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1   **Full paper**

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3   **Effectively promote wound healing of cellulose/gelatin sponges**

4   **constructed directly from cellulose solution**

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Abstract: Wound dressing is of critical importance for wound repair, and the traditional cotton gauze derived from cellulose has commonly been used in clinical practice for a long time. However, cotton gauze does not possess active healing ability. To search new wound dressings, in this work, cellulose sponge was fabricated directly from the cellulose solution in the NaOH/urea aqueous system with cooling, and then the cellulose/gelatin composite sponges were constructed successfully via a green and cost-effective pathway. The structure and physical properties of the sponges were characterized, and their cytocompatibility and *in vivo* wound healing were evaluated. The results indicated that, compared with cotton gauze, the cellulose sponge effectively promoted wound healing, as a result of the presence of the macro- and micro porous architecture. Furthermore, the gelatin and basic fibroblast growth factor (bFGF) were immobilized in the cellulose sponge through hydrogen bonding to retain their inherent biocompatibility, leading to the excellent repairing efficacy. Especially, for full-thickness coetaneous wound model, the complete wound healing time for the wounds treated with bFGF-loaded cellulose sponges was 7 days faster than that treated with gauze. The pores with thin wall in the cellulose composite sponges played an important role in achieving the highly effective wound healing, which could fit the requirements of oxygen permeability, controlled water vapor evaporation and wound exudates absorption.

Keywords: Cellulose sponge, wound dressing, porous architecture, gelatin, wound repairing efficacy.

1. Introduction

Skin, as the largest organ in the body, plays an important role in protection against invasion from the environment. On a global scale, about 234.2 million people undergo surgical procedures because of accident or healthy problems, resulting in huge demand for skin wound dressings [1]. Constructing effective dressing is essential for promoting wound healing in view of the health care issues. Basically, a desirable wound dressing should possess gas permeability and biocompatibility. It should keep moist at the injury interface as well as acting as a barrier to microorganisms [2]. Recently, natural [3], synthetic [4], and hybrid organic materials [5] have been employed to construct wound dressings. Synergistic compositions and novel structural design endow these dressings with functionality such as antimicrobial properties [6], growth factors [7] or drug release abilities [8], and biodegradable properties [9], thus helping to hasten up the healing process. Especially, the dressings were mostly designed to porous structure, benefiting to fit the requirements of gas permeability, controlled water vapor evaporation and wound exudates absorption. It is worth noting that natural polymers such as gelatin [10], collagen [11], silk fibroin [12], cellulose [13], alginate [14], chitosan, and chitin [2] have become good candidates for the fabrication of wound dressing due to their non-cytotoxicity and hydrophilicity. Cellulose is the most abundant renewable natural polymer, and it, as well as its derivatives, have been widely used for biological and medical application including scaffold [15,16], medical system development [17], drug diagnosis [18] and orthopedic biomaterial [19], showing good biocompatibility by *in vitro* and *in vivo* tests [20, 21]. Though the traditional cellulose

59   gauze based dressing has commonly been used in clinical practice for a long time, it

60   does not possess active healing capabilities, limited by its low efficiency to absorb the

61   exudates and to prevent bacteria from growth and migration. Thus, searching for new

62   wound dressing based on cellulose is essential for the effectively promote wound

63   healing. Recently, bacterial cellulose with nanofiber network has been reported as

64   wound dressing, and showed excellent biocompatibility and degradation rate

65   commensurate with new tissue formation [22], suggesting the effect of the network

66   structure. Currently, commercial regenerated cellulose dressings such as Cellstick®

67   and Cellspon® with porous structure are mainly produced by the viscose method [23, 24].

68   This method still dominates production methods in which pulp with CS_2 is converted

69   into cellulose xanthogenate with solubility in aqueous sodium hydroxide, which has

70   been forbidden in many developed countries due to the generation of hazardous

71   pollutions (CS_2, H_2S) during production. In our laboratory, NaOH/urea aqueous

72   solution precooled to -12°C has been used to dissolve cellulose [25], and from the

73   cellulose solution a series of cellulose materials have been successfully fabricated

74   such as films [26], hydrogels [27], fibers [28], microspheres [29], and aerogels [30], through a

75   green and low-cost process. Thus, a worthwhile endeavor would be to utilize the

76   cellulose solution to directly prepare a new cellulose dressing with porous structure,

77   such as sponges which have never been fabricated, and to compare with traditional

78   cotton gauze. Additionally, gelatin is a natural polymer consisted of complex proteins,

79   and has been widely used for wound dressing as well as absorbent pad in surgery as a

80   result of its low antigenecity, high bioabsorptivity, and commercial availability with

81   relatively low cost. [31] Meanwhile it is an outstanding carrier for growth factors (e. g.,

82   bFGF and EGF) [32], which can promote wound healing. However, gelatin is rarely used

83   as independent material without cross-linking since its gel-sol transition is at about

84   37°C in liquid media [33]. On the basis of the hydrogen bonding interaction between

85   gelatin and cellulose, an efficient strategy to construct composite sponges is by

86   blending the two natural polymers as wound dressing to avoid the safety problem of

87   the cross-linking agent usage.

88   In the present work, we provided a green pathway for the producing

89   cellulose/gelatin composite sponge as wound dressing by changing their aggregation

90   structure and morphology. The structure and properties of the composite sponges were

91   characterized by elemental analysis, Fourier transform infra-red (FT-IR) spectra,

92   solid-state ^{13}C NMR, scanning electron microscopy (SEM), mechanical testing, water

93   retention and swelling test. Aiming for wound healing, their growth factor release

94   ability, cytocompatibility and wound repair ability were also evaluated. A new

95   pathway to construct effective wound dressing based on cellulose materials is

96   important for the development and application of highly effective wound dressings to

97   ease the pain for patients.

98

99   **2. Materials and methods**

100   **2.1 Materials**

101   Cellulose (cotton linter pulp) with α-cellulose content of more than 95% was

102   provided by Hubei Chemical Fiber Co. Ltd. of China. Its weight-average molecular

103   weight (M_w) was determined by using an Ubbelohde viscometer in a LiOH/urea

104   aqueous solution at 25 ±0.05°C, and calculated by the equation $[\eta] = 3.72 \times 10^{-2} M_w^{0.77}$

105   [34] to be 1.1×10^5 g/mol. Gelatin used was derived from porcine skin (type A, powder

106   approx 300 Bloom, Sigma-Aldrich Co., USA). All other reagents were of analytical

107   grade and purchased from Sinopharm Chemical Reagent Co., Ltd. of China.

108   **2.2 Sponge Preparations**

109   Cellulose solution was prepared according to the previous method [26]. Cellulose

110   was dissolved in a mixed solution of NaOH/urea/H_2O (7:12:81, weight ratio)

111   precooled to -12°C with stirring vigorously for 5 min to obtain a transparent cellulose

112   solution with 2 wt% concentration. Epichlorohydrin (ECH, 1ml) as a cross-linking

113   agent was added to the cellulose solution (27g), and the mixture was stirred in an ice

114   bath for 1 h to yield a homogeneous solution, and then reacted at 65°C for 2 h. Finally,

115   the resulted cellulose gels were immersed in deionized water to remove the residual

116   reagents (NaOH, urea and unreacted ECH) for a week to obtain pure cellulose

117   hydrogels. The cellulose hydrogels were precooled at -20°C, and then freeze-dried by

118   using a lyophilizer at -45°C for 24h to obtain regenerated cellulose sponges material,

119   coded as RCS.

