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### **► To cite this version:**

Melika Nourbakhsh, Payam Zarrintaj, Seyed Hassan Jafari, Sayed Masoud Hosseini, Shayan Aliakbari, et al.. Fabricating an electroactive injectable hydrogel based on pluronic-chitosan/aniline-pentamer containing angiogenic factor for functional repair of the hippocampus ischemia rat model. *Materials Sciences and Engineering: C*, 2020, 117, pp.111328. 10.1016/j.msec.2020.111328 . hal-03491191

**HAL Id: hal-03491191**

**<https://hal.science/hal-03491191>**

Submitted on 22 Aug 2022

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# Fabricating an Electroactive Injectable Hydrogel Based on Pluronic-Chitosan/Aniline-Pentamer Containing Angiogenic Factor for Functional Repair of the Hippocampus Ischemia Rat Model

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35 **Abstract**

36 The hippocampus, a critical cerebral region involved in learning and memory formation, is  
37 especially vulnerable to ischemic defect. Here, we developed an injectable electroactive hydrogel  
38 based on pluronic-chitosan/aniline-pentamer with proper conductivity around  $10^{-4}$  S/cm to  
39 achieve the functional repair of the hippocampus following the ischemic defect. FTIR, DSC, and  
40 TGA measurements were performed to assess the chemical structure and thermal stability of the  
41 synthesized hydrogel. Aniline pentamer decreased the swelling capacity, degradation, and drug  
42 release rate. Further, contact angle, melting point, and gelation time of hydrogels were enhanced  
43 by addition of aniline oligomer. Moreover, it endowed the on-demand electro-responsive drug  
44 release. Injectability of hydrogel was evaluated by rheometry, exhibiting proper gelling time at  
45 the body temperature. The ionic/electrical conductivity and desired *in vitro* biocompatibility with  
46 PC12 cells were also achieved. Injection of VEGF-loaded electroactive hydrogel in the  
47 hippocampal ischemic animal model resulted in decreased infarction volume, improved  
48 hippocampal dependent learning, and memory performance. Taken all together, the results  
49 confirmed that fabricated injectable hydrogel would be a suitable candidate for ischemic defect  
50 treatment and can lead to new horizons to treat neurological disorders.

51 **Keywords:** Chitosan; Pluronic; Aniline oligomer; Injectable hydrogel; VEGF; Hippocampal  
52 ischemia

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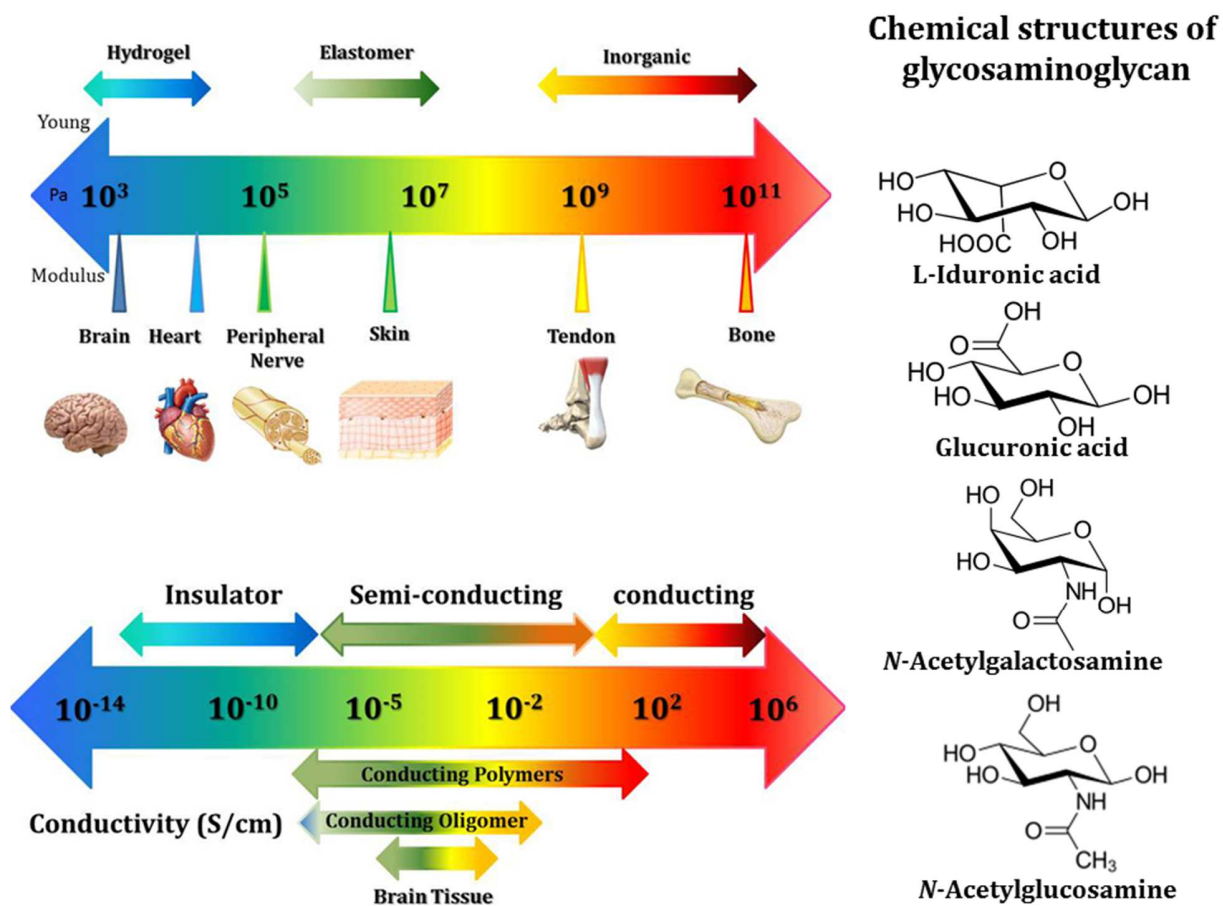
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## 57           **1. Introduction**

