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A porous sodium polyacrylate-grafted chitosan xerogel for severe hemorrhage control made from one-pot reaction

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Control of severe hemorrhage remains a challenge. Successful hemorrhage control depends on the speed and quality of blood clot formation. Fast deprivation of water from blood leads to the concentration of blood cells and coagulation factors and thus triggers blood clot formation. This inspired us to develop a new hemostatic material. In this study, we grafted sodium polyacrylate (SPA) onto the backbone of chitosan (CTS) and cross-linked with methacrylic anhydride–modified polyethylene glycol (MAAPEG) to provide flexible and elastic inter-chain connection between SPA and CTS chains in the presence of a blowing agent to achieve a porous structure. By a simple one-pot-reaction, we fabricated a soft, elastic porous xerogel sponge which could reach maximum water absorbency of 180 in less than 200 seconds. This SPA-co-Chitosan xerogel sponge demonstrated superior hemostatic effect in thromboelastography (TEG®) test and in a rabbit lethal extremity arterial bleeding model in comparison with zeolite granules, kaolin gauze and chitosan granules. Furthermore, this hemostat worked as a whole to transfer external pressure to the bleeding area and was adhesive to wet wound tissue to seal the bleeding site. In general, the SPA-co-CTS sponge demonstrated a fast and powerful hemostatic effect both *in vitro* and *in vivo*, which was superior over existing commercial products. It might be a promising first-aid device for severe hemorrhage control.

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1 **1. Introduction**

2 Hemorrhagic and its serious complications
3 remains the leading cause of half of all deaths on
4 the battlefield and the second leading cause of
5 civilian trauma deaths as it has been for

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6 centuries.[1-3] Uncontrolled hemorrhage is also
7 responsible for late morbidity and mortality.
8 Massive blood loss leaves victims vulnerable to
9 hypothermia, coagulopathy, infection, and
10 multiple organ failure.[4] Effective hemostatic
11 methods will improve survival and reduce the
12 long term complications of massive blood loss. It
13 is estimated that up to one-third of all deaths
14 from exsanguination could be prevented by more
15 effective hemorrhage control strategies.[5]
16 Research efforts of the last decade have
17 produced numerous hemostatic adjuncts that
18 exert their effect in a variety of ways. Some of
19 them have been approved by FDA for clinical
20 applications and demonstrated significant
21 improvement in hemorrhage control by US army
22 such as dry fibrin sealant dressing (DFSD)，
23 rapid deployment hemostat (RDH)， HemCon
24 Chitosan Dressing (HC)， QuikClot (QC) and
25 Combat Gauze (CG) and so on. However, none
26 of them can fulfill all of the qualities of ideal
27 hemostatic agent. An ideal hemostatic agent for
28 austere prehospital/battlefield use, whose
29 characteristics were described by Pusateri and
30 colleagues from the U.S. Army Institute for
31 Surgical Research (USAISR) and the Uniformed
32 Services University of the Health Sciences,[6] has
33 not been developed so far.

34 Biologically, blood coagulation is a process of
35 active interaction between blood coagulation
36 function and the flowing dynamics of the blood,
37 which to some extent, resembles thrombosis,
38 with common consensus as Virchow's triad: 1)
39 endothelial damage, 2) abnormal blood flow, and
40 3) hypercoagulability. The final arm of the triad,
41 hypercoagulability, although the mechanisms
42 underlying this category of risk are numerous
43 and often poorly-understood, is characterized by
44 higher concentration of coagulation factors, such
45 as thrombin, factor VIII, etc. During the
46 pathological process of thrombosis, the
47 progressively narrowed artery lumen leads to
48 slow-down of bloodstream flow which allows for
49 higher chance of circulating thrombin and
50 platelets to adhere to the rough surface of the
51 plaques and accumulate to higher
52 concentrations. Concentrated thrombin then
53 turns fibrinogens into fibrins which form a fibrosic
54 network and trap blood cells to form thrombus.

55 This inspired us to develop a new hemostatic
56 materials system that contains covalently bonded
57 chitosan (CTS), sodium polyacrylate (SPA) and
58 polyethylene glycol (PEG) in a porous network.
59 To prepare such a hemostatic sponge, we
60 grafted SPA onto CTS to achieve a
61 macromolecule in which CTS serves as the

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62 backbone while SPA provides super-absorbency. 63 Such an integrated system has not been 64 reported to the best of our knowledge in the 65 literature, although individual components have 66 been studied,[6-18] As presented here, the new 67 materials show greatly enhanced in vitro and in 68 vivo hemostatic properties over existing 69 commercial products.