120   Gelatin was dissolved in aqueous solutions at 45°C to prepare gelatin solutions

121   with concentration of 2, 8 and 15 wt%. The cellulose hydrogels mentioned above

122   were immersed in different gelatin solutions at 45°C for 24h to obtain

123   cellulose/gelatin (RCS/G) composite hydrogels. The RCS/G hydrogels were rinsed

124   with deionized water to remove the surface gelatin at RT. The hydrogels were

125   freeze-dried at -20°C by using a lyophilizer at -45°C for 24h to obtain the RCS/G

126   sponges. The RCS/G sponges were coded as RCS/G2, RCS/G8, and RCS/G15,

127   respectively, according to the gelatin concentrations from 2 to 15 wt%.

128   To fabricate basic fibroblast growth factor (bFGF, Sigma-Aldrich Co., USA) loaded

129   sponges, a solution of 1 μgml^{-1} bFGF in PBS was prepared. Dried cylindrical RCS

130   and RCS/G sponges with diameter of 15 mm and thickness of 2 mm were placed in

131   24-well plates, and were dropped into 500μl bFGF solutions. After absorption for 24h

132   at 25°C, the bFGF loaded sponges were taken out, freeze-dried, and coded as

133   bFGF-RCS and bFGF-RCS/G, respectively. The bFGF concentration was

134   quantitatively measured by an enzyme-linked immunosorbent assay (ELISA) kit

135   (R&D Systems Inc., USA) and enzyme-labeled instrument (Multiskan FC, Thermo

136   Fisher Scientific Inc., USA) at 450nm. The bFGF left in wells were rinsed by 10 ml

137   PBS, and their concentrations were analyzed to determine the amounts of bFGF

138   loaded in sponges.

139   **2.3 Characterizations**

140   For Fourier-transform infrared (FT-IR) measurement, the sponges were made into

141   powder, and then vacuum dried for 24h before characterizations. FT-IR spectra of the

142   samples pressed into KBr pellets were acquired with a FT-IR spectrometer

143   (NICOLET 5700, Thermo Fisher Scientific Inc., USA) in the range of 4000-500 cm^{-1}.

144   Solid-state[13]C NMR spectra of the sponge samples were recorded on a Bruker

145 AVANCE-300 NMR system (Bruker Co., Germany) operating at 75 MHz using the

146 combined technique of proton dipolar decoupling, magic angle spinning (MAS) and

147 cross-polarization (CP). The spinning rate was set at 5.0 kHz for all samples. The

148 contact time was 5 ms, the recycle delay 5 s and 5000 scans were accumulated for

149 each spectrum.

150 The morphologies of the RCS and RCS/G sponges with sputter coated with

151 platinum were observed by field emission scanning electron microscope (SEM,

152 Quanta 200, FEI Co., USA) with 15kV accelerating voltage. SEM element mapping

153 analysis was used to investigate the nitrogen distribution in the RCS/G sponges,

154 namely gelatin distribution. The pore size (d) of the sponges was determined by

155 measuring samples with 100 pores from the SEM images using the Image J computer

156 software (National Institute of Health, USA) and averaged.

157 The average nitrogen contents (W_N, wt %) in the RCS/G sponges were determined

158 by an elemental analyzer (CHN-O-RAPID, Heraeus Co., Germany). The W_N values in

159 different parts of RCS/G sponge were also analyzed to determine the distribution of

160 gelatin in sponge. The average protein content (W_{pro}, wt %) of the RCS/G sponges

161 was calculated by the Kjeldahl method

162
$$W_{pro} = W_N \times 6.25 \tag{1}$$

163 To measure the physical porosities, the RCS and RCS/G sponges were cut into

164 samples with a size of about 10cm × 10cm × 0.7 cm with avoiding excessive

165 deformation as a result of shear forces. The total volume (V) and weight of the dried

166   sponge (M) were measured at the same room temperature and humidity, and then the

167   average density (ρ) of the sponge was calculated. The gelatin content (W_G, wt %) is

168   equal to W_{pro}, and the rest of the content is equal to the cellulose content (W_{RCS},

169   wt %). The density of gelatin (ρ_G) and cellulose (ρ_C) are 1.35 g/cm^3 and1.62 g/cm^3 [35],

170   and then the solid volume of sponge (V_S) was calculated by the following equation:

171    $$V_S = M \left(W_G/\rho_G + W_{RCS}/\rho_C \right) \qquad (2)$$

172   The porosity of the sponge was calculated according to following equation:

173    $$P_r = 1 - V_S/V \qquad (3)$$

174   Where, P_r of each sponge is an average value of three measurements.

175   **2.4 Measurements of physical and mechanical properties**

176   The water retention and swelling degree of the composite sponges as well as gelatin

177   stability are important for the application. The water retention abilities of sponges

178   were measured by Ma's method [36]. The sponge samples in the dry state were weighed

179   and recorded as W_{dry}. The sample was immersed in phosphate buffered saline (PBS,

180   pH 7.4) at 37°C for 24h, and then the wet sample was put into a centrifuge tube which

181   contained filter paper at the button. After centrifugation at 500 rpm for 3 min to

182   remove the water in the pores, the wet sample was weighted and recorded as W_{wet}.

183   The water retention ratio (W_{wr}) of sample was calculated by the following formula:

184    $$W_{wr}(\%) = [(W_{wet} - W_{dry}) / W_{dry}] \times 100\% \qquad (4)$$

185   The value was an average value of three measurements for each sample.

186   The swelling properties of the sponges were measured by the method as described

187   as follows. The sample was dried under vacuum at 60°C for 2 h and cooled in a

188   desiccator. Subsequently, the long side length of sample was measured and coded as

189   L_d. The sample was then immersed in PBS (pH 7.4) at 37°C for 24h until the sample

190   reached a constant size. After immersion, the sample was removed from PBS

191   immediately, and the length of the longer side L_w was measured. The swelling ratio (S)

192   of the sample was calculated by the following equation [37]:

$$S\ (\%) = (L_w - L_d)/\ L_d \times 100 \qquad (5)$$

194   The value was an average value of three measurements for each sample.

195   To further study the gelatin stability in sponge, the RCS/G2 sponges with the same

196   area were immersed in PBS (pH 7.4) at 37°C for days. At different time intervals, the

197   samples were taken out and freeze-dried. The nitrogen content changes of samples

198   were analyzed to calculate the gelatin migration ratio accounting for sponge.

$$\text{The gelatin migration ratio} = (W_{N0} - W_{Nt}) \times 6.25 \qquad (6)$$

200   Where W_{N0} and W_{Nt} were the nitrogen content values of sample before and after

201   immersed in PBS for t days, respectively.