58   The hippocampus, a complex brain structure, is placed deep into the temporal lobe with the  
59   ability to regulate the hypothalamic activities [1], emotional behavior [2], and spatial navigation  
60   [3]. Many defects like ischemia and traumatic brain injury cause injury to the hippocampus,  
61   which can be partly restricted into one or some subareas [4]. For this, investigating the  
62   functionality of the hippocampus attracts significant attention. The hippocampus is also known  
63   as the region in the brain that is able to regenerate even in adulthood [5]. Any damage in  
64   different portions of the hippocampus alters the rate of neurogenesis, which causes anterograde  
65   amnesia for declarative memory and affects the relational and declarative memories formation  
66   [6]. Neurogenesis in adults usually occurs after any external stimulation that affects the functions  
67   of neurons. For the hippocampal regeneration, many therapeutic strategies, including the  
68   administration of antidepressant drugs, deep stimulation of the brain, and exercise, have been  
69   performed [7]. Recently, the achievements in applicable tools and increasing the knowledge  
70   about the process of nerve regeneration opened a new avenue to develop better strategies for  
71   optimal neuronal repair. For instance, by using nerve tissue engineering, it is possible to combine  
72   cells and materials in a substrate to provide proper support for the regeneration of nerve injuries  
73   [8-10]. Among various scaffolds that support the neural repair, hydrogels exhibit the better  
74   neural regeneration [11, 12]. Hydrogels are permeable to oxygen/nutrition and have low  
75   interfacial tensions that bypass the barriers against migrations of cells into the surrounding  
76   tissues [13]. Moreover, hydrogels are capable to deliver the encapsulated reagents e.g., growth  
77   factors, drugs, and cells to promote the desired function [14-18]. Among the wide range of  
78   hydrogels, electroactive hydrogels formed by conductive polymers are more used for nerve  
79   tissue engineering [19-21]. The need for using electroconductive polymers for better stimulations  
80   neural regenerate raises from the inherent electrical properties of nerve cells. Electroactive  
81   biomaterials have been introduced as a new generation of smart systems [22, 23]. The redox  
82   switching behavior of conductive polymers causes to control the drug release rate using electrical  
83   stimulation [24, 25]. Scrutinized survey on tissues mechanical and electrical properties lead us to  
84   select the proper material for tissue regeneration [26, 27]. It is obvious that the brain modulus is  
85    $\sim 10^4$  Pa [28], and brain tissue conductivity has been reported  $\sim 10^{-3}$  to  $10^{-4}$  S/cm [29] (Figure 1).  
86   Here, we fabricated an injectable hydrogel based on Pluronic-chitosan (CS)/aniline-pentamer.  
87   Pluronic (poly (ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide)) as a tri-block

88 copolymer contains hydrophilic and hydrophobic segments which hydrophobic interaction above  
89 lower critical solution temperature (LCST) between poly(propylene oxide) segments results in  
90 gel formation *in situ* (thermosensitive behavior) [30].

91 CS is a common polysaccharide produced by the deacetylation of chitin and formed by  
92 glycoside reactions between glucosamine and N-acetylglucosamine subunits. The primary amino  
93 groups of CS make them easily dissolve in acidic solutions and thus facilitate chemical  
94 modification [31-33]. CS as a natural polymer has been widely used in tissue engineering  
95 because of its acceptable interaction with cells and tissues [34-36]. It has been reported that the  
96 conductive matrix of CS increase the neural cell activities and regeneration [37-39]. Various type  
97 of conductive materials have been used in tissue engineering [40]. However, some conducting  
98 materials due to the arduous synthesis method, poor processability, and prolonged maintenance  
99 in the body resulted in some difficulties during their usage [41, 42]. Overcoming such problem,  
100 conductive oligomers have been utilized to fabricate the bio conductive materials [43]. In this  
101 study, CS was electro-activated using aniline-pentamer segments because aniline oligomer is  
102 highly stable, cost-effectiveness, biodegradable, and capable of switching between resistive and  
103 conductive states by doping/dedoping process [43, 44]. Furthermore, to improve the recovery of  
104 ischemia imperfection of the hippocampus, vascular endothelial growth factor (VEGF) was used.  
105 Localized VEGF delivery has proven greatly advantageous for tissue regeneration by enhancing  
106 neovascularization [45-47]. VEGF also act as a protein with neuroprotection and neurogenesis  
107 properties. VEGF has a central role in the reduction of infarct size, improving neurological  
108 performance, and angiogenesis promotion [48]. However, most of the growth factors are water-  
109 soluble, which are possible to rapidly diffuse into the culture media [49]. It was reported that  
110 reported that by intracerebral injection of alginate hydrogels, it was possible to control the  
111 release of VEGF in a rodent model of stroke. The alginate hydrogels containing VEGF protected  
112 the neuronal cells neurological and anatomically, which made this hydrogel promising for  
113 clinical treatment of stroke and CNS disease [50]. Here, we attempted to develop an ischemia  
114 hippocampus animal model to evaluate the effectiveness of pluronic-CS/aniline oligomer in  
115 repairing CNS. Based on our knowledge, it can be claimed that this study is the first research  
116 work of the hippocampus neurogenesis using a novel VEGF-loaded conductive injectable  
117 hydrogel.



