

Decellularized matrix from seaweed or lichen, scaffolds made therefrom and methods

FIELD OF INVENTION

The invention is in the field of natural materials and specifically concerns a chemically mild process for processing seaweed and related materials to obtain decellularized biocompatible matrix material with substantially intact extracellular structure that can be used as scaffolds or other exogenous extracellular matrix material for medical use.

TECHNICAL BACKGROUND

An acellular scaffold is a biological structure composed of a mixture of macromolecules, such as collagens, carbohydrates, and lipids. In addition there are multiple other smaller molecules and proteins that are important for cell to cell interaction and tissue regeneration such as glycosaminoglycans and proteoglycans [1]. There is a wide variety of scaffolds available from both organic and synthetic sources that have either natural or engineered microstructure. The microstructure provides an environment that supports cellular infiltration as well as providing a structure for cell adhesion during tissue healing [2][3]. Collagen based scaffolds derived from animals, are well known within tissue engineering (TE) and regenerative medicine (RM) and commonly used for wound healing and soft tissue reinforcement [4][5]. Despite the wide variety of available scaffolds, the quest for the ideal one is still ongoing with the attention extending into non-animal derived scaffolds. The use of the substantially intact brown seaweed as medical device is however new and unexplored. Brown seaweed is mainly composed of carbohydrate such as monosaccharide and polysaccharides 60-70% and proteins 6 -11%, ash (minerals) 25-32%, and polyphenols and other minor components. The use of polysaccharides extracted from processed algae for tissue engineering and regenerative medicine is well known and frequently used due to its biological, physical and chemical properties [6][7]. The alginates extracted from brown seaweed have been used in wound healing, to dispense medicine, in tissue engineering (TE) and regenerative medicine (RM). As such, cellulose, a polysaccharide, is the most abundant biomaterial available on the planet with terrestrial sources from plants, fungi and bacteria, and marine sources in marine plants and algae [8]. Terrestrial plants are largely composed of cellulose and lignin while seaweed will contain cellulose but no or negligent amount of lignin, [9]. The cellulose provides structure and strength to the organism with combination of cellulose Ia or cellulose Ib that forms a crystalline structure. In plants, cellulose Ib is dominate type in the crystalline structure while Ia is the main type for algae and bacterial cellulose [10][11]. Bacterial cellulose scaffolds have been a popular topic of study due to their distinctive origin and properties. Studies show promising results for this cellulose material both as soft tissue reinforcement [12] and vascular grafts [13]. Plant derived cellulose based scaffolds are also available but undergo harsh processing to purify and reconstitute the cellulose based part, mostly clear of any other natural ingredients of the source material.

Wahlström et al. [33] disclose extraction and characterization of cellulose from northern hemisphere green macroalgae *Ulva lactuca* (*Ulva fenestrata*) collected and cultivated indoors under controlled

conditions, followed by (2) its utilization in the production of lignin-free cellulose nanofibrils (CNF). Cellulose was extracted by sequential treatment with ethanol, hydrogen peroxide, sodium hydroxide, and hydrochloric acid, yielding a cellulose-rich insoluble fraction. The extracted cellulose was disintegrated into CNF using a mechanical homogenization process.

- 5 US 11,167,062 [34] describes use of apple to provide decellularized cell wall structure as scaffold material. The disclosed process is based on first treatment in detergent solution (0.1% SDS) and subsequently treating in 70% ethanol. The publication suggests generally to use plant materials and proposes also seaweed materials.

- 10 US2023364302A1 [35] discloses using decellularized macroalgae material as cellulose-based scaffold material, where the macroalgae material is first boiled in acetone bath (20% W/v) at 60°C order to remove pigments (chlorophyll) and proteins, residual biomass is boiled in acetate buffer containing sodium chlorite (NaClO₂) (20% w/v) spurring bleaching and the removal of simpler structure polysaccharides. The bleached seaweed residues were pH neutralized by washing with distilled water, and then alkylated in 0.5 M sodium hydroxide (NaOH) bath (20% w/v), at 60° C. for 8-10 h, to remove all excessive lipids.
- 15 Following the alkali treatment, the seaweed residues were pH neutralized by washing with distilled water, and then acidified in hydrochloric acid (HCl) (5% v/v), at 100° C. for 10 min (20% w/v), or until boiling started, then samples were rested overnight at room temperature to remove all excessive polysaccharides that might remain close to the cell wall. Finally, the samples were carefully rinsed repeatedly in distilled water.

20 SUMMARY OF INVENTION

- This invention provides a novel biocompatible scaffold material comprising decellularized seaweed or lichen that comprises a complex substantially intact extracellular matrix composed of at least complex carbohydrates, polysaccharides, monosaccharides and proteins. Also provided is a novel process for preparing decellularized (acellular) matrix material from seaweed and related material, such as in
- 25 particular the scaffold material of the invention. The scaffold material of the invention can advantageously be provided such that it substantially or significantly retains major extracellular matrix and also other beneficial substances, thus the scaffolds of the present disclosure advantageously retains one or more and preferably substantially or significantly all of the substances alginates, cellulose, and polysaccharides, as well as proteins, peptides and amino acids, i.e. not only large-
- 30 molecule substances tightly bound as building blocks of the extracellular matrix but also small molecule substances found in the native matrix material. This is readily achieved with the methods disclosed herein, which are mild methods designed so as to avoid harsh chemical treatment such as treatment by strong acid, strong base, organic solvent or high temperature. The novel acellular material obtained by the methods disclosed herein can advantageously be used as exogenous extracellular matrix
- 35 material, e.g., as scaffold matrix for biological applications, such as but not limited to implants and internal or external tissue engineering, wound dressing, and drug delivery implants.

Thus, in one aspect the disclosure provides and describes a biocompatible scaffold material, comprising decellularized seaweed or lichen, wherein the decellularized seaweed or lichen comprises a complex substantially intact extracellular matrix. The seaweed species should preferably be a seaweed with leaf-like structure and not too thick leaves, and can be, e.g., a brown seaweed species, a red seaweed species or a green seaweed species.

The scaffold material preferably substantially or significantly retains extracellular matrix substances including one or more of proteins, alginates, saccharides, and cellulose. Preferably the scaffold material of the invention has substantially same content of total protein as the source seaweed material, such as in the range 80-120 wt% of total protein content compared the source seaweed material (typically measured on dry weight basis, such as in the range from about 80% or from about 90%, to about 120% or to about 110 or to about 100%. Retention of biomaterials can be assessed by analysis of desired biological components, such as but not limited to total protein, amino acids, oligosaccharides and monosaccharides, alginates, etc. Scaffold material may in some embodiments show substantially similar or even higher content of some biomaterials than the source materials, after the material has been decellularized. Thus, in some embodiments the scaffold material shows similar or higher content of some or all amino acids than the source material. This is believed to enhance the potential of the scaffold material to support tissue recovery and healing. Substantially similar may in this context refer to a measured value of the scaffold material which is in the range of about 80% to about 120% or in the range from about 90% to about 100% of the measured value of the unprocessed source material, such as in the range of about 95% to about 100%, such as in a range from about 80% or from about 85% or from about 90% or from about 95%, to about 120% or to about 110% or to about 105% or to about 100%. Some substances of the natural extracellular matrix may be rinsed away from the substrate material to some degree in the process described herein, but are preferably retained to a substantial degree, which is referred to herein as significantly retaining natural substances. Thus, in some embodiments at least about 30 wt% of alginates are retained in the matrix material and more preferably at least about 40 wt%, or at least about 50 wt% and more preferably at least about 60% or at least about 70 wt%, and yet more preferably at least about 80 wt% or at least about 90 wt% of alginates, compared to the native untreated tissue.

The scaffold material of the present disclosure can be provided in any of various useful forms such as but not limited to a wound dressing, bandage, particularized form, suture material, or mesh material. In some embodiments the scaffold material comprises one or more added active or non-active agents. An added active agent may be selected from antibiotics, antiseptics, antimicrobial agents, antiviral agents, antifungal agents, antiparasitic agents, anti-inflammatory agents, antioxidants, drugs, proteins, peptides, and combinations thereof.

In useful embodiments the source organism is selected from brown seaweed, such as but not limited to seaweed of genus *Laminaria*, including the species *Laminaria abyssalis*, *Laminaria agardhii*, *Laminaria appressirhiza*, *Laminaria brasiliensis*, *Laminaria brongardiana*, *Laminaria bulbosa*, *Laminaria bullata*, *Laminaria complanata*, *Laminaria digitata*, *Laminaria ephemera*, *Laminaria farlowii*, *Laminaria groenlandica*, *Laminaria hyperborea*, *Laminaria inclinathorhiza*, *Laminaria longipes*, *Laminaria*

multiplicata, *Laminaria nigripes*, *Laminaria ochroleuca*, *Laminaria pallida*, *Laminaria platymeris*, *Laminaria rodriguezii*, *Laminaria ruprechtii*, *Laminaria sachalinensis*, *Laminaria setchellii*, *Laminaria sinclairii*, *Laminaria solidungula*, and *Laminaria yezoensis*. preferably of species *Laminaria Digitata* or *Laminaria Saccharina* (also referred to as *Saccharina latissima*).