202   Unconfined compressive test of the sponges was performed on a universal testing

203   machine (CMT6503, SANS Test Machine Co. Ltd., China) at room temperature with

204   a speed of 3 mm min^{-1}, according to ASTM 2166-66 [38]. Cylindral-shaped samples

205   with the size of 20 mm in diameter and 15 mm in height were used. All the samples

206   were provided with a uniform load, and the compressive strength and modulus values

207   were calculated within the linear range of the stress-strain curve. For each sample,

208   five specimens were tested and the results were averaged.

209   **2.5 *In vitro* bFGF release study**

210   The bFGF-loaded sponges were placed in PBS (pH 7.4) and incubated in a shaking

211   bath (150rpm) at 37°C for different time periods. Periodically, the suspensions were

212   collected for bFGF analysis, and the releasing media were added by fresh PBS.

213   Enzyme linked immunosorbent assay ELISA was used to determine the amounts of

214   bFGF releasing from sponges.

215   **2.6 Evaluation of cytocompatibility**

216   The fibroblasts cultures were used to evaluate the cytocompatibility of RCS and

217   RCS/G sponges. Fibroblasts were derived from rats as reported previously [39]. Briefly,

218   the skin area of the rats was shaved and disinfected using 75% ethanol. The skin

219   samples were aseptically removed from the rats and stored in Roswell Park Memorial

220   Institute (RPMI, Sigma-Aldrich Co., USA) medium, and were then minced into small

221   pieces to incubate for 3 h in 0.15% collagenase solution (37°C, 5% CO_2). After

222   incubation, the samples were centrifuged, the supernatant was discarded and the

223   precipitation was washed with Dulbecco's modified eagle medium (DMEM, Gibco

224   Co., USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco

225   Co., USA) for three times. The isolated cells were placed in DMEM medium. After 2

226   hours, the non-adherent cells were washed out. The cells were incubated in a

227   humidified incubator in an atmosphere of 5% CO_2 in air.

228   The RCS and RCS/G sponges (25mm in diameter and 2mm in thickness) were

229   sterilized by ethylene oxide gas at 55°C for 3h. The sterilized sponge samples were

230   incubated with DMEM medium for 24h before cell seeding, keeping the sponges

231   infiltrated with DMEM medium. Cells were seeded on each sponge with a cell density

232   of 1×10^4 cells per well and incubated in the incubator (37°C, 5% CO_2). After the

233   incubation time for 7 days, 14 days, 21 days, and 28 days, the proliferation of

234   fibroblasts on (or in) sponges were observed by SEM and fluorescence microscopy

235   separately.

236   The   cytotoxicity   of   the   sponges   was   measured   by   the   3-(4,

237   5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) method. After

238   incubation for 3 days, MTT (5mg/mL) solutions were added for 4 h, and then the

239   medium was replaced by Dimethyl Sulphoxide (DMSO, Sigma-Aldrich Co., USA).

240   The absorbance of DMSO solution was measured at 570 nm using the enzyme-labeled

241   instrument. The sample containing cells in the culture medium without sponges were

242   used as control. The relative cell viability in sponge was calculated using the

243   following equation:

244   Cell viability (%) = OD_{570} (sample) / OD_{570} (control) × 100   (7)

245   Where OD_{570} value (sample) is obtained in the presence of sponges and OD_{570} value

246   (control) is obtained in the absence of the sponges.

247   For the SEM observations, cell-seeded sponges were prepared as follows: firstly,

248   the cell-seeded samples were gently rinsed twice with PBS, followed by pre-fixation

249   with 2.5% glutaraldehyde (Sigma-Aldrich Co., USA) for 24h. Subsequently, the

250   samples were rinsed with PBS, followed by post-fixation treatment with 1% osmic

251   acid (Sigma-Aldrich Co., USA) for 2 h. After washed by PBS three times, the samples

252   were dehydrated in ethanol gradient (30%, 50%, 70%, 80%, 90%, 95% and 100%)

253   respectively. After drying in a critical point dryer, the cell-seeded samples were

254   sputter coated with gold, observed by field emission scanning electron microscope

255   (SIRION TMP, FEI Co., USA) and photographed. To prepare cell-seeded sample for

256   fluorescence microscopy observation, the cultured sponges were rinsed three times

257   with PBS, and the cells in sponges were labeled by calcein-AM and pro-pidium iodide

258   (PI) (Sigma-Aldrich Co., USA). Fluorescent images of the stained samples were

259   obtained using a florescent microscope (Axio Observer Z1, Zeiss Co., Germany).

260   **2.7 Animal Test**

261   The weights of twelve New Zealand rabbits (6 females and 6 males) were between

262   2.25 and 2.50 kg at the time of experiment, and were purchased from the Laboratory

263   Animal Center of Wuhan University and used in the animal test. The animals were

264   divided into two groups, each with 3 females and 3 males. Rabbits were anesthetized

265   by Nembutal injection (0.3ml/kg) for surgery and creation of wounds. Each group was

266   responsible for the partial-thickness and full-thickness cutaneous wound, respectively.

267   According to the depth of the wound, partial-thickness and full-thickness skin wounds

268   for group 1 and group 2 were separately prepared by removing skin area with surgical

269   scissors and forceps. For eliminating the interference of individual difference, four

270   skin wounds (20mm × 20mm) were made on dorsum of each rabbit and arranged

271 symmetrically. The wounds for rabbits were treated with RCS, RCS/G2 and

272 bFGF-RCS/G2 sponges with gauze as control, and then fixed with a polyurethane

273 film coated a binder material polyacrylic acid (Fulong Co., China) by surgical suture.

274 During the experiment, all rabbits showed good health condition. The wounds were

275 photographed to measure the areas of wounds at different time intervals. All animal

276 experiments in our study were carried out in accordance with the guideline and ethics

277 approval of the Laboratory Animal Center of Wuhan University.

278 **2.8 Histological Analysis**

279   At certain intervals, wound tissue samples were fixed in formalin and embedded in

280 paraffin (Sinopharm Chemical Reagent Co., Ltd., China) for routine histological

281 processing. The 3-5 μm sections of each paraffin block were stained with hematoxylin

282 and eosin (H&E) and observed by a light microscope.

283 **2.9 Statistical Analysis**

284   Statistical analysis was performed by the PASW Statistics 18 software package

285 (IBM Co., USA). The data were analyzed statistically with Duncan's multiple

286 range tests. The differences between groups were considered to be statistically

287 significant, P values <0.05 level, which indicates that the means are significantly

288 different when compared with the control group.

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3 Results and Discussion

3.1 The structure and morphology of sponges

The IR spectra of the RCS and RCS/G sponges are shown in Figure 1a. The OH stretching vibration bands around 3420 cm^{-1} in the RCS sponge were broadened and shifted to a lower wavenumber in the RCS/G sponges, suggesting the formation of new hydrogen bonds between the gelatin and cellulose macromolecules. Compared with RCS, the carbonyl group (amide I bond) at 1654cm^{-1} of gelatin in the RCS/G sponges shifted to a lower wavenumber. Moreover, the relative intensity at 1544cm^{-1} (amide Ⅱbond) of gelatin also appeared in RCS/G sponges, and the band broadened and shifted to lower wavenumbers, comparing with gelatin. These changes of relative intensity without new peak formation in RCS/G spectra indicated the existence of the intermolecular hydrogen bonds between gelatin and cellulose.

