameter

distribution of the final cross-linked beads was approximately 156 μm, with the narrowest size distribution of 0.123.

In Examples 16 to 18, natural cross-linking agents used for collagen cross-linking were selected to evaluate their effect on pumpkin seed protein. The relatively low efficacy of quercetin and flavanones may be attributed to their limited solubility in aqueous solutions. While quercetin may be solubilized in ethanol for cross-linking purposes, it may adversely affect cell growth or protein structure. TGase is a water-soluble cross-linker that is suitable for cell cultivation as food. Heat sterilization did not cause significant deformation or agglomeration of the cross-linked alginate microbeads, indicating the potential of pumpkin seed protein-sodium alginate microbeads cross-linked by TGase as a scaffold for cellular meat production.

APPLICATIONS

Embodiments of the disclosure provide a microcarrier, a method of making a microcarrier, a system for producing a cell cultured food product and a method of producing a cell cultured food product.

Advantageously, the microcarrier may provide a cost-effective alternative to commercial microcarriers. Commercial microcarriers or scaffolds for cell culture are fabricated by synthetic and inedible plastics and not ideal for commercial production of cell-based meat. The application of food grade (or edible) microbeads with plant protein coatings are attractive as microcarriers because there is no need to separate the grown cells from the carriers.

Even more advantageously, the use of plant-based materials in place of animal-derived materials may help to reduce the concern of spreading infectious diseases, growth factor contamination as well as batch-to-batch variation.

Even more advantageously, cultured meat may be identical to real meat both in nutritional composition and appearance. Using food grade material for microbeads fabrication can lower the cost, and upscale anchorage-dependent mammalian cells. Thus, embodiments of the microcarrier may have the potential to be used in different meat culture for culturing cells from pig, chicken, cows and even shrimps.

It will be appreciated by a person skilled in the art that other variations and/or modifications may be made to the embodiments disclosed herein without departing from the spirit or scope of the disclosure as broadly described. For example, in the description herein, features of different exemplary embodiments may be mixed, combined, interchanged, incorporated, adopted, modified, included etc. or the like across different exemplary embodiments. The present embodiments are, therefore, to be considered in all respects to be illustrative and not restrictive.

CLAIMS

1. A microcarrier comprising,

a core particle; and

a coating layer disposed on a surface of the core particle;

wherein the core particle and coating layer comprise one or more materials derived from plant-based sources.

2. The microcarrier according to claim 1, wherein the core particle is a hydrogel-based particle.

3. The microcarrier according to claim 1 or 2, wherein the core particle comprises one or more materials selected from the group consisting of alginate, cellulose, chitosan, gum, hemicellulose, lignin, pectin, soy, starch, and zein.

4. The microcarrier according to any one of claims 1 to 3, wherein the coating layer comprises a protein or a protein hydrolysate.

5. The microcarrier according to claim 4, wherein the protein hydrolysate has a degree of hydrolysis of from 1% to 30%.

6. The microcarrier according to any one of claims 1 to 5, wherein the coating layer comprises at least 3 amino acids selected from the group consisting of arginine, histidine, aspartic acid, glutamic acid, proline, serine, glycine, and alanine.

7. The microcarrier according to any one of claims 1 to 5, wherein the coating layer comprises amino acid sequences selected from the group consisting of RGD (SEQ ID NO: 1), IKVAV (SEQ ID NO: 6), YIGSR (SEQ ID NO: 7), DGEA (SEQ ID NO: 8), PHRSN (SEQ ID NO: 9), PRARI (SEQ ID NO: 10), permutations thereof or sequence order variations thereof.

8. The microcarrier according to any one of claims 1 to 7, wherein the coating layer is coupled to the core particle via a cross-linker.

9. The microcarrier according to claim 8, wherein the cross-linker comprises a natural cross-linker selected from the group consisting of flavanones, genipin, quercetin, tannic acid and transglutaminase.

10. The microcarrier according to any one of claims 1 to 9, wherein the coating layer exhibits self-fluorescence.

11. The microcarrier according to any one of claims 1 to 10, wherein the plant-based sources include one or more selected from the group consisting of broad bean, chickpea, chia seed, corn, lentil, mung bean, oat, pea, pumpkin seed, rapeseed, rice, soybean, wheat, and winter squash.

12. A method of making a microcarrier, the method comprising, providing a core particle; and forming a coating layer on a surface of the core particle to obtain the microcarrier, wherein the core particle and coating layer comprise one or more materials derived from plant-based sources.

13. The method according to claim 12, wherein providing the core particle comprising electrospraying a precursor solution to form the core particle in the form of a microbead.

14. The method according to claim 12 or 13, wherein forming the coating layer comprises immersing the core particle in a coating solution comprising a protein or a protein hydrolysate.

15. The method according to claim 14, wherein prior to forming the coating layer, the method further comprises preparing the coating solution comprising the protein hydrolysate.

16. The method according to claim 15, wherein the protein hydrolysate has a degree of hydrolysis of from 1% to 30%.

17. The method according to claim 15 or 16, wherein preparing the coating solution comprising the protein hydrolysate comprises hydrolyzing a protein using an enzyme-substrate concentration (E/S%) of from 0.1% to 50%.

18. The method according to any one of claims 12 to 17, further comprising immersing the core particle coated with the coating layer in a cross-linking solution.

19. The method according to claim 18, wherein the cross-linker comprises a natural cross-linker selected from the group consisting of flavanones, genipin, quercetin, tannic acid, and transglutaminase.

20. A system for producing a cell cultured food product, the system comprising,

a population of cells; and

a plurality of microcarriers configured to allow the population of cells to be grown thereon;

wherein the microcarrier comprises a core particle and a coating layer disposed on a surface of the core particle; and

wherein the core particle and coating layer comprise one or more materials derived from plant-based sources.

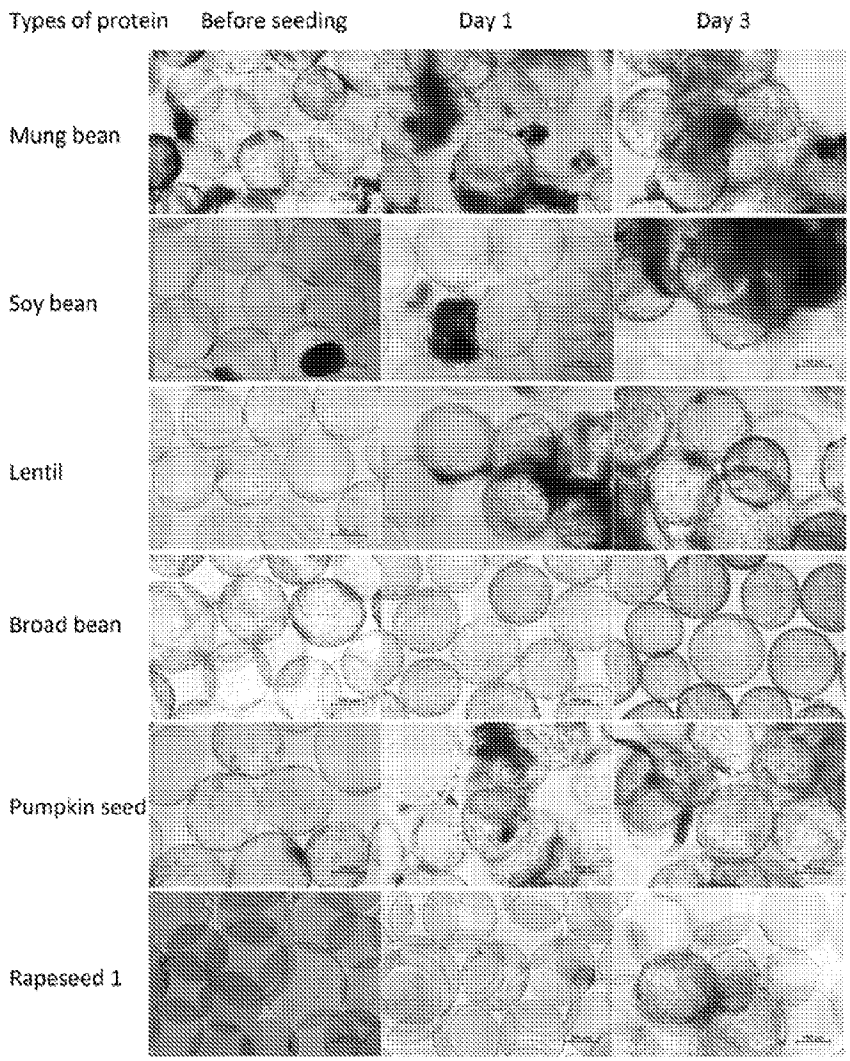

Types of protein
Before seeding
Day 1
Day 3
Mung bean
Soy bean
Lentil
Broad bean
Pumpkin seed
Rapeseed 1