- 5 In some embodiments, the seaweed is a green seaweed tissue, e.g., a tissue of a *Cladophora* species (*Cladophora* sp.) or a mixture thereof (*Cladophora* spp.). Examples of *Cladophora* spp. include, without being limited to, *Cladophora albida*, *Cladophora aokii*, *Cladophora brasiliensis*, *Cladophora catenata*, *Cladophora coelothrix*, *Cladophora columbiana*, *Cladophora crispata*, *Cladophora dalmatica*, *Cladophora fracta*, *Cladophora glomerata*, *Cladophora graminea*, *Cladophora montagneana*,
10 *Cladophora ordinata*, *Cladophora prolifera*, *Cladophora rivularis*, *Cladophora rupestris*, *Cladophora scopaeformis*, *Cladophora sericea*, *Cladophora socialis*, and *Cladophora vagabunda*.

- In some embodiments the seaweed species is a *Bangia* species (*Bangia* sp.) or a mixture thereof (*Bangia* spp.). Examples of *Bangia* spp. include, without limiting, *Bangia aeruginosa*, *Bangia amethystina*, *Bangia anisogona*, *Bangia annulina*, *Bangia atropurpurea*, *Bangia atrovirens*, *Bangia*
15 *biseriata*, *Bangia breviarticulata*, *Bangia callicoma*, *Bangia carnea*, *Bangia coccineopurpurea*, *Bangia condensata*, *Bangia confervoides*, *Bangia crispula*, *Bangia discoidea*, *Bangia dura*, *Bangia enteromorphoides*, *Bangia fergusonii*, *Bangia ferruginea*, *Bangia flocculosa*, *Bangia foetida*, *Bangia foetida*, *Bangia foliacea*, *Bangia fulvescens*, *Bangia fuscopurpurea*, *Bangia gloiopeltidicola*, *Bangia grateloupicola*, *Bangia halymeniae*, *Bangia harveyi*, *Bangia homotrichoides*, *Bangia intricata*, *Bangia*
20 *intricata*, *Bangia kerkensis*, *Bangia lacustris*, *Bangia lanuginosa*, *Bangia latissima*, *Bangia malacensis*, *Bangia maxima*, *Bangia punctulata*, *Bangia purpurea*, *Bangia quadripunctata*, *Bangia radicola*, *Bangia sericea*, *Bangia simplex*, *Bangia tanakae*, *Bangia tavarisii*, *Bangia tenuis*, *Bangia thaerasiae*, *Bangia trichodes*, *Bangia vermicularis*, *Bangia viridis*, and *Bangia yamadae*

- Other useful brown seaweed species that can be used include *Alaria esculenta* (Dabberlocks, Wing Kelp, Murlins), *Asperococcus fistulosus*, *Asperococcus bullosus*, *Colpomenia peregrina*, *Dictyopteris polypodioides*, *Dictyota dichotoma*, *Dilsea carnosa*, *Kallymenia reniformis*, *Petalonia fascia* (Sea Petals, Broad Leaf Weed), *Saccorhiza polyschides* (Furbellows, Furbellows, Sea Hedgehog), and *Undaria pinatifida* (Wakame). In some embodiments the source organism is selected from the genus *Schizymenia* including but not limited to *Schizymenia jonssonii* and *Schizymenia dubyi*.

- 30 Other useful red seaweed species that can be used include *Porphyra* and *Wildmania* such as but not limited to *Porphyra umbilicalis* and *Wildmania amplissima*.

- In other embodiments the scaffold material and process of the disclosure uses lichen. A lichen is a composite organism that arises from algae or cyanobacteria living among filaments of multiple fungi species in a mutualistic relationship. Preferred lichens for the invention include in particular so called microlichen, such as but not limited to *Usnea* and *Bryoria* lichens (e.g. *Bryoria fremontii*, *Cladonia* lichens (e.g. *Cladonia rangiferina*, *Ramalina* lichens, e.g. *Ramalina farinacea*, and *Cetraria* lichens such as but not limited to *Cetraria islandica* (Iceland moss)).

In another aspect the disclosure provides a process for producing a substantially decellularized biocompatible scaffold material from an organism selected from seaweed or lichen, the process comprising the steps of:

- a. removing said organism or portion thereof from its natural or cultivation habitat and arranging
5 said organism or portion of said organism in a body of water,
 - b. emitting electromagnetic radiation in the ultraviolet, visible and/or infrared part of the electromagnetic spectrum on said organism or portion over a sufficient period of time for decellularization of said portion to take place, to form a decellularized scaffold material, and
 - c. retrieving said decellularized scaffold material from the body of water or aqueous solution.
- 10 It follows that the organism is first taken from its natural environment (or alternatively it could be cultivated and taken from a cultivation); preferably, in particular when the organism is seaweed, the organism material is washed, e.g., rinsed with tap water.

The terms acellular and decellularized are used herein to describe material derived from biological tissue that has been made essentially free of cellular organelles and nuclei content, where the phrase
15 “essentially free of cellular organelles and nuclei content” as used herein with respect to the scaffold disclosed herein means that said scaffold is fully or substantially free of cell nuclei and other cellular organelles may comprise some residual cellular components such as DNA, mitochondria, and membrane-associated molecules including phospholipids, rather than being completely free of such material, considering that decellularization techniques usually cannot completely remove all such
20 material. The cellular components optionally comprised within the scaffold, i.e., left following the decellularization process utilized, can be measured quantitatively. As discussed above, it is an advantage of the present invention that some beneficial components are preferentially left in the material that has been decellularized with the process of the present disclosure.

Advantageously, the electromagnetic radiation in the process is preferably provided by light emitting
25 diodes (LEDs) which can be suitably arranged in panels. Such LED panels are preferably arranged so as to emit radiation from more than one direction, for example from opposite directions. Thus, in preferred embodiments the body of water is arranged in a transparent vessel or container and LED panels can be arranged on each side of said vessel or container or on multiple (more than two) sides. In some embodiments LED panels are configured in watertight enclosures and can thus be submerged
30 inside a vessel holding the body of water; for example, a vessel can thus contain series of parallel sandwiches of LED panels and alternating parallel rows of seaweed or lichen organisms or parts; in these embodiments the vessel need not be transparent but can be more advantageously from a material such as stainless steel or other suitable material, preferably a material that is reflective (such as stainless steel) so that emission that falls on the walls from the LED panels is reflected back into the
35 vessel.

The light intensity delivered by the radiation sources can suitably be characterized by a photon flux; in this invention, a suitable photon flux is in some embodiments in the range of 10 – 1500 $\mu\text{mol}/\text{m}^2/\text{s}$, such

as in the range of about 20 – 1000 $\mu\text{mol}/\text{m}^2/\text{s}$, such as in the range from about 50 or from about 60 or from about 80 or from about 100 or from about 120, to about 1000, or to about 800 or to about 600 or to about 500 $\mu\text{mol}/\text{m}^2/\text{s}$.

LED light panels are such known in the art. Preferably, the LED panels are arranged and configured to
5 emit desired and sufficient emission while not producing too much excess heat. The light panels can
comprise LED, QLED or OLED lights that are known in the art, and in the context herein are all
considered within the general term LED. For example, the panels can each comprise one or more LED
light boards or light engines, wherein each such light board comprises a plurality of lights that are
embedded on a circuit board that has electrical and mechanical fixings. Exemplary but non-limiting
10 LED light boards (light engines) are manufactured by Light Engines Europe (<http://www.leeltd.uk.com>).

Preferably the radiation is emitted continuously during a suitable selected period of time. The period of
time for emitting radiation is typically at least 12 hours and more preferably at least 24 hours or at least
48 hours, and yet more preferably at least one week. Thus, in some embodiments the period of radiation
emission is in the range from about 12 hours or from about 24 hours or from about 48 hours or from
15 about 72 hours or from about 96 hours or from about 1 week, to about two months or to about 45 days
or to about one month or to about 30 days or to about 20 days, or to about 14 days or to about 10 days.

The emitted electromagnetic radiation preferably comprises a radiation wavelength in the ultraviolet,
visible or infrared part of the electromagnetic spectrum and may comprise a plurality of wavelengths or
a band or spectrum of wavelengths. In some embodiments the radiation comprises one or more
20 wavelength in the range from about 380 to about 750 nm, such as one or more wavelengths in a range
from about 380 nm or from about 400 nm or from about 450 nm or from about 500 nm, to about 750
nm, or to about 700 nm or to about 650 nm. In some embodiments the radiation comprises one or more
wavelength in the infrared spectrum, such as in the near infrared spectrum, e.g., in the range from about
700 nm or from about 750 nm to about 3 μm or midrange infrared spectrum, from about 3 μm to about
25 30 μm , or far-infrared spectrum from about 30 μm to about 1000 μm wavelength. In some embodiments
the radiation may comprise one or more wavelengths in the ultraviolet part of the electromagnetic
spectrum, such as one or more wavelengths in the range from about 100 nm to about 400 nm. In some
embodiments the radiation may be combined of a plurality of wavelengths such as but not limited to
one or more visible light wavelengths, and/or one or more infrared wavelengths and/or one or more UV
30 wavelengths.