Figure 1b shows the solid-sate ^{13}C NMR spectra of RCS and RCS/G sponges. There were four significant peaks at 105.8, 88.0, 73.2 (75.0, 72.5), 63.3ppm in the spectrum of the RCS sponge, assigned to the C1, C4, C5 (C3, C2), C6 of the cellulose molecules. The peak assignment of gelatin was carried out in accordance with earlier reports [40]. No new peaks appeared in the spectra of the RCS/G sponge, suggesting that no derivatization occurred. The ^{13}C NMR spectra of RCS/G sponge revealed the same cellulose signal peaks, whereas that assigned to the glycine (Gly α) and hydroxyproline (Hyp γ) disappeared. Glycine and hydroxyproline were polar amino

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313   acids in gelatin, resulting in extraordinarily strong hydrogen bonds with cellulose. The

314   amino group of glycine could easily form hydrogen bonds with the hydroxyl group of

315   cellulose, leading to the decrease of signal expression in RCS/G sponge. On the basis

316   of the above results, it can be inferred that strong interactions between cellulose and

317   gelatin occurred through hydrogen bonding between the hydroxyl groups of cellulose

318   and the amino groups of gelatin in RCS/G.

319   The results of average nitrogen contents (W_N) and protein content (W_{Pro}) of the RCS

320   sponges are summarized in Table 1. The W_N value of RCS was 0, indicating that the

321   urea was completely removed from the hydrogel by washing treatment, so the W_N

322   values in RCS/G sponges could only be due to the existence of gelatin. With an

323   increase of the concentration of the gelatin solution, the W_N and protein content of the

324   RCS/G sponges increased. Therefore, the porous structure of cellulose hydrogels

325   could provide not only the cavities for the penetration of the gelatin molecules, but

326   also the compatible support to combine gelatin. Namely, cellulose hydrogel could

327   absorb abundant gelatin molecules, and the gelatin content of the RCS/G sponges can

328   be adjusted by changing the gelatin concentrations.

329   Figure 2 shows the photographs and surface SEM images of the RCS and RCS/G

330   sponges, and Figure S1 displayed the cross-section SEM images of sponge. All of the

331   sponges were of white color exhibited porous structure. The average pore size (d) of

332   the RCS sponge was about 97μm, and the pore size slightly increased with an increase

333   of the gelatin content (as shown in Table 1). This could be explained by the

334   hydrophilicity of gelatin which induced more H_2O molecules to be stored in the

335   RCS/G hydrogel, leading to the formation of bigger ice crystals in the RCS/G

336   hydrogel at -20°C, resulting in the larger pores. On the other hand, when gelatin

337   content achieved 46%, the average pore size of RCS/G15 sponge was reduced to 85

338   μm. It was not hard to imagine that large amount of gelatin attached on the pore walls

339   of cellulose, further leading to the increasing thickness of the pore walls and the

340   decreasing of pore space. Moreover, the porosity (P_r) values of the RCS and RCS/G2

341   sponges were above 90%, whereas excess gelatin filled into pores led to the sharp

342   decrease of P_r. According to the above results, the RCS/G sponge possessed macro-

343   and micro porous architecture, which could keep the gas exchange on the wound

344   interface.

345   To further determine the distribution of gelatin in RCS/G sponge, the cross-section

346   of the RCS/G2 sponges were divided as the external (①), internal (②) and internal

347   center (③) regions, as shown in Figure 3a. The W_N contents of samples had difference,

348   demonstrating that the gelatin distribution in the RCS/G2 sponge deceased from

349   external to center (Figure 3b). Setting RCS sponge as a control, the N signal in

350   element mapping images of the RCS/G sponge was from gelatin (Figure 3c). These

351   element mapping images were consistent with the results of element analysis. This

352   difference gelatin distribution in sponge owed to the gelatin diffusion gradient.

353   Combined with SEM images and element mapping images, large amount of gelatin

354   was found to attach on the pore walls of cellulose.

3.2 Water retention ability, gelatin stability and mechanical properties of sponges

356   The water retention ability of wound dressing is relative to absorb body fluids, to

357 transform cell nutrients and metabolites, and to keep a moist environment for

358 promoting wound repair. After absorption in the PBS solution for 2 min, the RCS/G

359 sponge keep abundant solution and exhibited flexibility, which was beneficial for

360 absorbing the exudates and fitting the wound surface (Figure 4a). It was noted that

361 after immersion in PBS, gauze displayed solution leakage due to its loose weave

362 structure (Figure S2). The water retention ratio values (W_{wr}) of the RCS and

363 composite sponges were in the range of 700-800% (Table 1), suggesting that the

364 sponges could hold water as much as several times of their own weight. Therefore, the

365 macro- and micro pores of the sponges could not only hold abundant liquids, and also

366 keep moist environment for effective wound repair. Furthermore, the presence of

367 gelatin with amino and carboxyl groups also increased the water retention ability

368 (Table 1). Usually, excessive change in size can induce separation of the dressing

369 from the skin outside of the wound area, and the leakage of fluid, leading to

370 enlargement of the wound due to the invasion of microorganisms.[41] In our findings,

371 the S values of RCS/G2 and RCS/G8 sponges were below 10%, which could be

372 accepted as wound dressings. Therefore, the RCS/G sponges could have both

373 excellent water retention capacity and dimensional stability, indicating the good

374 candidates for wound dressings. As in Figure S3 shown, the migration ratio of gelatin

375 migrated from sponge after immersed in PBS (pH 7.4) at 37°C for 14 days accounts

376 for 4.6% of the sponge. According to the result of the average nitrogen content of

377 RCS/G2 (29.2%), less than 16% gelatin was dissolved in PBS. The large amount

378 gelatin still kept stability in the composite sponge due to the hydrogen bonding

379   interactions between gelatin and cellulose, which could contribute to the combination

380   with growth factors.

381   As a candidate for wound dressings, the mechanical properties of sponges are

382   important. The results of mechanical testing are listed in Table 2. The compressive

383   strength values of RCS and RCS/G sponges were much higher than that of chitosan [42],

384   alginate [43, 44], collagen [42] and silk [45]. The compressive moduli and strength of the

385   composite sponges increased with the increase of the gelatin content, indicating

386   further the strong interactions between gelatin and cellulose. Therefore, the RCS and

387   RCS/G sponges exhibited excellent mechanical properties, which were beneficial to

388   be used as dressing or scaffold for wound healing.

389   **3.3 The bFGF sustained release and cytocompatibility of sponges**

390   Basic fibroblast growth factor (bFGF) can stimulate the proliferation of fibroblasts

391   and endothelial cells to promote angiogenesis and wound healing. However, the direct

392   use of bFGF usually results in relatively low activity due to the short half-life of

393   bFGF [46]. By ELISA assay and calculations, as given the same amount of bFGF

394   (500ng) for RCS and RCS/G2 sponges with the same size (cylindrical shape of

395   sponges with diameter of 15 mm and thickness of 2 mm), the bFGF can connect with

396   them, and the loading on the RCS/G2 sponge (330±10 ng) was higher than that on

397   RCS sponge (274±12 ng). This could be explained that the gelatin containing amino

398   acids   has   more   bonding   points   with   bFGF   through   the   electrostatic   and

399   hydrogen-bond interactions [47,48]. To figure out the bFGF release from the sponges and

400   evaluate the possibility as a functional wound dressing with releasing growth factors,

401   their release abilities were evaluated by the *in vitro* test. Compared with RCS, bFGF

402   in RCS/G2 exhibited a sustained and steady release and the cumulative release mass

403   was approximately 84% until 21 days (as shown in Figure 4b). Therefore, sustained

404   release of bFGF in RCS/G sponge contributed to the strong interactions between

405   gelatin and bFGF. For clinical applications, the RCS/G2 sponge was found to be more

406   desirable since the release of the growth factor matched the wound healing period and

407   provided a sustained release to the target area.