70 2. Materials and methods

71 2.1 Materials

72 Chitosan (degree of deacetylation ≥95%) was 73 obtained from Qingdao Haihui Biotechnology 74 Co., Ltd, China. polyethylene glycol (PEG MW: 75 ~20,000) was obtained from Beijing Seasky 76 biotechnology Co.Ltd. Methacrylicanhydride (MA, 77 chemically pure) was obtained from Beijing 78 HengyeZhongyuan Chemicals Co., Ltd, 79 China.Acrylic acid, hexane, sodium bicarbonate, 80 ammonium persulphate (APS), dichloromethane 81 and sodium hydroxide (chemically pure) were 82 obtained from Sinopharm Chemical Reagent 83 Co.,Ltd, China. Avitene (Microfibrillar Collagen 84 Hemostat) was a commercial product of Davol 85 Inc., Subsidiary of C. R. Bard, Inc. USA, Kaolin 86 gauze (QCCG, QuikClot Combat Gauze) and 87 Zeolite granules (QCZG, QuikClot) were 88 commercial products from Z-Medica, USA, 89 chitosan granules (Celox) was a commercial 90 product from SAM Medical Products, USA.

91 2.2 Preparation of SPA-co-CTS xerogel 92 sponge in one-pot reaction manner

93 1% PEG (v/v) and 2% CTS (v/v) respectively 94 reacted with 1mL methacrylic anhydride (MAA) 95 through N-acylation of anhydride groups to 96 produce MAAPEG, a derivative of PEG, and 97 MAACTS, a derivative of chitosan, both 98 containing C=C double bonds group. SPA was 99 then grafted onto the backbone of MAACTS at 100 $60^{o}C$ via melt free radical copolymerization using 101 ammonium peroxo-disulfate (APS) as initiator 102 and MAAPEG as crosslinker. $NaHCO_3$ was 103 incorporated as the blowing agent by reacting 104 with residual acids brought into the reaction 105 system as dissolvent of chitosan and leftover of 106 incomplete copolymerization reaction(**Fig 1 (1～ 107 5)**). The sponge was washed three times by in 108 50%, 70% and 90% ethanol at room temperature 109 to remove salt ions and unreacted acids. The 110 end product was PEG-crosslinked, sodium 111 polyacrylate grafted CTS which was labeled as 112 SPA-co-CTS xerogel sponge.

113 2.3 Characterization

114 FT-IR spectra of SPA-co-CTS sponge were 115 taken using Perkin Elmer spectrum 100 FT-IR

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116 Spectrometer (American Perkin Elmer Co.). To
117 exclude the influence of water, samples were
118 fully dried prior to submission to FTIR analysis.
119 Morphology of the sponge with/without contact
120 with fresh blood was examined under scanning
121 electron microscope (SEM, Czech Republic FEI
122 Co.Ltd, operating at 10kV).

123 **2.4 Quick water absorption efficiency and**
124 **swelling in vitro**

125 In vitro water absorption study of sponge was
126 carried out in different aqueous environment
127 including deionized water (H_2O), Normal Saline
128 (NS), phosphate buffer (PBS), simulated body
129 fluid (SBF) and fetal calf serum (FCS). Briefly, for
130 swelling kinetics examination, dried xerogel
131 sponge samples were immersed in the five
132 above-mentioned medium at 37°C for a
133 predetermined time, and then wiped with
134 moistened filter paper and weighed. For pH-
135 sensitivity test, dried xerogel sponge samples
136 were placed in media with various pH values
137 (prepared by mixing deionized water with HCl or
138 NaOH) at 37°C for 24 h to reach equilibrium,
139 then wiped with moistened filter paper and
140 measured using pre-equilibrated acidimeter.

141 The swelling ratio (SR) is calculated from the
142 following equation (SR-1):

143

144
$$SR = \frac{W_s - W_d}{W_d} \quad \text{SR-1}$$

145

146 Where W_d and W_s are the weights of dried and
147 swollen xerogel sponge samples, respectively.

148 All the above experiments were carried out in
149 triplicate, and the swelling ratios are reported as
150 the average of three separate experiments ± SD
151 (n=3).

152 **2.5 Hemostatic property assay in vitro**

153 Fresh blood samples were drawn from 4
154 healthy volunteers (There was informed consent
155 before we took the blood samples from the
156 volunteers. Moreover, these experiments were
157 approved by the Ethics Committee of 307
158 Hospital of Chinese PLA). Whole blood
159 thromboelastogram (TEG-5000, Haemoscope
160 Corp, US) analysis was run in a CFMS^{TM}
161 thromboelastography. Because hemostatic
162 sponge induces blood clotting very quickly within
163 the material, only the free blood away from the
164 clot can be used for TEG analysis, we had the
165 protocol modified. A final concentration of 0.1%
166 of xerogel sponge was mixed with 2 ml of fresh
167 blood from healthy donors and 300 μl of free
168 blood was aspirated for TEG analysis 30

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169 seconds after mixing. All TEG analyses were
170 performed by one technician within 10 minutes
171 after sample collection. The tests for negative
172 control were performed without xerogel sponge,
173 and positive control was performed with Avitene.
174 The modified TEG assay only reflects the
175 secondary effect of xerogel sponge on the
176 coagulation property of whole blood after initial
177 clot formation.