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## 2. Material and methods

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### 2.1 Materials

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Dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), carbodiimide crosslinking agent (N, N-di cyclohexyl carbodiimide (DCC) and N-hydroxysuccinimide (NHS)), ammonium peroxodisulfate (APS), camphor sulfonic acid, and succinic anhydride were purchased from Merck. Medium molecular weight CS, 4-aminodiphenylamine, benzene-1,4-diamine, Pluronic F127, 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum

131 (FBS), Dulbecco Modified Eagle's Medium (DMEM), streptomycin, triphenyltetrazolium  
132 chloride, and penicillin were purchased from Sigma-Aldrich Company.

## 133 **2.2 Experimental methods**

### 134 **2.2.1 NHS-capped Aniline Pentamer Synthesis**

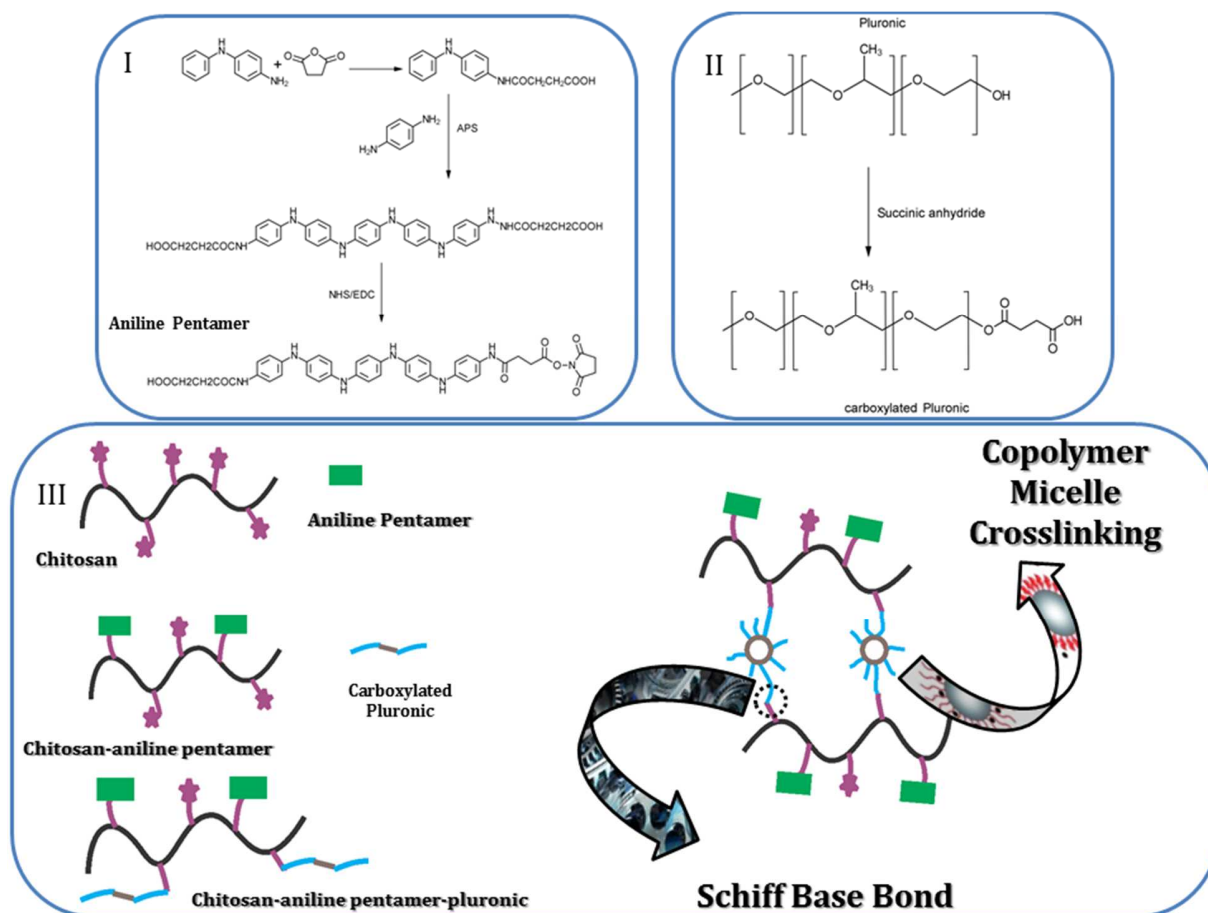
135 4-Aminodiphenylamine was reacted with succinic anhydride to achieve the aniline dimer with  
136 carboxylic end group. To achieve the aniline pentamer (AP) with carboxylic end groups  
137 benzene-1,4-diamine was mixed with carboxylic-capped aniline dimer solution and APS was  
138 poured to the mixture slowly to proceed the reaction. Reacting of aniline pentamer with CS  
139 necessitated activation of AP end groups. In this regards, NHS/DCC was added to the carboxyl-  
140 capped AP to achieve the AP with NHS activated end groups [51].