In some embodiments the emitting of radiation is arranged to comprise at least two different temporal
phases of radiation, wherein in a first phase radiation comprising at least one first wavelength is emitted,
and in a second phase radiation comprising at least one second wavelength is emitted. In such
embodiment, the radiation in the first phase may preferably comprise one or more wavelength in the
35 range 600-750 nm and/or wavelength in the infrared part of the electromagnetic spectrum, such as a
comprising a wavelength of about 600 nm or about 650 nm or about 700 nm, and/or a wavelength in
the infrared spectrum, such as in the above-mentioned ranges.

In some embodiments radiation in the second phase may comprise one or more wavelength in the range 400-550 nm such as comprising a wavelength of about 450 nm, about 500 nm, or about 550 nm, and/or ultraviolet part of the electromagnetic spectrum, such as the above mentioned.

5 It is postulated by the present inventor that in the invention, radiation of wavelengths in the visible spectrum, in particular comprising one or more wavelength in the "red" or "orange" part of the spectrum and/or wavelengths in the infrared spectrum may enhance breakdown of cellular material and thus affect the decellularization part of the process of the invention, whereas wavelengths in the "blue", "purple" or "green" part of the visible spectrum and/or wavelengths in the UV spectrum may affect crosslinking of susceptible molecules in the material and thus enhance mechanical strength of the
10 material.

In some embodiments the order of different wavelengths can be different, thus in some embodiments, a first temporal phase of radiation may comprise one or more wavelength in the range from about 400 nm to about 550 nm, e.g. about 450 nm or about 500 nm, and a second temporal phase of radiation may comprise one or more wavelength in the range from about 600 nm to about 750 nm, e.g. about
15 600 or about 650 or about 700 nm.

The time period of the first phase of radiation is preferably in the range from about 12 hours, and more preferably from at least 24 hours or from at least 48 hours, to about two months or to about 45 days or to about one month or to about 30 days or to about 20 days, or to about 14 days or to about 10 days, such as e.g. about 7 days, about 10 days, about 12 days or about 14 days.

20 The time period of the second phase of radiation is preferably in a range such as any of the above listed ranges and may be substantially the same time length as the first phase or of different time length.

It is preferred and beneficial to maintain in the body of water or solution circulation of the water or solution, preferably substantially continuous circulation. Thus, in preferred embodiments the process includes a step of flowing water (or aqueous solution) into the body of water and allowing water/solution
25 to drain off from the body, preferably substantially continuously throughout the process. This is preferably tap water but in case an aqueous solution (e.g. water with dissolved minerals etc.) is used in the body of water, then typically the same solution (compositionally) would be used for circulation. For simplicity, whenever the term "body of water" is used herein it may refer to pure water such as clean tap water or water from other source such as taken from natural aquifer or other natural source, or an
30 aqueous solution, e.g. water with dissolved minerals etc., such as but not limited to sea water or similar mixed solutions. Note that "circulation" as used herein does not necessitate *re*-circulation such that the same water or solution is circulated back to the body of water or solution. Circulation is found to inhibit or substantially eliminate microalgal growth (slime) on the organism. The body of water may be maintained at a suitable temperature such as in the range of about 5-15°C, e.g. a range from about 5°
35 or from about 6° or from about 8°, to about 15° or to about 12° or to about 10°C.

The process preferably includes bubbling gas or a gas mixture into the body of water, preferably substantially directed towards said organism or portion(s) arranged in the body of water and may preferably be from the bottom of the vessel/container containing the body of water. In one embodiment

the gas or gas mixture is air. The bubbling of gas through the body of water aids keeping the organism or portion upright and typically creates continuous movement of the organism in the body of water, which ensures more even distribution of the emission of radiation on to the organism. Thus, in some embodiments the bubbling is provided by air nozzles arranged in the bottom or near the bottom of the vessel.

The seaweed or lichen material may be pre-processed, such as but not limited to rinsed, washed or frozen, and accordingly the process of the invention may in some embodiments comprise a pre-treating step of freezing and defrosting the seaweed or lichen material prior to arranging it in said body of water and irradiating.

As mentioned, the disclosure provides substantially decellularized scaffold matrix from an organism selected from seaweed or lichen, which can advantageously be obtained with the process of the invention. The substantially decellularized scaffold matrix comprises a native extracellular structure originating from at least a portion of said organism, wherein said structure has undergone a decellularization process as described herein. As mentioned above the decellularization process preferably removes substantially all cellular structure (i.e. intracellular structure) of the organism, e.g. as determined by microscopical/ histological analysis, but in some embodiments a substantial portion of major organelles and microscopic internal structures of the cells are no longer present. Preferably, however, at least a portion of advantageous small molecule chemical components from the extracellular matrix and cells remain in the scaffold structure, such as oligosaccharides, alginates, smaller saccharides, and cellulose.

The term decellularized as used herein referring to the decellularized matrix material of the disclosure can mean that the matrix comprises less than 10% of the original cell count of the portion of said organism, preferably less than 5% of the original cell count, more preferably less than 1% of the original cell count. "Cell count" as used herein refers to cells being visible in microscope and with substantially intact cellular walls. However, preferably, the remaining cells, or at least a substantial portion thereof such as at least 75% or least 90% or at least 95% or at least 99% or substantially all, are cell remnants, i.e. cells without a nucleus.

It will be appreciated that the decellularized matrix of the invention is biocompatible, meaning that it is not harmful or toxic to living tissue and can be implanted into a human or animal. Accordingly, the matrix is suitable and preferably intended for use as a scaffold for medical use such as regenerative medicine, for example but not limited to internal or external tissue engineering, wound dressing, or agent delivery. The term agent as used herein is meant to encompass any type of drug or bioactive compound.

The decellularized matrix of the invention is preferably from brown seaweed, such as in particular brown seaweed from any of the above-mentioned genera and species. In other useful embodiments the matrix is from red seaweed or green seaweed. In other embodiments the matrix is from lichen, such as but not limited to any of the above-mentioned lichen and preferably lichen of the species *Cetraria islandica*.

The decellularized scaffold matrix of the invention is preferably comprised of porous material, with improved mechanical strength compared to seaweed portion or tissue of the organism being used (i.e.

which has not been treated according to the invention). In some embodiments, the increased mechanical strength of the decellularized scaffold matrix of the invention can be quantified as at least 50% higher ultimate tensile strength (UTS) and preferably at least 100% higher ultimate tensile strength and yet more preferable at least 150% higher ultimate tensile strength.

- 5 In some embodiments, the decellularized scaffold matrix of the invention has ultimate tensile strength (UTS) in the range of about 5 MPa or from about 8 MPa, to about 20 MPa or to about 18 MPa or to about 16 MPa or to about 12 MPa. In some embodiments the decellularized scaffold matrix is from *Laminaria Digitata* and has a UTS in the range from 10-20 MPa such as in the range 12-18 MPa. In some embodiments the decellularized cellulose matrix is from *Laminaria Saccharina* and has a UTS in the range from 6-15 MPa such as in the range 8-12 MPa.

- 10 Mechanical strength of the decellularized scaffold matrix can also be indicated by its suture pull strength or knot-pull tensile strength which indicates the resistance of the material being torn by a needle or suture extending through the material, where the material preferably has a higher suture pull strength knot-pull tensile strength compared to the corresponding untreated material, such as preferably by at least 50% or at least 100% or at least 150% higher.

- 15 It is an advantage of the invention that the decellularized scaffold matrix has a suitable porosity for use as a biological scaffold. In some embodiments the matrix has an average pore size (average diameter) in the range 75-250 μm , such as in the range from about 75 μm or from about 100 μm or from about 120 μm , to about 250 μm or to about 200 μm or to about 180 μm . This may however depend on the seaweed or lichen species selected. Thus, a matrix obtained from *Laminaria Digitata* may in useful embodiments exhibit pore size in the mentioned range while a matrix made from *Laminaria Saccharina* may in some embodiments have a pore size with an average diameter in the range from about 25 to about 100 μm , such as from about 25 μm or from about 30 μm or from about 35 μm , to about 100 μm or to about 85 μm or to about 75 μm or to about 65 μm .

- 25 As mentioned, this disclosure provides a biocompatible decellularized extracellular matrix material, suitable for use as a biocompatible exogenous scaffold, for use in medical treatment, where said extracellular matrix material comprises an extracellular structure originating from an organism selected from seaweed or lichen. which is preferably and advantageously a decellularized scaffold matrix according to the invention, wherein said matrix structure has undergone a decellularization process to remove substantially all cellular structure of the organism, as discussed above. Preferably the decellularization process is the herein defined and disclosed process of the present disclosure.

- 30 Biocompatibility can be assessed with a variety of methods, many of which are applicable to determine and confirm biocompatibility of the decellularized matrix and exogeneous extracellular matrix of the invention. Accordingly, in preferred embodiments the decellularized extracellular matrix substrate of the invention does not exhibit cytotoxicity, as determined with standard protocols in the art and preferably does not induce inflammatory reaction or scar tissue formation.