178 **2.6 Hemostatic property assay in vivo**

179 The hemostatic efficacy of xerogel sponge was
180 evaluated in a model of lethal extremity arterial
181 bleeding.[19, 20] All experiments were performed in
182 accordance with the Academy of Military Medical
183 Sciences Guide for Laboratory Animals. Thirty
184 New Zealand white rabbits (wt 3 kg) were used
185 at the age of 4 months and fasted 24 h before
186 assay. The rabbits were anesthetized by
187 intravenous injection of sodium pentobarbital (45
188 mg/kg) and then a unilateral femoral artery was
189 exposed (**Fig 5A**). A severe extremity arterial
190 hemorrhage was then produced by puncturing
191 the femoral artery with a 16 G needle (**Fig 5B**).
192 Free bleeding was allowed for 5 s and
193 hemostatic materials were applied with manual
194 compression employed immediately and the
195 compression was hold for 2 minutes (**Fig 5C** and
196 **D**). 32-layer standard gauze (SG) as well as
197 commercial products Kaolin gauze (QCCG,
198 QuikClot Combat Gauze, Z-Medica, USA),
199 Zeolite granules (QCZG, QuikClot, Z-Medica,
200 USA) and chitosan granules (Celox, SAM
201 Medical Products, USA) served as controls to
202 TiHS. Macroscopic observation of immediate
203 bleeding and secondary bleeding during the
204 subsequent 10 minutes of observation time was
205 recorded. All animals received only one piece of
206 hemostatic materials and one compression. At
207 the end of the study period, each groin was
208 opened and visually examined (**Fig 5E** and **F**).
209 Liquid and clotted inguinal blood was suctioned
210 or absorbed by pre-weighted cotton pad to weigh
211 the blood loss. The samples that resulted in
212 successful hemorrhage control were carefully
213 removed and immediately freeze-dried and
214 sectioned for examination of blood infiltration and
215 clot formation (**Fig 5G** and **H**).

216 **2.7 Biocompatibility of SPA-co-CTS sponge**

217 The samples of hemostatic sponge were
218 immerged in serum-free DMEM (1 g sample in
219 100 ml medium) and incubated at 37 ^{o}C for 48 h.
220 Murine fibroblasts were cultured in medium
221 containing 0%, 1%, 10% and 100% of
222 hemostatic sponge extracts and supplemented
223 with 10% FCS. MTT was used to evaluate cell
224 viability and proliferation. Standard tests for

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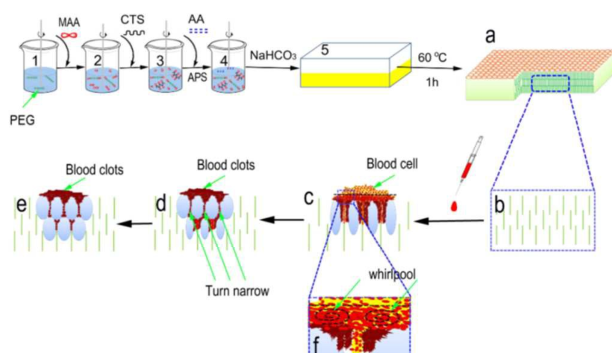
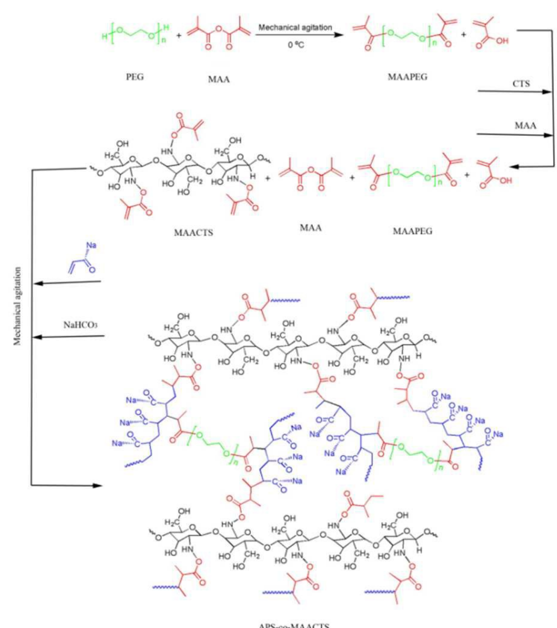
225 irritation and delayed-type hypersensitivity were
226 performed using hemostatic sponge extracts in
227 Guinea pigs (wt 200g) according to ISO 10993-
228 10:2010 Biological evaluation of medical devices
229 Part 10.



230

231 **Fig.1** Synthesis and schematic diagram of
232 hemostasis mechanism of hemostatic sponge.
233 (Hemostatic sponge was synthesized in a one-
234 pot-reaction manner(1~5), and the sponge
235 exerted its hemostatic effects(c~e)).