408   To evaluate the applicability of sponges for wound healing, the cytocompatibility of

409   sponges was studied by *in vitro* cell culture test. The morphologies of fibroblasts

410   cultured on sponges for 3 days are shown in Figure 5a-d. The fibroblasts attached and

411   spread well both on the RCS and RCS/G sponges, indicating that the environment of

412   the sponges was beneficial for cells growth. To further clarify their cytocompatibility,

413   the MTT test was also performed (Figure S4). The cell viability values of the RCS,

414   RCS/G2 and RCS/G8 sponges were all above 80%, confirming that these sponges did

415   not have acute cytotoxic effect. Moreover, the cell viability values of RCS/G2 and

416   RCS/G8 were higher than RCS, showing the effect of gelatin to promote cell growth.

417   However, RCS/G15 sponge with 46% gelatin content exhibited only 70% cell

418   viability. This could be explained that the excess gelatin can block the pathways for

419   transformation of cell nutrients and metabolites. As shown in Figure 5e-h, the cells

420   displayed more profuse proliferation on RCS and RCS/G2 sponges and almost

421   occupied the pore walls of sponges after culture for 14 days, as a result of the role of

422   the porous structure. To further assess the cell growth in sponges, fluorescence

423   microscopy was used to observe the calcein-AM and PI labeled fibroblasts in sponges

424   (Figure S5). The sponges displayed a number of live cells, in consistent with the result

425   of MTT test and SEM observation. Therefore, the gelatin incorporation with an

426   appropriate amount (< 46 wt%) into cellulose could further promote cell growth,

427   leading to good biocompatibility.

428   **3.4 Evaluation of *in vivo* wound healing**

429   The healing of wounds treated with RCS, RCS/G2 and bFGF-RCS/G2 sponges

430   were evaluated by *in vivo* wound healing experiments on rabbits, using gauze as a

431   control (Figure S6). Figure 6a-b shows the wound healing observation and the

432   changes of the partial-thickness wound area treated with sponges. The healing rate of

433   gauze-treated wound was the slowest, compared with all other sponges. After 12 days,

434   the area of wound treated with bFGF-RCS/G2 sponges was 0.6 cm^2, exhibiting 85%

435   wound closure. In comparison with RCS sponge, the bFGF and RCS/G2 sponges

436   exhibited, respectively, the best and better healing effect. This could be due to the

437   excellent ability of bFGF in promoting wound healing, and gelatin still retained some

438   signals such as Ary-Gly-Asp (RGD) sequence of collagen, promoting cell adhesion,

439   proliferation and differentiation [49]. Figure 6c-d shows the wound healing observation

440   and the changes of the full-thickness wound area treated with sponges. Clearly, the

441   healing rate of the gauze-treated wounds was also lower than all sponges. The wound

442   treated with the bFGF-RCS/G2 sponge also displayed the fastest healing rate.

443   Interestingly, the complete wound healing time for the wounds treated with

444   bFGF-loaded sponges was 7 days faster than that treated with traditional cotton gauze

445　for full-thickness coetaneous wound model, which could substantially ease the pain

446　for the patients. On the whole, cellulose based-sponges (RCS, RCS/G2 and

447　bFGF-RCS/G2) exhibited much more effective wound repair abilities than gauze.

448　Especially, since the RCS sponges were derived from cellulose, same as gauze. Why

449　is there such clear difference in wound repairing between them? It was not hard to

450　imagine that the macro- and micro-porous structure with thin pore wall in the sponges

451　played an important role in the improvement in absorbing the wound exudates, the gas

452　exchange and to maintain a moist environment for wound repair. However, gauze

453　weaved from cellulose fibers with small specific area, which not only ineffectively

454　absorbs excess exudates, but also fails in water retention (Figure S2).

455　　As shown in Figure 7a-b, the RCS/G2 sponge was removed easily from the wound,

456　keeping intact the wound surface. However, cotton gauze adhered to the wound

457　surface when removed, leading to secondary injuries (Figure 7c-d). The excellent

458　water retention ability of the sponge and the minimal contact interface between the

459　sponge and the wound (due to thin pore wall of about 1-5μm estimated by SEM) were

460　the possible reasons that facilitated the easy-peeling ability of sponges as showed in

461　(Figure 7e). Very different from sponge, the minimal contact interface between gauze

462　and skin wound was a cotton fiber with the diameter of about 300-400 μm.

463　**3.5 Histological Analysis**

464　　Generally, wound healing is a complex process involving a series of interrelated

465　and overlapped steps, such as hemostasis, inflammation, cell migration and

466　proliferation, angiogenesis, neovascularization, extracellular matrix (collagen)

467   production and remodeling [12]. In this work, the wound tissues were biopsied and

468   processed for histological examination to evaluate the progress of wound healing.

469   Figure 8 shows the histopathological changes of the wound tissues as a function of

470   time. For partial-thickness wounds on day 3, the wounds treated with bFGF-RCS/G2

471   sponges exhibited less inflammation cells and neovascularization scores than the

472   wound treated with cotton gauze and RCS sponge. Furthermore, compared with

473   cotton gauze, more new capillaries around the inflammatory cells were observed in

474   the wounds treated with RCS sponges, indicating more effective wound repair by

475   sponges. After 7 days, a continuous nascent epithelial layer was formed, namely

476   epidermis, on the wounds treated with the RCS, RCS/G2 and bFGF-RCS/G2 sponges

477   with an appearance more mature than those treated with gauze. Especially, after

478   treated with the bFGF-RCS/G2 sponge, the bundles of collagen fibers were observed

479   to be loose and wavy in the dermis tissue, suggesting that the excellent promotion

480   provided by bFGF. For full-thickness wounds, a large number of inflammatory cells

481   were observed in the wounds after 3 days. However, a continuous nascent epithelial

482   layer and some collagen fibers started to form over the wounds with the treatments of

483   RCS/G2 and bFGF-RCS/G2 sponges after 19 days, indicating complete healing. In

484   particular, the great formation of blood vessels and well-proliferated fibroblasts

485   occurred on the wound for bFGF-RCS/G2 sponges. The inflammatory cell number on

486   the wounds treated with RCS sponge was clearly decreased on day 19. However, the

487   wound treated with cotton gauze had many inflammatory cells, indicating the healing

488   process was still in the inflammatory phase. On the basis of the results mentioned

489 above, both the full thickness and partial-thickness wounds indicated that the RCS/G2

490 and bFGF-RCS/G2 sponges as potential dressings could provide suitable environment

491 for a rapid and complete wound healing. It was demonstrated that the macro- and

492 micro-pores with thin wall in the sponges provided the cavities and channels for gas

493 exchange, exudates absorption and water retention, this is very important for the

494 wound healing. In addition, gelatin in the sponge not only promoted fibroblasts

495 proliferation and differentiation, but also facilitated the sustained release of bFGF,

496 resulting in the formation of neovascularization and collagen fiber.