In some embodiments of the invention the decellularized matrix and exogeneous extracellular matrix of the invention exhibit biocompatibility as shown with the indirect media extract method (following protocol

according to ISO 10993), where the materials exhibit at least 70% viability of the tested cell culture, preferably at least 80% viability and more preferably at least 90% viability.

The biocompatible exogeneous extracellular matrix, which can be in the form of a scaffold, including a dressing or other matrix form, is in some embodiments suitable and intended for use in regenerative medicine, in internal or external tissue engineering, wound dressing, or agent delivery, such as but not limited to treatment of acute and chronic wounds, drug delivery (agent delivery), tissue regeneration such as nerve regeneration, repair and induction of cartilaginous or bone tissue, for repair and support of soft tissue weakness such as hernia and esophageal perforation.

It is an advantage of the methods presented herein that the source materials undergo mild treatment not involving strong acids or bases, organic solvents or high temperatures. This is believed to result in higher retention of advantageous biomaterials such as proteins, amino acids, and oligosaccharides. Accordingly, in some embodiments the biocompatible scaffold material of the present disclosure has not undergone chemical treatment that includes any of acid treatment at pH below 3.5, alkaline treatment at pH above 9.5, and organic solvent treatment.

15

BRIEF DESCRIPTION OF FIGURES

Figure 1 shows a device for producing the matrix material of the invention.

Figure 2 shows an alternative device for producing the matrix material of the invention.

Figure 3 shows another device for producing the matrix material of the invention.

20 Figure 4 shows another device for producing the matrix material of the invention.

Figure 5 shows results comparing three different protocols with red, blue or red and blue light emitted on seaweed specimen, all starting with red light.

Figure 6 shows results comparing three different protocols with red, blue or red and blue light emitted on seaweed specimen, all starting with blue light.

25 Figure 7 shows results comparing three different protocols with red, blue or red and blue light emitted on seaweed specimen, all starting with mixed red and blue light.

Figure 8 shows SEM images seaweed (panel A) and matrix scaffold (panel B) noting the internal chamber microstructure, of the *L. digitata* (LD) and *L. saccharina* (LS) and the difference of the seaweed vs scaffold made therefrom.

30 Figure 9 shows SEM images of LD seaweed (panel A) and matrix scaffold (panel B) made therefrom.

Figure 10 shows SEM images of LS seaweed (panel A) and matrix scaffold (panel B) made therefrom.

Figure 11 shows average thickness seaweeds and scaffolds (Sc.) before and after rehydration.

Figure 12 shows thickness swelling ratio of the seaweed and scaffolds before and after rehydration

Figure 13 shows weight increase of scaffolds of the invention, from LD and LS following rehydration.

Figure 14 shows measured UTS values for seaweed (LD and LS) and for scaffolds made therefrom.

Figure 15 shows measured Youngs modulus values for seaweed (LD and LS) and for scaffolds made therefrom.

- 5 Figure 16 shows stress vs strain curves for the LD (upper panel) and LS (lower panel) seaweed and scaffolds in the highest UTS outliers of all samples.

Figure 17 shows results from cytotoxicity study (see Example 7). Columns show reduction in Alamar Blue (AB) in cell culture with media extracts from the LS and LD scaffolds.

- 10 Figure 18 shows the percentage amount of amino acids in LD and LS seaweed and scaffold dry samples.

Figure 19 shows healing rate of LD and LS scaffolds used for full thickness treatment in rat model.

Figure 20 shows the results of histological evaluation at day 14 from rat wound trail, comparing LD and LS scaffolds to SOC (standard of care) and CAP (commercially available product).

15 DETAILED DESCRIPTION

- The main source of brown seaweed is from kelp forests. Kelp forests are found in temperate to cold nutrient rich oceans spanning across 25% of the world coastline mostly in the polar, subpolar and temperate regions around the globe. Common members of the brown seaweed family include *Laminaria digitata* (*L. digitata*) and *Laminaria saccharina* (*L. saccharina*), which are found in abundance around the Icelandic coastline.

- Both the *L. saccharina* and *L. digitata* seaweed are anchored to the seafloor with a holdfast that extends to a blade. The *L. digitata* short stripe extends to a thick flat blade that bifurcates into finger like projection [18] while the *L. saccharina* short or long stripe connects to undulating blade with bullae without midrib [19]. Cross-sectional microscopic inspection of the brown seaweed blade reveals three layers, the outermost layer or the meristoderm or epidermis, a second layer the cortex, and the medulla at the center. These layers are interconnected via plasmodesma pit fields that are essentially conduits that connect cells from the epidermis to the medulla allowing for transportation of nutrition and cell communication [20]. The cellular components of each layer differs significantly, the epidermis has a layer of compact closely bound cells, the cortex contains larger cells that are more loosely organized while the medulla at the center of the cross section, is composed of disorganized tissue without compartments [21]. The cell walls are composed of at least two layers, the first a layer of microfibrils mostly composed of cellulose providing strength and rigidity, and the second layer composed mostly of alginate that lends flexibility to the structure [22]. The *L. digitata* and *L. saccharina* carbohydrates include alginate, laminarin, cellulose and mannitol that together account for 70.7 ± 11.6 % and 63.1 ± 11.4 % of the seaweeds dry weight respectively [23]. The use of polysaccharides extracted from processed algae for

tissue engineering and regenerative medicine is well known and frequently used due to its biological, physical and chemical properties [24].

Sufficient tensile strength is especially important for scaffolds when the intended application is hernia repair or breast reconstruction. The origin and biophysical profile of the scaffold material contributes to its characteristics and affects the indications for use in clinical settings and other intended use. For instance, permanent synthetic mesh usually have high mechanical strength [29] and are commonly used for hernia repair. However, research shows higher instances of infections and complications when compared with biological scaffolds [29], [30]. Vice versa, biological scaffolds for soft tissue reinforcement have been shown to both support rapid tissue integration and can be used when the tissue has been compromised due to infection but may have less favorable mechanical strength profile and higher risk of hernia recurrence [31]. Though the clinical benefits of biological scaffolds as reinforcement are evident, its use has been limited due to high production cost, immunological sensitivity and cultural/religious concerns due to the origin of the human or animal-derived products [32].

Figure 1 illustrates an example of an apparatus configured for applying the method of the invention, where one or more seaweed pieces (3) have been arranged in a vessel (2) that holds a body of water (1). Light panels (4) with light emitting diodes (LEDs) (5) are shown on opposite sides of the vessel, and the vessel is from a material which is transparent to the radiation emitted. The light panels could also be more than two, to emit radiation from more than two directions; alternatively, if the vessel is elongated (horizontally) series of light panels can be arranged on each side of the vessel, to distribute emission into the vessel along its long sides. Such vessel can house a series of tissue portions, e.g., seaweed pieces. The seaweed or lichen portions are held in place by any suitable means, e.g., by tying to a string, wire or other fixture, or holding with a clamp or clip.

The vessel has a solution inlet (7) and an outlet (10), and water or an aqueous solution (8) are circulated through the vessel, preferably continuously. An air intake (9) is arranged to introduce a gas or gas mixture (typically compressed air) into the body of water, to aerate the water or solution in the vessel. A lid (11) is placed on top of the vessel to close it, to shield from dust etc.

Figure 2 shows an alternative embodiment with a horizontally arranged vessel (2) with a body of water (1). A frame or support line (20) is arranged along the vessel to fix the seaweed (3) onto. A surrounding panel "drum" is arranged around the vessel supporting a plurality of LEDs (5) that emit light (6) towards the vessel. A water intake (7) is on the left end and an outlet (drain) (10) on the right end. A series of air intakes/nozzles (9) is arranged along the bottom of the vessel. The diodes are shown connected to a control unit (12) which can be a PLC computer or PC computer.

Figure 3 shows another embodiment, with a rectangular shaped vessel (2). A frame (22) is arranged in the vessel which is arranged with fixtures for seaweed pieces (3) that are supported vertically by the frame. LED panels (4) in this embodiment are arranged in water-tight enclosures such that they can be inserted into the vessel (see arrows). A plurality of air nozzles (9) is arranged along the bottom of the vessel.

Figure 4 shows another embodiment, with a rectangular shaped vessel (2). A frame (22) is arranged in the vessel which is arranged with fixtures for seaweed pieces (3) that are supported vertically by the frame. Light panels (4) with light emitting diodes (LEDs) (5) that emit light (6). Air feed lines (23) are connected all along to the frame emitting air from nozzles near the bottom of the vessel. This vessel has a solution inlet (7) and a solution outlet (10).

EXAMPLES

Example 1: Sample collection

Samples of brown seaweed of the type *Laminaria digitata* (LD) and *Laminaria saccharina* (LS) were collected from the coastline of Reykjanes in Iceland during summer and autumn. Seaweed samples of *L. saccharina* were also collected in Eyjafjörður of the coast of Hauganes. Samples were cleaned with cool tap water, sorted by hand, and stored in a freezer at -14 to -20°C.

The effect of different lights (wavelengths) and the temporal order of the different lights was tested on the seaweed samples. Each treatment was conducted for 4 weeks, either with continuous emission of the same wavelength or divided in two phases of emission of different wavelength.