236

237 **Scheme1.** One-pot reaction synthesis of
238 hemostatic sponge. PEG and CTS reacted with
239 MAA in the same system to derive MAAPEG and
240 MAACTS (1～3). In presence of APS as initiator,
241 MAAPEG as crosslinker and $NaHCO_3$ as blowing
242 agent, SPA was grafted onto MAACTS, and a
243 porous SPA-co-MAACTS supermacromolecule
244 was developed (4～6), which had a porous
245 morphology of sponge (7,8).

246 *Statistics:* All quantitative data were expressed
247 as means ± standard deviations. The data were
248 assessed using one-way analysis of variance
249 (ANOVA), comparing the differences between
250 groups by Student's t-tests (t-tests). *p*-values
251 less than 0.05 were considered statistically
252 significant.

253 3. Results and discussion

254 3.1 Structural, chemical and morphological 255 characterization

256 The hemostatic sponge we fabricated is
257 flexible, elastic, but mechanically resistant to
258 compression, bending and stretching. It has a
259 porosity of 71 ± 0.534% (**Fig 2B**). Adjacent
260 channels are interconnected and, what's more,
261 tapered channels ranging 200~500 μm in

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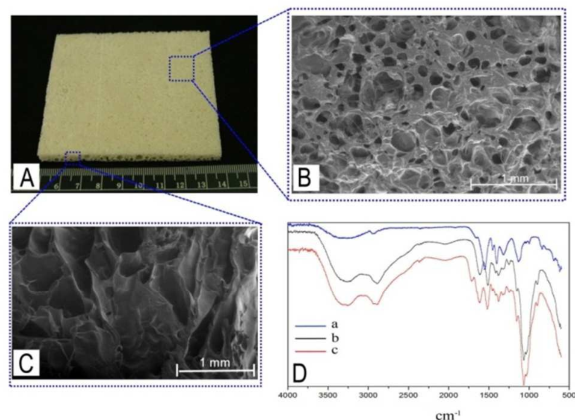
262 diameter formed naturally due to the bottom-to-
263 top gas escape pathway (**Fig 2C**).

264 SPA-co-CTS was characterized using FTIR
265 spectra pure chitosan and MAACTS were also
266 analyzed for comparison (**Fig 2D**). The
267 absorption bands for pure chitosan were
268 identifiable with vibrations, i.e. NH stretching at
269 3350 cm^{-1}, C-H stretching at 2890 cm^{-1}, NH_2
270 bending at 1513-1614 cm^{-1}, and C-O stretching
271 at 894-1153 cm^{-1}. Furthermore, the spectrum for
272 MAACTS contained ester bond with
273 characteristic absorption peak at 1714 cm^{-1}, C=O
274 characteristic absorption peak and N-H bending
275 vibrations at 1617 cm^{-1} and 1519 cm^{-1} in amide
276 bond. There was C-O-C stretching at 1101 cm^{-1}
277 from PEG in the SPA-co-CTS sponge, apart from
278 amide bond.

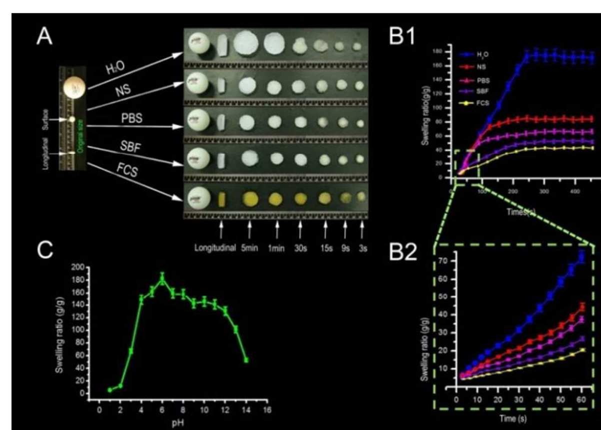


279

280 **Fig.2** Characterization of xerogel sponge. (A:
281 The photo of hemostatic sponge, B: SEM
282 images of the surfaces section morphology,
283 C: SEM images of the longitudinal section
284 morphology, D: The FTIR of SPA-co-CTS)

285 **3.2 Water absorbability and swelling**

286 The SPA-co-CTS proved to be highly
287 superabsorbent (**Fig 3**). The maximal swelling
288 ratio was 180 in distilled water. No significant
289 superabsorbent capacity variation was found in
290 water with different pH levels. Significant
291 decrease of absorbency only occurred at pH
292 levels less than 2 and above 12 (**Fig 3C**). Ionic
293 environment had some effects on the water
294 absorbency of the hemostatic sponge (**Fig 3B**).



