



(51) International Patent Classification:

C07K 14/78 (2006.01) **C07K 1/00** (2006.01)
A61K 38/39 (2006.01)

(21) International Application Number:

PCT/IN2016/000211

(22) International Filing Date:

18 August 2016 (18.08.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

201631001353 14 January 2016 (14.01.2016) IN

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(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, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, 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, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, 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, LI, LU, LV, MC, 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).

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

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

— with amended claims and statement (Art. 19(1))

(54) Title: A PROCESS FOR EXTRACTION OF COLLAGEN FROM FISH SCALE AND POLYELECTROLYTE BASED BIO-ACTIVE SUPER-ABSORBENT MATERIALS

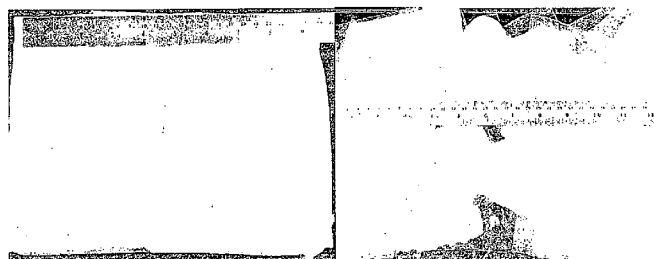


Figure 1

(57) Abstract: Polyelectrolyte based bioactive super-absorbent material ionotropically crosslinked/neutralized involving polyvalent carboxylic acids, citrate, Kojic Acid, Alpha Arbutin along with fish scale collagen cross linked with other polyelectrolyte biopolymers preferably selected from chitosan, alginate or their combinations is used in this invention. The advancement is also directed to process of extraction of collagen of high purity from fresh water fish scale by salt and alkaline washing, crushing followed by continuous dialysis thereby minimizing the chance of collagen degradation. Also disclosed are different forms of collagen-chitosan composite biomaterial using citrate as the neutralizing buffer in combination with antimicrobial agent, antioxidant, skin plumper and melanin reducer wherein the different forms of collagen chitosan particularly sheet, flakes, powder, gel, particles, fiber, film, spray etc. reveal efficient wound healing properties. The advancement is thus directed to find wide application in various dermal wound healing, tissue engineering, 3D cell culture and expansion, cosmetics and different other health care applications.



TITLE: A PROCESS FOR EXTRACTION OF COLLAGEN FROM FISH SCALE AND POLYELECTROLYTE BASED BIOACTIVE SUPER-ABSORBENT MATERIALS

5 FIELD OF THE INVENTION

The present invention relates to a process for extraction of collagen from fish scale and to polyelectrolyte based bioactive super-absorbent material. More specifically, the present invention provides the process for extraction of collagen of high purity from fish scale and the manufacture
10 of polyelectrolyte based bioactive super-absorbent material such as chitosan-collagen based composite biomaterial. Advantageously, the polyelectrolyte based bioactive super-absorbent material of the advancement is found to have efficient wound healing property and thus finds beneficial application in dermal wound dressing and other health
15 care applications like tissue engineering scaffolds, drug delivery system, cell delivery vehicles and transient 3D matrix for cell expansion.

BACKGROUND ART

Collagen is the most abundant protein representing nearly 30% of total
20 proteins in the animal body. It is the major component of extracellular matrices and is vital for mechanical protection of tissues, organs, and physiological regulation of cellular environment. Collagen and collagen based forms in combination with other synthetic, semi-synthetic and biopolymers have broad applications in wound healing, tissue
25 engineering, regenerative medicine moisturiser, food industry, laboratory reagents, health drink, etc.

Aquatic source of collagen is of great importance worldwide. Marine source of Type I collagen are fishes such as codfish [K.A. Piez, *Biochemistry*, 1965 Dec;4(12):2590-2596], salmon, skin of Big eye
30 snapper, Large fin long barbel catfish, Seaweed pipefish, Brown backed toadfish, Ocellated puffer fish, Squid, Cuttlefish, scales of Lizard fish, Horse mackerel, Grey mullet, Flying fish, Yellowback seabream etc. [T.H.

Silva, et.al. Mar. Drugs 2014, 12, 5881-5901], or invertebrate marine animals, such as marine sponges, star fish, octopus or jellyfish [T. Nagai, et.al., J Sci Food Agric 79:855-858;1999]. The marine sources contains higher amount of minerals and therefore, removing of them is costly, time consuming and cumbersome and usually ethylenediaminetetraacetic acid (EDTA) is used for this. However, remnant EDTA in collagen to be used for medical devices is very harmful for skeletal tissues and can cause to decay of bones.

Further, the denaturation temperature (T_d) of collagen from marine source is low. The denaturation temperature (T_d) of edible jelly fish collagen is 26.0 °C [T. Nagai, J Sci Food Agric 79:855-858, 1999] and that of starfish is 24.7 °C [B. Wang, Mar. Drugs 2013, 11, 4641-4661].

The mineral content in fresh water fish scale is relatively less. Moreover, this source is free from the fat or turbid matter. Fish scale is abundant and easily available, particularly in Indian subcontinent, East and South-east Asia because fresh water fish is a food for a large number of populations of these regions. The scales and other external part of fish, considered to be biological wastes, are rich sources of collagen and the extraction procedure of collagen from these sources is simple, less time consuming and could be extracted using less chemical treatments. This collagen of fresh water origin has relatively higher denaturation temperature (T_d) mainly due to high content of hydroxyproline associated with nature of their habitat.

It is well known that the extraction of collagen from fish scale usually comprises either of the enzymatic method/ hot water extraction method/ methanol/ EDTA or a combination of them.

CN 103773828 discloses method for extraction of collagen from fish scale by conducting crushing, hot water and enzymatic treatment using 1-3% of protease assisted by ultrasonic wave. Without acid-base pre-treatment, the method provided has no pollution to the environment can achieve good separation and purification effect, and does not affect the biological activity of collagen.

CN 1814782, mentions an enzyme engineering technology including taking scales as the main raw material, drying and crushing them to be sieved, mixed with water, enzymolyzed with proteinases then to eliminate;enzymes and filter them to getthe solution to be concentrate
5 d, dried to get the productand extracting small molecular collagens from the scales by a biological enzyme cutting technology where hydrolysis is carried out at 35-70 °C for 3 to 12 hours followed by heating reaction liquid at 90 °C for constant temperature enzyme inactivation.

10 **CN 103570827** discloses preparation methods of fish scale collagen protein comprising four methods: an acid method, alkali/ salt method, an enzymatic method and a hot-water, extraction method. It describes that use of ultrasonic wave improves extraction of collagen from fish scale.

US20070231878 discloses a similar method of protein hydrolysis using
15 enzyme and treating in warm water to extract collagen of fish scale. This is followed by centrifugation of the hydrolyte; taking out the supernatant of the hydrolyte rice; and drying the supernatant to become collagen powder.

US20070231878 (pg 3 para 0047) discloses extraction of collagen using
20 acetic acid, and the method disclosed produces 90 % pure collagen.

Pati et al. has reported extraction of collagen from fresh water fish scales of Rahu and Katla with higher thermal stability and ~5% yield by EDTA based dissolution [F. Pati, B. Adhikari, S. Dhara, Isolation and characterization of fish scale collagen of higher thermal stability,
25 Bioresource Technology, 101, 3737-3742, 2010]. However, the collagen yield was found not of desired level and was limited to up to 5% of the scales by EDTA treatment.

CN 1888075 describes a method of extracting collagen from fish scale comprising of steps of defatting fish scale with mixture solution of
30 methanol and distilled water, desalting with 0.075 mol/L concentration EDTA solution, enzymolysis with pepsin, centrifuging and spray drying to obtain crude product.

CN 1332981 discloses extraction method of collagen by stirring the scale with 14-fold warm water in a water vessel for 1 hour and this process is repeated.

CN 104004086 A also discloses pulsed ultrasound assisted hot water treatment where the temperature of the water is 40-80 °C and treatment time is 20-70 minutes.

Importantly, the concept of the modern development of dressing has undergone a fundamental change and now its function is not limited to cover the wound bed only but it should also promote tissue repair functions, provide habitat for the new organization to accelerate wound healing, reduce scarring and prevent bacterial contamination, and with its biodegradability, reduced the number of dressing change.

Chitosan, an amino-polysaccharide having resemblance with glycosaminoglycan, is not present in living bodies and apparently a foreign body to living bodies. A method of improving biocompatibility of chitosan by chemically modification or by blending with bioactive derivative is necessary. Composite biomaterial consisting of chitosan and collagen for artificial skin, artificial blood vessel, wound-coating material, or adhesion preventive agent has been studied by many researchers (JP Patent Publication (Kokai) Nos. 56-133344 A (1981) and 63-59706 A (1988)).

Alginate, a polysaccharide based polymer known for wound healing applications, could also be combined with chitosan, different organic acids and/ or collagen to form hydrogel, superabsorbent materials for wound healing, external wound dressing and as cell delivery vehicles. In particular, hyaluronic acid, alginate, cellulose, chitosan and, to a lesser extent other polysaccharides, received attention for used for the development of several biomedical applications such as tissue engineering and controlled release of drugs and pharmaceutical proteins.

CN 1167471 describes the low cost biological composite dressing comprising of non-woven fabrics, chitosan, collagen I, and calcium alginate to be used in wound healing, has good biocompatibility,

permeability, elasticity, flexibility and used as drug carriers, dosing dressing, improved for clinical use.

CN 1381274 discloses a composite biologic adhesive bandage composed of pressure-sensitive adhesive hydro-entangled cloth, hygroscopic pad, non-woven cloth, chitosan, collagen type I and medicine preparation. Its advantages are high penetrability, elasticity, flexibility, bioactivity, biodegradability, and cost-effectiveness.

EP0200574 discloses a biologically compatible material comprising composite materials of N-succinyl chitosan and collagen for preparing wound dressing materials, vascular pro-artificial skins and hemostatic agents.

EP 1115432 relates to the fabrication of dermal scaffold and bioartificial dermis using neutralized chitosan sponge, neutralized chitosan/collagen mixed sponge or neutralized chitosan/collagen mixed sponge containing chitosan fabrics is mentioned and these are extremely useful for wound healing therapy.

However, there is a need for a simpler, environmentally friendly process for extraction of collagen of high purity with minimum chemical treatment which makes the extracted collagen free from toxic chemicals and cost effective. In another aspect it is also necessary to work on advancements related to polyelectrolyte based bioactive super-absorbent having efficient wound healing property.

OBJECTS OF THE INVENTION

It is thus the primary object of the present invention to provide a process for extraction of collagen of high purity from biological wastes of different fresh water fish with minimum chemical treatment.

Another object of the present invention is to provide a cost effective and environmentally friendly process of collagen extraction by two step alkali

and crushing treatment thereby minimizing the chance of collagen degradation due to chemical and /or hot water treatment.

Yet another object of the present invention is to provide collagen obtained following the above process with yield exceeding 22% total fish scale weight basis and high purity in the range of 92-95%.

A further object of the present invention is to provide polyelectrolyte based super-absorbent having efficient wound healing property and its process for manufacturing.

Yet another object of the present invention is to provide polyelectrolyte based super-absorbent having efficient wound healing property and its process for manufacture involving ionic liquids with possible interpenetrating network (IPN) based gel with significant ion-pair mediated crosslinking.

According to another aspect the present advancement is directed to the process for preparing polyelectrolyte complex including chitosan-collagen, chitosan-collagen-citrate or chitosan-collagen-alginate, chitosan-collagen-alginate-citrate or chitosan-collagen-polymaleate etc. based composite biomaterial based super-absorbent with or without antimicrobial (iodinated form of unsaturated acid/aldehyde/amine like maleic acid, fumaric acid, di-aldehyde etc.), antioxidant (Kojic Acid), depigmenting agent (Alpha Arbutin) having efficient wound healing property and its process for manufacture.

It is still another object of the present invention is to provide for polyelectrolyte based composite biomaterials in various forms including particles, powder, flake, bead, sheet, films, fibres, spray, gel, composite based contact lenses and delivery vehicle and methods of their manufacturing.

SUMMARY OF THE INVENTION

Thus, according to the basic aspect of the present invention there is provided a process for extraction of collagen from fish scale comprising the steps of:

- i) washing and softening the fish scale;
- ii) subjecting the thus softened fish scale to crushing and smashing to achieve punching and tearing of the scales;
- iii) treating the crushed and smashed fish scale with acetic acid such as to solubilize protein molecules;
- iv) carrying out filtration of the acetic acid treated crushed fish scale and thus separating the solution of soluble and the residue therefrom;
- v) subjecting the solution obtained to sodium chloride salt treatment followed by filtration to again generate solution and residue therefrom;
- vi) subjecting the residue thus obtained to acetic acid treatment; followed by
- vii) carrying out dialysis of solution for purification; and finally
- viii) obtaining therefrom purified collagen.