The samples were cut into 6 cm x 12 cm pieces and placed into glass tube that was filled with water, with air injected from the bottom as described in previous sections. The seaweed samples are indicated as LD (*Laminaria digitata*) and LS (*Laminaria saccharina*).

Samples following protocol I received 4 weeks of red light, samples with protocol G received 2 weeks red light followed by 2 weeks of mixed red and blue light, samples following protocol J received 2 weeks of red light and 2 weeks blue light.

Accordingly, in this test, phase one was red light (about 630 nm) for 2 weeks and phase 2 was either blue (about 450 nm), mixed red and blue (R&B), or (continued) red light. Here the most effective decellularization method for LS is G, which however is the least effective for LD. The most effective decellularization for LD is the method J, which is only slightly less effective for LS than for LD, and slightly less effective than method G for LS. The results are shown in FIG. 5.

The decellularization (cell removal success) was evaluated by evaluating the number of cells in histological slides stained with H&E (hematoxylin and eosin). The slides were evaluated under a light microscope, each slide evaluated at 6 different sites, by counting the number of cells or cell debris observed in the sited. If no cells or cell debris were seen, the site was given a score 0. If on the other hand the site had cells or cell debris the site was scored up to the maximum score of 4, according to the following criteria:

Score scale

- 0: No cells or cell debris
- 1: Some cell debris but less than 50 cell remnants
- 2: Obvious cell debris but no cells with nucleus, more than 50 cell remnants

- 3: Few cells with nucleus (less than 50)
- 4: Many cells with nucleus (more than 50)

5 The average score of the six sites for each slide was calculated and then calculated as percentage of cell removal success:

$$\text{Cell removal success} = ((\text{max score} - \text{average evaluation score}) / \text{max score}) \times 100$$

10 LED light panels were used that could emit red light (630 ± 20 nm), blue light (450 ± 20 nm) or both. The experiment was run in a windowless room, thus not affected by daylight and only very limited room light for setup and periodical observation.

Example 2:

15 Samples collected and pretreated as described above. Three more light sequences (protocols) were compared, all starting with blue light:

- C: Blue light 4 weeks
- E: Blue light 2 weeks followed by red light 2 weeks
- D: Blue light 2 weeks followed by mixed red-blue light 2 weeks

20 Setup was otherwise the same as in Example 1.

Results are shown in FIG. 6, showing that sequence D gave the best results for LS species whereas sequence E gave the best results for species LD.

25 Example 3:

Three more light sequences were compared, all starting with mixed red-blue light:

- H : R&B light 4 weeks
- K : R&B light 2 weeks followed by red light 2 weeks
- 30 A : R&B light 2 weeks followed by blue light 2 weeks

Setup was otherwise the same as in Examples 1 and 2. Results are shown in FIG. 7, showing best results for sequence K for both LD species and LS species (sequence K marginally better than sequence H for LS).

35 Example 4:

SEM analysis – Microstructure

Scanning electron microscopy (SEM) of LD and LS seaweed and scaffolds therefrom shows chambers of the internal structure separated by cell wall. This distinct structure becomes more evident when viewing the scaffolds were smaller compartments within the bigger partitions become evident, see Figure 8.

- 5 FIG. 8 shows SEM images in planar view of the seaweed and scaffolds therefrom, showing compartmentation of the internal structure separated by cell wall. Scanning electron microscopy (SEM) reveals differences in the microstructure of the LD and LS seaweed and scaffold. Overview of Surface Chamber Diameter (SCD), Cross-Sectional Pore Diameter (CSPD), the surface chamber area (SCA) and the Surface Chamber Wall Thickness (SCWT) is found in Table 1. The average SCD for the LD
10 seaweed and scaffold was 6.33 ± 1.25 vs 8.77 ± 1.80 μm respectively. For the LS seaweed and its corresponding scaffold, the SCD was 10.29 ± 1.72 vs 9.45 ± 1.75 μm respectively. The average SCA of the LD seaweed and scaffold was 22.4 ± 9.72 μm and 42.68 ± 11.15 μm respectively, revealing increased chamber area of the scaffold compared to the seaweed. For the LS the average SCA of the seaweed and scaffold was 76.9 ± 12.9 μm vs 36.73 ± 11.03 μm respectively, or increased chamber
15 area of the seaweed in comparison to the scaffold.

- FIG. 9 shows SEM images, cross-sectional and planar view, of *Laminaria digitata* (LD) seaweed (panel A) and scaffold therefrom (panel B) made according to the invention. The cross-sectional images show the scaffold as highly porous in comparison to the structure of the unprocessed seaweed. The LD seaweed has a compact center with Cross-Sectional Pore Diameter (CSPD) of 52.77 ± 19.03 μm ,
20 mainly found in the outer layers. The scaffold on the other hand has evenly distributed pores from the outer layer into the center, average CSPD 149.25 ± 83.5 μm .

FIG. 10 shows SEM images, cross-sectional and planar view, of *Laminaria saccharina* (LS) seaweed (panel A) and scaffold therefrom (panel B) made according to the invention.

- The LS seaweed cross-sectional image shows a more compact overall structure than the scaffold. The
25 average LS seaweed CSPD was 42.25 ± 30.97 μm , and the CSPD of the scaffold was 46.18 ± 15.83 μm . Pores in the LS specimen were found both at the center and in the outer layers of both the seaweed and scaffold.

The determined average of Surface Chamber Diameter (SCD), CSPD, the surface chamber area (SCD) and the Surface Chamber Wall Thickness (SCWT) is found in Table 1.

- 30 Table 1: The mean value of microstructures of the seaweed and scaffolds, *L. digitata* and *L. saccharina*.

	LD (μm)		LS (μm)	
	Seaweed	Scaffold	Seaweed	Scaffold
Surface chamber diameter (SCD)	6.33 ± 1.25	8.77 ± 1.80	10.29 ± 1.72	9.45 ± 1.75
Surface chamber area (SCA)	22.40 ± 9.72	42.68 ± 11.15	76.9 ± 12.90	36.73 ± 11.03
Surface chamber wall thickness (SCWT)	NA	2.09 ± 0.45	NA	2.15 ± 0.48
Cross-section pore diameter (CSPD)	52.77 ± 19.03	149.25 ± 83.5	42.25 ± 30.97	46.18 ± 15.83

The average SCD for the LD seaweed and scaffold was 6.33 ± 1.25 vs 8.77 ± 1.8 μm respectively. While for the LS the seaweed and scaffold, the SCD was 10.29 ± 1.72 vs 9.45 ± 1.75 μm respectively or substantially the same. The average SCA of the LD seaweed and scaffold was 22.4 ± 9.72 μm vs 42.68 ± 11.15 μm respectively, revealing increased SCA of the scaffold in comparison to the seaweed.

5 While for the LS the average SCA of the seaweed and scaffold was 76.9 ± 12.9 μm vs 36.73 ± 11.03 μm respectively, or increased area of the seaweed SCA in comparison to the scaffold.

Example 5: Absorption abilities of matrix material

This experiment tests absorption abilities of two different types of brown seaweed scaffold, from *L. digitata* (LD) *L. saccharina* (LS) to see if the material can aid in exudate control during wound management. Thickness and swelling rate of processed (scaffold) vs natural (control) samples from LD and LS were compared. Freeze dried samples of processed (scaffolds MMdx-9) and natural version of LD and LS seaweeds were compared by direct thickness measurements at dry state vs wet state. Wet state was established by 2h soaking in excess volume of 0.9% saline (Baxter) at room temperature. Thickness measurements were performed on six samples (n=6) of each type of variant. Both processed and natural samples were measured by caliper (Micrometer Gauge) for thickness. Measurements were done on the same spot of the sample prior and after soaking. Each measurement was done 3 times on the same spot and the median number used as the thickness number for the sample being tested.

Results

Thickness measurements of freeze-dried seaweed and scaffold showed no difference of thickness for LD, were both the seaweed and the scaffold measured at 0.2 mm. However, LS scaffold was measured 30% thinner on average than the seaweed (0.08 vs 0.1 mm). Following rehydration, the thickness of the biomaterial grows with noticeable changes in the thickness of the seaweed and its scaffold versions.

Before rehydration, the calculated average thickness of lyophilized LD seaweed and scaffold was the same or 0.2 mm. This was not the case for lyophilized LS seaweed and scaffold. The LS scaffold was on average 30% thinner than the seaweed, 0.08 mm vs 0.1 mm respectively. However following rehydration of the seaweed, up to fourfold thickness increase was noted in both seaweed types. However, observed thickness increase for the scaffolds of both types was much less. For the LD scaffold thickness increase after rehydration was 50%, and 30% increase for the LS scaffold.