295

296 **Fig.3** Super absorbency property of SPS-co-CTS
297 xerogel sponge. Xerogel sponge displayed
298 different swelling ratio in water, normal
299 saline(NS), PBS, SBF and fetal calf serum (FCS)
300 at 37°C (A). The sponge could reach equilibrium
301 of absorbency within 240 seconds in all the
302 media tested (B1). In the initial 60 seconds the
303 sponge could reach from 20 to 70 times of its

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304 original mass in different media tested (B2). pH-
305 sensitivity of hemostatic sponge swelling in
306 various pH values (C).
307

308 In PBS without Ca^{2+}, the xerogel sponge
309 reached maximal swelling equilibrium at 82, in
310 simulated body fluid (SBF) and fetal calf serum
311 (FCS) with Ca^{2+} supplement at physiological
312 level, the maximal swelling equilibrium was 50
313 and 41, respectively, indicating a minor decrease
314 in comparison with PBS. This was similar to
315 other superabsorbent macromolecules.[21]
316 However, we had particular interest in the
317 swelling dynamics of our xerogel sponge,
318 especially in the initial minutes or even seconds.
319 We were happy to see that the xerogel sponge
320 could absorb water very quickly, reaching
321 maximal swelling capacity within 200 seconds
322 (**Fig 3B1**). In our study, the prepared SPA-co-
323 CTS sponge might work through a multimodal
324 mechanism. In detail, firstly, similar to that of
325 zeolite absorbent, the hemostatic capacity of
326 SPA-co-CTS sponge was correlated with the
327 absorption of water from blood flowed in the
328 wound site, which would assist in primary
329 hemostasis by concentrating the clotting factors
330 to accelerate the turnover of coagulation
331 cascade and the subsequent formation of blood
332 clot. The mechanism of absorbs water was
333 formed in two stages. The first stage could be
334 attributed to the interconnective channel and
335 high specific surface area of the water absorbing
336 core made of channel in the sponge (as shown
337 in Fig. 2 B and C). When the sponge
338 encountered the blood, the interconnective
339 channels fleetly absorbed the blood. The second
340 stage could be attributed to the abundant
341 carboxyl groups on the molecule of SPA which
342 could combine with molecule of water with
343 hydrogen bond to form hydration water. When
344 the interconnective channels fleetly absorbed the
345 blood in the first stage, the water in the blood
346 was chained by carboxyl groups on the molecule
347 of SPA and thus constricted the blood.

348

349 **Table 1 .** Analysis of blood coagulation
350 efficiency

materials	*R* [d] *(min)*	*K* [e] *(min)*	*Angle deg* [f]	*MA* [g] *(mm)*
a	7.63 ± 0.45	3.80 ± 0.22	51.47 ± 1.52	52.20 ± 2.41
b	3.41 ± 0.24	2.63 ± 0.33	57.33 ± 0.71	54.51 ± 2.41
c	3.56 ± 0.25	2.23 ± 0.28	60.50 ± 0.65	59.70 ± 1.45

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351 a Negative control were performed without materials

352 b Positive control were performed with Avitene.

353 c The SPA-co-CTS sponge xerogel samples

354 d Represents period of time latency from start of test to
355 initial fibrin formation. This represents the standard
356 clotting studies.

357 e Represents –time taken to achieve a certain level of
358 clot strength (where r-time = time zero) –equates to
359 amplitude 20 mm.

360 f Measures the speed at which fibrin build-up and cross-
361 linking take place, and hence assesses the rate of clot
362 formation.

363 g MA is a direct function of the maximum dynamic
364 properties of fibrin and platelet bonding via GPⅡb / Ⅲ
365 a and represents the ultimate strength of the fibrin clot.

366

367 **3.3 Whole blood coagulation efficiency**

368 Because blood coagulation happens so 369 quickly and blood clot forms immediately within 370 and on the surface of the xerogel sponge upon 371 contacting with blood, classical blood tests like 372 prothrombin time (PT), activated partial 373 thromboplatin time (APTT) and thrombin time 374 (TT) were difficult to manage. We then tried 375 thromboelastography (TEG®) test, a point-of- 376 care hematological test that was used to define 377 the viscoelastic properties of whole blood.[22,23] 378 Although blood clot formed immediately within 379 the material and only the rest blood could be 380 used for TEG analysis, the TEG value of R-time 381 and K-time were significantly reduced by xerogel 382 sponge samples compared with the negative 383 control group ($p < 0.05$), and the Angle deg and 384 maximum amplitude (MA) value were also 385 increased. We ascribed this coagulation- 386 promoting phenomenon to the sustainable water 387 absorption effect of xerogel sponge that resulted 388 in hypercoagulability. Considering the fact that 389 the TEG test only partially reflected the 390 hemostatic effect of xerogel sponge, we could 391 see that xerogel sponge could induce blood 392 clotting in a dramatically shortened period of time. 393 Contact activation of the blood plasma 394 coagulation cascade by xerogel sponge was 395 investigated by TEG test (**Table 1**). Experimental 396 results showed that the measured R-time and K- 397 time were significantly reduced by xerogel 398 sponge samples compared with the standard 399 gauze control ($p < 0.05$), and Angle deg and MA 400 were slightly increased. Comparably, the xerogel 401 sponge samples exhibited the same pronounced 402 effect on R-time, K-time, Angle deg and MA 403 values with Avitene, where no statistically 404 significant difference was observed between 405 them ($p > 0.05$).