### 141 **2.2.2 Chitosan graft-NHS capped AP synthesis route**

142 CS amine groups reacted with carboxylic end groups of AP using carbodiimide reaction. About  
143 0.5 gr of CS was dissolved in 20 ml camphor sulfonic acid. Also, 0.1 gr NHS-capped-AP were  
144 dissolved in 5 ml DMSO. Subsequently, the two solutions were mixed and stirred for 24h at 50  
145 °C under the nitrogen atmosphere [31].

### 146 **2.2.3 Synthesis of CS-graft- NHS capped AP / Pluronic F127**

147 Carboxylated Pluronic was synthesized with succinic anhydride dimethylaminopyridine (DMAP)  
148 and triethylamine which was dissolved and stirred overnight. The solvent was removed and  
149 diethyl ether was used to precipitate residue. Finally, activated Pluronic was achieved with  
150 product vacuum drying. The CS-AP was reacted with 0.5, 0.25, and 0.12 gr activated-Pluronic  
151 using carbodiimides reaction (EDC/NHS) to achieve the CS-AP-Pluronic (CAP1, CAP2, and  
152 CAP3).



153

154 **Figure 2.** Chitosan-aniline pentamer- Pluronic synthesis route. I) synthesis carboxyl-capped AP: succinic anhydride  
 155 reacted with 4-aminodiphenylamine to achieve the aniline dimer with carboxylic end groups. After that, the product  
 156 was reacted with benzene-1,4-diamine and APS to obtain carboxyl-capped AP II) Pluronic hydroxyl group was  
 157 activated using succinic anhydride to facilitate the reaction with chitosan, III) AP reacted with chitosan and then  
 158 activated Pluronic grafted to the CS-AP. Pluronic tended to self-assembly and acted as a crosslinking agent with  
 159 enhancing temperature.

160

## 161 2.3 Characterization and experiments

### 162 2.3.1 Fourier transformed infrared spectroscopy (FT-IR)

163 Chemical interaction of the component and CAP chemical structure was studied using FTIR  
 164 spectra (Bruker instrument with the KBr disc, made by Germany, working in the range of 4000-  
 165 600  $\text{cm}^{-1}$  at ambient temperature).



### 166 2.3.2 Electro activity and conductivity

167 UV-visible (UV-Vis) was utilized to evaluate the AP transition states in doped and undoped state  
168 (Camphor sulfonic acid (CSA) was utilized to dope AP) of the copolymer with Shimadzu  
169 spectrophotometer (Kyoto, Japan). DMSO/deionized water (1:1) mixture was used to dissolve  
170 AP and the spectra of the doped and undoped AP using camphor sulfonic acid were assessed at  
171 340 nm [52].

172 Cyclic voltammetry (CV) was applied to evaluate the CAP electroactivity impedance.  
173 Spectroscopy was also assessed to determine the electrochemical behavior of the samples using  
174 micro auto lab (III). Carbon paste electrode (CPE), platinum wire, and Ag/AgCl were worked as  
175 electrode, counter electrode, and reference electrode, respectively. One M HCl solution was used  
176 as medium at a scan rate of 50 mVs<sup>-1</sup>. Also, EIS was done in 0.01 Hz. EIS by the neural model  
177 which consists of one capacitance and two resistors. With EIS records, Nyquist plot and  
178 conductivity properties were obtained [53].

179 The conductivity of the hydrogel was determined using four-probe method [54]. Pellet like shape  
180 of the sample was formed. After that, volt and amperes were applied and conductivity was  
181 calculated using the Eq. (1):

$$182 \quad \sigma = \frac{1}{R} \frac{d}{S} \quad (1)$$

183 Where  $\sigma$  is conductivity,  $d$  is thickness,  $R$  is resistance, and  $S$  is area of the sample. Swollen  
184 samples were utilized to determine the ionic conductivity at different temperatures using Eq. (2):

$$185 \quad \sigma = \frac{\sigma_0}{T} \exp\left(-\frac{E_a}{RT}\right) \quad (2)$$

186 Where,  $\sigma_0$  is conductivity of sample before exposing to swelling situation,  $T$  is absolute  
187 temperature,  $E_a$  is activation energy, and  $R$  is constant (molar gas). Conductivity is a subordinate  
188 of mobility and hole concentration according to Eq. (3):

$$189 \quad \sigma = q \times \mu_p \times p \quad (3)$$

190 Where  $P$ ,  $\mu_p$ , and  $q$  and are hole concentration, mobility, and electronic charge value ( $1.6 \times 10^{-19}$   
191 C), respectively [55, 56].

192

### 193 **2.3.3 Gelation time and rheological characterization**

194 The gelation time and storage modulus of hydrogels were measured using MCR 300 Anton Paar  
195 Rheometer with cylinder geometry. Temperature sweep test was done in the range of 15°C to  
196 70°C, a frequency 0.1 Hz and strain of 1%. Time sweep experiment was conducted with a  
197 frequency 1 Hz and strain of 1% at 30 °C. Also, a dynamic frequency sweep experiment was  
198 performed (frequency ranging from 1 to 100 rad s<sup>-1</sup> at shear amplitude of 0.1%).