Table 2: Absorption of scaffolds. Saline absorption is given as percentage of increase in mass (mg) over 24 hours

	L.D (μm)		L.S (μm)	
	Seaweed	Scaffold	Seaweed	Scaffold
Lyophilized Thickness (mm)	0.16 ± 0.08	0.17 ± 0.05	0.12 ± 0.03	0.08 ± 0.02
Rehydrated Thickness (mm)	0.88 ± 0.12	0.30 ± 0.02	0.37 ± 0.12	0.13 ± 0.03
*Thickness ratio	450%	76%	208%	63%
**Absorption ratio	NA	$1050.7 \pm 103.6\%$	NA	$1494.3 \pm 96.5\%$
**Water as % of total weight after soaking	NA	94%	NA	91%

Figure 11 shows the average thickness of seaweeds and scaffolds made therefrom according to the invention, before rehydration (that is, thickness of lyophilized sample) and after rehydration. Solid columns show dry thickness and checkered columns show wet thickness. Scaffolds are indicated as "Sc".

- 5 The swelling ratio of the seaweeds shows increases upto fourfold and twofold for LD and LS seaweed types respectively, while there was a less increase in scaffold thickness following rehydration, or 76% and 63% for LD and LS respectively. (Fig. 12)

.Absorption test for *L. digitata* (LD) and *L. saccharina* (LS) scaffolds shows a steady weight increase over the first 5 hours with slight drop in measured weight after 24 hours. The LS scaffold consistently absorbs
10 more saline than the LD scaffold, reaching 1494.3 % and 1050.7 % of its initial weight, respectively, after 24 hours (FIG. 13).

FIG. 13 shows weight increase of scaffolds of the invention, from *L. digitata* and *L. saccharina*. following rehydration for upto 24 hours. Values on the x axis refer to number of hours soaked in 0.9% saline solution (NaCl).

- 15 The *L. digitata* scaffold weight increased by 1051% over 24 hours and the *L. saccharina* scaffold by 1494%, see Table 2. A critical aspect of tissue healing is the maintenance of an optimal moisture level. The medical community has widely recognized that maintaining appropriate hydration levels leads to accelerated healing, diminished discomfort, and enhanced cosmetic outcomes [15]. The high hydrophilicity property of the seaweed scaffold is demonstrated in FIG. 13 showing more than 10-fold
20 increase in weight after 5 hours of soaking in 0.9% saline solution. (Solid columns represent LD specimen and striped columns LS specimen.)

- Both types of processed seaweed from LD and LS (version MMdx-9) showed well over 1000% absorption rate after 5h soaking in saline. A small drop in absorption was noticed after 24h. Comparison of swelling or thickness gain after soaking in saline showed that the processed versions gain less
25 thickness than the natural seaweed, but still can absorb over 90% weight gain in fluid. The less swelling of the processed samples is beneficial for wound management where the material can absorb over 90% of fluid compared to its own weight without becoming too bulky in the wound area.

Example 6: Mechanical properties

- The mechanical properties of LD and LS seaweed and scaffolds were evaluated by measuring the
30 tensile strength using tensile testing machine (UniVert, Cellscale). Additionally, the elastic modulus (E, Young's modulus) was calculated from the stress vs strain curve obtained from the tensile testing. Lyophilized seaweed and scaffolds were soaked for 2 hours prior to the tests. Samples were then cut into dog bone shapes according to ASTM standards D638-14. All samples were stored in plastic bags to keep them moist for the duration of the testing session. Each sample was marked with a black pen
35 to indicate the testing area. Samples that did not break within the testing area were excluded from the final data set. Tensile testing was conducted at a displacement rate of 7 mm/min using 20-kgf load cell.

The stress-strain diagrams were obtained to identify the Young's modulus (E) and the ultimate tensile strength (σ_{uts}). The Young's Modulus was determined from the initial linear portion of the stress-strain curve, where the material behaves elastically. The following equations were used for the calculations and construction of the stress-strain diagrams:

$$\sigma_{uts} = F_{max}/A_0 \quad (3)$$

where F_{max} is the force at breaking (N) and A_0 is the initial cross-sectional area (mm²). The strain (ϵ) is defined according to:

$$\epsilon = \delta/L \quad (4)$$

where δ is the elongation (mm) and L is the initial length (mm)

$$E = \sigma/\epsilon \quad (5)$$

where σ is the stress (MPa) and ϵ the strain (mm/mm).

The result from the tensile test shows higher average ultimate tensile strength (UTS) for both types of scaffolds LD and LS when compared with the seaweed.

Figure 14 shows the measured UTS values for seaweed (LD and LS) and for scaffolds made therefrom according to the invention. Solid columns represent seaweed specimens, striped columns the corresponding scaffolds.

Figure 15 shows the Young's modulus for each group, showing similar Young's modulus for the LD seaweed and its corresponding scaffold of 8.7 vs 9.84 MPa, respectively. Notably, the LS scaffold demonstrated the highest Young's modulus among groups tested, at 16.6 ± 8.3 MPa.

Figure 16 shows stress vs strain curves for the LD (upper panel) and LS (lower panel) seaweed and scaffolds in the highest UTS outliers of all samples.

Sufficient tensile strength, flexibility and elasticity is important when applying medical devices onto wounds. The device should not rip during application, conform to underlying structures and maintain its structural integrity under stress.

Table 2 shows comparison of the ultimate tensile strength (UTS), Young's modulus and strain between seaweed, and scaffold. All samples were tested wet.

Table 2

	Sample	σ_{uts} (MPa)	Young's Modulus (MPa)	Strain at σ_{uts}
LD	Seaweed	11.22 ± 3.79	8.66 ± 3.44	0.65 ± 0.09

	Scaffold	16.02 ± 4.37	9.84 ± 2.99	0.6 ± 0.06
LS	Seaweed	6.42 ± 0.68	8.79 ± 4.57	0.65 ± 0.09
	Scaffold	9.64 ± 3.25	16.58 ± 8.29	0.44 ± 0.07

- All samples initially showed little resistance to stress and absorbed the applied energy. The elastic region that follows is linear for all samples; however, the upward slope of the curve for the scaffolds was steeper. This demonstrates greater resistance to deformation, indicating that the scaffold material is stiffer compared to the seaweed. The considerable difference in tensile strength between the L.D seaweed and scaffold - 11.22 vs 16.02 MPa - may be due to differences in composition, microstructure, and cross-linking caused by the decellularization process resulting in stiffer material. Similar trends were observed in the L.S samples, where the tensile strengths of the seaweed and scaffold were 6.42 MPa, and 9.64 MPa, respectively.
- The strain at UTS reflects the L.D and L.S ability to deform before failure demonstrating ductility and flexibility. For the L.D group the seaweeds strain was slightly higher than the scaffolds 0.65 vs 0.60, respectively indicating that the decellularization process increases the stiffness of the scaffolds. However, for the L.S group the difference between seaweed and scaffolds was more pronounced, 0.65 vs 0.44 indicating that following decellularization the scaffold becomes less flexible and more resistant to deformation. The decrease in strain at UTS for both types of scaffolds corresponds with the observed increases in their Young's Modulus values. Specifically, the LS scaffold exhibited a significant increase in stiffness, 8.79 to 16.58 MPa, supporting the observed reduced deformation property before failure. For clinical application L.D scaffolds could provide strength and flexibility in situations where the required biomaterial should be able to deform without failure within given parameters. The L.S scaffolds provide greater stiffness and reduced ductility and would be suitable for structural support where minimal deformation is ideal.

Example 7: Biocompatibility – Indirect Cytotoxicity

- The cytotoxicity for both seaweed scaffolds *L. digitata* (LD) and *L. saccharina* (LS), was determined by the indirect media extract method, applied to keratinocyte cells cultured in cell-culture dishes. Relative change of Alamar Blue (AB) fluorescence signal, directly reflects the metabolic activity of the cell culture, and was evaluated after 24, 48 and 72 h incubation with 30% and 100% media extract concentration.

- AB is a cell viability assay reagent which contains the cell permeable, non-toxic, and weakly fluorescent blue indicator dye resazurin. Resazurin is used as an oxidation-reduction (REDOX) indicator that undergoes colorimetric change in response to cellular metabolic reduction.

Keratinocytes are the main skin cell type constituting 90% of the epidermis and playing a critical role in wound healing and skin repair as structural cells communicating with immune cells in the wound healing

process. Considering the importance of keratinocytes in wound healing, in vitro biocompatibility test was conducted using keratinocytes.

The cytotoxicity of the scaffolds was determined by the indirect media extract method (ISO 10993), applied to keratinocytes. Alamar Blue (AB) was added to the media and the reduction of the AB after 4 hours measured at the time points 48 and 72 h. The metabolic activity of the keratinocytes in the culture is measured by relative change of AB fluorescence signal at the time points where the cells were incubated with 30%, 50% and 100% concentrates of media extracts. There were two control groups, a normal group were keratinocytes normal media without extract, and a group that got methanol (known to kill cells). Each group had 3 clones n=3, seeded with 10 000 cells per well, and additional group of 3 clones n=3, seeded with 75.000 cells per well for additional confirmation of none-cytotoxicity. The cell plates are the same throughout the test as AB itself is not cytotoxic. Therefore, all test groups contain the same cells at all timepoints.

After each time point (24h, 48h, 72h) treatment, each plate was examined under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Recorded changes in the morphology of the cells due to cytotoxic effects of the test sample extract, but these records are not used for any quantitative measure of cytotoxicity. Undesirable growth characteristics of control cells can indicate experimental error and can cause rejection of the assay.