Another aspect of the present invention provides a process wherein the residue in step iv) also having some collagen content is subjected to:

- i) crushing and smashing;
- ii) treating with acetic acid and pepsin;
- iii) carrying out filtration to generate a solution of soluble and residue;
- iv) recycling the solution thus obtained to carry out said steps v) to viii) and the residue to carry out said steps ii) to viii).

In another aspect, the present invention provides a process wherein said step of dialysis comprises continuous dialysis process.

In yet another aspect, the present invention provides a process comprising continuous dialysis process preferably carried out involving hollow fibre membrane and dialyzer of desired molecular cut off and controlling inlet and outlet pressure at different points whereby different protein present in the solution can be separated according to their molecular weights.

A further aspect of the present invention provides a process comprising continuous dialysis process wherein said inlet pressure of the hollow fibre membrane input (collagen containing solution) is 300 to 1000 mbar and outlet pressure of the same is at 100 to 600 mbar, inlet pressure of the dialysate (water) input is maintained at 1-200 mbar below the outlet pressure of the hollow fiber membrane with continuous flow of dialysate.

In a further aspect, the present invention relates to a process wherein in the step (i) fish scales are washed with salt solution selected from NaCl or KCl, followed by softening of the scales by treating with alkali selected from NaOH or KOH, at a temperature below 15°C, preferably 4-8 °C for 24-48 h under stirring with shaking speed of 60-100 rpm.

In yet another aspect, the present invention relates to a process wherein the crushing and smashing such as to decalcify/ demineralise the scale for subsequent treatment at a low temperature up to 20 °C preferably at 6-15 °C

In yet another aspect, the present invention relates to a process wherein the crushed /smashed fish scale is treated with acetic acid of 0.3 to 0.5 M at temperature 6 to 15 °C by stirring with shaking speed of 60-100 rpm.

Another aspect of the present invention relates to a process wherein the hollow membrane dialyzer has molecular cut off 3-80 kDa and the dialysis is continued till the pH of the solution is 5.5-6.5.

In another aspect, the present invention provides collagen obtained following the above process with yield exceeding 22% total fish scale weight basis and high purity in the range of 92-95%.

A further aspect of the present invention relates to a process for the manufacture of a polyelectrolyte based bioactive super-absorbent material comprising:

- i) providing a solution of at least one polyelectrolyte and treating with acetic acid;
- ii) selectively neutralizing the polyelectrolyte solution using citrate buffer with i) selective pH controlling ionic liquid based neutralizing/buffer system with or without polyelectrolyte under continuous stirring till

involving selective control of desired gelation for the formation of the crosslinked polyelectrolyte based bioactive super-absorbent including step of controlled gelation selectively for (a) slow gelation maintaining pH in the range of pK_1 to pK_2 (in the range of 3.13 to 4.7 for citrate) and (b) 5 instantaneous gelation maintaining pH in the range of pK_2 or above (in the range of 4.76 or higher up to 12 especially for citrate buffer).

Yet another aspect of the present invention relates to a process for the manufacture of a polyelectrolyte based bioactive super-absorbent material wherein said ionic liquid include unsaturated acids including 10 organic acids containing more than one pK_a values selected from citric, oxalic, fumaric, ascorbic, lactic, pyruvic, succinic, lipid, maleic, malonic, glucaric, succinic, adipic, pamelic, subaric acid, Kojic Acid, Alpha Arbutin having several pK_a values and with varied pH for desired selective used in the form of ionic liquid for neutralizing and forming ion-pairs mediated 15 cross-linked material selected from hydrogel, film, particles, beads, fibers, powder, flakes, sponge, sheet, particle suspension spray, in-situ gelation during spraying;

Said polyelectrolytes including polymers preferably are selected from polyvinyl alcohol, silk alginate, chitosan, polyfumaric acid, poly maleic 20 acid etc and other synthetic biopolymers and semi-synthetic polymers.

A further aspect of the present invention relates to a process for the manufacture of a polyelectrolyte based bioactive super-absorbent material wherein for forming interpenetrating network (IPN) based gel with significant ion-pair mediated crosslinking both the neutralizing 25 solutions/buffer contains polyelectrolyte, said ion-pair providing a highly interconnected porous template facilitating anyone or more of protein adsorption, growth factor adhesion and cell adhesion.

Another aspect of the present invention provides a process for the manufacture of a polyelectrolyte based bioactive super-absorbent 30 material wherein said cross linked polyelectrolyte based bioactive super-absorbent is obtained including covalent or ionic cross linking preferably including in situ iodination during crosslinking for generating iodinated crosslinkined polymer dressing system.

In yet another aspect of the present invention is related to a process for the manufacture of a chitosan based bioactive super-absorbent material comprising:

5 i) providing a solution of at least one of (a) chitosan and (b) a mix of chitosan-collagen in the weight ratio of 20 : 0.25–10 wt % and treating with acetic acid;

10 ii) treating the solution of i) above with polycarboxylic acid based system under continuous stirring till involving selective control of desired gelation for the formation of the chitosan based bioactive super-absorbent including step of controlled gelation selectively for (a) slow gelation maintaining pH in the range of pK_1 to pK_2 (3.13 to 4.7 for citrate) and (b) instantaneous gelation maintaining pH within the range of pK_2 or above (in the range of 4.76 or higher up to 12 for citrate).

15 Another aspect of the present invention relates to a process for the manufacture of a chitosan based bioactive super-absorbent material comprising:

20 i) providing a mix of chitosan-collagen in the weight ratio of 20 : 0.25–10 wt % by adding 0.1 to 1 M acetic acid to the final concentration of the solution 5 to 10 wt% depending upon the molecular weight of chitosan used;

ii) neutralizing the solution by addition of citrate buffer in the range of 1 to 10 wt% under continuous stirring to thereby reduce the charge density of chitosan gradually until the solution was completely neutralized.

25 In a further aspect of the present invention is provided for a process for the manufacture of a chitosan based bioactive super-absorbent material for producing selectively anyone or more of chitosan-collagen particles, powder, flakes and beads comprising:

30 (i) carrying out said neutralization of chitosan-collagen solution by mixing with citrate buffer under continuous stirring maintaining pH range of citrate buffer more than 3.13 preferably at 7.4;

(ii) casting the neutralized solution;

(iii) storing the thus cast solution in freezer at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs; followed by;

(iv) lyophilization at $<-30^{\circ}$ under vacuum $1 \times 10^{-1} - 5 \times 10^{-2}$ mbar or below until the beads or freezing solution has dried substantially or completely;

(v) crushing the dried mix to obtain therefrom selectively anyone or more of bioactive super-absorbent chitosan-collagen particles, powder, flakes and beads.

A further aspect of the present invention relates to a process for the manufacture of a chitosan based bioactive super-absorbent material for producing selectively anyone or more of chitosan-collagen particles, powder, flakes and beads comprising:

(i) carrying out said neutralization of chitosan-collagen solution by drop wise addition of citrate buffer under continuous stirring maintaining pH range of citrate buffer in the range of 4.76–10 preferably at 7.4 to thereby obtain neutralized beads;

(ii) storing the thus neutralize beads in freezer at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs; followed by;

(iv) lyophilization at $<-30^{\circ}$ under vacuum $1 \times 10^{-1} - 5 \times 10^{-2}$ mbar or below until the beads or freezing solution has dried substantially or completely optionally involving temperatures of $40-80^{\circ}\text{C}$

(v) obtaining from the dried beads selectively anyone or more of bioactive super-absorbent chitosan-collagen particles, powder, flakes and beads.

In another aspect, the present invention relates to a process for the manufacture of a chitosan based bioactive super-absorbent material wherein said dried beads directly or following crushing, sieved in meshes of varied mesh sizes to thereby obtain selectively anyone or more of bioactive super-absorbent chitosan-collagen particles, powder, flakes and beads.

A further aspect of the present invention relates to a process for the manufacture of a chitosan based bioactive super-absorbent material for producing chitosan-collagen sheets comprising:

- i) providing a solution of at least one of (a) chitosan and (b) a mix of chitosan-collagen in the weight ratio of 20 : 0.25-10 wt %;
- ii) neutralizing the solution by mixing said chitosan or chitosan-collagen solution with citrate buffer under continuous stirring till the solution was completely neutralized to different extent;
- (ii) casting the neutralized solution;
- (iii) storing the thus cast solution in freezer at -20 °C to -80 °C preferably at -80 °C for 10 to 24 hrs preferably at 12 hrs; followed by;
- (iv) providing neutralized collagen solution;
- (v) pouring the neutralized collagen solution on top of the said frozen chitosan solution or said frozen chitosan-collagen to provide a collagen layer followed by
- (vi) freezing 20 °C to -80 °C and preferably at -80 °C for 10 to 24 hrs preferably at 12 hrs and lyophilization at <-30 °C under vacuum 1×10^{-1} – 5×10^{-2} mbar or below into sponge;
- (vii) pressing the sponge to obtain sheets therefrom.

- In another aspect , the present invention relates to a process wherein the said step of casting was carried out in a tray preferably a Teflon coated tray and stored in freezer at -20 °C for 12 hrs; and opposite to the collagen layer of said sheet there is provided a non-adhesive mesh either by pressing or involving cellulose based adhesive to provide support to the sheet, form a non-sticky barrier and retain the shape of the sheet.

In a further aspect of the present invention is related to a process for the manufacture of a chitosan based bioactive super-absorbent material for producing chitosan-collagen sheets comprising:

- i) providing neutralized chitosan solution;
- ii) mixing the neutralized chitosan solution with alginate-citrate buffer to form gel;
- (iii) casting the gel in tray;
- 5 (iv) storing the thus cast solution in freezer at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs; followed by;
- (v) providing neutralized collagen solution;
- (vi) pouring the neutralized collagen solution on top of the said frozen gel to provide a collagen layer; followed by
- 10 (vii) lyophilization at $<-30^{\circ}\text{C}$ under vacuum $1 \times 10^{-1} - 5 \times 10^{-2}$ mbar or below into sponge.

In a still further aspect, the present invention relates to a process for the manufacture of a chitosan based bioactive super-absorbent material for producing chitosan-collagen films comprising:

- 15 (i) carrying out said neutralization to different extent of chitosan-collagen solution with citrate buffer under continuous stirring maintaining pH range of citrate buffer in the range of 3.13 to 4.76 maintaining pH of neutralized solution in the range of 5-7;
- (ii) casting the neutralized solution and drying under sterilized condition
- 20 preferably under air flow until solution dried completely to form film.

In another aspect, the present invention relates to a process for the manufacture of a chitosan based bioactive super-absorbent material for producing chitosan-collagen fibers comprising:

- preparing said chitosan-collagen solution by adding 0.1–1.0 M acetic acid
- 25 using 0.1–1.0 M acetic acid to the solution of 0.5–5 wt%; filtering the solution and
- wet spinning the filtrate in polycarboxylic acid bath at varied pH with pKa values higher than at least two carboxylated group ionization (above pKa_2) to thereby obtain the chitosan-collagen fibers.

Yet another aspect of the present invention relates to a process wherein the filtrate was wet spun in a citric acid bath with pH between 4.76 and 10 preferably at 7.4; followed by washing the fibers and drying.

5 In a further aspect of the present invention is related to a polyelectrolyte based bioactive super-absorbent material comprising: polyelectrolyte based bioactive super-absorbent which is ionotropically crosslinked/neutralized involving polyvalent carboxylic acids.

10 In yet another aspect, the present invention relates to a polyelectrolyte based bioactive super-absorbent material comprising collagen-biopolymer which is ionotropically crosslinked/neutralized involving polyvalent carboxylic acids.

15 In a further aspect, the present invention relates to a polyelectrolyte based bioactive super-absorbent material comprising variable pH based neutralized and formed ion pair mediated cross linked products including selectively anyone or more of dressing system including iodinated crosslinkined polymer as antimicrobial agent, antioxidant along with Kojic Acid and Alpha Arbutin as skin plumper and melanin reducer in the form of beads, sheet, Film and fiber blanket.

20 Yet another aspect of the present invention provides a polyelectrolyte based bioactive super-absorbent material comprising fish scale collagen based wound healing/dressing including partial, full thickness, diabetic, chronic, traumatic, ulcer, exudating wounds, bone cartilage healing, cell/drug/different bioactive molecules cytokines, kemokines delivery vehicles, scaffold for tissue engineering, 3D cell expansion media, 25 collagen delivery in dry eye, collagen based health drink and food, intra ocular lens, contact lens, oral patch, ingredients for capsules, mineral delivery, moisturizer, ointments, facial creams.

30 A further aspect of the present invention provides a polyelectrolyte based bioactive super-absorbent material comprising fish scale collagen cross linked with other polyelectrolyte biopolymers preferably selected from chitosan and alginate.

A still further aspect of the present invention relates to a polyelectrolyte based bioactive super-absorbent material which is in-situ gelleable and cross linked on wound site.

5 DETAILED DESCRIPTION OF THE INVENTION

As discussed herein before the present advancement provides a simple process for collagen extraction from fish scales without using toxic chemicals. Moreover the present advancement also provides a process for manufacture of collagen-chitosan composite biomaterial in various forms
10 which is useful in wound dressing and wound healing.