### 199 **2.3.4 Swelling/deswelling behavior of hydrogels**

200 The swelling ratio of hydrogels with similar size and shapes was measured over a day at room  
201 temperature in distilled water (pH=7.2). First, hydrogels were freeze-dried and then were  
202 immersed in distilled water. After specified time intervals samples were taken out, and weighted  
203 after removing excess water. The swelling ratio of the sample was determined using equation (5),  
204 in which SR, W<sub>s</sub>, W<sub>d</sub> were swelling ratio of sample, the swollen weight of sample at specified  
205 time interval, and sample weight in dry state, respectively.

$$206 \quad SR = \frac{W_s - W_d}{W_s} \times 100 \quad (4)$$

207 The equilibrium water content (EWC) and the rate parameter were intended according to  
208 equation (6) and (7).

$$209 \quad EWC = \frac{W_e - W_d}{W_e} \times 100 \quad (5)$$

$$210 \quad S_t = S_e(1 - e^{-t/\tau}) \quad (6)$$

211 Equation (7) is called Voigt equation, and W<sub>e</sub>, S<sub>t</sub> and S<sub>e</sub> are the weight of swollen hydrogel at  
212 equilibrium state, swelling ratio at time t and equilibrium the swelling ratio.

213 In order to assess deswelling behavior, after hydrogels were reached swelling equilibrium, the  
214 samples were weighted immediately in specific periods. The water retention (WR) was measured  
215 from Equation (8) where W<sub>t</sub> is the hydrogel weight at a certain time of deswelling

$$216 \quad WR = \frac{W_t - W_d}{W_e - W_d} \times 100 \quad (7)$$

### 217 **2.3.5 Shape memory**

218 Polymers with suitable shape memory can deform under the loading and recover their initial  
219 shape after removing the loading [57]. To measure shape memory, the deformation of cylindrical  
220 samples under compression with 200g load for 5 min was measured. The load after 5 min was

221 increased to 400, 600, 800, and 900 g. To examine whether the compressed hydrogels could  
222 recover the enforced deformation or not, the hydrogels under the final load were immersed in  
223 water to absorb the lost water. Then the load was decreased and deformation was recorded at the  
224 end of each interval [55].

### 225 **2.3.6 In vitro degradation of hydrogel**

226 The degradation assay was done using phosphate-buffered saline (PBS) under pH 7.4 and 37 °C.  
227 Samples were drowned in PBS, the PBS was refreshed every week and at the specified time  
228 intervals the samples were removed from immersing water and washed with DI water to remove  
229 salinity. After drying in an oven at 60 °C, samples were weighed to calculate the degradation rate  
230 using the following equation.

$$231 \text{ weight remaining ratio (WRR)} = \frac{W_t}{W_0} \times 100 \quad (8)$$

232 where  $W_t$  and  $W_0$  are the hydrogel weight at specified times and the initial hydrogels weight,  
233 respectively.

### 234 **2.3.7 Thermal properties**

235 Thermal characteristics of the samples were conducted using differentiation scanning calorimetry  
236 apparatus (DSC) under the atmosphere of nitrogen. The details are available in Supporting  
237 Information.

### 238 **2.3.8 Contact angle**

239 Contact angles of hydrogels at room temperature were evaluated using OCAH 200 device (data  
240 physics contact angle system, static mode). The details are available in Supporting Information.

### 241 **2.3.9 Morphology and porosity determination**

242 Surface morphology, porous structure, and cell adhesion of the freeze-dried hydrogel were  
243 studied using scanning electron microscopy (SEM). The details are available in Supporting  
244 Information.

### 245 **2.3.10 In vitro drug loading and drug release behavior**

246 To evaluate the drug release behavior of the samples in stimulated and passive conditions, 50 mg  
247 of dexamethasone as drug model was dissolved in methanol, and 0.5 g of dry samples were  
248 added to the solution and stirred for 24h at ambient temperature. Then, the drug-loaded hydrogel

249 was washed to remove the un-bonds drugs, and the sample was immersed in PBS (pH 7.4, and  
250 37 °C). At particular interval times, the same amount of fresh PBS was replaced. To evaluate the  
251 drug release profile, UV-Vis ( $\lambda=237$  nm) was utilized. In stimulated drug release, electrical  
252 current was applied to the hydrogel and then the release pattern was determined. In order to  
253 further study of release mechanism, the Korsmeyer-Peppas exponential equation (equation (9))  
254 was utilized [58]. The mean dissolution time (MDT) was used to evaluate the drug release rate,  
255 which was determined by Mockel and Lippold (equation (10)) [59].

$$256 \quad \frac{M_t}{M_f} = k \cdot t^n \quad (9)$$

$$257 \quad MDT = (n/n + 1) \cdot k^{-1/n} \quad (10)$$

### 258 **2.3.11 Antibacterial assay**

259 Minimum inhibitory concentration (MIC) of CAP2 for *Staphylococcus aureus* (ATCC 29213)  
260 and *Escherichia coli* (ATCC 8739) was assessed. The details are available in Supporting  
261 Information.

### 262 **2.3.12 In vitro evaluation of hydrogel cell viability**

263 Hydrogels biocompatibility was carried out using the MTT assay. The samples were sterilized  
264 using UV and ethanol. After sterilization, samples were washed and culture media was poured on  
265 sample overnight. Pheochromocytoma (PC12) cells in DMEM with 10% FBS were seeded on  
266 the samples and then placed in incubator (37 °C, 95% moisture, and 5% CO<sub>2</sub>). The MTT test was  
267 performed in 1, 3, and 5 days [60].