FIG. 1A

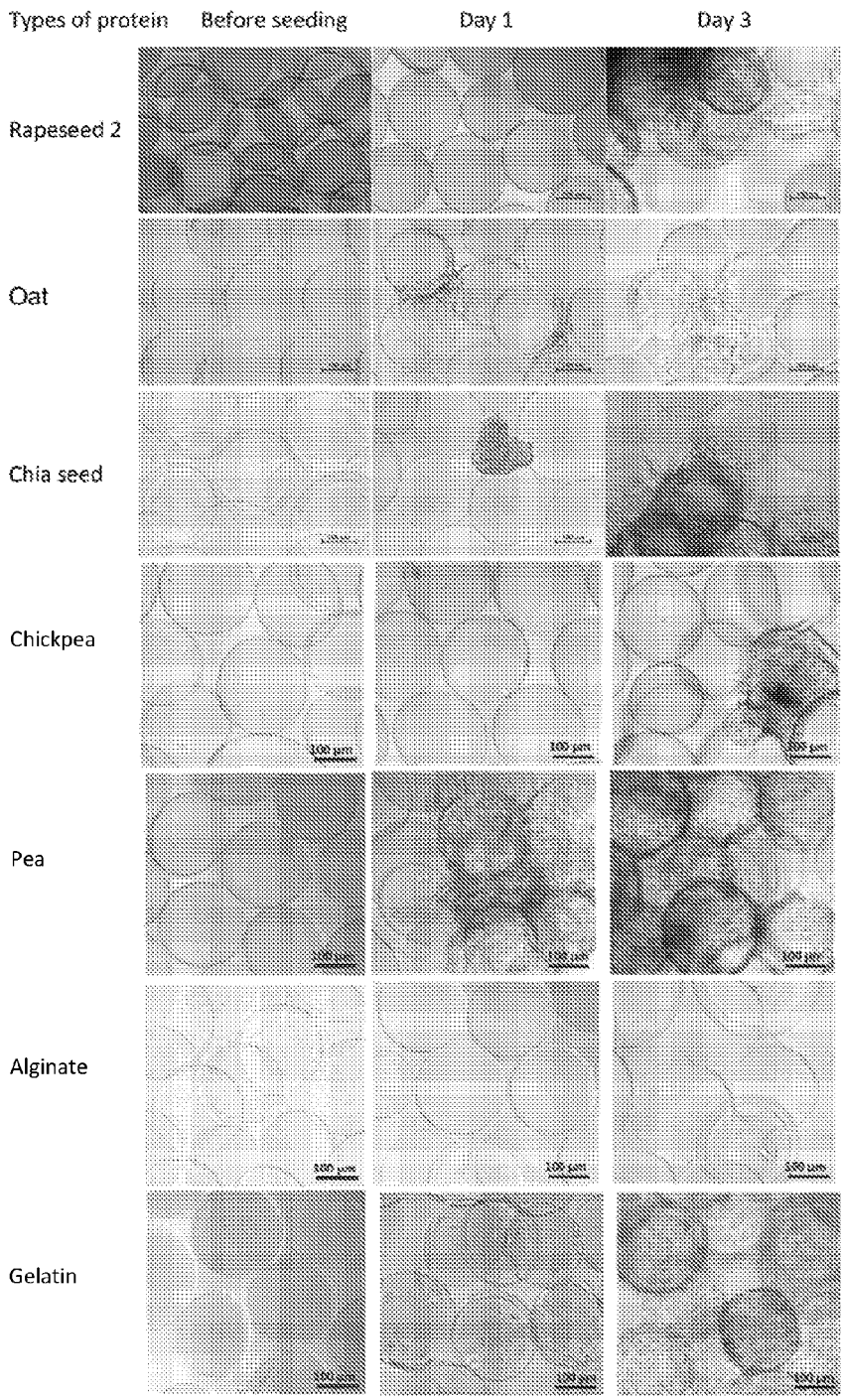


FIG. 1A (continued)