Results

The average reduction of Alamar Blue was calculated for the extraction media 100%,50% and 30% as well as normal media and media mixed with methanol for comparison. The results from both 10 000 and 75 000 seeded plates (upper panel vs. lower panel in FIG. 17) indicate that the LD and LS extracts are not cytotoxic.

Results are shown in Figure 17. Columns at each timepoint represent, from left to right: LD 100%, LD 50%, LD 30%, normal control (negative control), methanol control (positive control), LS 50%, LS 30%, and LS 100%. Values show % Alamar Blue reduction.

At the beginning of the test, the AB absorbance measurements show similar cell growth (t0) for all groups with variations in group LD 100% and LS 100%, due to the slightly different levels of cell coverage in each well. The AB reduction was measured above 90% in all subsequent time-points in all groups with 30%, 100% media extract. These results show more than 70% viability of the cell culture, confirming that the scaffolds are non-cytotoxic.

Example 8: chemical composition

Biocompatibility of biological scaffolds is most commonly achieved through chemical methods utilizing bleaching agents, soaking agents or acid and alkaline solutions. Through such chemical processing the scaffolds undergo significant changes in their chemical composition, commonly only leaving a specific part of the scaffold such as isolated cellulose (see e.g. [35]).

For the chemical analysis of the scaffolds of the present invention, approximately 10 g of processed scaffolds and freeze-dried unprocessed seaweed was homogenized by milling to a fine powder under liquid nitrogen using a Retsch ZM200 mill.

For total Protein analysis duplicates of about 0,3 g of each milled sample were analysed using CHN-analysator. The total protein content was determined using the nitrogen-to-protein conversion factor 4.12 as recommended by Biancarosa et al. [36]

For amino acid profiling duplicates of about 10 mg of each milled sample were hydrolysed using 6 M HCl at 110°C for 20 h. The lyophilized hydrolysates were resolubilized in 20 mM HCl and derivatized using the AccQ-Tag-reagent (Waters). The labelled amino acids were analysed using LC/UV/MS and quantified by an external standard.

The results are shown in Table 3 and demonstrate conservation of protein content between unprocessed and fully processed LS and LS species. Total protein is shown as % of dry weight. Values for the indicated amino acids are provided as % of total amino acid content.

For LS there is a measurable increase in the ratio of total protein content. Furthermore all essential Amino acids are also preserved through the processing of the Scaffold, see figure 18.

Table 3

Seaweed Species	Condition	Total Protein (%)	Lys* (%)	Met* (%)	Val* (%)	Ile* (%)	Leu* (%)	Phe* (%)	Thr* (%)	His* (%)
Laminaria	Seaweed	5.4	3.1	1.0	1.0	1.9	2.2	2.5	2.6	0.46
Digitata	Scaffold	5.1	2.0	1.2	1.0	2.7	3.1	2.9	3.4	0.29
Laminaria	Seaweed	3.6	1.2	0.30	2.2	0.9	1.0	1.4	2.6	0.21
Saccharina	Scaffold	6.4	3.0	1.2	1.4	2.7	3.1	3.2	3.6	0.29

Proteins are essential for tissue regeneration and repair, providing the building blocks (amino acids) for cell proliferation, collagen synthesis, and immune response modulation. It is postulated that the increased protein content post-processing of Laminaria Saccharina from 3.6% to 6.4% may enhance the potential of the material to support tissue recovery.

Essential amino acids are critical for wound healing due to their role in cellular processes.

Lysine (Lys): Supports collagen cross-linking and strength in newly formed tissue. Both species show reasonable lysine content, with processed Laminaria Saccharina providing higher levels (3.0%).

Methionine (Met): A precursor for cysteine and sulfur-containing compounds necessary for antioxidant defense and tissue repair. Methionine levels increased post-processing in both species (L. Saccharina: 0.30% → 1.2%; L. Digitata: 1.0% → 1.2%).

Leucine (Leu) and Isoleucine (Ile): Promote tissue regeneration by stimulating protein synthesis and reducing degradation. Both amino acids are enriched in processed samples.

Threonine (Thr): Vital for glycoprotein synthesis, which is essential for extracellular matrix and wound repair. Its levels increased in processed samples.

- 5 The Processing method improves the concentration of several key amino acids in Laminaria Saccharina, making it more suitable for applications where enhanced nutritional support is needed for tissue recovery.

Polysaccharide assessment of Cellulose and Alginates

- 10 For Cellulose assessment the non-soluble polysaccharide is hydrolysed and then measured as percentage ratio of cellulose to the total dry starting material. The cellulose ratio is preserved throughout the processing as shown in Table 4.

- 15 For Alginate assessment, duplicates of approximately 1 g of the milled samples were washed by 20 ml ethanol and leached by 50 ml MQ-water at 90° C for 20 h. The water fraction was used for further analysis while the solid fraction was further extracted using 50 ml acid water (pH 1.47, HCl) at 100° C for 2 h. The acidic water fraction was dialysed (MwCO = 1 kDa) against water and lyophilized. A portion each of the water fractions and resolubilized solids after lyophilisation of the acid fractions were first hydrolysed by addition of H₂SO₄(100° C, 10 min). The released uronic acids were then estimated using 0.125 % carbazol in ethanol followed by measuring absorbance at 530 nm. The uronic acids were quantified using GlcA as external standard and corrected for degree of hydrolysis using alginic acid from brown algae as reference. The alginate content is retained in the scaffold version, especially in LD (Table 4).
- 20

Table 4. Cellulose and alginates content

Seaweed Species	Condition	Cellulose (% of dry weight)	Alginates (% of dry weight)
Laminaria Digitata	Seaweed	34	8.5
	Scaffold	31	6.4
Laminaria Saccharina	Seaweed	26	6.3
	Scaffold	46	2.8

Element assessment, Metals and Halogens

- 25 Metals: Duplicates of the milled samples (0.5 g) were digested in microwave oven using 4 ml HNO₃, 2 ml, H₂O₂ and 2 ml MQ-water. The digested samples were further diluted in water before analysis. Part of the sample solutions were analysed using ICP-MS for determination of levels of B, Al, Cr, As, Cd and Pb. Another part of the same solutions were used for determination of Fe, Sr, Zn, Ca, Mg, K and Na using ICP-OES. External standards were used for quantification of each element.

Mercury: About 0.1 g of milled sample was used for determination of Hg using DMA-80 Mercury analyser.

Iodine: The milled samples were leached using approximately 0.15 g sample in 10 ml MQ-water and 2 ml of 25 % tetramethyl ammonium hydroxide. Digested samples were filtered through 0.45µm filters and diluted to 50 g before analysis by ICP-MS. The iodine was quantified using an external standard.

Chloride: Duplicates of about 0.1 g of each milled sample was digested by combustion, resolubilized and analysed using C-IC. The chloride contents was determined using an external standard.

The processing step reduces metal and halogen impurities as shown in Table 5 below.

Table 5. Metal and Halogen content

Seaweed Species	Condition	*Elements (% of dry weight)
Laminaria Digitata	Seaweed	7.2
	Scaffold	4.8
Laminaria Saccharina	Seaweed	8.6
	Scaffold	5.1

*Metals and Halogens

Example 9: Rat model full thickness wound study

Biological scaffolds derived from the brown seaweed Laminaria Digitata (LD) and Laminaria Saccharina (LS) were tested as a treatment for full-thickness wounds in a rat model. The study examined various aspects of wound healing, including thickness of neo-tissue vs uninjured tissue, scar index assessment, clinical (using digital software ImageJ) and histological assessment of the wound re-epithelialization, granulation, inflammatory response, re-vascularization, fibroblast infiltration, and foreign body reactions. The study evaluated the effectiveness of LD and LS scaffolds compared to standard moist wound care and a commercially available cellulose wound product.

Four full-thickness excisional skin defects (diameter 8 mm) were created using a dermal cutter on the back/dorsum of 24 young male Sprague Dawley rats in the weight range of 250-300 gr.

The wounds were splintered with a silicone ring that was secured in place with sutures and a tissue glue to allow for increased granularization and epithelialization rather than contraction as per published results of Galiano et al rodent wound mode [37].

The wound treatments with LD and LS scaffolds, commercially available (cellulose) product (CAP), and standard of care (SOC) were administered randomly into the wounds. The secondary dressing for the LD, LS and CAP treated wounds was Tegaderm film. SOC wounds were only covered with Tegaderm film. The animals were monitored daily for wellness and possible complications.

All wounds were photographed while the animals were under general anesthesia (isoflurane) and subsequently, the software ImageJ used to measure the wound area.

The animals were sacrificed on days 7, 11 and 14. Histological samples were collected following euthanasia, using a biopsy pen to remove the wound and place it into 4% formalin. The samples were processed for histology with hematoxylin/eosin staining for evaluation under light microscopy. All evaluations and measurements were conducted blindly.

Results

The graph in Figure 19 shows the rate of wound area closing for all test groups. The LS scaffold achieves the largest reduction in wound area at all timepoints, followed by SOC and LS. The CAP group has the slowest rate of wound area closure.