### 268 **2.3.13 Animals**

269 Adult male Wistar rats (250-300 g) were purchased from the Pasteur Institute of Iran. They were  
270 habituated for at least 6 days in the animal facility before starting the experiments. Rats were  
271 caged in the group of four with arbitrary access to nutrients and maintained on a 12h light/dark  
272 cycle (light on at 07:00 AM) and at a temperature of 23±1 °C. We endeavored to utilize the  
273 minimum number of animals and reduce their suffering during the experiments. The experiments  
274 were performed according to the Review Board and Ethics Committee of Pasteur Institute of Iran  
275 and conform to the European Communities Council Directive of 24 November 1986  
276 (86/609/EEC).

#### 277 **2.3.14 Unilateral hippocampal ischemia induction**

278 Unilateral hippocampal ischemia was induced according to the previously described method [61,  
279 62]. Briefly, after achieving the proper level of anesthesia using an incision in the femoral  
280 region, the femoral vein was determined and isolated from the femoral nerve and artery. Then,  
281 the distal section of the vein was strongly fastened and the proximal end was loosely tied using  
282 two pieces of thread. Using small scissors, an incision was lined on the vein to insert a  
283 polyethylene tubing (PE-50) in the femoral vein. The catheter was fixed in the vein by tightening  
284 the loose tie. In accordance with Paxinos atlas [63], a guide cannula (23 gauge stainless steel)  
285 was implanted on the upward section of the hippocampal fissure (AP= 5.64 mm, L= 5.2 mm and  
286 DV= 2.6 mm) and fixed to the skull with dental cement. To preserve a root for drugs  
287 microinjection, the cannula was sealed with a 29-gauge stainless steel stylet. After recovery, the  
288 upward section of the hippocampal fissure was illuminated by a 523 nm laser diode (CNI laser)  
289 attached to an optical fiber (200 $\mu$ m) for 25 min. Along the first two-minute of illumination  
290 periods, 2 mL/kg of Rose Bengal (20 mg/ml, Sigma-Aldrich Co., US) was gently injected into  
291 the femoral vein through polyethylene tube. Finally, the polyethylene tube and optical fiber were  
292 detached and the femoral region was sutured.

#### 293 **2.3.15 Hydrogel microinjection**

294 The animals were randomly divided into five groups. Sham group (underwent surgery without  
295 hippocampal ischemia induction), control group (hippocampal ischemia induction plus 10  $\mu$ L/rat  
296 PBS injected in the site of ischemia), hydrogel treated group (hippocampal ischemia induction  
297 plus 10  $\mu$ L/rat hydrogel injected in the site of ischemia), VEGF treated group (hippocampal  
298 ischemia induction plus 10  $\mu$ L/rat VEGF (1 mg/mL) injected in the site of ischemia), and  
299 hydrogel containing VEGF treated group (hippocampal ischemia induction and 10  $\mu$ L/rat VEGF  
300 (1 mg/mL) loaded hydrogel injected in the site of ischemia). After animal acclimatization, the  
301 photothrombotic model was used to make unilateral selective hippocampal ischemia [64]. Three  
302 days after hippocampal ischemia induction, the stainless-steel stylet was discarded from guide  
303 cannula and the mixture was microinjected, with the rate of 1 $\mu$ L/min, into the ischemic site in  
304 conscious rats using a 25- $\mu$ L Hamilton syringe connected a 29-gauge injection cannula through a  
305 PE-10 polyethylene tube. The volume injected was 10  $\mu$ L/rat. After injection, the needle remained  
306 for 2 min in the injection site to minimize drug backflow. Infarcted volume (six rats in each

307 group) and behavioral evaluations (eight rats in each group) were done 5 days after drug  
308 injection.

### 309 **2.3.16 Infarct volume measurement**

310 Five days after injection, animals were sacrificed, and brains were dissected and coronally  
311 sectioned into 2-mm-thick slices using a stainless-steel brain matrix. Sliced brain sections were  
312 stained with triphenyltetrazolium chloride solution (2% in normal saline) for 20 min at 37 °C.  
313 The unstained section of the brain section was the criterion of ischemic lesions [65]. The infarct  
314 volume was quantified using the ImageJ software. The infarct volume (%) was determined using  
315 the following formula to attain the actual volume of injury [66, 67].

$$316 \text{ Infarct volume (\%)} = \frac{\text{Volume of the left hemisphere} - \text{Non-infarct volume of the right hemisphere}}{\text{Volume of the left hemisphere}} \times 100$$

### 317 **2.3.17 Behavioral testing**

318 Memory impairment was evaluated in separate experimental groups (n=8) through passive  
319 avoidance and Morris water maze (MWM) paradigms which assessed fear-motivated memory  
320 and spatial memory performance, respectively.