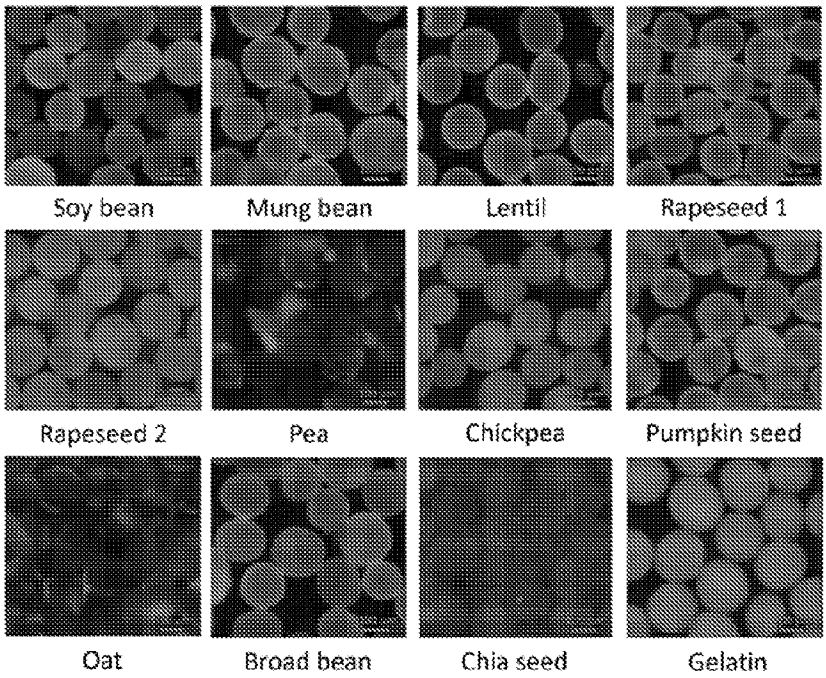


FIG. 1B

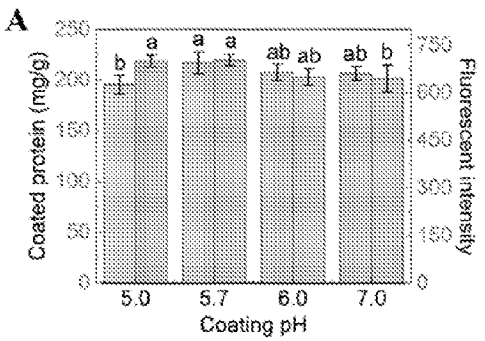


FIG. 2A

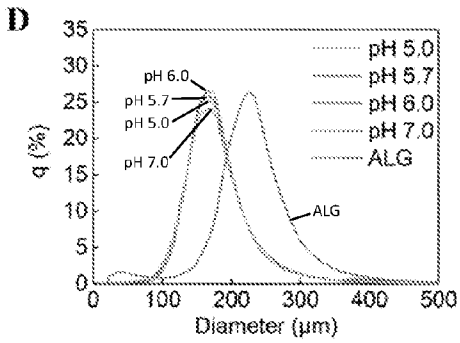


FIG. 2D

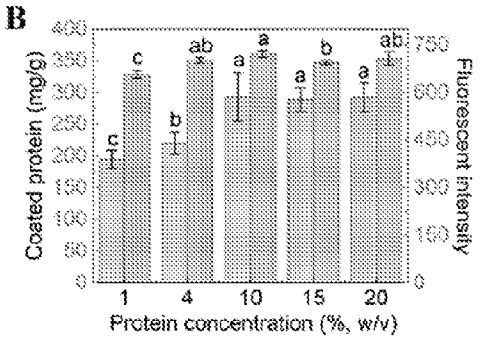


FIG. 2B

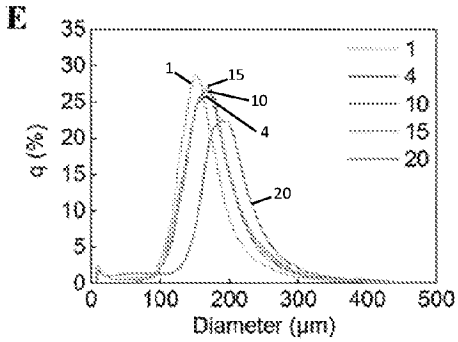


FIG. 2E

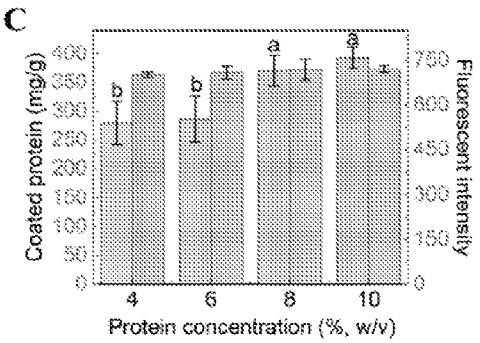


FIG. 2C

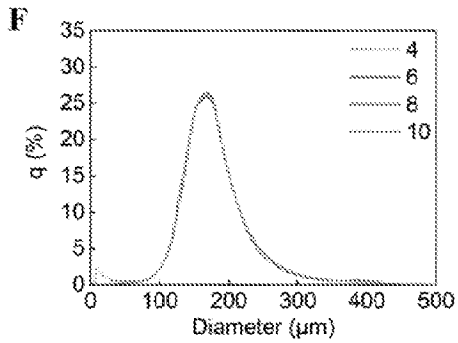


FIG. 2F

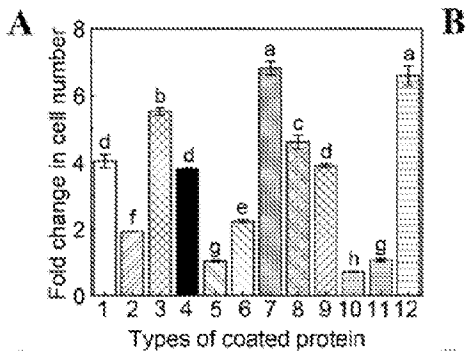


FIG. 3A

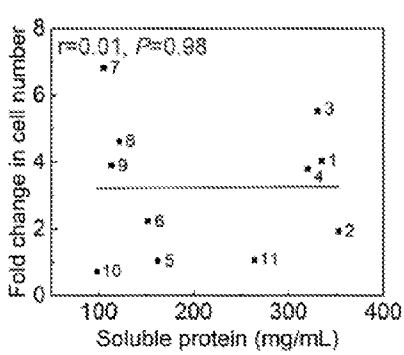


FIG. 3B

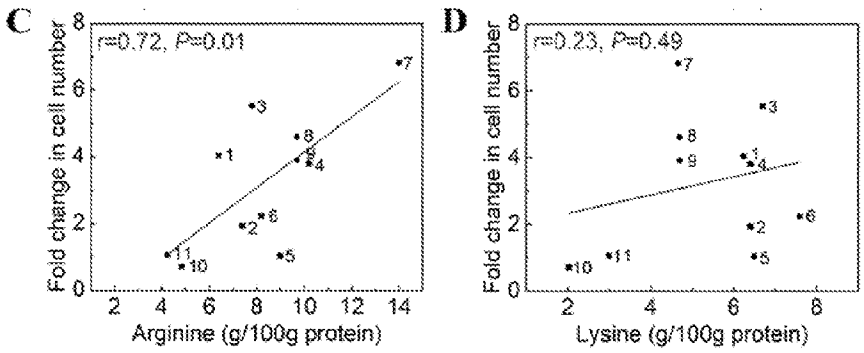


FIG. 3C

FIG. 3D

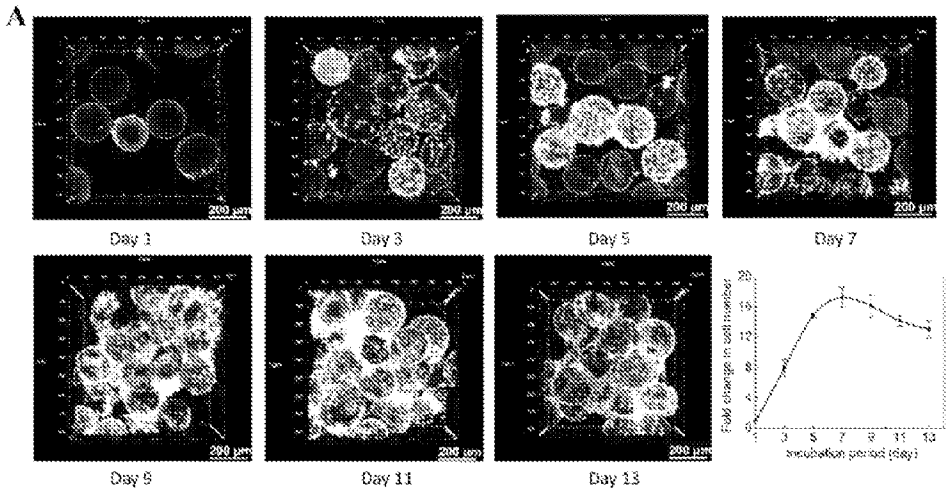


FIG. 4A

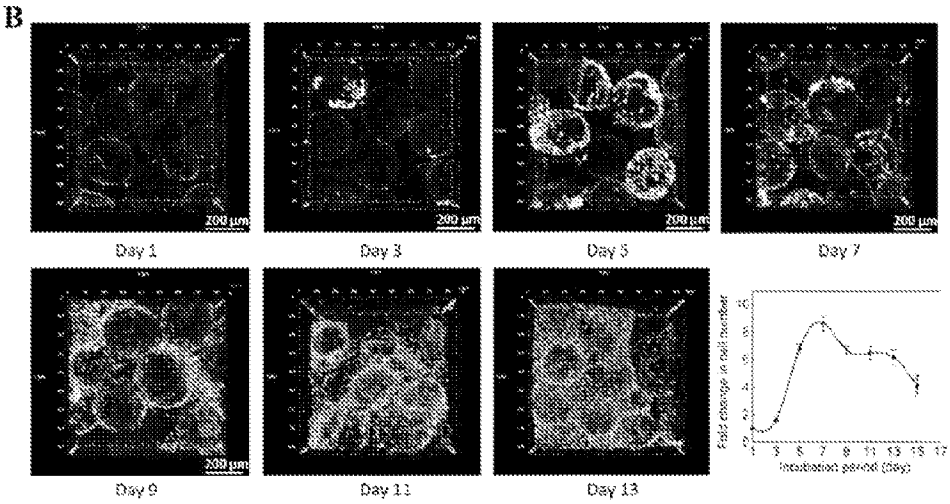


FIG. 4B