497   In view of the results mentioned above, a scheme describing the mechanism that

498 bFGF-RCS/G sponge promotes wound healing is proposed in Figure 9. The

499 bFGF-RCS/G sponge was consisted of cellulose as porous support and gelatin as filler,

500 supported by SEM observation in Figures 2b1-c4 and S1. The macro- and micro-

501 pores of the sponges induced the gas exchange, absorb excess wound exudates and

502 maintain moist environment around the wound area, supported by the results in

503 Figures 6-8. Gelatin combined to the pore walls of cellulose not only promoted cell

504 growth, but also achieved a sustained release of bFGF to stimulate collagen synthesis

505 [50], promoted cell proliferation and neovascularization [51], leading to a rapid and

506 effective wound repair (as supported by the results in Figures 3-8). The bFGF and

507 gelatin were immobilized in the cellulose sponge through hydrogen bonding, retaining

508 their inherent biocompatibility, so they can significantly promote wound healing.

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4. Conclusion

513   The cellulose/gelatin composite sponges for wound healing were successfully

514   fabricated by immersing the cellulose hydrogels in the gelatin solutions and followed

515   by freeze-drying process via a simple, green and cost-effective pathway. Compared

516   with cotton gauze, the cellulose sponge having the same chemical structure effectively

517   promoted wound healing, as a result of the macro- and micro porous architecture.

518   Furthermore, the gelatin and basic fibroblast growth factor were immobilized in the

519   cellulose sponge through hydrogen bonding to retain their inherent biocompatibility,

520   leading to the excellent repairing efficacy. The incorporation of gelatin not only

521   promoted cell growth, but also achieved the sustained release of growth factors from

522   sponge, resulting in rapid wound healing. For the full-thickness coetaneous wound

523   model, the complete wound healing time for the wounds treated with bFGF-loaded

524   cellulose sponges was 7 days faster than that treated with gauze. The pores with thin

525   wall in the sponges played an important role to hasten up the healing process, as a

526   result of satisfying the requirements of oxygen permeability, controlled water vapor

527   evaporation and wound exudates absorption. The cellulose composite sponges

528   exhibited the highly effective wound healing, this is important for the successful

529   application of the wound dressings based on cellulose materials.

530

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Figure captions

611

Figure 1. FT-IR (a) and solid state ^{13}C NMR (b) spectra of RCS, gelatin and RCS/G sponges.

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Figure 2. The photographs of RCS and RCS/G sponges at the dry state (a), and the surface SEM images of RCS(b1, c1), RCS/G2 (b2, c2), RCS/G8 (b3, c3) and RCS/G15 (b4, c4), the bar for b1-b4:20 μm and the bar for c1-c4:10μm.

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Figure 3. Scheme to describe the external (①), internal (②) and internal center (③) regions of the cross-section of the RCS/G2 sponge (a), the nitrogen content (W_N, wt %) (b), the SEM images and the nitrogen element mapping images (c) of the RCS and RCS/G2 sponges, corresponding to the ①, ② and ③ regions in (a).

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Figure 4. The photograph of RCS/G2 sponge after being immersed in PBS solution for 2 min (a), the bar is 1 cm. Cumulative bFGF release from RCS and RCS/G2 sponges (b), and individual points represent the mean values ± standard deviation (SD) from three sponges samples.

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Figure 5. RCS (a, e), RCS/G2(b, f), RCS/G8(c, g) and RCS/G15(d, h) sponges seeded with fibroblasts for 3 days (a-d) and 14 days (e-h) of culture. The bar is 50μm.

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Figure 6. The wound closure of partial-thickness (a) and full-thickness (c) wounds treated with gauze, RCS sponge, RCS/G2 sponge and bFGF-RCS/G2 sponge for days. The initial area of all wounds on each rabbit was 4 cm^2, and the bar is 2 cm. Evaluation of the partial-thickness (b) and full-thickness wound area (d). The data represent the mean ± SD of six rabbits. (* $P<0.05$, compared to control for same day).

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Figure 7. RCS/G2 sponge has easy-peeling property (a), preventing the secondary injury when removed from the wound (b); cotton gauze adheres to the wound surface when peeled (c), leading to the secondary injury (d); the schematic of interface between the sponge and the skin wound (e).

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Figure 8. Histological images of skin tissues stained by hematoxilin and eosin (H&E) method dissected in the post-operative day 3 (a1-d1) and day 7 (a2-d2) for partial-thickness wound, and in day 3 (a3-d3) and day 10 (a4-d4) for full-thickness wound, respectively. The groups were RCS (b), RCS/G2 (c), bFGF-RCS/G2 (d) sponges treated wound, and the medical cotton gauze (a) treated

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638   wound was used as control. Scar bar is 100μm. The arrow, triangle and star represent blood vessel,

639   epidermis, collagen fibers, respectively.

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641   sponges.

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643   (P_r, %), water retention ratio (W_{wr}, %), the average pore size (d, μm) and swelling ratio (S, %) of