406 When dropping fresh artery blood onto the 407 surface of the xerogel sponge, we observed that, 408 upon contacting with blood, the porous 409 multichannel structure of the sponge which 410 provided maximum contact surface between 411 blood and the sponge, immediately sucked in the 412 blood (**Fig 4A**). The blood was soon absorbed

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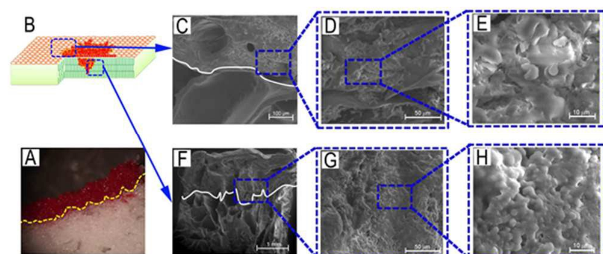
413 but neither spread too much horizontally nor
414 penetrated too much vertically, although the
415 structure of the hemostatic sponge was porous
416 and well-interconnected and the material itself
417 was highly hydrophilic. Instead, as shown in **Fig**
418 **4B,C,F,** water in the blood was absorbed into the
419 wall of the channels within seconds and the
420 blood became highly concentrated and blood clot
421 formed very quickly, making the blood could no
422 longer spread or penetrate. Meanwhile, the wall
423 of the channels became thicker and thicker, just
424 like the atherosclerosic plaque-occupied artery
425 lumen, due to the continuous absorbency of
426 water. As a result, the channels were narrowed
427 until closed and the blood was concentrated to
428 clotting. Blood cells were entrapped in
429 concentrated plasma fibrins, adhered strictly to
430 the material surfaces and could no longer be
431 distinguished from their original morphology (**Fig**
432 **4D, E, G, H**).

433 

434 **Fig.4** Blood clot formation on hemostatic sponge.
435 Blood drop on hemostatic sponge coagulated
436 immediately (A, B). Clear boundary could be
437 seen between clots and the sponge material
438 either from the vertical (C, D, E) or horizonal (F,
439 G, H) direction. Blood cells were entrapped in a
440 fibrin mesh and coalesced into a blood clot (D, E,
441 G, H).

442 **3.4 Hemostatic property assay in vivo**

443 QuikClot zeolite granules (QCZG), QuikClot
444 Combat Gauze (QCCG) and Celox have been
445 well tested in animal models and clinical trials.[24-
446 31] QCZG could rapidly absorb water in a non-
447 chemical reaction at the site of bleeding, which
448 effectively concentrated platelets and clotting
449 factors and promoted coagulation. The
450 disadvantages in causing burns from exothermic
451 reactions and difficulties of being completely
452 removed from the wound following application,
453 led to the formation of inflammatory granulomas
454 or abscesses because of foreign body
455 reactions,[32] it was proved reliable in the
456 treatment of uncontrolled life-threatening
457 hemorrhage during Afganstan war and Iraq
458 war.[33,34] QCCG absorbs water into internal pores
459 in a non-chemical reaction and activates Factor
460 XII to initiate platelet adhesion, thus triggers the
461 clotting cascade.[35] QCCG has no adverse effect
462 of exothermic burns and is recommended by the
463 US military's Committee on Tactical Combat