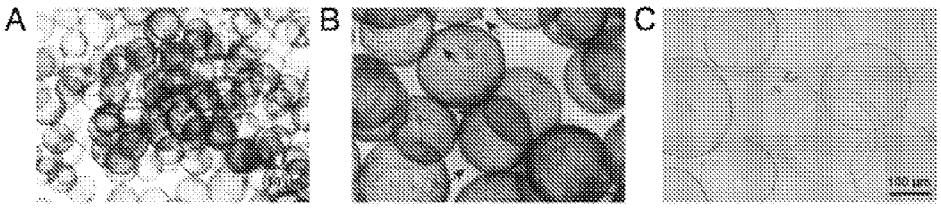


FIG. 5A FIG. 5B FIG. 5C

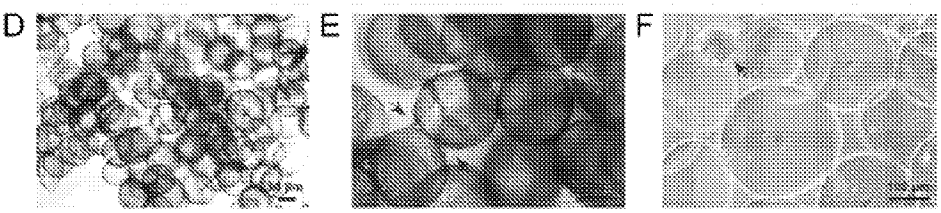


FIG. 5D FIG. 5E FIG. 5F

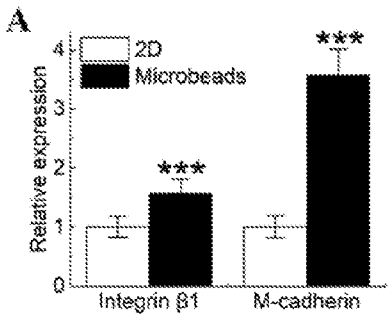


FIG. 6A

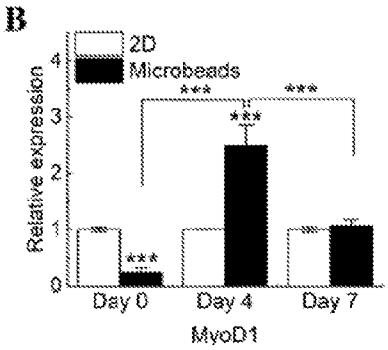


FIG. 6B

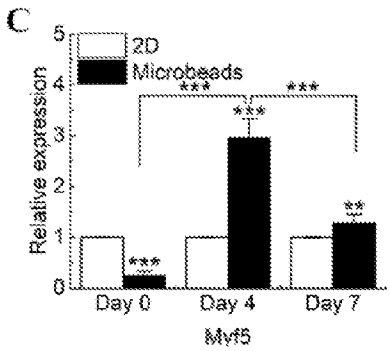


FIG. 6C

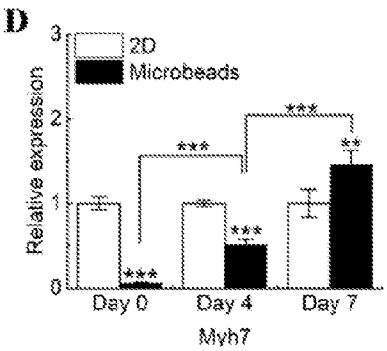


FIG. 6D

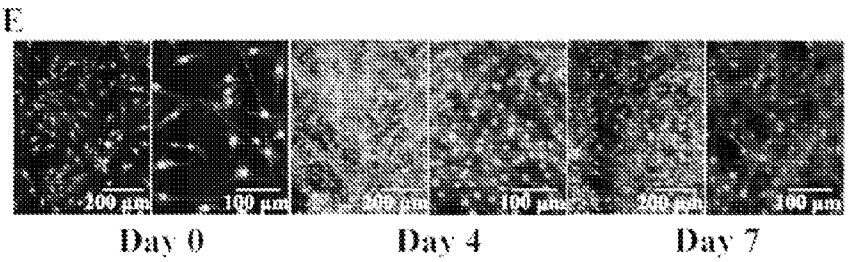


FIG. 6E

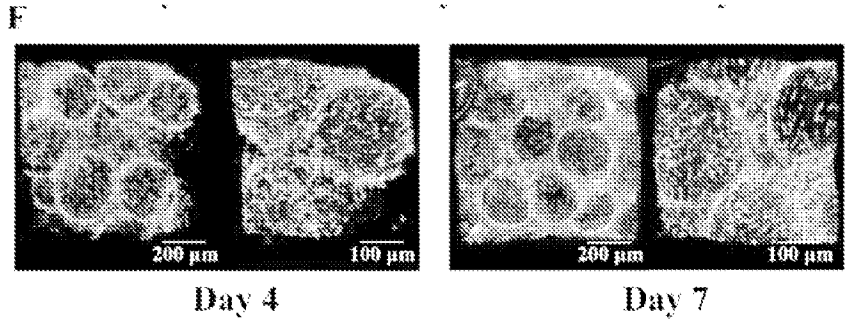

F
200 µm
100 µm
200 µm
100 µm
Day 4
Day 7

FIG. 6F

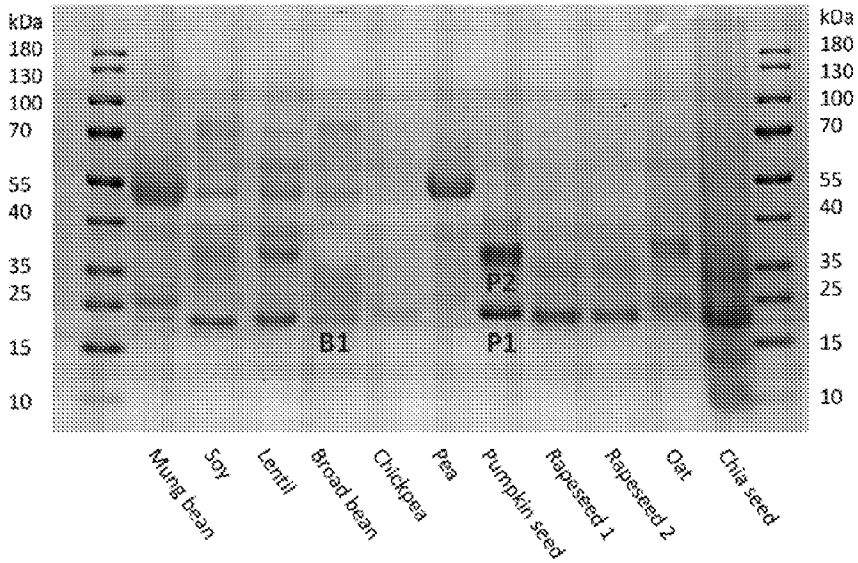

kDa
180
130
100
70
55
40
35
25
15
10
B1
P2
P1
Mung bean
Soy
Lentil
Broad bean
Chickpea
Pea
Pumpkin seed
Rapeseed 1
Rapeseed 2
Oat
Chia seed