#### 321 **2.3.18.1 Passive avoidance test**

322 Five days after drug injection, the passive avoidance (PA) test was performed using a shuttle box  
323 equipment consisting of a bright and a dark compartment (20 cm × 20 cm × 20 cm each) floored  
324 by stainless-steel rods connecting to an electrical stimulator. A guillotine door (8 cm × 8 cm)  
325 separated the two compartments. Briefly, following two habituation sessions (30 min inter-  
326 session interval), the animals were trained to perform the task. In training trial, after entering the  
327 dark compartment the guillotine door was closed and immediately the animals were given  
328 electric foot shock (1.5 mA for 3s). Thirty seconds after applying foot shock, they were returned  
329 to the home cage. After two min, the animals were retested. They were placed in the light  
330 compartment and successful training was achieved if they did not go into the dark compartment  
331 over two min. Otherwise, training trials were repeated until the rat learned the task. Twenty-four-  
332 hour later, the animal was located in a light chamber, the guillotine door was opened and its  
333 behavior was monitored for 300s. The latency of entrance to the dark compartment known as  
334 step-through latency (STL) and total time elapsed in dark chamber (TDC) were registered.

### 335           2.3.18.2   *Morris water maze test*

336 Spatial learning and memory were assessed in the animals based on the previously described  
337 method [68]. MWM consisted of a dark water tank (155 cm) loaded with water ( $20 \pm 2$  C) up to  
338 10 cm from the tank edge. Four quadrants (North, East, West, and South) were determined for  
339 the tank using a software system (Ethovision, versionXT7, the Netherlands). A hidden stage (10  
340 cm diameter) was positioned in the target quadrant, Q1, 1.5 cm beneath the water surface. The  
341 lab room walls were designed with specific shapes as an extra maze or spatial cues. Using a  
342 charge-coupled device (CCD) camera the swim path of animals was tracked in the pool and the  
343 locomotion was analyzed using Ethovision software 7. At first, animals were accommodated to  
344 the pool by swimming in the tank for 60 min. The platform was located in the Q1 and animals  
345 were put in the tank water from different directions to train the animals. Rats were trained for  
346 three successive days. Each day, they were trained by four trials with ten-minute inter-trial  
347 intervals. The rats were supposed to locate the hidden platform over 60s. In case the rats failed to  
348 find the platform until 60s, they were directed to it and permitted to stay there for 10s. This  
349 procedure was repeated in the second and third days. Twenty-four hours after the last session, the  
350 platform was taken out, the animal was placed in the pool from the opposite side of the platform  
351 and the motor behavior was monitored for 60s. Escape latency, distance to platform and duration  
352 in the target quadrant and swim speeds were analyzed.

### 353   2.3.18   *Statistical analysis*

354 The quantitative data were analyzed as a mean  $\pm$  standard deviation. Statistical comparisons were  
355 done by using ANOVA version 16.0 (SPSS, USA). The level of significance was considered if P  
356 values were  $<0.05$ .

## 357           3.   **Result and discussion**

### 358           3.1   *Synthesis and characterization*

359 The FTIR spectra of samples (CS, AP, CS-AP, and CAP) are illustrated in **figure 3(I)**. The  
360 spectrum of CS displays typical adsorption peaks at  $3435.73 \text{ cm}^{-1}$  related to the distinctive peak of  
361 amine ( $--\text{NH}_2$ ) and hydroxyl group ( $--\text{OH}$ ) stretching vibrations [69]. Peaks of  $1602.27 \text{ cm}^{-1}$  and  
362  $1423.89 \text{ cm}^{-1}$  in CS correspond to the amide bond ( $--\text{CONH--}$ ) and amine bond ( $--\text{NH}_2$ ) stretching  
363 and bending vibrations. Peaks of  $1081.88 \text{ cm}^{-1}$  and  $1157.82 \text{ cm}^{-1}$  are related to pyranose ( $--\text{C—O—}$   
364  $--$ ) stretching vibrations from CS [70]. The spectrum of AP illustrates peaks at  $1626.45 \text{ cm}^{-1}$  and

365 1574.34  $\text{cm}^{-1}$  due to (C=C) absorption of the benzene ring (--N—B—N) and the quinoid ring (--  
366 N=Q=N--) of AP. The 1312.06  $\text{cm}^{-1}$  band is attributed to C—N stretching in the proximity of  
367 quinoid rings [71]. The peak at 1738.34  $\text{cm}^{-1}$  in curve CAP is related to the C=O asymmetric  
368 stretching mode of ester in Pluronic [72]. The data confirmed the successful crosslinking and  
369 grafting reaction of the hydrogel.

### 370 **3.2 Electroactivity and conductivity**

371 Oligoanilines and polyaniline possess three oxidation states: leucoemeraldine (LE), emeraldine  
372 (EM), and pernigraniline (PN). **Figure 3(II-A)** shows the oxidation states of aniline oligomer.  
373 Polyaniline and the oligoaniline conductivity occur in the oxidation state. The UV-Vis spectra of  
374 undoped sample showed peak at 356 nm attributed to the benzene ring  $\pi$ - $\pi^*$  transition. By  
375 doping with camphor sulfonic acid, peak at 580 nm was observed and the blue shift of benzene  
376 ring because of the polaron formation (solvatochromism effect) resulted in a peak at 338 nm  
377 (**Figure II-B**) [73-76]. Cyclic Voltammetry (CV) was used to investigate the electroactivity of  
378 hydrogels. As it is illustrated in **figure 3(II-C)**, it exhibited three pairs of quasi-reversible  
379 oxidation/reduction peaks and the mean peak potentials ( $E_{1/2} = (E_{pa} + E_{pc})/2$ ). Peaks refer to the  
380 transition of different states of the aniline oligomer (transition between leucoemeraldine state,  
381 emeraldine state I and II, and pernigraniline state). The results indicated that the hydrogels  
382 showed good electroactivity. Also, molecular resonance is depicted in **figure 3(II-A)**. The  
383 electrochemical behavior of PC12 cells was evaluated by Kafi et al. [77]. It was observed that the  
384 PC12 cells exhibited the two redox peaks with current ratio larger than 1 which demonstrated the  
385 quasi-reversible characteristics of the cell. Hydrogel CV behavior look likes CV behavior of  
386 PC12 cell which this similarity can be inferred as an electrochemical behavior mimicking of  
387 neural cells with synthesized hydrogel.