The process of the present advancement replaces a number of steps of the existing processes by two steps comprising alkali treatment followed by crushing/smashing making the process simple, fast, economic, and minimizes the chances of collagen degradation as a result of previously
15 reported chemical and/or hot water treatments.

Advantageously, according to a preferred aspect the advancement involves dialysis of the collagen containing solution with hollow fiber membrane which would effectively replace the batch type membrane based osmosis by enabling a continuous hollow fiber membrane based
20 reverse osmosis. Thus for protein purification, the reverse osmosis based hollow fiber dialysis membrane is utilized via continuous flow based dialysis process where collagen is separated from solution containing collagen, NaCl, Acetic acid and other minor impurities of small molecules and ions by dialysate (water in this process).

25 Importantly, using dialyzer of different molecular cut off and controlling the inlet and outlet pressure of the dialyzer at different points, the different proteins present in the solution can be separated according to their molecular weight. The process as a whole (sequential NaCl, NaOH treatment followed by smashing/crushing and treatment of the
30 smashed/crushed scale by acetic acid and pepsin-acetic acid alternatively) accelerated the dissolution of the collagen molecules from scale. The

complete dissolution of the scale can be achieved in 15–18 weeks. After the first three dissolution steps, the collagen yield increased significantly.

Collagen is an important protein containing 'RGD' moiety for adhesion of cells via surface integrin receptors and can also be utilized for cross-linking mediated entrapment and slow release via ion exchange for different health care applications. Collagen has isoelectric point (IEP = 5.1) like any other proteins and acts as a charged molecule at pH away from IEP.

Chitosan, being a polycataionic amino polysaccharide, has structural resemblance with GAGs having wound healing efficacy, mucoadhesivity, biocompatibility and non-toxicity. Chitosan bio-polymer has various applications in the field of hydrogels formation, tissue engineering, wound dressings and drug delivery systems. Under dissolved state in organic acid like acetic acid, chitosan becomes positively charged through protonation in the amine functional group (pKa ~ 6.5).

Several organic acids (e.g. citric, oxalic, fumaric, lipid, maleic, malic, glucaric, ascorbic, lactic, pyruvic, succinic, subaric, Kojic Acid, Alpha Arbutin etc.) containing polycarboxylic acids are used in formulating ionic liquids and are degradable via metabolic pathway *in vivo*. These acids also have antioxidant properties which are effective in scavenging reactive oxygen species. Different polyelectrolytes (like alginate, chitosan, polyfumaric acid, poly maleic acid) could be neutralized by opposite charge ions at suitable pH range that can be detected via potentiometric or conductometric titration forming ionotropic gelation, neutralization, complexation. If both the neutralizing solutions/buffer contains polyelectrolyte, they also form interpenetrating network (IPN) based gel with significant ion-pair mediated crosslinking. These ion-pair network containing highly interconnected porous template acts as zwitter ions for ultra low fouling activity preventing non-specific protein adsorption and facilitates necessary protein, growth factor and cell adhesion. Ion-pair mediated crosslinked gel is dried under super critical temperature for formation of superabsorbent and the ion-pairs could promote absorption of very high weight percentage of polar solvent medium like water. In fact animal extracellular matrices are also naturally

occurring interpenetrating gel network. IPNs, semi- or full-IPN may also have different types of physical, ionic, covalent interactions depending on nature of the material used.

Different organic acids including mono, di, tri and polycarboxylic acids (like Kojic Acid, Alpha Arbutin, ascorbic, lactic, pyruvic, succinic, citric, oxalic, polymaleic, polyfumaric, tartaric, fumaric, maleic, malic, subaric etc.) have several pKa values. These molecules with varied pH could be efficiently used in the form of ionic liquid for neutralizing and forming ion-pairs mediated cross-linked hydrogel, film, particles, beads, fibers, powder, flakes, sponge, sheet etc. In case of citric acid, citrate has pK_a (pK_{a1} = 3.13, pK_{a2} = 4.76, pK_{a3} = 6.39) values which vary within 3.13 to 6.39. The citric acid solution with pH value within range of 3.13 to 4.76 could be used only for neutralization, whereas pH values ranging from 4.76 to 6.39 and higher than that could be used for neutralization and citrate mediated crosslinking. The hydrogels can also be formed by using combination of two or more electrolytes, molecules types and/or in combination with other types of cross linking like covalent, hydrogen bonding etc.

The details of the invention, its objects and advantages are explained hereunder in greater detail in relation to the following non-limiting accompanying figures and examples.

BRIEF DESCRIPTION OF ACCOMPANYING FIGURES

Figure 1: illustrates optical images of collagen sponge obtained after lyophilisation;

Figure 2: illustrates the flow diagram of collagen extraction from fresh water fish scale;

Figure 3: illustrates electrophoretic patterns of Type I collagen obtained from fish scale of Rahu, Catla, Mrigel and mixed species;

Figure 4: illustrates collagen based different forms; (a) sheet (b) film (c) sponge (d) particles (e) fibers and (f) beads;

Figure 5: illustrates fluorescence micrographs of seeded cells on sponge, Sheet and Film after 48 h;

Figure 6: represents SEM micrographs of seeded cells on a) sponge b) sheet and c) film after 72 h;

Figure 7 illustrates images of wound healing kinetics of control (A), collagen-chitosan;

5 Film (B), and collagen-chitosan Sheet (C) on 0th day and after 5, 10, 15 and 18th days of implantation;

Figure 8: illustrates the wound healing rates of the rats in different groups (control, collagen-chitosan sheet and collagen-chitosan film);

Figure 9: illustrates H&E staining after 14 days of implantation (A) control group (B) collagen-chitosan sheet group and (C) collagen-chitosan film group. [Mag-20x: scale bar denote 100 µm];

Figure 10: illustrates masontrichrome staining after 14 days of implantation (A) control group, (B) collagen-chitosan sheet group compared and (C) collagen-chitosan film group [mag-20x: scale bar denote 100 µm];

Figure 11: illustrates distribution of newly formed blood vessels using a type IV collagen (green) in (A) control group, (B) collagen-chitosan sheet group and (C) collagen-chitosan film group after 14 days post implantation. Arrows denote the formation of newly formed blood vessels. Sections were counterstained with DAPI, which stains nuclei blue. Scale bars represent 200 µm.

Fresh water fish origin of Indian subcontinent, East and South-east Asia (such as Golden Carp, Silver Carp (*Hypophthalmichthys molitrix*), Grass carp (*Ctenopharyngodon idella*), Labeo Bata, Lata, Catla (*Catla catla*), Rahu, Mrigel (*Cirrhinus cirrhosis*), Shol, Talapia (*Oreochromis mossambicus*), Nilontika, Hilsa, Bhetki (*Lates calcarifer*), Balichura (*Psilorhynchus balitora*), Barali (*Barilius barila*), Bata/Bangna (*Labeo ariza*), Bele (*Awaous grammepomus*), Rupchand, Cypinus, Chital (*Chitala chitala*), Foli/Chitol (*Notopterus notopterus*), Chuna (*Trichogaster chuna*), Common carp (*Cyprinus carpio carpio*), Darkina (*Rasbora daniconius*), Gechua (*Channa gachua*, *Channa orientalis*), Gozar (*Channa marulius*), Ilish (*Tenuialosa ilisha*), Kalibaus (*Labeo calbasu*), Nilotica (*Oreochromis niloticus niloticus*), Japanese Pnuti, Koi (*Anabas testudineus*), Putitor mohashoul (*Tor putitora*), Mohashoul (*Tor Tor*), Shorpunti (*Puntius*

sarana), Tapse (*Sarotherodon melanotheron heudelotii*), Mourala etc.) including fish scale are good source of collagen.

- Scales and other biological wastes of Fresh water fish of individual species (Rahu, Katla, Mrigel) and the mix scales of the above mentioned species (henceforth will be referred as mixed species) were collected from fish market of Cotai, East Midnapur, West Bengal, India.

EXAMPLE 1: EXTRACTION OF COLLAGEN TYPE I FROM FISH SCALE

- 10 Fresh water fish scale of 1000 g was taken and washed with water thoroughly. It was again washed with 0.2-0.5M NaCl solution. After washing, the fish scale is kept in a container and soaked in 0.2-1.0 M preferably 0.5M NaOH solution for 24 to 48 h in a BOD incubator shaker with a shaking speed of 60-100 r.p.m and at temperature below 10 °C; 15 preferably at 4 °C.

After soaking, alkali treated scale was washed with distilled water and smashed /crushed in a grinder/crusher while maintaining the temperature below 20 °C by adding ice/ ice water intermittently.

- The smashed/ crushed scale was kept in a container along with 0.5 M 20 acetic acid solution (acid solution: scale = 10-20:1 ratio by weight) for 7 days in a BOD incubator shaker at <10 °C and 60-100 r.p.m. After 7 days, the solution was filtered with nylon/cotton net of porosity 300-1000 µm to recover the scale. The filtered solution (Part I) was collected in a container. The residue (Part II) is also collected in another container.

- 25 In the solution (Part I), NaCl was added pinch by pinch and was gently shaken in a magnetic stirrer at r.p.m. <150. The dissolved collagen precipitated out form the solution continuously. Salt addition process was continued until the concentration of NaCl in the solution became 0.9 M. Then the precipitate was separated from the solution by a specially 30 designed filter system where filtration was carried out in a single step using cloth filter (in the range of 50-500 µm). After filtration, the precipitate was collected and the filtrate was discarded.

The collected precipitate was re-dissolved in 0.3–0.5M acetic acid by gentle stirring. The dissolved solution was dialyzed in a hollow fiber membrane dialyzer (molecular cut off 3 –80 kDa). The inlet pressure of the solution was kept at 300–1000 mbar and outlet pressure was at 100–
5 600 mbar. The inlet pressure of water was kept at 0–200 mbar below the outlet pressure of the solution. The dialysis was continued until the pH of the solution reached in the range of 5.5 to 6.5. The solution obtained after dialysis was frozen at –20 °C for 12 h followed by lyophilizing at <–
10 30°C for 24 to 48 h under vacuum. After freeze drying the purified collagen sponge was collected (Fig. 1).

The collected residue (Part II) was smashed/ crushed in ice-water mixture in a grinder /crusher and the ground/crushed scale was kept in a container. 0.5 M acetic acid (acid solution: scale = 10–20:1 ratio by weight) and 0.1% pepsin were added in the bottle. The bottle containing
15 scale and chemicals was kept under shaking condition for 3 days in a BOD incubator shaker at <10 °C at 60–100 r.p.m. After 3 days, solution and residue were collected separately. Same treatment, as given to part I solution, was applied to this solution and the same procedure was followed to obtain collagen from this solution. The residue was recycled
20 and again smashed/ crushed followed by acetic acid treatment and filtration to obtain part I and part II, as described above. The process of extraction collagen from fish scale is shown schematically in Fig. 2.

Washing in NaCl removes the odour and colour/ pigment from the scale and therefore, high purity collagen isolation is possible by minimizing the
25 filtration which is significantly time consuming. The removal of pigment subsequently helps to reuse the hollow fiber membrane for more number of times.

NaOH treatment makes the scale softer with significant swelling characteristics to facilitate grinding/ crushing without significant increase
30 in the temperature and protein degradation.

This step of grinding/ crushing promotes exposure of collagen into acidic environment during acetic acid treatment which further facilitates easy dissolution of collagen without demineralization of scale.

The hollow fiber membrane was used for dialysis and purification which could be advantageously reused several times (4-10 times) and collagen production rate increased significantly.

Thus, in this method, the minerals were not attempted to be demineralized by EDTA treatment as demineralization by chelating is time consuming and costly affair, which also eliminates the possibility of presence of remnant EDTA (a chelating agent for Ca^{+2} , Fe^{+2} , Fe^{+3} causing demineralization) in the isolated collagen.

Example 2: Characterization of the extracted collagen obtained from Ex 1

i) SDS-PAGE

Electrophoretic patterns of Type I collagen from fish scale of Rahu, Catla, Mrigel and mixed species is shown in Fig. 3. In all the cases (i.e. Rahu, Catla, Mrigel and mix sources) the mobility of α -chains was obtained as expected, based on the molecular weight markers.

The estimated molecular weight for a chain of these collagens, using globular proteins as standards was approximately 120–150 kDa. As in type I collagen from calf skin which was compared with the isolated collagens comprised of at least two different α -chains ($\alpha 1$ and $\alpha 2$) with different mobility, which indicated that the collagens from these two species are of type I collagen.

ii) FTIR analysis

In the FTIR spectra of fish scale collagen of mixed species, the amide I band found is in the range from 1600 cm^{-1} to 1700 cm^{-1} . This is mainly associated with stretching vibrations of the carbonyl groups (C=O bond) along the polypeptide backbone, which is a sensitive marker of the peptide secondary structure. The ratio of absorption intensity between 1240 cm^{-1} (amide III) and 1463 cm^{-1} (amide II) band is approximately equal to 1.0, which confirms the triple helical structure of collagen.

iii) Amino acid analysis by HPLC

The amino acid composition of the collagen isolated from mixed species of fresh water fish scale was expressed as amino acid residues per 1000 total amino acid residues and is shown in Table 1. As collagen is triple helical in nature with characteristic amino acid repeat, (Gly-Pro-Hyp), glycine (Gly) was the most abundant with the amount of 340 of the total amino acid present in collagen extracted from mixed species. The total amount of imino acids per 1000 total amino acid residues, proline (Pro) and hydroxyproline (Hyp), are 120 and 80, respectively, which is likely to have effect on the stability of the collagen fibers and influences the denaturation temperature as well. Absence of cysteine in collagen emphasizes presence of type I collagen. The hydroxyproline content in the collagen isolated from scale of Katla, Rahu, Bata, Mrigel etc. (fresh water fish origin) had marginal variation which was significantly higher than the marine source and thus significantly higher thermal stability.