FIG. 7

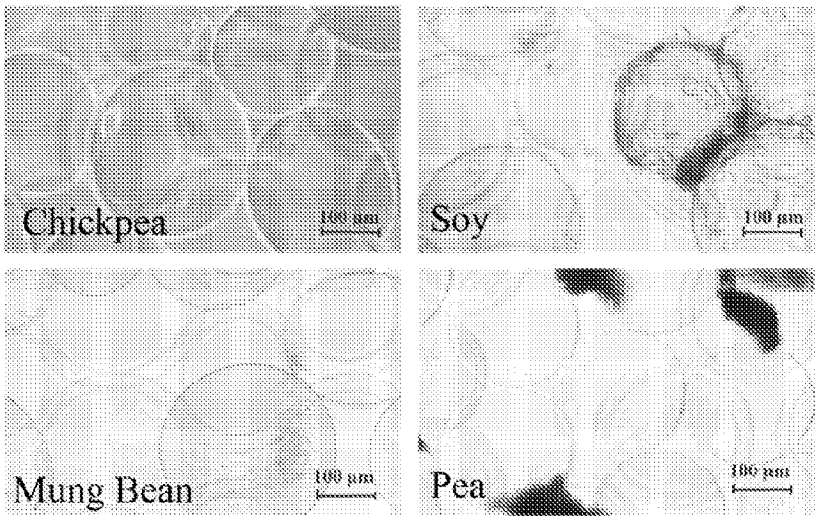


FIG. 8A

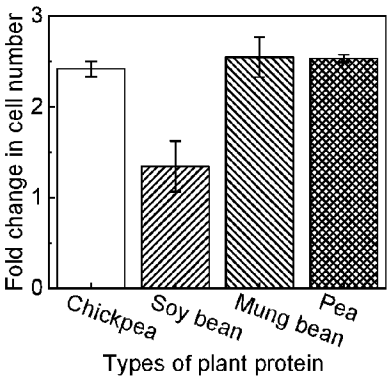


FIG. 8B

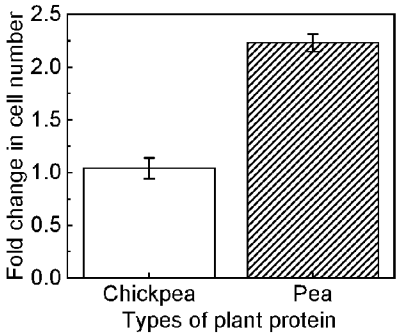


FIG. 8C

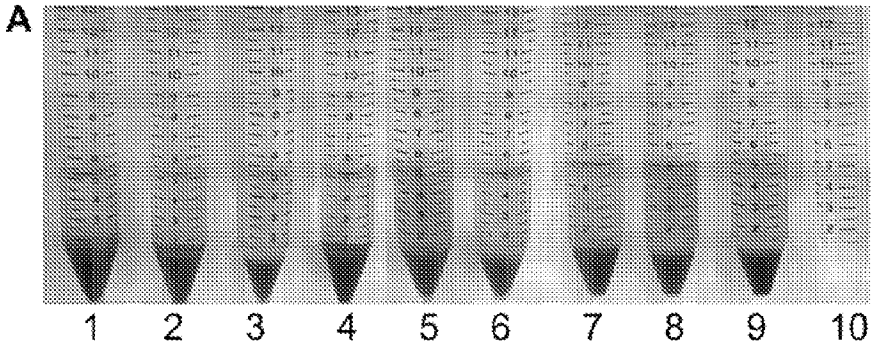


FIG. 9A

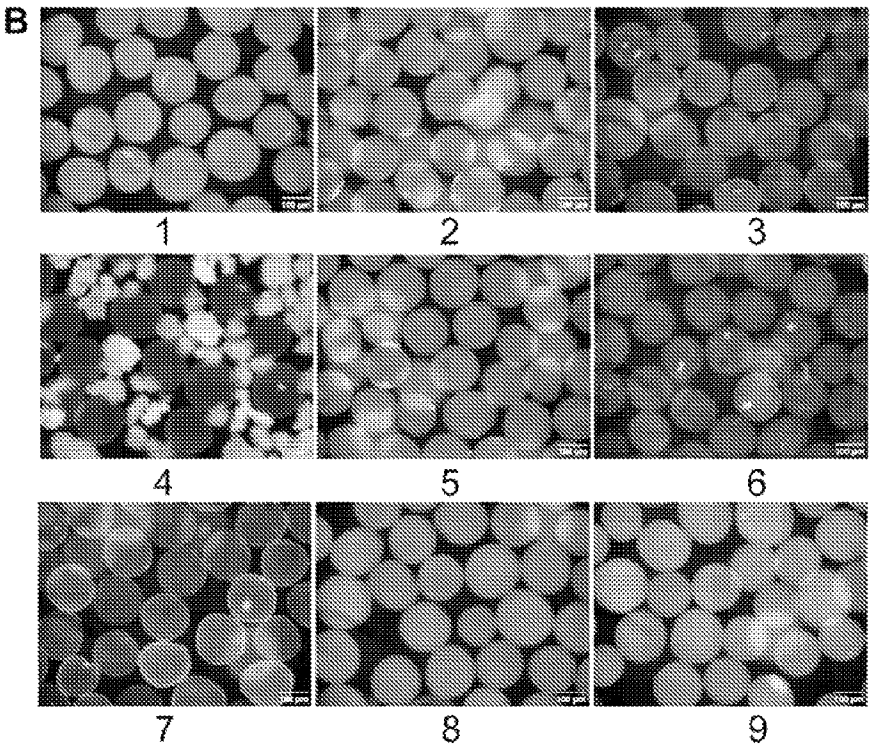


FIG. 9B

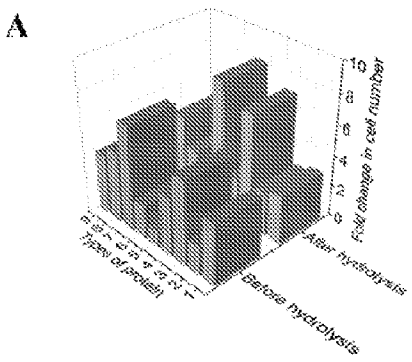


FIG. 10A

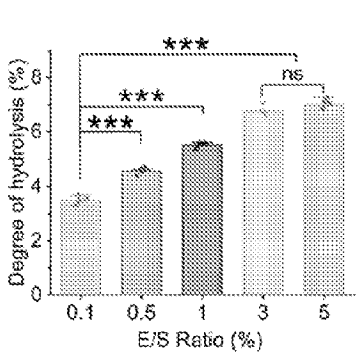


FIG. 10B

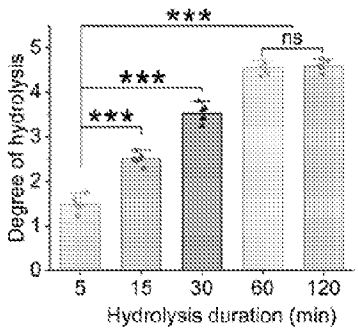


FIG. 10C

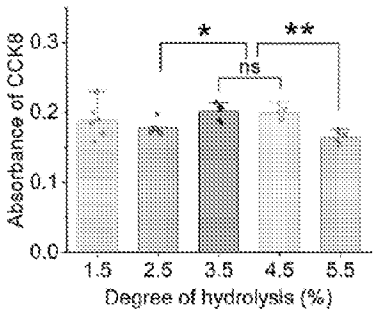


FIG. 10D

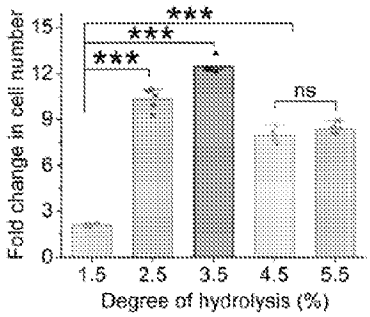


FIG. 10E

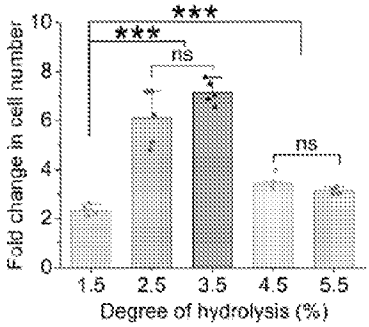


FIG. 10F

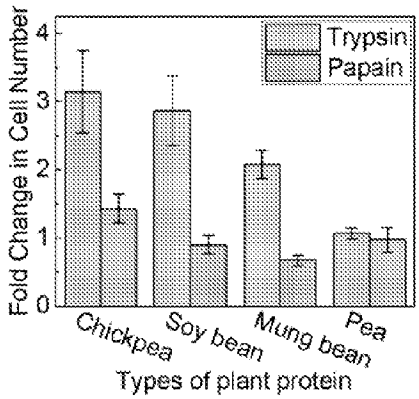


FIG. 11

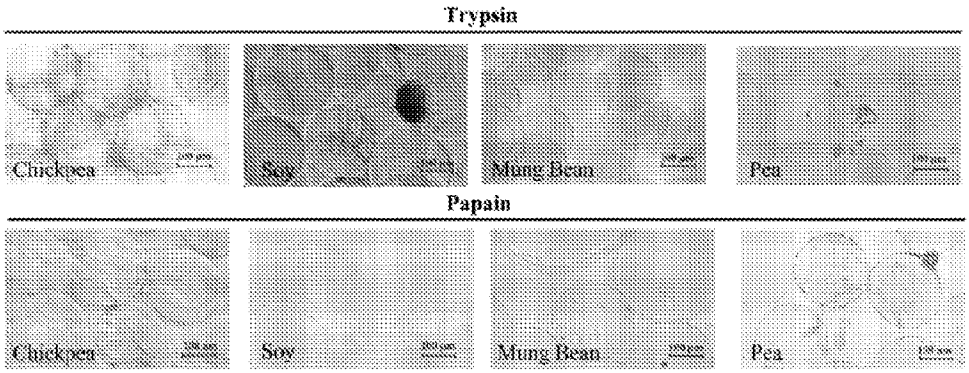


FIG. 12

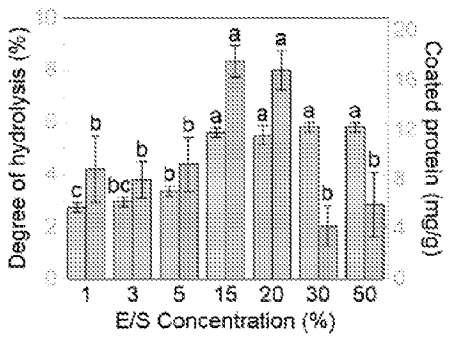


FIG. 13A

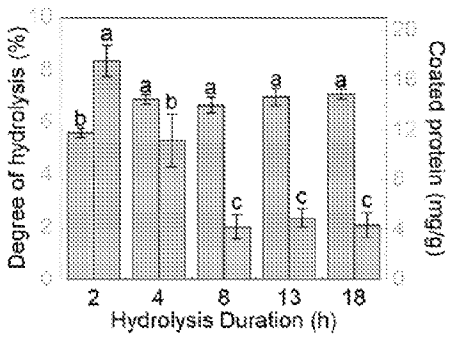


FIG. 13B

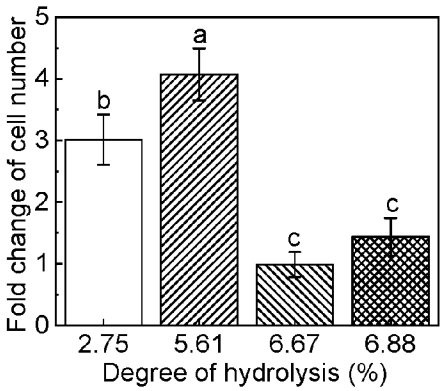


FIG. 14

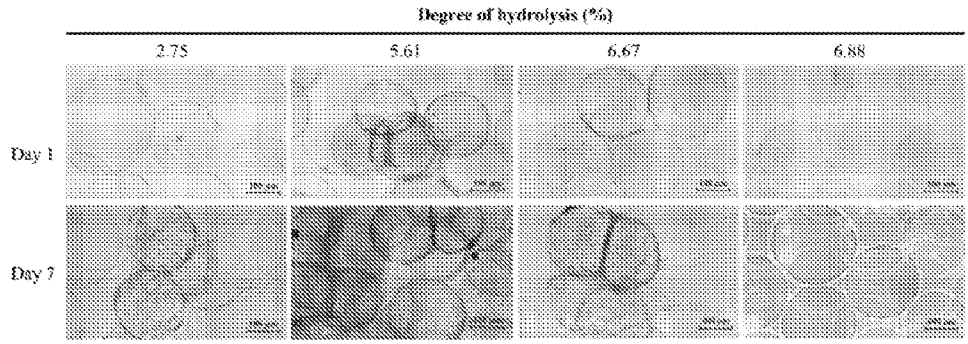


FIG. 15

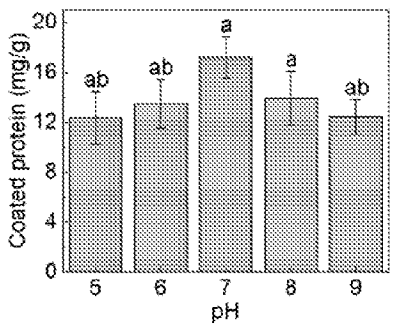


FIG. 16A

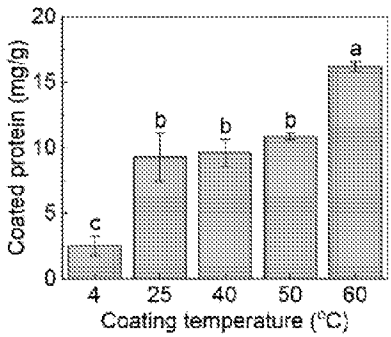


FIG.16B

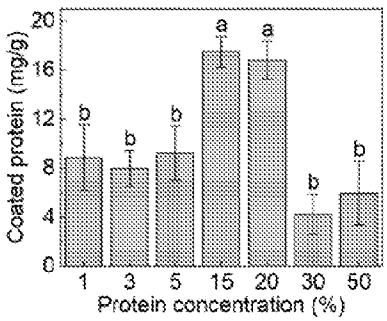


FIG. 16C

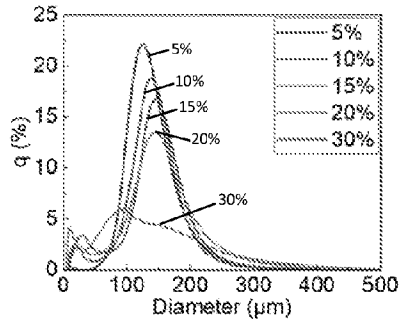


FIG.16D

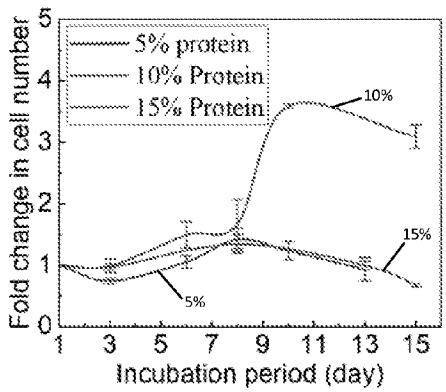


FIG. 17

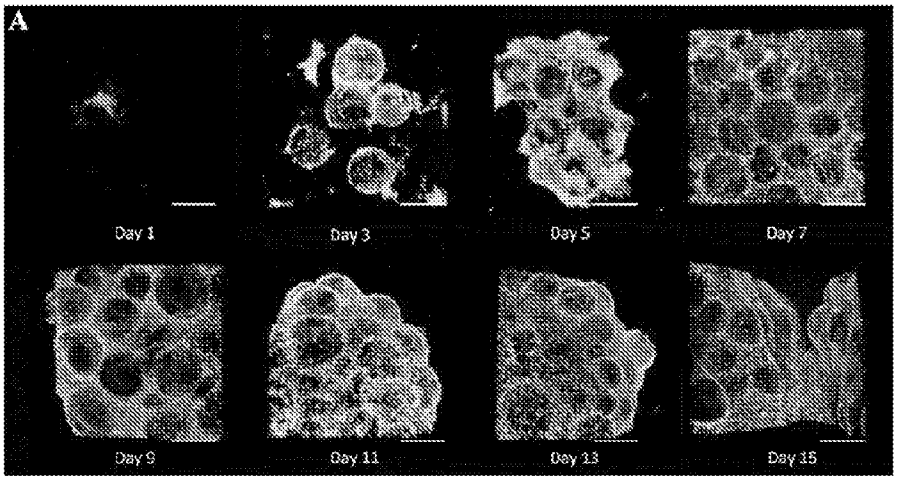


FIG. 18A

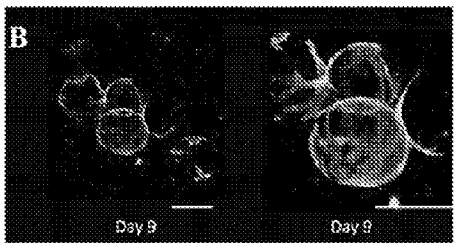


FIG. 18B

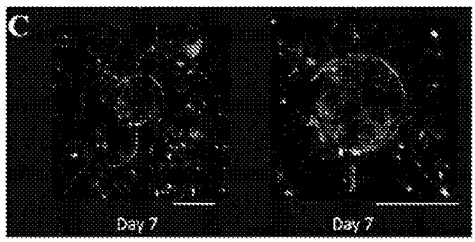


FIG. 18C

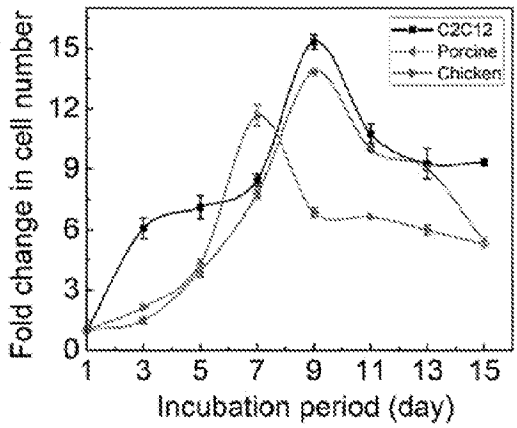


FIG. 18D

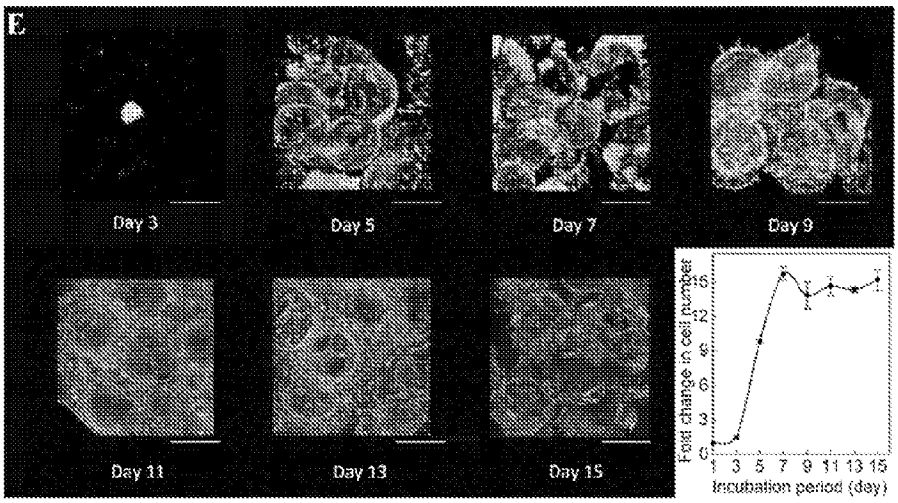


FIG. 18E

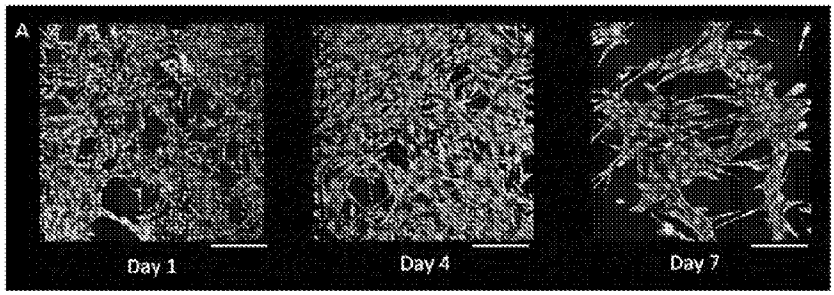


FIG. 19A