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464 Casualty Care (CTCCC) as the first-line
465 hemostatic agent for use in treatment of severe
466 hemorrhage that cannot be controlled by a
467 tourniquet.[36,37] Celox is a chitosan-based product
468 that performs more excellent hemostatic effect
469 than HemCon bandage.[39-40] As shown in **Table 2**,
470 in SG-treated group, hemostasis was not
471 achieved and no animals survived at the end of
472 the experiment. In QCCG-treated group,
473 hemostasis was achieved in 3 of 6 rabbits but
474 secondary bleeding occurred 5 minutes after
475 manual pressure was removed. Complete blood
476 penetration of the gauze was obvious in all the 6
477 animals. In QCZG-treated group, hemostasis
478 was achieved in 4 of 6 rabbits but secondary
479 bleeding occurred in 1 of the 4 rabbits within 10
480 minutes after manual pressure was removed. In
481 Celox-treated group, hemostasis was achieved
482 in 4 of 6 rabbits but secondary bleeding occurred
483 in 2 of the 4 rabbits within 10 minutes after
484 manual pressure was removed and hematoma
485 was observed in one of the 2 rabbits without
486 secondary bleeding. In the hemostatic sponge-
487 treated group, hemostasis was successfully
488 achieved (**Fig 5A-C** and supporting video
489 information), no secondary bleeding occurred
490 during the subsequent 10 minutes of observation
491 time course and after the sponge was carefully
492 removed from the injury site (**Fig 5D**).
493 Significantly higher amount of blood loss was
494 observed in the successful cases of the QCCG,
495 QCZG and Celox groups than the hemostatic
496 sponge group. We also noticed that, different
497 from the control materials, hemostatic sponge
498 adhered to the wound tissue very strongly, and
499 sealed the wound area completely, leaving no
500 access for further bleeding. Although sticky to
501 the wound tissue, the sponge could be removed
502 easily from the bleeding site and the wound
503 tissue was neat and clean, leaving no visible
504 blood clot (**Fig 5E, F**). This was very important
505 because complete sealing of the bleeding site
506 decreased the unnecessary contact of the rest
507 part of the sponge with blood and thus
508 contributed in several aspects to the successful
509 control of hemorrhage. First, it kept the central
510 area of the sponge as the only battle field to fight
511 bleeding and reserved the hemostatic capacity of
512 the rest part of the sponge, making the
513 hemostatic effect sustainable. Second, it was
514 beneficial for the sponge to keep its mechanical
515 property and work as a whole to fully transfer
516 external pressure on the sponge to the bleeding
517 site. Finally, the complete sealing of the bleeding
518 site resulted in significant decrease of blood loss
519 and thus played an important role in maintaining

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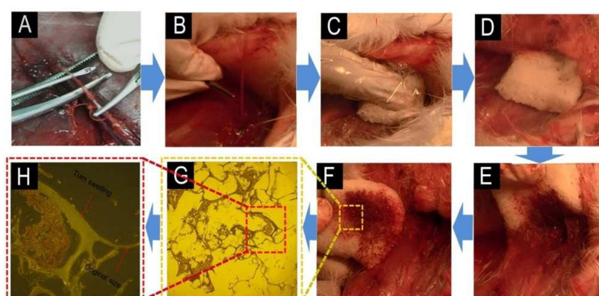
520 blood pressure and reducing risk of hemorrhagic
521 shock as well as infection. These data
522 demonstrated that the hemostatic sponge we
523 developed was superior in controlling severe
524 hemorrhage compared with currently most-
525 recommended commercial products.

526 

527 **Fig.5** Hemostasis in a rabbit lethal extremity
528 arterial bleeding model. The femoral artery was
529 exposed and punched with a 16 G needle (A) to
530 cause a spurting bleeding (B). The wound was
531 covered and squeezed with hemostatic sponge
532 to staunch bleeding in inguinal cavity (C). No
533 bleeding could be observed after 2 minutes of
534 manual pressure (D).The sponge was removed
535 from the injury site at 10 min post-treatment and
536 sticky sealing of the wound area was visualized
537 (E). Clean and neat wound was observed after
538 removal of hemostatic sponge (F) and only a
539 small area of the sponge that directly contacted
540 with the bleeding site was found partially
541 penetrated with blood (F). Microscopy images of
542 freeze-dried hemostatic sponge sections
543 indicated swelling of the channel walls of the
544 sponge and blood clots formed within the
545 channels (noted that most blood cells were lost
546 during sectioning of the freeze-dried blood-
547 containing hemostatic sponge as showed in G
548 and H).

549 All the *in vitro* and *in vivo* studies
550 demonstrated that, the SPA-co-CTS xerogel
551 sponge exerted its hemostatic effect through a
552 dynamic embolization process. Upon contacting
553 with blood, the porous structure of the sponge
554 provided the maximal surface contact, the blood
555 that flowed into the tapered channels of the
556 sponge was soon concentrated by the
557 superabsorbent sponge, and the swollen sponge
558 further narrowed the channels until they were
559 completely blocked. The water-sucked sponge
560 began to gel and became sticky, elastic and hard
561 to penetrate so that the saturation of water-
562 absorbency only happened in the very thin layer
563 of the blood-contacting part. While the rest of the
564 sponge remained unsaturated or even
565 untouched with blood, keeping great potency for
566 further hemostasis and the ability of continuously
567 transferring water from saturated area to
568 unsaturated area to stabilize blood clot (**Fig 4C**).
569 At the same time, when the sponge was pressed
570 onto the bleeding site, the remaining blood or

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571 tissue fluid in the wound tissue made the sponge
572 highly adhesive to the wet tissue and thus sealed
573 the peripheral tissue, leaving great force of
574 resistance for bleeding.
575

576 **Table 2.** Hemostatic efficacy in a rabbit lethal
577 extremity arterial bleeding model.