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Table 1. Amino acid composition of ASC from scale of Mixed species (expressed as residues per 1000 total amino acid residues)

Amino acid	Residues/1000 residues
Aspartic acid/ Asparagine	47
Cysteine	0
Glycine	340
Hydroxyproline	80
Glutamic acid/ Glutamine	77
Alanine	115
Proline	120
Lysine	26
Valine	22
Methionine	14
Isoleucine	10
Leucine	23
Serine	35
Threonine	21

Tyrosine	3
Phenylalanine	13
Tryptophan	0
Histidine	4
Arginine	50

Example 3: Yield percentage of different fish scales

Using this procedure, the mixture of scale from different species yielded collagen up to 22% which is substantially higher than the most of the reported values.

The collagen yield was up to 5% of the scales by EDTA treatment and acetic acid dissolution as discussed in the F. Pati et al without crushing of the scales.

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Example 4: COLLAGEN BASED WOUND DRESSING

Collagen extracted by the Example 1 is used to prepare different forms of products for wound dressing by combining with a polysaccharide such as chitosan, alginate or their combination followed by citrate buffer neutralization. The basic steps comprise:

i) Chitosan-collagen solution at different weight ratio (20 : 0.25–10 wt % on total weight basis) was prepared individually or as a mix by adding 0.1–1 M acetic acid to the final concentration of the solution 0.5–5 wt% depending on the molecular weight of chitosan used.

ii) The solution was further neutralized to a different extent by drop wise addition of 5 wt% citrate buffer (in the range of 1–10 wt%) at varied pH but higher than 3.13 (preferably at pH 7.4) under continuous stirring by mechanical/magnetic stirrer (200–300 r.p.m.) to reduce the charge density of chitosan gradually.

iii) When pH of the solution reached to 7.0 the appearance became white and the solution was completely neutralized.

iv) The neutralized solution was then cast in a tray (preferably in Teflon coated tray) and stored in freezer at -20°C for 12 h followed by

lyophilisation at $<-30\text{ }^{\circ}\text{C}$ under vacuum of $1\times 10^{-1} - 5\times 10^{-2}$ mbar or below until the freeze solution dried substantially.

v) The dried mix was crushed and sieved by nylon/cotton mesh of varied mesh sizes.

- 5 vi) The different forms (particles, powder, flake, bead etc.) obtained after sieving were stored in an air tight bag under sterilized condition at room temperature or below for final applications.

These downstream products like collagen based sheet, film, sponge, particles, fibres and beads are shown in Fig. 4.

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Example 5a: Preparation of chitosan-collagen powder, particles, flakes and beads

- To form beads, citrate buffer (in the range of 1–10 wt%) at pH in the range of 4.76 to 10 (preferable at pH 7.4) is added to the solution of
- 15 Example 4 following under continuous stirring by mechanical means (200–500 r.p.m.) to reduce the charge density of chitosan and form beads. When pH of the solution was closed to 7.0 the appearance of neutralized beads turned into white and looked like precipitate. The beads were collected, washed and dried to a different extent and by different
- 20 means. The neutralized beads were stored in freezer at $-20\text{ }^{\circ}\text{C}$ for 12 h followed by lyophilisation at $<-30\text{ }^{\circ}\text{C}$ under vacuum of $1\times 10^{-1} - 5\times 10^{-2}$ mbar or below until the beads dried substantially or completely. The drying of beads was also attempted under vacuum ($1\times 10^{-1} - 5\times 10^{-2}$ mbar or below) or in a temperature controlled oven (at $40-80\text{ }^{\circ}\text{C}$). Finally
- 25 the beads were either crushed followed by sieving or directly sieving in nylon/cotton mesh of varied mesh sizes and depending upon the mesh size different forms like powder, particles, flakes and beads were obtained.

Example 5b: Preparation of chitosan-collagen sheet

- 30 The differentially neutralized chitosan /chitosan-collagen solution obtained in Example 4 was then cast in a tray (preferably in Teflon coated tray) and stored in freezer at $-20\text{ }^{\circ}\text{C}$ for 12 h.

2.5 wt% collagen solution was prepared separately by adding collagen in 0.3 M acetic acid solution (250 mg collagen in 10 ml acetic acid solution). This solution was neutralized by adjusting the pH with 5 wt% citrate buffer.

- 5 The collagen/neutralized collagen solution was poured on the top of the frozen mix followed by similar freezing and lyophilisation (at $<-30^{\circ}\text{C}$ under vacuum of $1\times 10^{-1} - 5\times 10^{-2}$ mbar or below) into sponge. The sponge was pressed into different extent to form sheet of required thickness. Opposite to collagen layer of the sheet, a non-adhesive mesh
10 (nylon or any other similar materials) was fixed either by pressing or by using cellulose based bio-adhesive in order to provide support to the sheet, form a non-sticky barrier and retain the shape of the sheet.

Optionally, 1–5 wt% alginate solution was prepared with distilled water by shaking in any mechanical means. 5 wt% citrate buffer was added (ratio
15 of alginate solution to citrate buffer was 5–10:1 by volume) drop wise to the solution by continuous mechanical/magnetic stirring (100–300 rpm) to increase charge density of the solution.

The separately prepared neutralized chitosan (by the method of Example 4) solution was mixed with alginate-citrate buffer to form gel. The gel was
20 cast in a tray (preferably in Teflon coated tray) and subsequently frozen in a freezer at -20°C for 12 h. Then the neutralized collagen solution was poured on the top of frozen mix in the tray and lyophilized at $<-30^{\circ}\text{C}$ under vacuum of $1\times 10^{-1} - 5\times 10^{-2}$ mbar or below. Sponge was formed after complete lyophilization.

- 25 The sponge was pressed into different extent to form sheet of required thickness. Opposite to collagen layer of the sheet, a non-adhesive mesh (nylon or any other similar materials) was fixed either by pressing or by using cellulose based bio-adhesive in order to provide support to the sheet, form a non-sticky barrier and retain the shape of the sheet. The
30 sheet was stored in an air tight bag under sterilized condition at room temperature or below for further applications.

Example 5c: Preparation of Chitosan–collagen Film

The neutralized solution of Example 4 was cast in a tray (preferably in metallic (like stainless steel)/glass/polymer based tray) and dried under sterilized condition preferably under air flow until the solution dried completely to form the film. The film was carefully removed and stored
5 under sterilized sealed bag at room temperature or below for further applications.

Example 5d: Preparation of Chitosan-collagen fiber

Chitosan-collagen solution obtained from Step 1 of Example 4 was filtered and the filtrate was wet spun in polycarboxylic acid bath at varied pH with
10 pKa values higher than at least two carboxylated group ionization (above pKa₂). The filtrate was also wet spun in a citric acid bath with pH between 4.76 and 10 preferably at 7.4. The fibers were washed, dried and kept in air sealed bag to store at below 30 °C preferably at 15 °C under sterilized condition for final applications.

Example 5e: Preparation of chitosan-collagen spray

The chitosan-collagen powder prepared in 5a was suspended in aqueous/dilute acetic acid medium and could be sprayed on the wound bed prior to covering by occlusive dressing. The collagen-citrate based solution/suspension could also be sprays on the assaulted area.

Example 5f: Preparation of chitosan-collagen spray-gel

Chitosan-collagen mix (20 : 1-10 wt% on total weight basis) solution using 0.2 mol% acetic acid with pH below 6 along with citric acid (1-10 wt%) solution at pH above 5 were promoted for in-situ gelation during spraying using a special spraying device which facilitated mixing of both
25 the solution into gel at the specific site of interest.

Example 5g: Preparation of collagen based transparent film, curved contact lenses, delivery vehicles

Blending collagen with other polymer like Polyvinyl alcohol, silk or chitosan (20: 1-10 wt% on total weight basis) followed by co-
30 valent/ionotropic crosslinking. Covalent or ionic crosslinking using unsaturated acid/aldehyde/amine etc. also favours in situ iodination during crosslinking using iodine as blend. This iodinated crosslinkined

polymer dressing system is efficient in antimicrobial activity due to slow release of iodine into the wound site.

**Example 6: CHARACTERIZATION OF CHITOSAN-COLLAGEN BASED
5 WOUND DRESSING**

i) Swelling study

Collagen, chitosan-collagen, and chitosan-alginate-collagen samples (in example 5a to 5g) showed a steady state of swelling in 48 h of soaking period without any disintegration or dissolution in the medium and
10 preserved their physical integrity. The sponge and sheet samples showed a rapid swelling in first 10–24 h because of rapid diffusion of water molecules. Then it gradually attained equilibrium in about 36 h and maintained equilibrium swelling state. The sheet showed higher swelling than the other forms and after 24 h it reached to ~ 6000%.

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ii) Degradation study

The enzymatic degradation of raw collagen and collagen based different samples (in example 5a to 5g) were investigated by monitoring the residual mass percent as a function of exposure time to collagenase
20 solution. The collagen samples degraded rapidly compared to the chitosan-collagen samples. Within 24 h, the residual mass reduced to 18.4% of the initial mass for sponge/sheet (example 5b).

iii) Cross-linking density

25 Ninhydrin assay was used to determine the degree of cross-linking of collagen and collagen-chitosan based samples. From this assay, it was evident that the crosslinking density was varied from 6 to 50 % with different extent of neutralization. Chitosan or chitosan-neutralized collagen in combination with citric acid at varied pH ionotropically cross-
30 linked to hydrogel.

iv) MTT Assay

The cytocompatibility of the samples (in example 5a to 5g) was evaluated by MTT assay using primary fibroblast cells isolated from rat skin.

Cell viability of cross-linked samples was evaluated to compare cellular growth kinetics. The rate of proliferation in all the samples like film, sponge and sheet, beads, fibers was much better compared to the control which was tissue culture plate (TCP) in this study. This is due to the bioactivity of the collagen (RGD moiety binds with cell surface integrin receptor) which improved the cell viability and proliferation.

v) Cell Morphology by Fluorescence and Scanning Microscopy

The comparative attachment and proliferation rate of fibroblast cells on samples were observed under a fluorescence microscope after staining with Rhodamine Phalloidin and DAPI staining, at predetermined times.

The morphology of fibroblast cells cultured on collagen sponge, sheet and film was evaluated after 48 hours by rhodamine-phalloidin and DAPI staining (Fig. 5). It is evident that the morphology of the cells cultured samples exhibited stretched morphology and the cells had elongated cytoskeleton indicating they are cytocompatible and nontoxic in nature.

A complete confluent layer of cells on collagen sponge, sheet and film is seen under SEM as shown in figure 6 a, b and c. Polygonal morphology of cells and presence of well spread cytoskeletal filaments were observed on sponge and sheet compared to film due to their 3D morphology. Collagen sponge and sheet supported a greater number of cellular adhesion, cell-cell contacts and proliferation. Compared to the cellular extensions spanned across the surface of sponge and sheet, the cells tried to establish contacts amongst themselves. After 72 hours of culturing on collagen based sponge, sheet and film matrixes, the cells found to be confluent and giving rise to a cell-scaffold construct.

vi) In vivo animal study (Full-thickness cutaneous wound models)

Thirty-six rats (male wistar weighing ~ 220 g) divided into two groups were studied for wound healing under animal ethical clearance using standard protocol. A full-thickness square defect (2 cm x 2 cm) was

created in the upper back area of each rat and then implanted with or without a chitosan-collagen film/sheet. Wounds were wrapped with dressing for protection. Wound closure was measured every other day after wounding. Animals were euthanized after 14 days and tissues were harvested for analysis. All the tissue samples were cut into a full-thickness manner from the wounded sites, and then were used for histological analyses. Photographic images were also captured to understand the healing process. The entire studies were carried out under ethical clearance.

10 **Measurement of wound healing**

The wound healing process after treatment with a collagen-chitosan sheet and film and its comparison with the healing process of the control is shown in Fig. 7. During the first day post-operation, the collagen-chitosan sheet and film efficiently absorbed wound exudates and tightly attached to the wound surface.