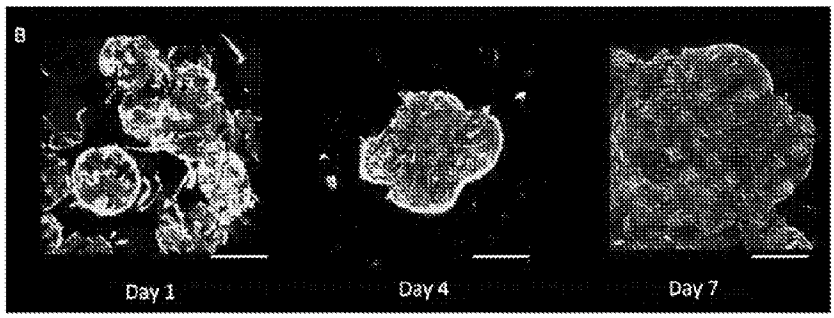


FIG. 19B

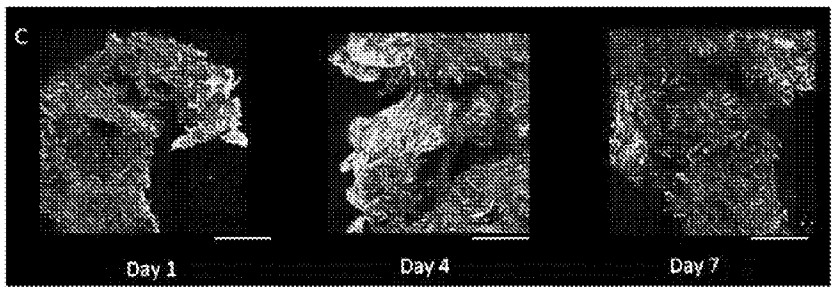


FIG. 19C

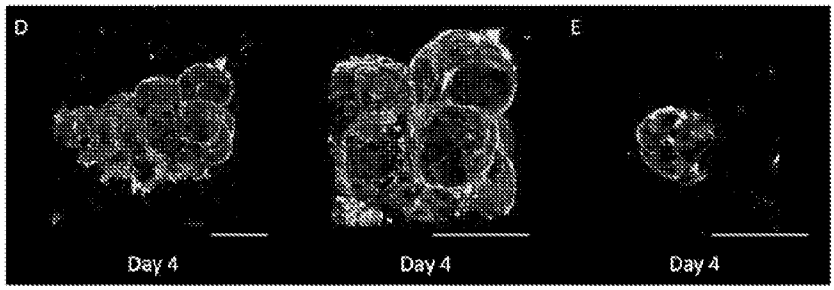


FIG. 19D

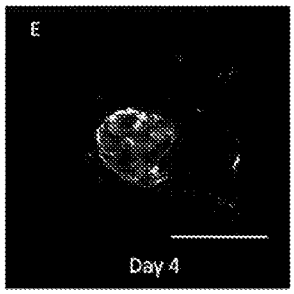


FIG. 19E

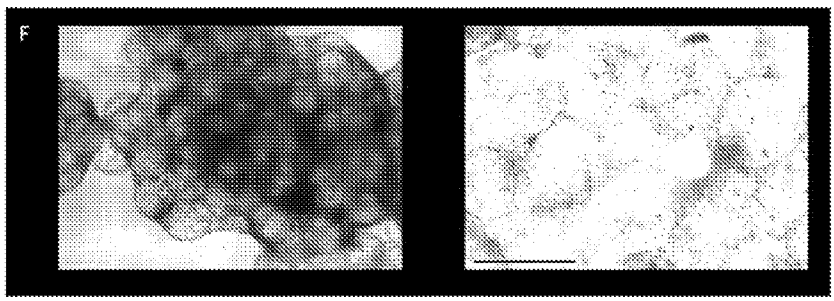


FIG. 19F

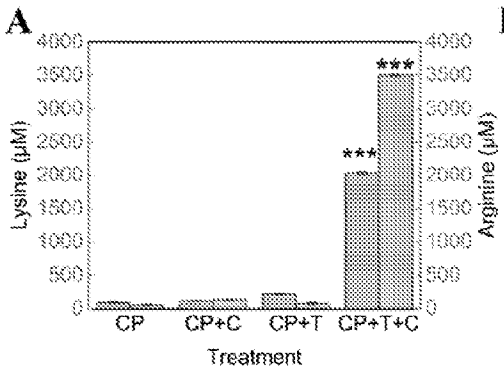


FIG. 20A

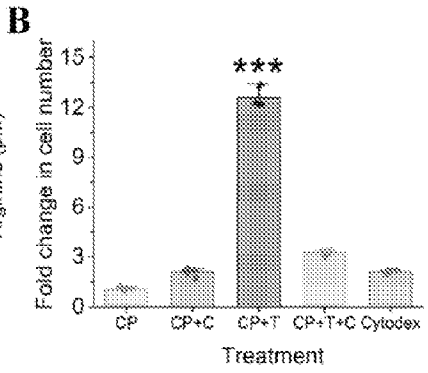


FIG. 20B

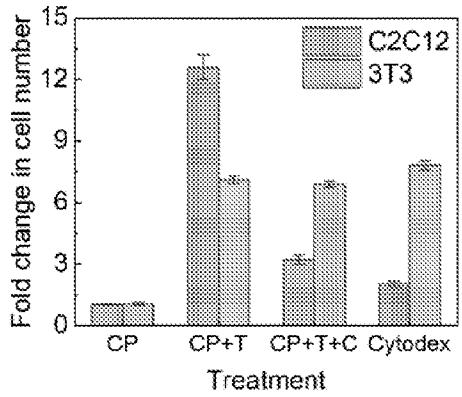


FIG. 20C

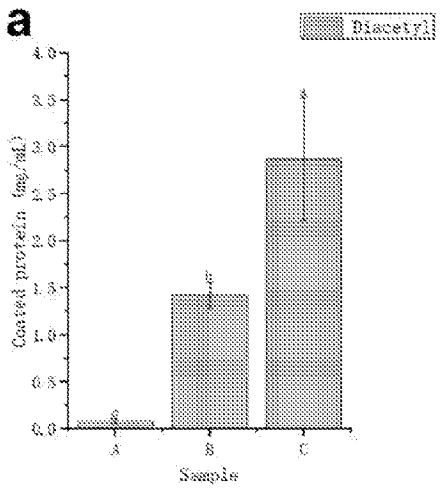


FIG. 21A

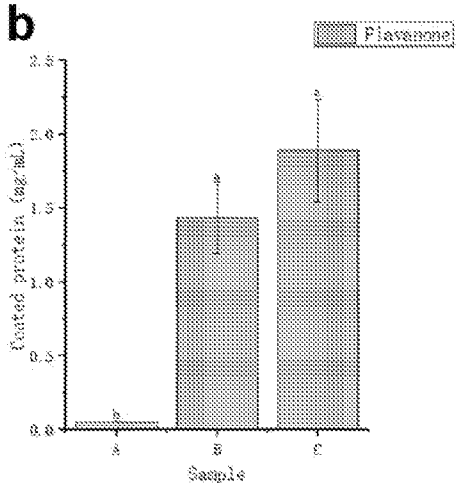


FIG. 21B

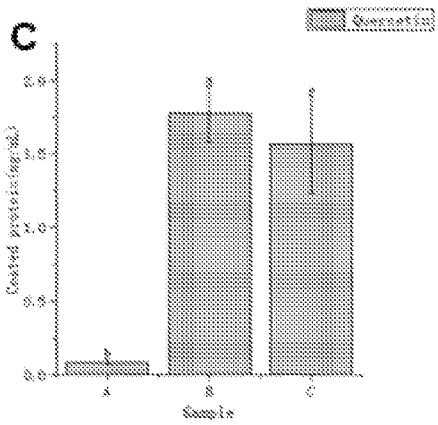


FIG. 21C

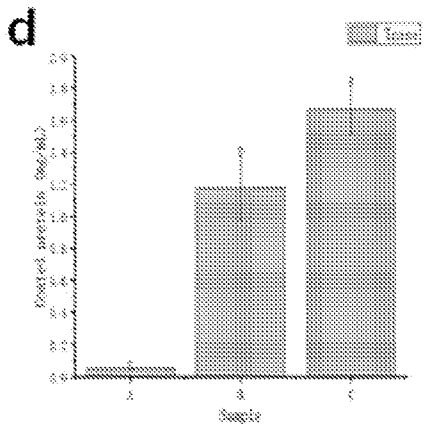


FIG. 21D

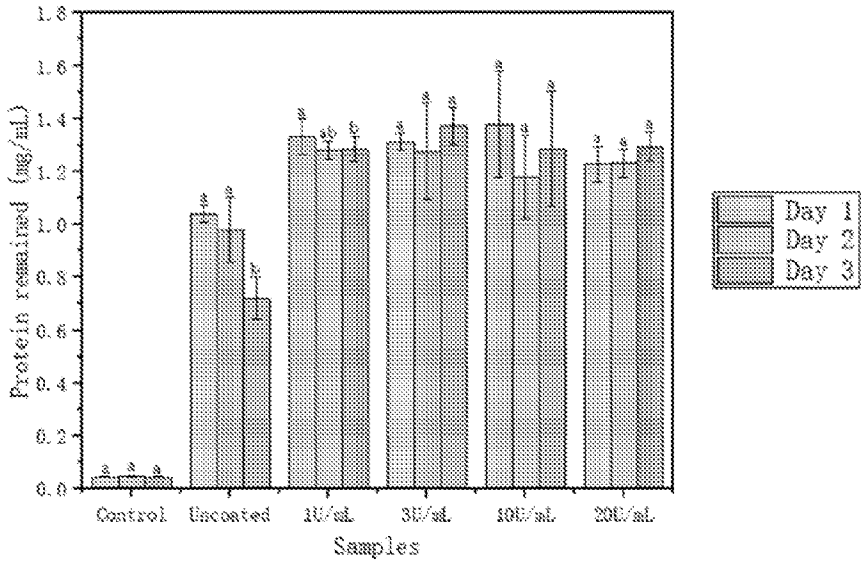


FIG. 22A

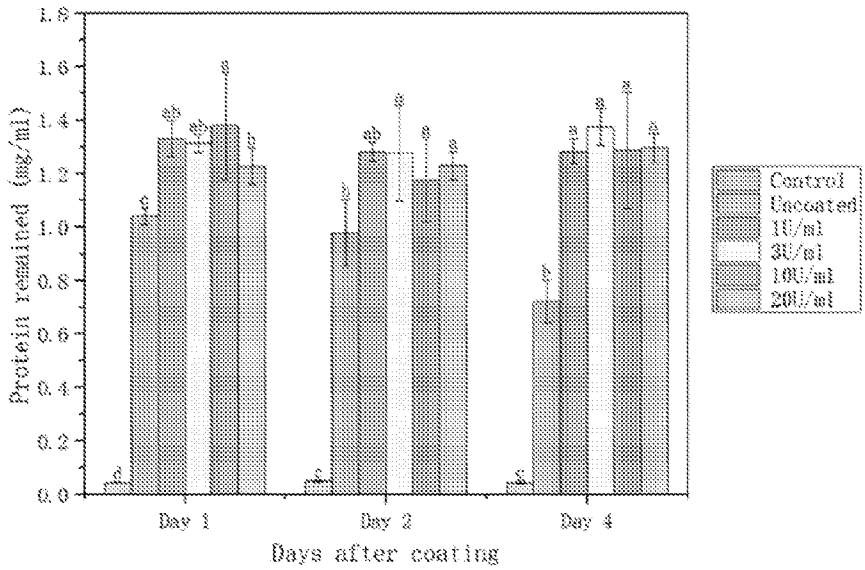


FIG. 22B

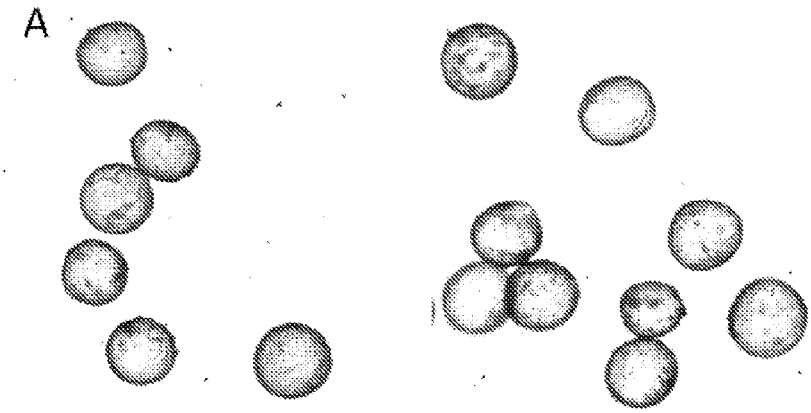


FIG. 23A

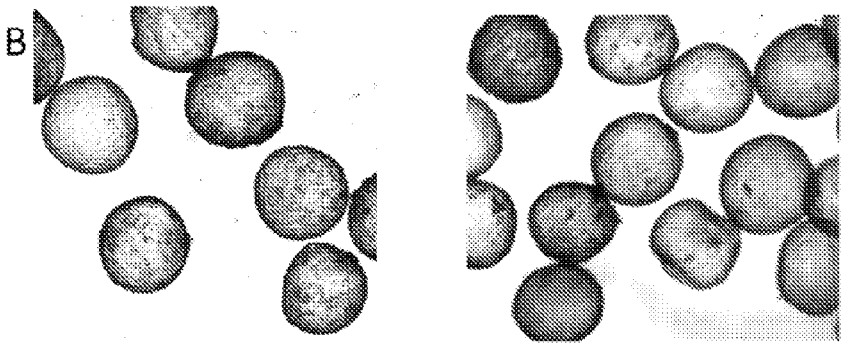


FIG. 23B

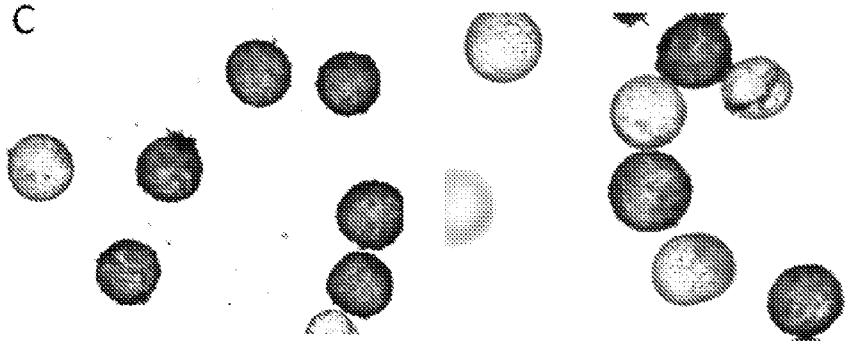


FIG. 23C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2023/050688

A. CLASSIFICATION OF SUBJECT MATTER

A23L 13/00 (2016.01) ***A23L 33/185 (2016.01)*** ***C12N 5/077 (2010.01)*** ***A23J 3/34 (2006.01)***

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EMBASE, MEDLINE, BIOSIS, FAMPAT: microcarrier, coating layer, plant-based, cultured meat, and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KONG Y. ET AL., Functional composite microbeads for cell-based meat culture: effect of animal gelatin coating on cell proliferation and differentiation. *J. Phys. D: Appl. Phys.