The re-epithelialization score (0=none, 1=partial and 2=complete) is favorable for the seaweed scaffolds with a score for LS=1.8, LD=1.75, SOC 1.5 and CAP 1.25 (see Figure 20 lower left panel). Similarly Neo-tissue thickness is higher for LS (1.52) and LD (1.73) compared to CAP and SOC (1.46 and 1.34) (Figure 20 lower right panel).

Calculated Normal Scar Elevation Index (SEI) is a thickness between 95-105% for uninjured skin. The results for both LD (99.32 ± 8.51) and LS (100.33 ± 14.57) groups fall within the normal range. The CAP and SOC group values (87.29 ± 19.35 , 80.71 ± 14.45) indicate hypoplasia (<95%), see Figure 20, upper panel.

Throughout the study, LD and LS groups maintained low inflammatory responses and minimal foreign body reactions. On day 7, LD showed slightly lower inflammation reaction (higher score= less reaction) (2.67) than LS (2.50), but by day 14, both groups exhibited similar inflammatory scores. The minimal foreign body reaction and favorable healing outcomes demonstrate good biocompatibility of the seaweed scaffolds as a medical device, aligning with prior in vitro cytotoxicity assessments that confirmed their non-toxic nature per ISO 10993-5 and 10993-12 standards.

This study demonstrates that decellularized brown seaweed scaffolds derived from *Laminaria digitata* and *Laminaria saccharina* effectively enhance wound healing in a full-thickness rat model. The LD and LS scaffolds promoted faster wound closure, superior re-epithelialization, and normal scar formation compared to a commercial product and standard care. These findings suggest that seaweed scaffolds are a viable alternative to traditional collagen-based dressings, offering benefits such as biocompatibility and alignment with cultural or ethical considerations regarding animal-derived products.

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CLAIMS

1. A biocompatible scaffold material, comprising decellularized seaweed or lichen, wherein the decellularized seaweed or lichen comprises a complex substantially intact extracellular matrix.
- 5 2. The biocompatible scaffold material according to claim 1, wherein the biocompatible scaffold material substantially retains extracellular matrix substances including proteins and cellulose.
3. The scaffold material according to claim 1 or 2, wherein the biocompatible scaffold material has substantially same content of total protein as the source seaweed material.
- 10 4. The scaffold material according to any of claims 1 to 3, wherein the scaffold material is in the form selected from at least one of a wound dressing, bandage, particularized form, suture material, and mesh material.
- 15 5. The scaffold material according to any of the preceding claims, wherein the scaffold material further comprises one or more added active or non-active agents.
6. The scaffold material according to any of the preceding claims, wherein the added active agent is selected from the group consisting of antibiotics, antiseptics, antimicrobial agents, antivirals, antifungals, antiparasitics, anti-inflammatory agents, antioxidants, drugs, proteins, peptides, and combinations thereof.
- 20 7. The biocompatible scaffold material according to any of the preceding claims, comprising seaweed or lichen that has been treated with electromagnetic radiation, in a body of water or aqueous solution with continuous flow.
- 25 8. The biocompatible scaffold material according to any of any of the preceding claims, which has not undergone chemical treatment that includes any of acid treatment at pH below 3.5, alkaline treatment at pH above 9.5, and organic solvent treatment.
- 30 9. The biocompatible scaffold material according to any of any of the preceding claims, for use in medical treatment selected from regenerative medicine including tissue regeneration such as nerve regeneration, internal or external tissue engineering including repair and induction of cartilaginous or bone tissue, as wound dressing, for agent delivery, treatment of acute and chronic wounds, and repair and support of soft tissue weakness such as hernia and esophageal perforation.
- 35 10. A process for producing a substantially decellularized biocompatible scaffold material from an organism selected from seaweed or lichen, said process comprising steps of:

- 5
- a. removing said organism or portion thereof from its natural or cultivated habitat and arranging said organism or portion of said organism in a body of water or aqueous solution;
- b. emitting electromagnetic radiation portion in the ultraviolet, visible and/or infrared part of the electromagnetic spectrum on said organism or portion thereof over a period of time for decellularization of said organism or portion to form a decellularized scaffold material; and
- c. retrieving said decellularized cellulose matrix from said body of water or aqueous solution.
- 10
11. The process according to claim 10, wherein said organism is *Cetraria islandica* lichen.
12. The process according to claim 10, wherein said organism is selected from brown seaweed, red seaweed and green seaweed.
- 15
13. The process according to the preceding claim, wherein said organism is brown seaweed of genus *Laminaria*, preferably of species *Laminaria Digitata* or *Laminaria Saccharina*.
- 20
14. The process according to any of claims 10 to 13, wherein said organism or portion thereof has not undergone chemical treatment that includes any of acid treatment at pH below 3.5, alkaline treatment at pH above 9.5, and organic solvent treatment
- 25
15. The process according to any of claims 10 to 14, wherein said electromagnetic radiation is emitted from one or more light-emitting diodes (LEDs).
16. The process according to any of claims 10 to 15, wherein said electromagnetic radiation is emitted continuously on said portion of said organism.
- 30
17. The process according to any of claims 10 to 16, wherein said period of time is at least 12 hours, at least 24 hours, at least 48 hours, a week, a fortnight, a month or two months.
- 35
18. The process according to any of claims 10 to 17, wherein said step of emitting electromagnetic radiation comprises at least two temporal phases of emitting electromagnetic radiation, wherein a first phase comprises emitting electromagnetic radiation comprising at least one first wavelength for a first period of time and a subsequent second phase comprises emitting electromagnetic radiation comprising at least one second wavelength for a second period of time, wherein said first and second wavelengths are different.
- 40
19. The process according to the preceding claim, wherein said electromagnetic radiation emitted in the first phase comprises red light having a wavelength in the range 600-750 nm or wavelength in the infrared part of the electromagnetic spectrum.

20. The process according to claim 18, wherein said electromagnetic radiation emitted in the second phase comprises blue light having a wavelength in the range from about 400 to about 550 nm or wavelength in the ultraviolet part of the electromagnetic spectrum.
- 5
21. The process according to claim 18, wherein said first period of time is in the range from 12 hours to 2 months, preferably in the range from 24 hours to two weeks.
22. The process according to claim 18, wherein said second period of time is in the range from 12 hours to 2 months, preferably in the range from 24 hours to two weeks.
- 10
23. The process according to any of claims 10 to 22, wherein said process further comprises the step of continuously flowing water or said aqueous solution into and out of said body of water or aqueous solution.
- 15
24. The process according to any of claims 10 to 23, wherein said process further comprises the step of continuously bubbling gas or gas mixture into said body of water, preferably substantially directed towards said organism or portion thereof arranged in said body of water.
- 20
25. The process according to the preceding claim, wherein said gas or gas mixture is air.
26. A substantially decellularized substantially intact extracellular matrix from an organism selected from seaweed or lichen, said matrix comprising:
- 25
- a. an extracellular structure originating from at least a portion of said organism, wherein said extracellular structure has undergone a decellularization process to remove substantially all intracellular structure of the organism.
27. The decellularized extracellular matrix according to the preceding claim, wherein said matrix has not undergone chemical treatment that includes any of: acid treatment at pH below 3.5, alkaline treatment at pH above 9.5, and organic solvent treatment.
- 30
28. The decellularized extracellular matrix according to claim 26 or 27, substantially retains extracellular matrix substances including proteins, alginates, saccharides, and cellulose.
- 35
29. The decellularized extracellular matrix according to any of claim 26 to 28, wherein the biocompatible scaffold material has substantially same content of total protein as the source seaweed material.
- 40
30. The decellularized extracellular matrix according to any of claim 26 to 29, wherein said decellularized extracellular matrix is biocompatible.

31. The decellularized extracellular matrix according to any of claim 26 to 30, for medical use such as regenerative medicine, preferably as a matrix scaffold.
- 5 32. The decellularized extracellular matrix according to any of claim 30 to 31, wherein said biocompatible decellularized matrix is suitable for use in internal or external tissue engineering, wound dressing, or agent delivery.
- 10 33. The decellularized extracellular matrix according to any of claims 26 to 32, wherein said organism is brown seaweed.
- 15 34. The decellularized extracellular matrix according to the preceding claim, wherein said organism is brown seaweed of genus *Laminaria*, preferably of the species *Laminaria Digitata* or *Laminaria Saccharina*.
35. The decellularized extracellular matrix according to any of claims 26 to 32, wherein said organism is lichen preferably of species *Cetraria islandica*.
- 20 36. The decellularized extracellular matrix according to any of claims 26-35, wherein said decellularized cellulose matrix comprises less than 10% of the original cell count of the portion of said organism, preferably less than 5% of the original cell count, more preferably less than 1% of the original cell count.
- 25 37. The decellularized extracellular matrix according to the preceding claim, wherein remaining cells are cell remnants, i.e., cells without a nucleus.
- 30 38. The decellularized extracellular matrix according to any of claims 26-37, wherein said decellularized cellulose matrix is porous material comprising improved mechanical strength compared to untreated organism.