After 5 days of post-operation, scabs were observed in both groups (Fig 7). After 10 days post-operation, the scabs were falling off the wounds, and the wounds were mostly filled with restored skin. It is notable that the wounds of the collagen-chitosan sheet and film group were greatly reduced in size as compared to the control which is more evident after 15 days. Complete wound closure was observed in collagen-chitosan sheet treated group after 18 days. The restored skin treated with a collagen-chitosan sheet and film was similar to normal skin, while an elongated scar was still observed in the skin after healing of a control group due to excessive contraction.

The defects treated with collagen-chitosan sheet demonstrated superior healing and the skin defect was reconstructed with a good cosmetic outcome. The rate of wound healing is shown in Fig. 8. The wound area decreased at an accelerated rate for the wounds treated with collagen-chitosan sheet and collagen-chitosan film at the end of 14th day compared to the control. The difference between the control group and the collagen-chitosan sheet/film group showed significant healing compared to the control after 14 days of treatment ($p < 0.05$). From this animal study, the

positive role of collagen and citric acid in accelerating wound healing is evident.

vii) Tensile strength of regenerated skin

Assessment of regenerated skin tissues was done by measuring the mechanical properties of the regenerated tissues.

Analysis of the all biomechanical parameters (mainly ultimate tensile strength and young's modulus) demonstrates that biomechanical property of collagen-chitosan sheet and collagen-chitosan film treated group was better compared to the control group.

viii) Histological and immunofluorescence studies

Histological analyses were performed to assess the regenerated tissues guided by collagen-chitosan based samples (in example 5a to 5g) in rats, and the results are presented in Fig. 9 (H&E staining) and Fig. 10 (MT staining). H&E staining of wound sections after 14 days post-surgery revealed distinct differences in maturity of the epidermis/dermis and quality of granulation tissue between collagen-chitosan sheet/film-group and the control group (Fig. 9). Both collagen-chitosan sheet and collagen-chitosan film-group demonstrated accelerated maturation and a well formed epidermis with compact orthokeratosis, while control group displayed more inflammatory granulation tissue and partially re-epithelialized epidermis with overlying serum crust. Evaluating of collagen deposition in control, collagen-chitosan sheet and collagen-chitosan film is shown in Fig. 10. Collagen-chitosan film treated wounds demonstrated pale but more mature collagen bundles than the controls. In contrast, collagen-chitosan sheet treated wounds displayed well-organized compact collagen bundles that were oriented parallel to the epidermis. Furthermore, trichrome semi-quantitative analysis revealed significantly increased collagen intensity in collagen-chitosan sheet treated wounds compared to all other wounds ($p < 0.05$; Fig. 10).

Type IV collagen antibody staining which stains the blood vessels was used to demonstrate the vascularization of each experimental group after 2 weeks of implantation. From Fig. 11, it can be observed that higher

number of blood vessel/density in the collagen-chitosan sheet/film implanted group than in the control group (Fig. 11A, B, and C). A large number of micro-vessels were observed in the collagen-chitosan sheet and film (Fig. 11B and C) implanted group, and the micro-vessel density was much higher compared with the control group. This finding could be attributed to the presence of bioactive cues in the collagen-chitosan samples along with its porosity improved the activation and facilitated the endothelial cells to form new blood vessels in the wound bed.

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We Claim:

1. A process for extraction of collagen from fish scale comprising the steps of:
 - i) washing and softening the fish scale;
 - 5 ii) subjecting the thus softened fish scale to crushing and smashing to achieve punching and tearing of the scales;
 - iii) treating the crushed and smashed fish scale with acetic acid
 - iv) carrying out filtration of the acetic acid treated crushed fish scale and thus separating the solution of soluble and the residue therefrom;
 - 10 v) subjecting the solution obtained to salt treatment followed by filtration to again generate solution and residue therefrom;
 - vi) subjecting the residue thus obtained to acetic acid treatment; followed by
 - vii) carrying out dialysis based purification; and finally
 - 15 viii) obtaining therefrom purified collagen.
2. A process as claimed in claim 1 wherein the residue in step iv) also having some collagen content is subjected to:
 - i) crushing and smashing;
 - ii) treating with acetic acid and pepsin;
 - 20 iii) carrying out filtration to generate a solution of soluble and residue;
 - iv) recycling the solution thus obtained to carry out said steps v) to viii) and the residue to carry out said steps ii) to viii).
3. A process as claimed in anyone of claims 1 or 2 wherein said step of dialysis comprises continuous dialysis process.
- 25 4. A process as claimed in anyone of claims 1 to 3 comprising continuous dialysis process preferably carried out involving hollow fibre membrane and dialyzer of desired molecular cut off and controlling inlet and outlet

pressure at different points whereby different protein present in the solution can be separated according to their molecular weight.

5. A process as claimed in claim 4 wherein said inlet pressure of hollow fiber membrane input (collagen containing solution) is 300 to 1000 mbar and outlet pressure of the same is at 100 to 600 mbar, inlet pressure of the dialysate (water) input is maintained at 1 to 200 mbar below the outlet pressure of the hollow fiber membrane with continuous flow of dialysate.
6. A process as claimed in claim 1, wherein in the step (i) fish scales are washed with salt solution selected from NaCl or KCl followed by softening of the scales by treating with alkali selected from NaOH or KOH, at a temperature below 15 °C preferably 4-8 °C for 24-48 h with a shaking speed of 60-100 rpm.
7. A process as claimed in claims 1-2, wherein the crushing and smashing is done at a low temperature up to 20 °C preferably 10 °C
8. A process as claimed in claim 1 wherein the crushed /smashed fish scale is treated with acetic acid of 0.3 to 0.5 M at temperature 6 to 10 °C by stirring with shaking speed of 60-100 rpm.
9. A process as claimed in claim 1 wherein the hollow membrane dialyzer has molecular cut off 3-80 KDa and the dialysis step is continued till the pH of the solution is 5.5 to 6.5.
10. Collagen obtained following the process as claimed in anyone of claims 1 to 9 with yield exceeding 22% total fish scale weight basis and high purity in the range of 92-95%.
11. A process for the manufacture of a polyelectrolyte based bioactive super-absorbent material comprising:
- i) providing a solution of at least one polyelectrolyte and treating with acetic acid;
 - ii) selectively neutralizing the polyelectrolyte solution using citrate buffer
- i) with selective pH controlling ionic liquid based neutralizing/buffer system with or without polyelectrolyte under continuous stirring till

involving selective control of desired gelation for the formation of the crosslinked polyelectrolyte based bioactive super-absorbent including step of controlled gelation selectively for (a) slow gelation maintaining pH within the range of pK_1 to pK_2 (in the range of 3.13 to 4.7 for citrate) and
 5 (b) instantaneous gelation maintaining pH within the range of pK_2 or above (in the range of 4.76 or higher up to 12 for citrate).

12. A process for the manufacture of a polyelectrolyte based bioactive super-absorbent material as claimed in claim 11 wherein

said ionic liquid include unsaturated acids including organic acids selected
 10 from citric, oxalic, fumaric, lipid, maleic, malic, glucaric, succinic, adipic, ascorbic, lactic, pyruvic, succinic, pamelic, subaric, Kojic Acid, Alpha Arbutin having several pKa values and with varied pH for desired selective used in the form of ionic liquid for neutralizing and forming ion-pairs mediated cross-linked material including those selected from hydrogel,
 15 film, particles, beads, fibers, powder, flakes, sponge, sheet particle suspension spray, in-situ gelation during spraying;

said polyelectrolytes including polymers preferably those selected from polyvinyl alcohol, silk, alginate, chitosan, polyfumaric acid, poly maleic acid and other synthetic biopolymers and semi-synthetic polymers.

20 13. A process for the manufacture of a polyelectrolyte based bioactive super-absorbent material as claimed in anyone of claims 11 or 12 wherein for forming interpenetrating network (IPN) based gel with significant ion-pair mediated crosslinking both the neutralizing solutions/buffer contains polyelectrolyte, said ion-pair providing a highly interconnected porous
 25 template facilitating anyone or more of protein adsorption, growth factor adhesion and cell adhesion.

14. A process for the manufacture of a polyelectrolyte based bioactive super-absorbent material as claimed in anyone of claims 11 to 13 wherein said cross linked polyelectrolyte based bioactive super-absorbent is
 30 obtained including covalent or ionic cross linking preferably including in situ iodination during crosslinking for generating iodinated crosslinked polymer dressing system.

15. A process for the manufacture of a chitosan based bioactive super-absorbent material comprising:

- 5 i) providing a solution of at least one of (a) chitosan and (b) a mix of chitosan-collagen in the weight ratio of 20 : 0.25–10 wt % and treating with acetic acid;
- ii) treating the solution of i) above with polycarboxylic acid based system under continuous stirring till involving selective control of desired gelation for the formation of the chitosan based bioactive super-absorbent including step of controlled gelation selectively for (a) slow gelation
10 maintaining pH in the range of pK_1 to pK_2 (in the range of 3.13 to 4.7 for citrate) and (b) instantaneous gelation maintaining pH within the range of pK_2 or above (in the range of 4.76 or higher up to 12 for citrate).

16. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in claim 15 comprising:

- 15 i) providing a mix of chitosan-collagen in the weight ratio of 20 : 0.25–10 wt % by adding 0.1 to 1 M acetic acid to the final concentration of the solution 5 to 10 wt% depending upon the molecular weight of chitosan used;
- ii) neutralizing the solution by addition of citrate buffer in the range of 1
20 to 10 wt% under continuous stirring to thereby reduce the charge density of chitosan gradually until the solution was completely neutralized.

17. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in anyone of claims 15 or 16 for producing selectively anyone or more of chitosan-collagen particles, powder, flakes
25 and beads comprising:

- (i) carrying out said neutralization of chitosan-collagen solution by mixing with citrate buffer under continuous stirring maintaining pH range of citrate buffer more than 3.13 preferably at 7.4;
- (ii) casting the neutralized solution;
- 30 (iii) storing the thus cast solution in freezer at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs; followed by;

(iv) lyophilization at $<-30^{\circ}\text{C}$ under vacuum $1\times 10^{-1} - 5\times 10^{-2}$ mbar or below until the beads or freezing solution has dried substantially or completely;

(v) crushing the dried mix to obtain therefrom selectively anyone or more of bioactive super-absorbent chitosan-collagen particles, powder, flakes and beads.

18. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in anyone of claims 14 or 16 for producing selectively anyone or more of chitosan-collagen particles, powder, flakes and beads comprising:

(i) carrying out said neutralization of chitosan-collagen solution by drop wise addition of citrate buffer under continuous stirring maintaining pH range of citrate buffer in the range of 4.76-10 preferably at 7.4 to thereby obtain neutralized beads;

(ii) storing the thus neutralized beads in freezer at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs; followed by;

(iii) lyophilization at $<-30^{\circ}$ under vacuum $1\times 10^{-1} - 5\times 10^{-2}$ mbar or below until the beads or freezing solution has dried substantially or completely optionally involving temperatures of $40-80^{\circ}\text{C}$;

(iv) obtaining from the dried beads selectively anyone or more of bioactive super-absorbent chitosan-collagen particles, powder, flakes and beads.

19. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in anyone of claims 14 or 18 wherein said dried beads directly or following crushing sieved in meshes of varied mesh sizes to thereby obtain selectively anyone or more of bioactive super-absorbent chitosan-collagen particles, powder, flakes and beads.

20. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in claim 15 for producing chitosan-collagen sheets comprising:

- i) providing a solution of at least one of (a) chitosan and (b) a mix of chitosan-collagen in the weight ratio of 20 : 0.25-10 wt %;
- ii) neutralizing the solution by mixing said chitosan or chitosan-collagen solution with citrate buffer under continuous stirring till the solution was neutralized to different extent;
- (iii) casting the neutralized solution;
- (iv) storing the thus cast solution in freezer at -20 °C to -80 °C preferably at -80 °C for 10 to 24 hrs preferably at 12 hrs; followed by;
- (v) providing neutralized collagen solution;
- (vi) pouring the neutralized collagen solution on top of the said frozen chitosan solution or said frozen chitosan-collagen to provide a collagen layer; followed by
- (vii) freezing at -20 °C to -80 °C preferably at -80 °C for 10 to 24 hrs preferably at 12 hrs and lyophilization at <-30 °C under vacuum 1×10^{-1} – 5×10^{-2} mbar or below into sponge;
- (viii) pressing the sponge to obtain sheets therefrom.

21 A process as claimed in claim 20 wherein the said step of casting was carried out in a tray preferably a Teflon coated tray and stored in freezer at -20 °C for 12 hrs; and opposite to the collagen layer of said sheet there is provided a non-adhesive mesh either by pressing or involving cellulose based adhesive to provide support to the sheet, form a non-sticky barrier and retain the shape of the sheet.

22 A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in claim 15 for producing chitosan-collagen sheets comprising:

- i) providing neutralized chitosan solution;
- ii) mixing the neutralized chitosan solution with alginate-citrate buffer to form gel;
- (iii) casting the gel in tray;

(iv) storing the thus cast solution in freezer at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs; followed by;

(v) providing neutralized collagen solution;

(vi) pouring the neutralized collagen solution on top of the said frozen gel
5 to provide a collagen layer; followed by

(vii) lyophilization at $< -30^{\circ}\text{C}$ under vacuum $1 \times 10^{-1} - 5 \times 10^{-2}$ mbar or below into sponge.

23. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in claim 15 for producing chitosan-collagen
10 films comprising:

(i) carrying out said neutralization to different extent of chitosan-collagen solution with citrate buffer under continuous stirring maintaining pH of citrate buffer in the range of 3.13 to 4.76 maintaining pH of neutralized solution in the range of 5-7;

15 (ii) casting the neutralized solution and drying under sterilized condition, preferably under air flow until solution dried completely to form film.

24. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in claim 15 for producing chitosan-collagen fibers comprising:

20 preparing said chitosan-collagen solution using 0.1–1 M acetic acid to the solution of 0.5–5 wt%; filtering the solution and

wet spinning the filtrate in polycarboxylic acid bath at varied pH with pKa values higher than at least two carboxylated group ionization (above pKa_2) to thereby obtain the chitosan-collagen fibers.

25 25. A process as claimed in claim 24 wherein the filtrate was wet spun in a citric acid bath with pH between 4.76 to 10 preferably at 7.4; followed by washing the fibers and drying.

26. A polyelectrolyte based bioactive super-absorbent material comprising polyelectrolyte based bioactive super-absorbent which is

ionotropically crosslinked/neutralized involving polyvalent carboxylic acids.

27. A polyelectrolyte based bioactive super-absorbent material as claimed in claim 26 comprising:

- 5 collagen-biopolymer which is ionotropically crosslinked/neutralized involving polyvalent carboxylic acids.

28. A polyelectrolyte based bioactive super-absorbent material as claimed in anyone of claims 26 or 27 comprising variable pH based neutralized and formed ion pair mediated cross linked products including selectively
10 anyone or more of dressing system including iodinated crosslinkined polymer as antimicrobial agent, antioxidant along with Kojic Acid and Alpha Arbutin as skin plumper and melanin reducer in the form of beads, sheet, film and fiber blanket.

29. A polyelectrolyte based bioactive super-absorbent material as claimed
15 in anyone of claims 26 to 28 comprise fish scale collagen based wound healing/dressing including partial, full thickness, diabetic, chronic, traumatic, ulcer, exudating wounds, bone cartilage healing, cell/drug/different bioactive molecules cytokines, kemokines delivery vehicles, scaffold for tissue engineering, 3D cell expansion media,
20 collagen delivery in dry eye, collagen based health drink, intra ocular lens, contact lens, oral patch, ingredients for capsules, mineral delivery, moisturizer, ointments, facial creams

30. A polyelectrolyte based bioactive super-absorbent material as claimed in anyone of claims 26 to 29 comprising fish scale collagen cross linked
25 with other polyelectrolyte biopolymers preferably selected from chitosan and alginate.

31. A polyelectrolyte based bioactive super-absorbent material as claimed in anyone of claims 26 to 30 and/or in combination of live cells in other healing agents which is utilized in the form of spray or injectable in-situ
30 gelation on wound site.

AMENDED CLAIMS

received by the International Bureau on 12 May 2017 (12.05.2017)

We Claim :

1. A process for extraction of collagen from fish scale comprising the steps of:
 - i) washing and softening the fish scale involving washing with salt solution selected from NaCl or KCl followed by depigmenting and further softening of the scales by treating with alkali selected from NaOH or KOH, at a temperature below 15 °C preferably 4-8 °C for 24-48 h with a shaking speed of 10-100 rpm;
 - ii) subjecting the thus softened fish scale to crushing and smashing at a low temperature up to 20 °C preferably 10 °C to achieve disintegration through punching, grinding and tearing of the scales;
 - 10 iii) treating the crushed and smashed fish scale with acetic acid such as to favour collagen dissolution directly from said fish scales in said acetic acid solution;
 - iv) carrying out filtration of the acetic acid treated crushed fish scale and thus separating the solution of soluble collagen thus obtained and the residue therefrom;
 - v) subjecting the solution of soluble collagen obtained to salt treatment to thereby precipitate the dissolved collagen from said solution followed by filtration to again generate solution and residue therefrom;
 - 15 vi) subjecting the residue thus obtained to acetic acid treatment; followed by
 - vii) carrying out dialysis based purification; and finally
 - viii) obtaining therefrom purified collagen through supercritical drying.
- 20 2. A process as claimed in claim 1 wherein the residue in step iv) also having some collagen content is subjected to:
 - i) crushing and smashing;
 - ii) treating with acetic acid and pepsin;
 - iii) carrying out filtration to generate a solution of soluble and residue;
 - 25 iv) recycling the solution thus obtained to carry out said steps v) to viii) and the residue to carry out said steps ii) to viii).
3. A process as claimed in anyone of claims 1 or 2 wherein said step of dialysis comprises continuous dialysis process.

4. A process as claimed in anyone of claims 1 to 3 comprising continuous dialysis process preferably carried out involving hollow fibre membrane and dialyzer of desired molecular cut off and controlling inlet and outlet pressure at different points whereby different protein present in the solution can be separated according to their molecular weight.

5. A process as claimed in claim 4 wherein said inlet pressure of hollow fiber membrane input (collagen containing solution) is 300 to 1000 mbar and outlet pressure of the same is at 100 to 600 mbar, inlet pressure of the dialysate (water) input is maintained at 1 to 200 mbar below the outlet pressure of the hollow fiber membrane with continuous flow of dialysate.

6. A process as claimed in claim 1 wherein the crushed /smashed fish scale is treated with acetic acid of 0.3 to 0.5 M at temperature 6 to 10 °C by stirring with shaking speed of 10-100 rpm.

7. A process as claimed in claim 1 wherein said dialysis based purification is carried out involving hollow membrane dialyzer having molecular cut off 3-80 KDa and the dialysis step is continued till the pH of the solution is 5.5 to 6.5.

8. Collagen obtained following the process as claimed in anyone of claims 1 to 7 with cumulative yield exceeding 22% total fish scale weight basis and high purity in the range of 92-95% through supercritical drying.

9. A process for the manufacture of a polyelectrolyte based bioactive super-absorbent material comprising:

i) providing a solution of at least one polyelectrolyte preferably selectively from chitosan, chitosan-collagen solublized in acetic acid;

ii) selectively neutralizing the above polyelectrolyte solutions (i) using ionic solution of di, tri or higher polycarboxylic acids preferably citrate buffer at different pH range above pK_{a1} through continuous stirring

involving selective control of desired gelation for the formation of the crosslinked polyelectrolyte based bioactive super-absorbent including the step of controlled gelation selectively for (a) slow gelation maintaining pH of neutralizing buffer within the range of pK_1 to pK_2 (in the range of 3.13 to 4.7 for citrate) and (b) faster gelation maintaining pH of neutralizing buffer within the range of pK_2 or above (in the range of 4.76 or higher up to 12 for citrate) for achieving desired ionotropic neutralization and crosslinking.

10. A process for the manufacture of a polyelectrolyte based bioactive super-absorbent material as claimed in claim 9 wherein said ionic liquid include dicarboxylic acid, tricarboxylic acid or higher polycarboxylic acid, unsaturated acids including organic acids
 5 selected from citric, oxalic, fumaric, lipid, maleic, malic, glucaric, succinic, adipic, ascorbic, lactic, pyruvic, succinic, pamelic, subaric, Kojic Acid, Alpha Arbutin, alginate, polyfumaric acid, poly maleic acid, alginate used as neutralizing buffer having several pKa values and with varied pH specially at higher than pK_{a2} for neutralizing polyelectrolyte and forming ion-pairs mediated cross-linked material including those
 10 selected from hydrogel, film, particles, beads, fibers, powder, flakes, sponge, sheet particle suspension spray, in-situ gelation during spraying;

said polyelectrolytes including polymers preferably those selected from polyvinyl alcohol, silk, chitosan, collagen and other synthetic, biopolymers and semi-synthetic polymers alone or in combination.

11. A process for the manufacture of a polyelectrolyte based bioactive super-absorbent material as claimed in anyone of claims 9 or 10 wherein for forming interpenetrating network (IPN) based gel with significant ion-pair mediated crosslinking both the neutralizing solutions/buffer contains polyelectrolyte dried under super critical temperature for formation of superabsorbent, said ion-pair providing a highly
 20 interconnected porous zwitter ionic template facilitating ultra low fouling activity preventing non-specific protein adsorption and necessary protein, growth factor and cell adhesion.

12. A process for the manufacture of a polyelectrolyte based bioactive super-absorbent material as claimed in anyone of claims 9 to 11 wherein said cross linked polyelectrolyte
 25 based bioactive super-absorbent is obtained including covalent or ionic cross linking preferably including in situ iodination during crosslinking for generating iodinated crosslinkined polymer dressing system.

13 A process for the manufacture of a chitosan based bioactive super-absorbent material comprising:

30 i) providing a solution of at least one of (a) chitosan and (b) a mix of chitosan-collagen in the weight ratio of 20 : 0.25–10 wt % and treating with acetic acid;

ii) treating the solution of i) above with polycarboxylic acid based system under continuous stirring till involving selective control of desired gelation for the formation of

the chitosan based bioactive super-absorbent including step of controlled gelation selectively for (a) slow gelation maintaining pH in the range of pK_1 to pK_2 (in the range of 3.13 to 4.7 for citrate) and (b) rapid gelation maintaining pH within the range of pK_2 or above (in the range of 4.76 or higher up to 12 for citrate).

5 14. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in claim 13 comprising:

i) providing a mix of chitosan-collagen in the weight ratio of 20 : 0.25–10 wt% by adding 0.1 to 1 M acetic acid to the final concentration of the solution 5 to 10 wt% depending upon the molecular weight of chitosan used;

10 ii) neutralizing the solution by addition of citrate buffer in the range of 1 to 10 wt% with pH above pK_{a2} under continuous stirring to thereby reduce the charge density of chitosan gradually until the solution was completely neutralized.

15 15. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in anyone of claims 13 or 14 for producing selectively anyone or more of chitosan-collagen particles, powder, flakes and beads comprising:

(i) carrying out said neutralization of chitosan-collagen solution by mixing with citrate buffer under continuous stirring maintaining pH range of citrate buffer more than 3.13 preferably at 7.4;

(ii) casting the neutralized solution;

20 (iii) storing the thus cast solution in freezer at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs; followed by;

(iv) lyophilisation at $<-30^{\circ}\text{C}$ under vacuum $1 \times 10^{-1} - 5 \times 10^{-2}$ mbar or below until the beads or freezing solution has dried substantially or completely;

25 (v) crushing the dried mix to obtain therefrom selectively anyone or more of bioactive super-absorbent chitosan-collagen particles, powder, flakes and beads.

16. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in anyone of claims 13 to 15 for producing selectively anyone or more of chitosan-collagen particles, powder, flakes and beads comprising:

30 (i) carrying out said neutralization of chitosan-collagen solution by drop wise addition of citrate buffer under continuous stirring maintaining pH range of citrate buffer in the range of 4.76-10 preferably at 7.4 to thereby obtain neutralized beads;

(ii) storing the thus neutralize beads in freezer at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs; followed by;

(iii) lyophilization at $<-30^{\circ}$ under vacuum $1 \times 10^{-1} - 5 \times 10^{-2}$ mbar or below until the beads or freezing solution has dried substantially or completely optionally involving temperatures of $40-80^{\circ}\text{C}$;

(iv) obtaining from the dried beads selectively anyone or more of bioactive super-absorbent chitosan-collagen particles, powder, flakes and beads.

17. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in anyone of claims 13 to 16 wherein said dried beads directly or following crushing sieved in meshes of varied mesh sizes to thereby obtain selectively anyone or more of bioactive super-absorbent chitosan-collagen particles, powder, flakes and beads.

18. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in claim 13 for producing chitosan-collagen sheets comprising:

i) providing a solution of at least one of (a) chitosan and (b) a mix of chitosan-collagen in the weight ratio of 20 : 0.25–10 wt%;

ii) neutralizing the solution by mixing said chitosan or chitosan-collagen solution with citrate buffer under continuous stirring till the solution was neutralized to different extent;

(iii) casting the neutralized solution;

(iv) storing the thus cast solution in freezer at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs; followed by;

(v) providing neutralized collagen solution;

(vi) pouring the neutralized collagen solution on top of the said frozen chitosan solution or said frozen chitosan-collagen to provide a collagen layer; followed by

(vii) freezing at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs and lyophilization at $<-30^{\circ}\text{C}$ under vacuum $1 \times 10^{-1} - 5 \times 10^{-2}$ mbar or below into sponge;

(viii) pressing the sponge to obtain sheets therefrom.

19. A process as claimed in claim 18 wherein the said step of casting was carried out in a tray preferably a Teflon coated tray and stored in freezer at -20°C for 12 hrs; and

opposite to the collagen layer of said sheet there is provided a non-adhesive mesh either by pressing or involving cellulose based adhesive to provide support to the sheet, form a non-sticky barrier and retain the shape of the sheet.

20. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in claim 13 for producing chitosan-collagen sheets comprising:

i) providing neutralized chitosan solution;

ii) mixing the neutralized chitosan solution with alginate-citrate buffer to form gel;

(iii) casting the gel in tray;

(iv) storing the thus cast solution in freezer at -20°C to -80°C preferably at -80°C for 10 to 24 hrs preferably at 12 hrs; followed by;

(v) providing neutralized collagen solution;

(vi) pouring the neutralized collagen solution on top of the said frozen gel to provide a collagen layer; followed by

(vii) lyophilization at $< -30^{\circ}\text{C}$ under vacuum $1 \times 10^{-1} - 5 \times 10^{-2}$ mbar or below into sponge.

21. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in claim 13 for producing chitosan-collagen films comprising:

(i) carrying out said neutralization to different extent of chitosan-collagen solution with citrate buffer under continuous stirring maintaining pH of citrate buffer in the range of 3.13 to 4.76 maintaining pH of neutralized solution in the range of 5-7;

(ii) casting the neutralized solution and drying under sterilized condition, preferably under air flow until solution dried completely to form film.

22. A process for the manufacture of a chitosan based bioactive super-absorbent material as claimed in claim 13 for producing chitosan-collagen fibers comprising:

preparing said chitosan-collagen solution using 0.1–1 M acetic acid to the solution of 0.5–5 wt%; filtering the solution and

wet spinning the filtrate in polycarboxylic acid bath at varied pH with pKa values higher than at least two carboxylated group ionization (above pKa_2) to thereby obtain the chitosan-collagen fibers.

23. A process as claimed in claim 22 wherein the filtrate was wet spun in a citric acid bath with pH between 4.76 to 10 preferably at 7.4; followed by washing the fibers and drying.

24. A polyelectrolyte based bioactive super-absorbent material comprising polyelectrolyte based bioactive super-absorbent which is ionotropically crosslinked/neutralized product and obtained of involving di, tri, or polyvalent carboxylic acids as neutralizing buffer at pH above pK_{a2} through slow addition under stirring into chitosan-collagen followed by drying such as to include interpenetrating network (IPN) based gel with significant ion-pair mediated crosslinking providing a highly interconnected porous template of zwitter ions for ultralow fouling activity preventing non-specific protein adsorption and facilitates necessary protein, growth factor and cell adhesion facilitating protein favouring anyone or more of protein adsorption, growth factor adhesion and cell adhesion.

25. A polyelectrolyte based bioactive super-absorbent material as claimed in claim 24, which is produced by the process as claimed in anyone of claims 9 and 13 comprising variable pH specifically above pK_{a1} based neutralized and formed ion pair mediated cross linked products at pH values above pK_{a2} including selectively anyone or more of dressing system including iodinated crosslinked polymer as antimicrobial agent, antioxidant along with Kojic Acid and Alpha Arbutin as skin plumper and melanin reducer in the form of beads, sheet, film and fiber blanket.

26. A polyelectrolyte based bioactive super-absorbent material as claimed in anyone of claims, 24 and 25 comprise fish scale based collagen-chitosan dissolved in aqueous acetic acid neutralized with polyvalent carboxylic acid buffer at pH above pK_{a2} as super-critically dried matrices as wound healing/dressing including partial, full thickness, diabetic, chronic, traumatic, burn, ulcer, exudating wounds, bone cartilage healing, cell/drug/different bioactive molecules cytokines, chemokines delivery vehicles, scaffold for tissue engineering, 3D cell expansion media, collagen delivery in dry eye, collagen based health drink, intra ocular lens, contact lens, oral patch, ingredients for capsules, mineral delivery, moisturizer, ointments, facial creams.

27. A polyelectrolyte based bioactive super-absorbent material as claimed in anyone of claims, 24 to 26 and/or in combination of live cells in other healing agents which is utilized in the form of spray or injectable in-situ gelation on wound site and matrices for in vitro 3D cell culture and expansion.

STATEMENT UNDER ARTICLE 19 (1)

Amended claims are directed to clarify and qualify inventive aspects of the present advancement providing a cost-effective process for direct extraction of fish-scale collagen of high purity at low temperature with minimum chemical treatment, a polyelectrolyte based bioactive super-absorbent biopolymer manufacturing process involving the said high-purity collagen and the said polyelectrolyte based bioactive super-absorbent material. The said biopolymer is effective in wound healing/dressing including partial/full thickness, diabetic, chronic, traumatic, burn, ulcer, exudating wounds, bone/cartilage healing, cell/drug/different bioactive molecules cytokines/chemokines delivery vehicles, tissue-engineering scaffold, 3D cell-expansion media, collagen delivery in dry eye, collagen based health drink, intra-ocular/contact lens, oral patch, ingredients for capsules, mineral delivery, moisturizer, ointments, facial creams.

The amendment also brings out the qualifying steps of collagen extraction process comprising alkali treatment followed by crushing/smashing at low temperature below 15°C minimizing the chances of collagen degradation, and efficient softening and depigmentation. Acetic acid treatment of the crushed, smashed fish scale dissolves collagen directly from said fish scales in said acetic acid solution, which on subsequent filtration separate the solution of soluble collagen and the residue that undergoes acetic acid treatment and filtration. Salt treatment and filtration precipitate the dissolved collagen, subsequently re-dissolved in acetic acid, and is purified to more than 90% purity through continuous-flow based dialysis process involving 3-80 KDa molecular cut-off hollow fibre membrane and dialyzer and controlling inlet, outlet pressure at different points separating different proteins present in the solution based on molecular weights.

The amendment also highlights manufacturing process of the polyelectrolyte based bioactive super-absorbent material comprising the special steps of i) selectively neutralization of a at least one polyelectrolyte solution (chitosan, chitosan-collagen) dissolved in acetic acid, using polyvalent carboxylic acids like citrate buffer at different pH range above pK_{a1} ; ii) Selective control of desired gelation for the crosslinked polyelectrolyte based bioactive super-absorbent formation for (a) slow gelation maintaining neutralizing buffer pH within the range of pK_1 - pK_2 (3.13 - 4.7 for citrate) (b) faster gelation maintaining neutralizing buffer pH within the range of pK_2 or above (4.76 - 12 for citrate) for achieving desired ionotropic neutralization and crosslinking. In the said process for forming interpenetrating network based gel with significant ion-pair mediated crosslinking, both the neutralizing solutions/buffer containing polyvalent carboxylate at pH higher than pK_{a2} are slowly added under stirring to acetic acid based polyelectrolyte for significant ion-pair mediated neutralization and crosslinking. These ion-pair network leads to highly interconnected porous template of zwitter ions for ultra-low fouling activity preventing non-specific protein adsorption and facilitates necessary protein, growth factor and cell adhesion. Ion-pair mediated crosslinked gel is dried under supercritical temperature forming superabsorbent and the ion-pairs promote absorption of very high weight percentage of polar solvent medium like water.

(1/9)

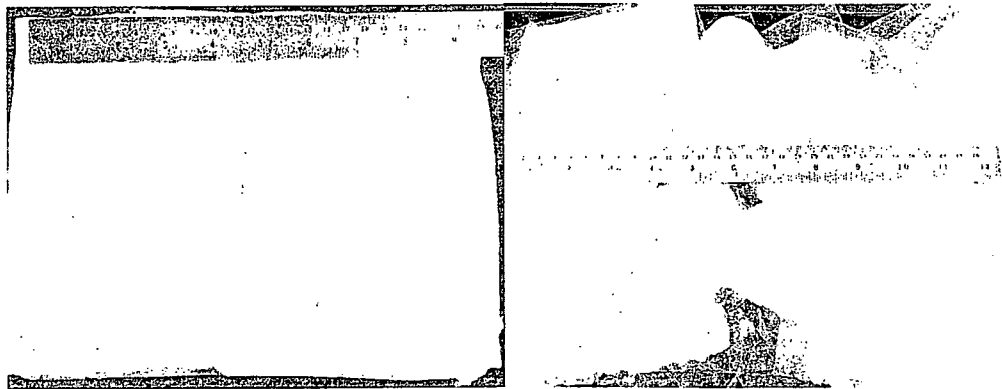


Figure 1

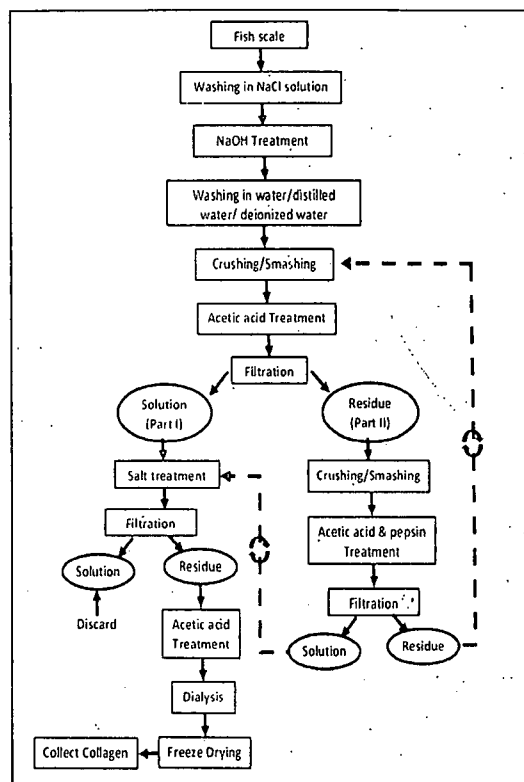


Figure 2

(2/9)

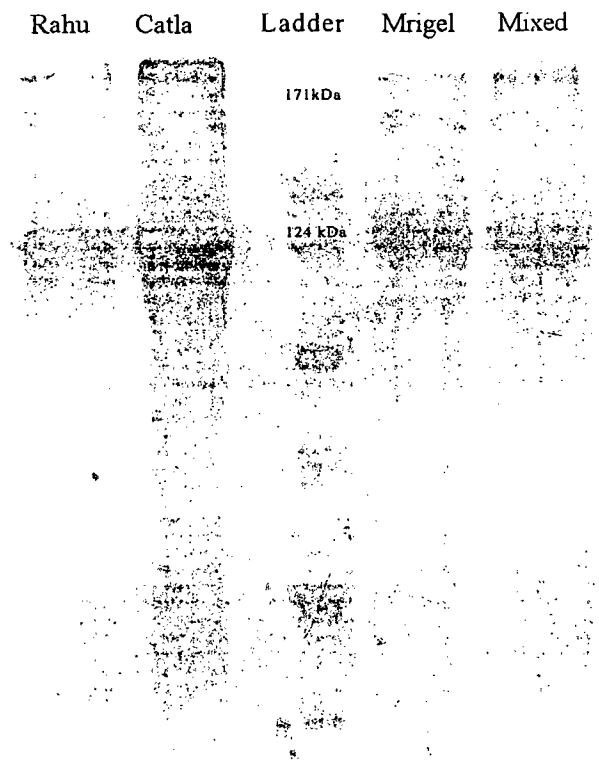


Figure 3

(3/9)

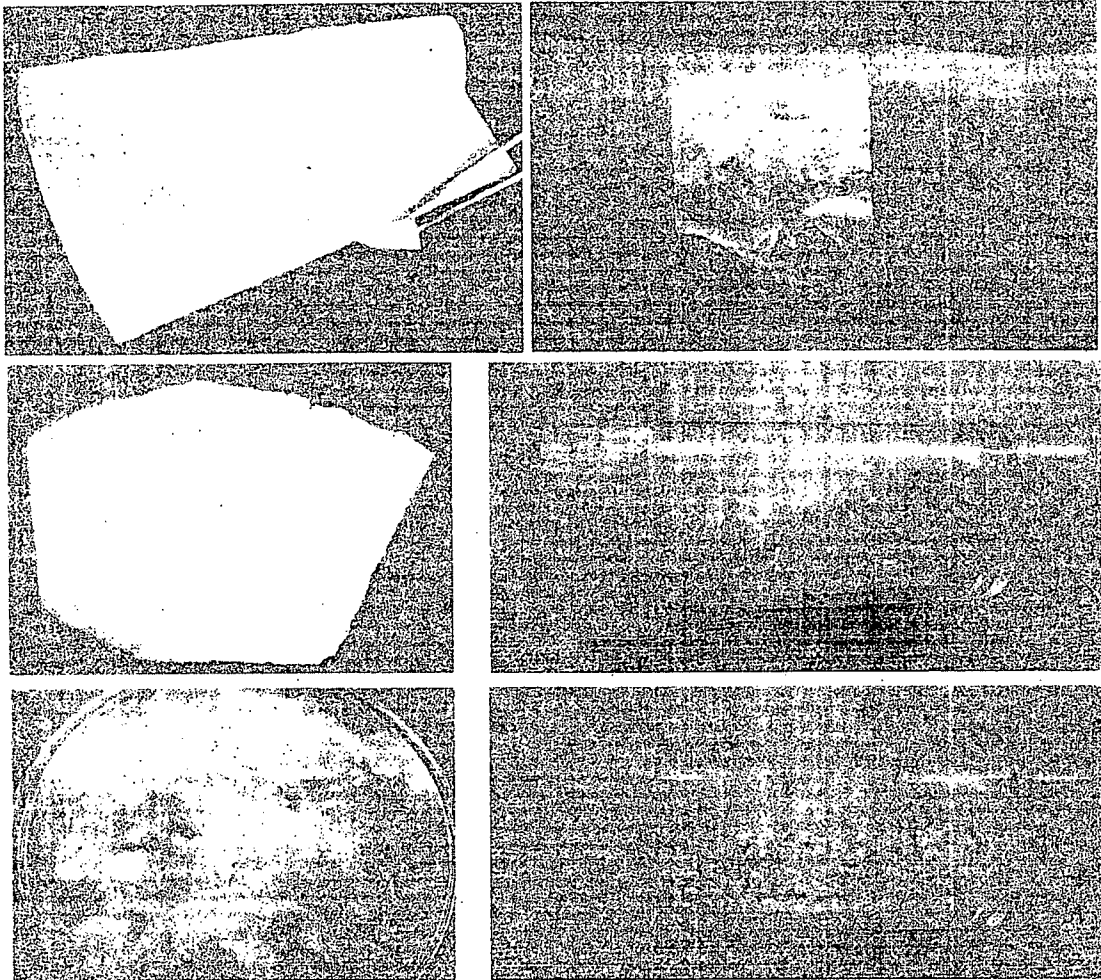


Figure 4

(4/9)