*, 9 June 2022, Vol. 55, No. 34, Article 345401, pages 1-12 [Retrieved on 2024-01-26] <DOI: 10.1088/1361-6463/AC7011> Pg. 2 left col. first para., pg. 2 right col. second para. - third para., pg. 3 right col. third para., pg. 4 right col. last para. - pg. 5 left col. first para., pg. 10 right col. first para.	1-20
Y	DUTTA S. D. ET AL., Bioengineered Lab-Grown Meat-like Constructs through 3D Bioprinting of Antioxidative Protein Hydrolysates. *ACS Appl Mater Interfaces*, 18 July 2022, Vol. 14, No. 30, pages 34513-34526 [Retrieved on 2024-01-26] <DOI: 10.1021/acsami.2c10620> Abstract, pg. 34516 left col. first para.; Fig. 5a-5c, Fig. S1a	1-20
A	CN 114317394 A (SHANGHAI FOOD NON BIOTECHNOLOGY LTD COMPANY) 12 April 2022 Para. [0009]-[0018] of the machine translation	-

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

*Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26/01/2024 *(day/month/year)*	29/01/2024 *(day/month/year)*

Name and mailing address of the ISA/SG

Intellectual Property Office of Singapore
1 Paya Lebar Link, #11-03
PLQ 1, Paya Lebar Quarter
Singapore 408533

Email: pct@ipos.gov.sg

Authorized officer

Woo Chern Chiuh (Dr)

IPOS Customer Service Tel. No.: (+65) 6339 8616

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2023/050688

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015/038988 A1 (MODERN MEADOW, INC.) 19 March 2015 Para. [0045]-[0047]	-