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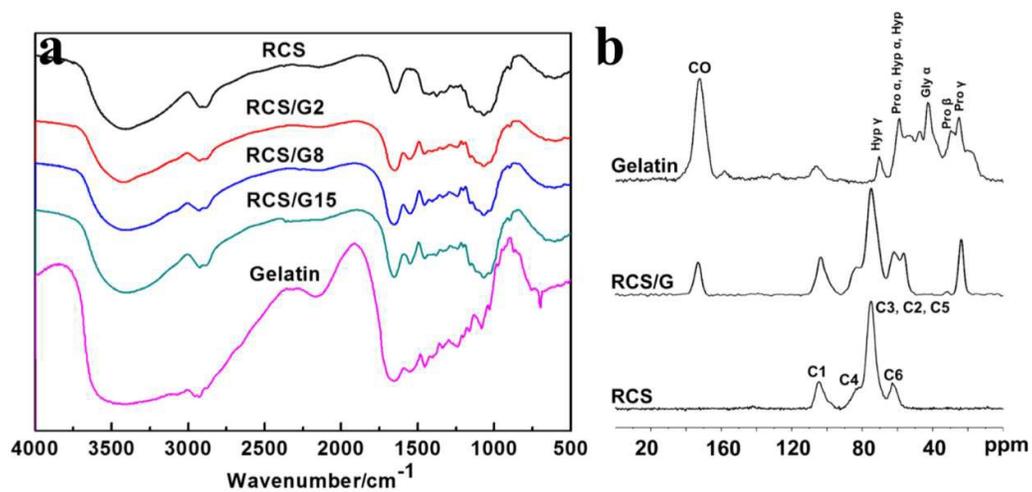
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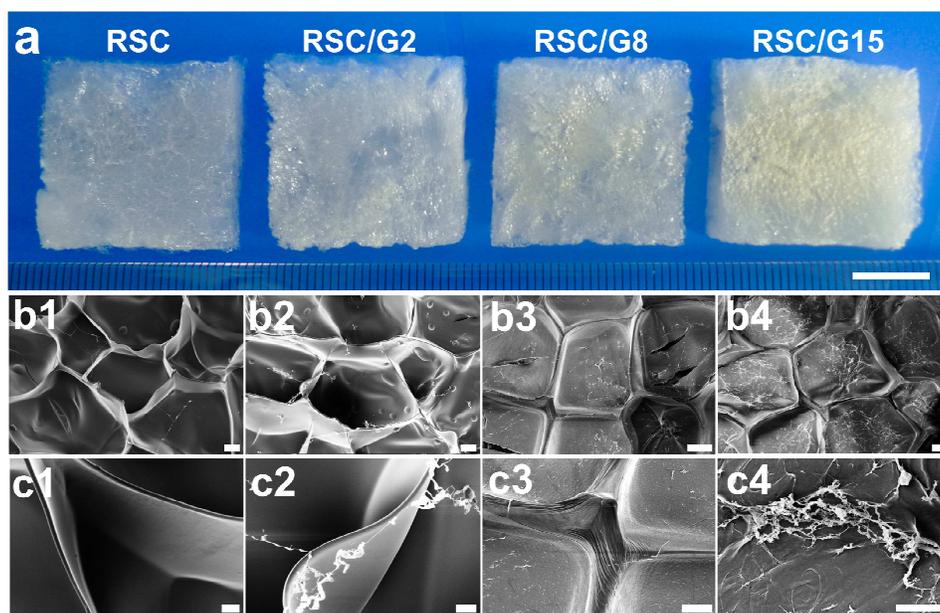
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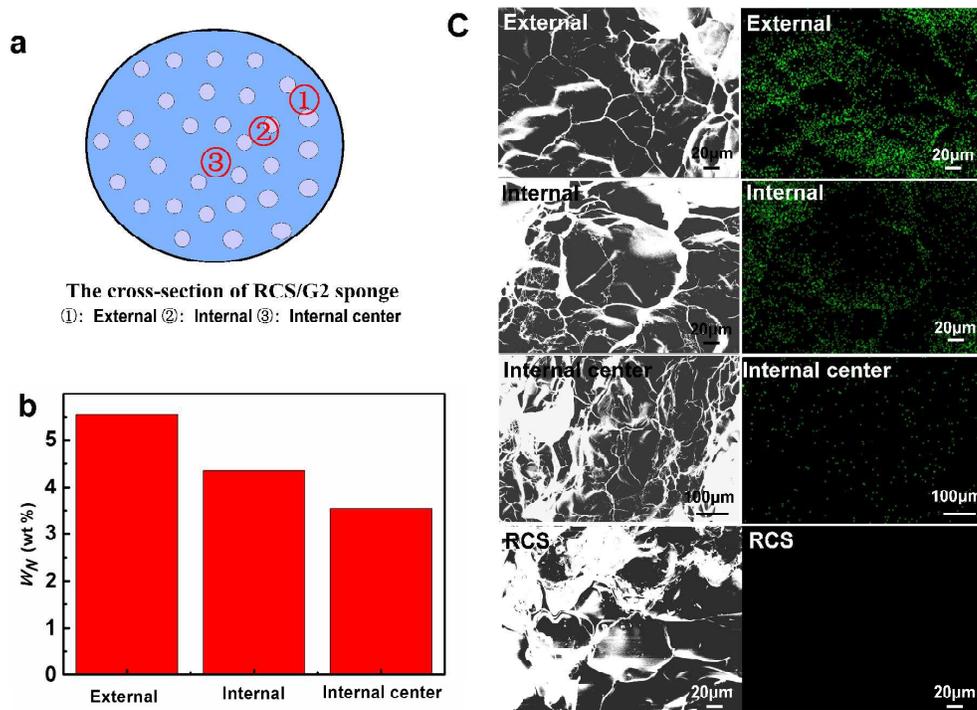
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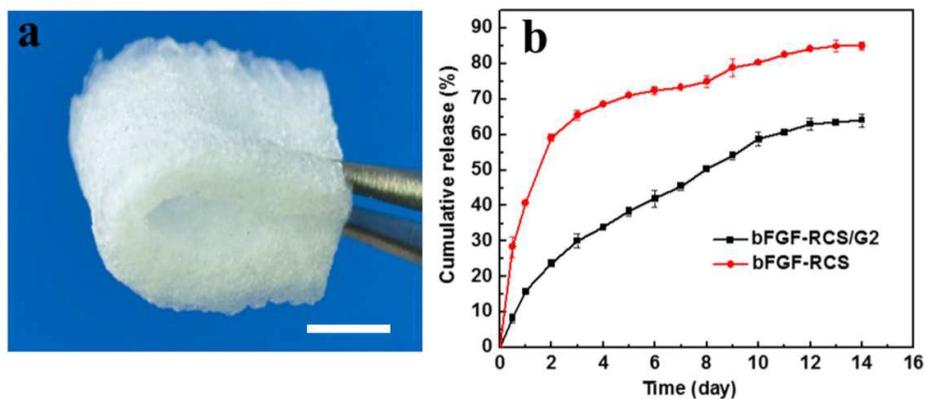
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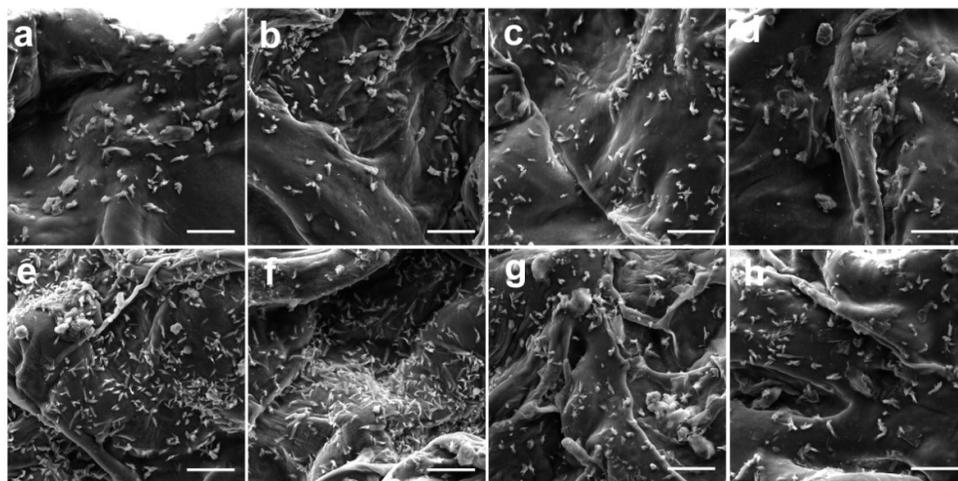
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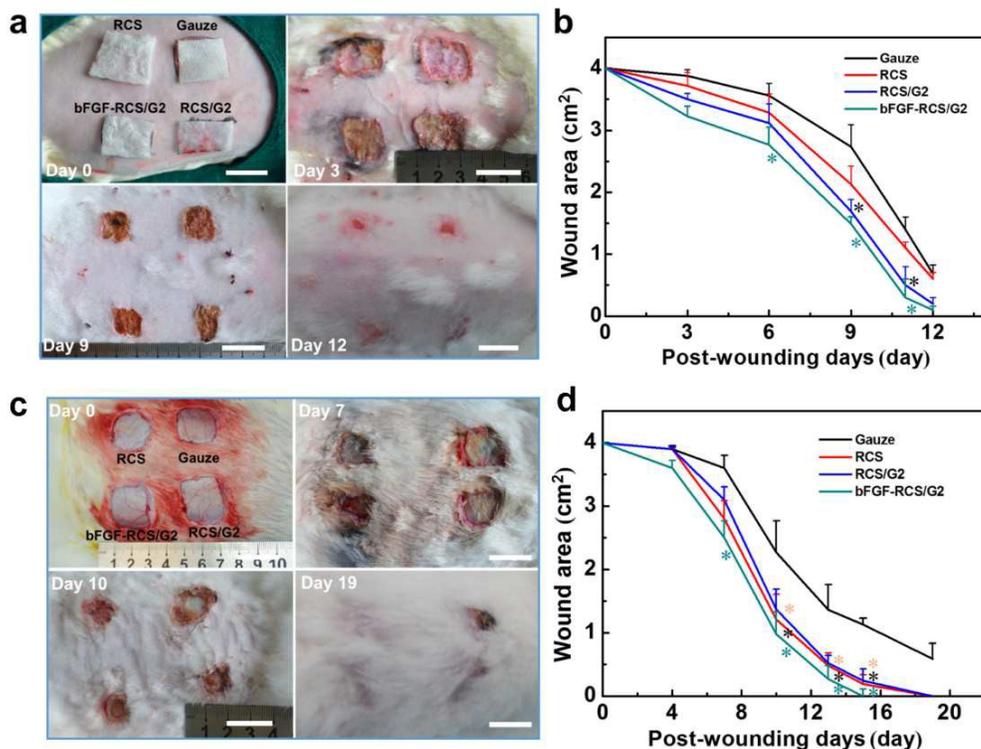
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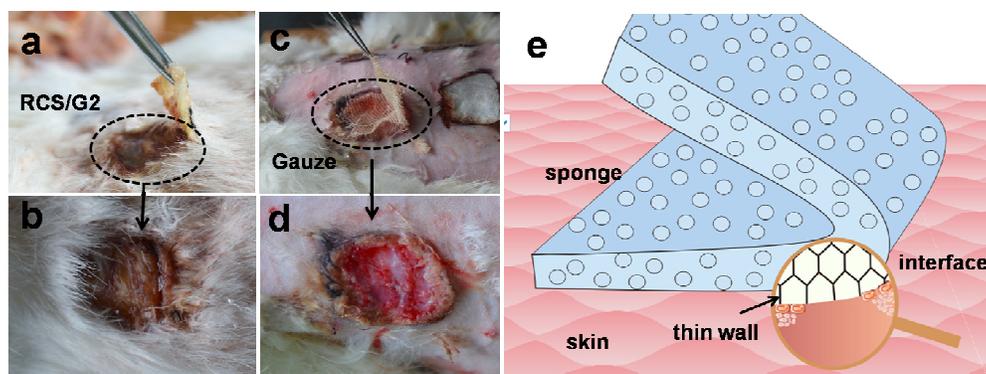
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706　the RCS and RCS/G sponges.