	Hemostatic materials				
	SG [a]	QCCG [b]	QCZG [c]	CELOX [d]	TiHS
1)	0/6	3/6	4/6	4/6	6/6
2)	6/6	3/6	4/6	5/6	6/6
3)	–	2/3/6	1/4/6	2/4/6 [e]	0/6
4)	--	21.4 ± 1.9*	20.4 ± 1.9*	20.2 ±1.2*	7.1 ± 0.7

578 1) Survival
579 2) Complete hemostasis
580 3) Secondary hemorrhage
581 4) Blood loss (g)
582 [a] 32-layer standard gauze
583 [b] Kaolin gauze (QuikClot Combat Gauze, Z-Medica,
584 USA)
585 [c] Zeolite granules (QuikClot, Z-Medica, USA)
586 [d] Celox (SAM Medical Products, USA)
587 [e] Hematoma formed in one of the two rabbits without
588 secondary hemorrhage

589 Additionally, the xerogel sponge works as a
590 whole during the whole process of application.
591 This is especially important when used for
592 control of severe artery bleeding which needs
593 mechanical force for compression to stop blood
594 flow.[41] In such cases, the xerogel material should
595 be physically resistant to compression and thus
596 transfer external force onto the bleeding site.
597 This feature not only makes this xerogel sponge
598 an ideal material to combine external pressure
599 and internal hemostatic capacity for severe
600 hemorrhage control, but also favors complete
601 removal from the wound site after use, leaving
602 nothing that may cause safety concerns. The
603 adhesive property of hemostatic sponge is also
604 potentially useful as a temporary sealing material
605 for rupture of visceral organs.

606 Furthermore, the xerogel sponge can be
607 fabricated in a one-pot-reaction manner, making
608 the scalable production easier and more
609 economic. No toxic or environment pollution
610 waste is released during the manufacture of the
611 hemostatic sponge product. This may
612 significantly lower the cost and improve the
613 stability of the quality of end products. Once
614 sealed in a pack away from light and water, the
615 hemostatic sponge may have a long shelf life.
616 Taken together, the hemostatic sponge we
617 developed fulfills all the critical standards of ideal
618 hemostatic agent for pre-hospital/battlefield use,

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as Pusateri and colleagues have described: (1) capability to stop large arterial vessels and venous bleeding within 2 mins of application when applied to an actively bleeding wound through a pool of blood; (2) no requirement for mixing or pre-application preparation; (3) simplicity of application by wounded victim, companions, or medical care personnel; (4) light and durable; (5) long shelf life in extreme environments; (6) safe to use with no risk of injury to tissues or transmission of infection; (7) inexpensive.[6]

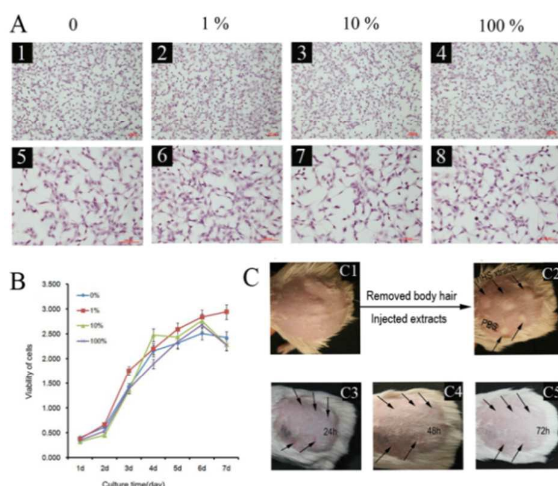

Fig.6 Biocompatibility of hemostatic sponge. A: Microscopy images of mouse fibroblasts cells cultured with 0%, 1%, 10% and 100% water extracts at three days. B: Murine fibroblasts cultured in medium containing 0%, 1%, 10% and 100% water extracts of TiHS displayed no significant difference in cell viability and proliferation. C: Intradermal injection of 100% water extracts of hemostatic sponge did not induce significant tissue inflammation in comparison with normal saline (NS) during observation time of 24, 48 and 72 h.

We also examined the biocompatibility and toxicity of hemostatic sponge both *in vitro* and *in vivo*. Hemostatic sponge extracts demonstrated no adverse effect on the morphology and proliferation rate of 3T3 cells (**Fig 6**). Intradermal injection of hemostatic sponge extracts also elicited no redness or edema. These data indicated that the hemostatic sponge had good biocompatibility and was safe as a medical device.

4. Conclusions

In conclusion, we have developed a new hemostatic materials system that can be prepared via a simple one-pot-reaction and used as a first aid device for control of severe arterial bleeding. This SPA-co-MAACTS copolymer system concentrates platelets and exerts its hemostatic effect in a dynamic thrombosis manner. Comparing with existing commercial products, this material shows greatly enhanced properties of blood concentration, wound sealing and external pressure application as well as after-use removal. Although further preclinical

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667 and clinical studies are required for validation of
668 its superiority of hemostatic efficacy than other
669 materials available, hemostatic materials is
670 highly promising as a hemostatic agent for
671 control of severe hemorrhage in both military and
672 civilian trauma settings.

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