P,X	KONG Y. ET AL., Plant proteins as the functional building block of edible microcarriers for cell-based meat culture application. *Crit Rev Food Sci Nutr*, 17 November 2022, pages 1-11 [Retrieved on 2024-01-26] <DOI: 10.1080/10408398.2022.2147144> Whole document	1-20
P,X	KONG Y. ET AL., Pumpkin seed proteins rival animal gelatin in increasing the cytoaffinity of edible microbeads for cell-based meat culture. *Food Res Int*, 22 March 2023, Vol. 168, Article 112750, pages 1-11 [Retrieved on 2024-01-26] <DOI: 10.1016/J.FOODRES.2023.112750> Whole document	1-20
P,X	KONG Y. ET AL., Trypsin-treated chickpea protein hydrolysate enhances the cytoaffinity of microbeads for cultured meat application. *Food Res Int*, 27 July 2023, Vol. 173, Pt2, Article 113299, pages 1-10 [Retrieved on 2024-01-26] <DOI: 10.1016/J.FOODRES.2023.113299> Whole document	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2023/050688

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed.

   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),

      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

   Although a sequence listing has been filed or furnished, it was not used for the purposes of this search.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2023/050688

Note: This Annex lists known patent family members relating to the patent documents cited in this International Search Report. This Authority is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 114317394 A	12/04/2022	NONE	
WO 2015/038988 A1	19/03/2015	US 2015/0079238 A1	19/03/2015