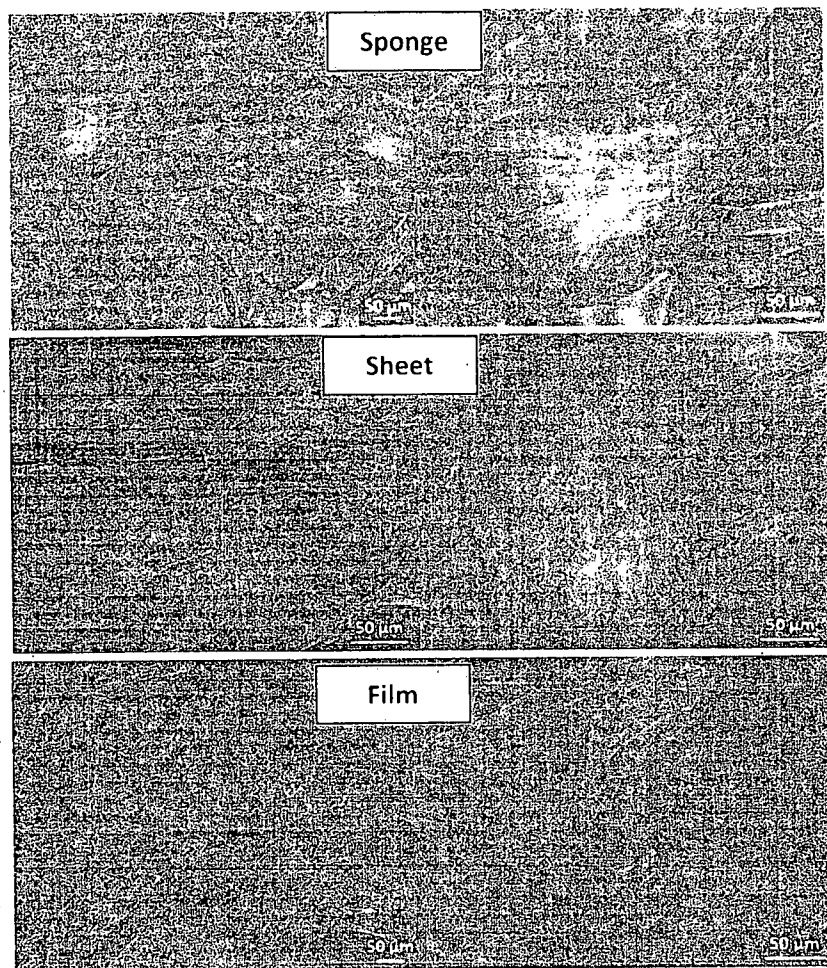


Figure 5

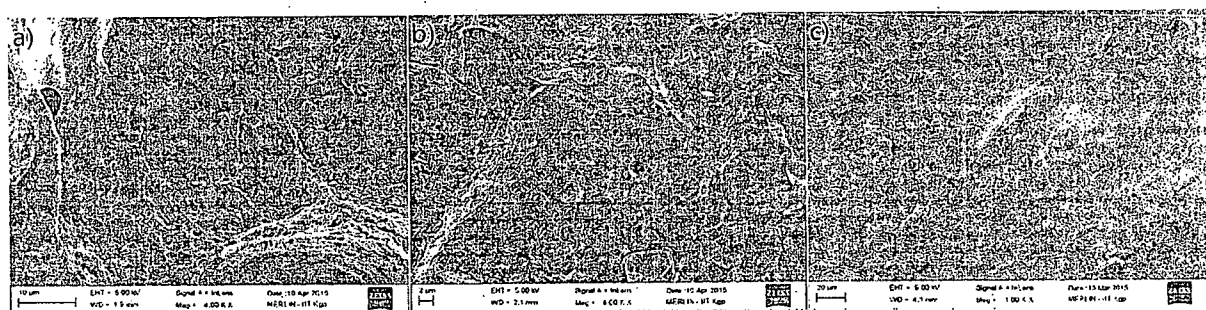


Figure 6

(5/9)

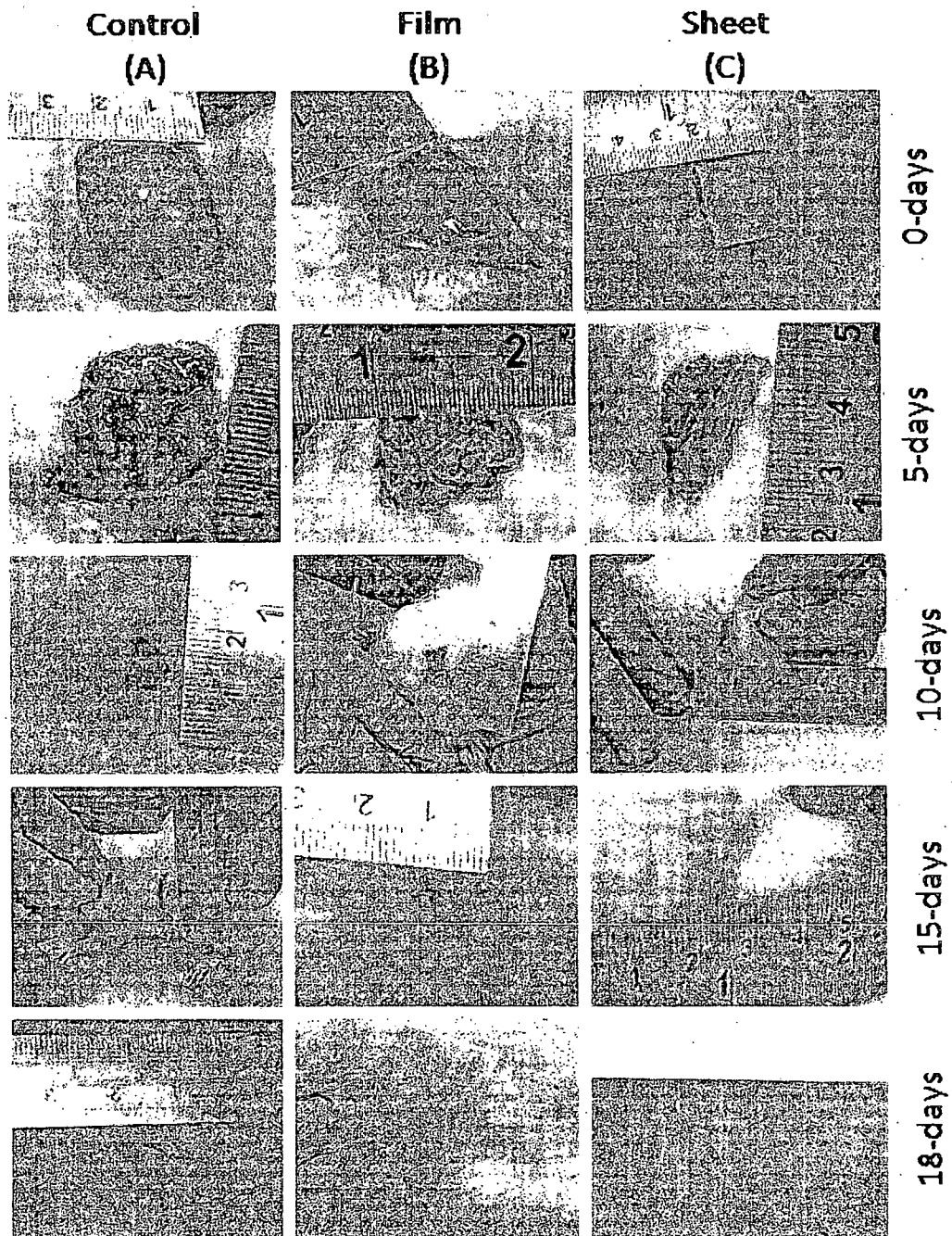


Figure 7

(6/9)

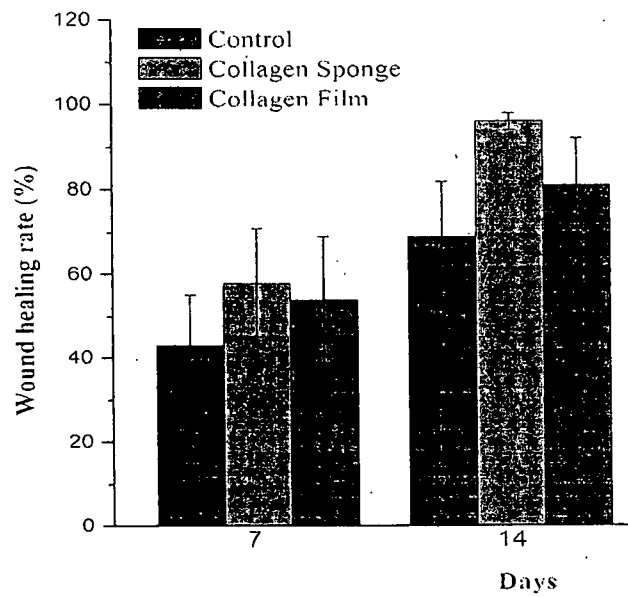


Figure 8

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Figure 9

(8/9)



Figure 10

(9/9)

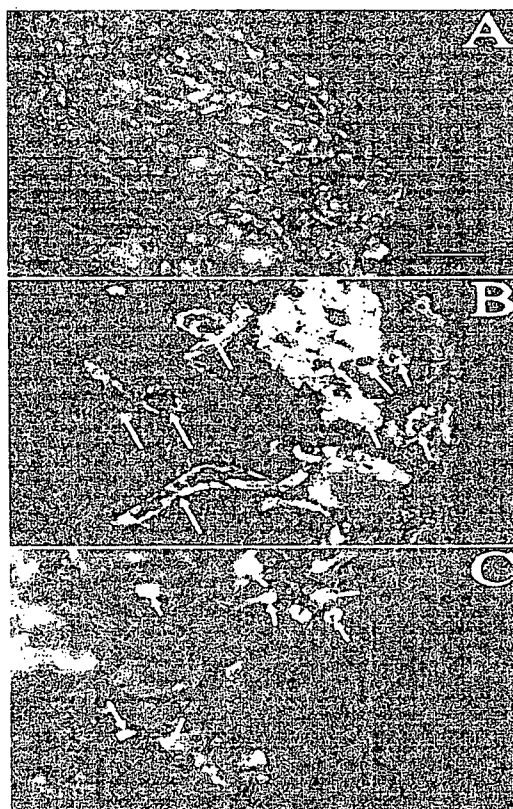


Figure 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2016/000211

A. CLASSIFICATION OF SUBJECT MATTER
C07K14/78, A61K38/39, C07K1/00 Version=2016.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

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)

Patseer, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FENGXIANG ZHANG et al, "Preparation and Characterisation of Collagen from Freshwater Fish Scales," Food and Nutrition Sciences, 2011, Vol. 2, pages: 818-823. page 818, right column, section material and methods; page 819, left column, section 2.4: Extraction of Acid- and Pepsin-Solubilized Collagens (ASC and PSC)	1-10
Y	B. D. SHRINIWAS et al, "Membrane Technology in Fishery Industry-A State of the Art," Clean Technology," 2010, pages: 368-371. abstract, whole document	1-10
Y	WO 2013096448 A1, (AGENTA BIOTECHNOLOGIES, INC), 27-06-2013 (27 June 2013). abstract, page 10, last para; claim 1; claim 6	11-25
Y	Le Y et al, Recent developments in fibres and materials for wound management, Indian Journal of Fibre & Textile Research Vol.22, December 1997, pp.337-347. page 344, left column, para 2; page 340, left column para 1; page 343, right	11-25



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

"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

26-12-2016

Date of mailing of the international search report

26-12-2016

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2016/000211

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