Samples	c (%)	W_N (%)	W_{Pro} (%)	P_r (%)	d (μm)	W_{wr} (%)	S (%)
RCS	0	0	0	97.3	97.0	717	1.00
RCS/G2	2	4.67	29.2	94.4	102	768	5.00
RCS/G8	8	5.61	35.1	85.7	110	774	8.60
RCS/G15	15	7.36	46.0	70.8	117	787	17.0
Gelatin	100	15.1	94.3	-	-	-	-
Gauze	-	-	-	-	-	110	-

707　*c*: the concentration of gelatin solution .

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710   **Table 2.** The compressive moduli (kPa) and compressive strength (kPa) of RCS sponge, RCS/G

711   sponges, and porous scaffolds or sponges derived from other natural polymers.

Samples	Compressive moduli (kPa)	Compressive strength (kPa)
RCS	160±10	110±5
RCS/G2	490±12	266±7
RCS/G8	600±10	360±9
RCS/G15	750±16	400±10
Collagen[42]	150	15
Collagen/Chitosan[42]	500	30
Chitosan[42]	750	45
Aginate[43, 44]	230-1300	-
Silk[45]	170-220	20-80

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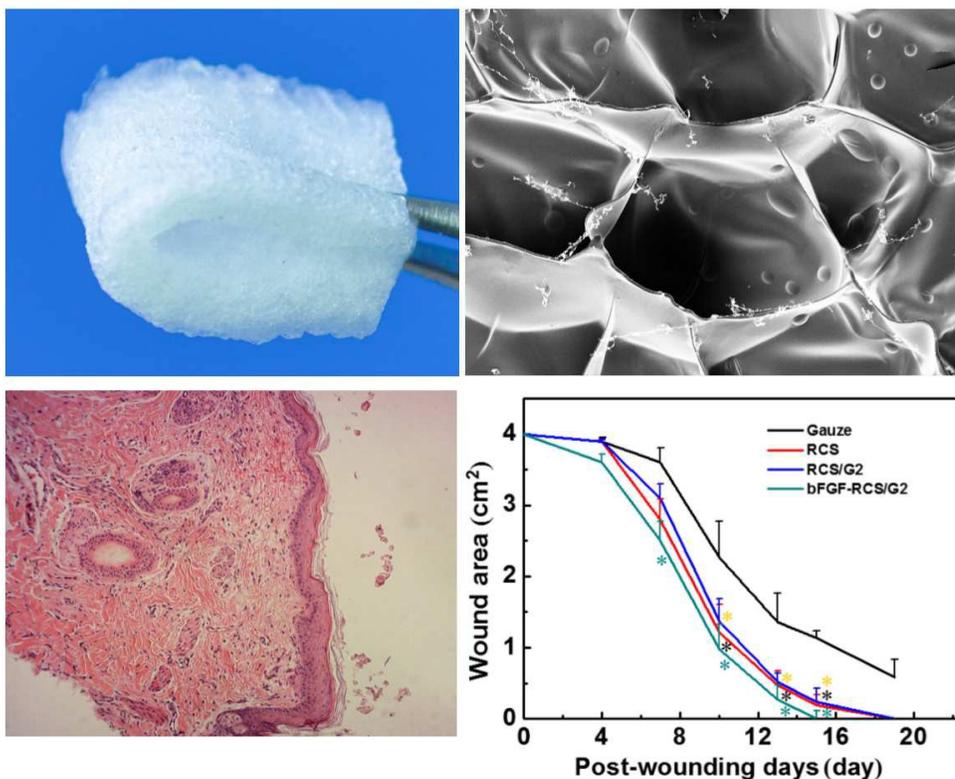
The cellulose sponges loading gelatin and fibroblast growth factor as wound dressing were constructed directly from cellulose solution via a simple, green and cost-effective pathway. The cellulose sponges effectively promoted wound healing, as a result of the macro- and micro porous architecture.