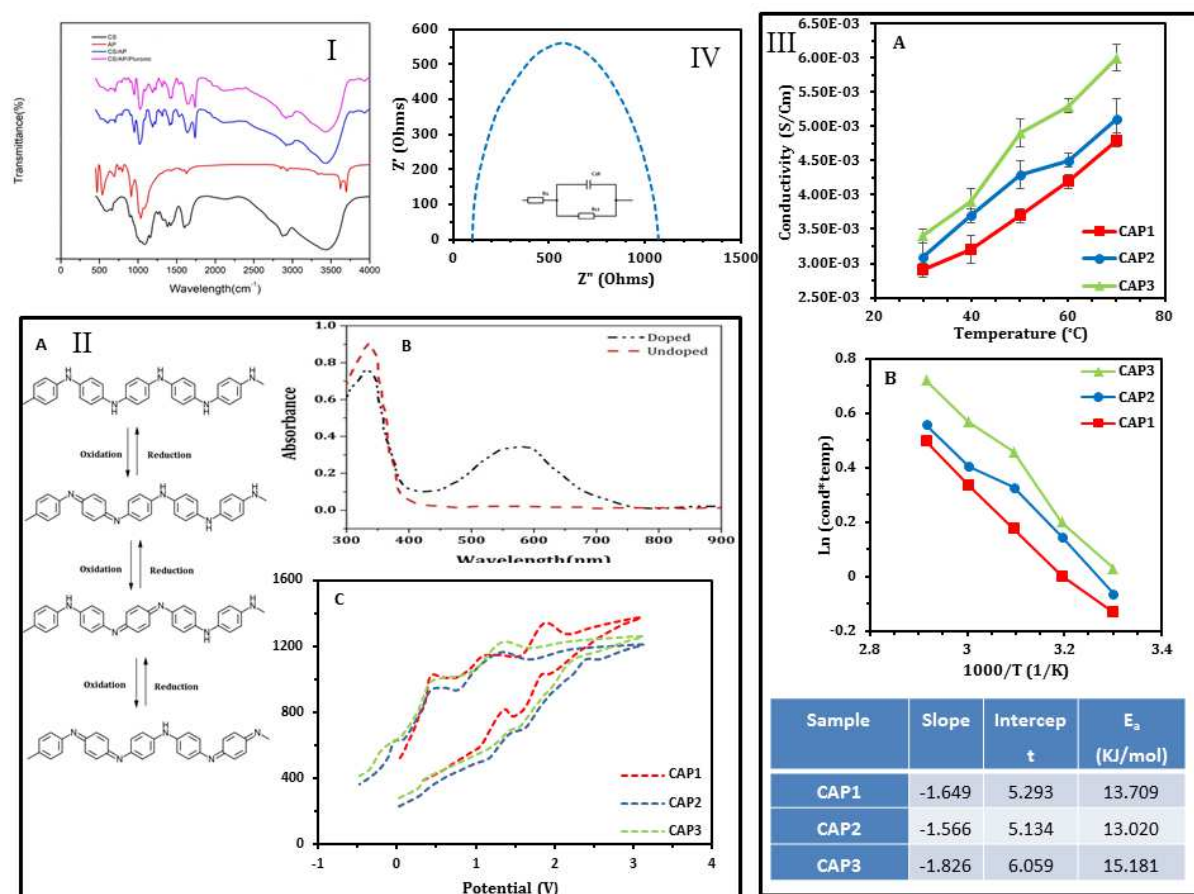
388 Ionic conductivity was evaluated using four-probe method (**figure 3(III-A)**). The ionic  
389 conductivity increased by increasing the temperature due to the accelerated ionic mobility at  
390 elevated temperatures. Also, the increment of AP content enhanced the absolute conductivity  
391 because AP provided a higher number of ions for the hydrogel. According to **figure 3(III-A)** the  
392 conductivity of hydrogels at 37 °C is ca.  $3 \times 10^{-3}$  which is adequate for the cellular response  
393 (proliferation or/and differentiation) [29]. Such resemblance between the hydrogel and neural  
394 cells demonstrated the electrical mimicking of cells using synthesized hydrogel. The  
395 conductivity of hydrogels is related to two factors: the number of ions and their mobility. **Figure**



396 **3(III-B)** shows a line where the slope of the plot multiplied by R represents the activation energy  
397 for conductivity and the exponential of the intercept displays the pre-exponential factors which  
398 represents the number of ions in the hydrogel. According to equation (2), the measurements are  
399 given in Table within **figure 3-III** and it can be understood that higher pre-exponential factor  
400 and low activation energy lead to more conductivity. The pre-exponential factor increases with  
401 the increment of the AP content. Also, activation energy declines with increasing the of AP  
402 percentage in hydrogel because less conductive hydrogel required more energy for ion transfer.  
403 According to equation (3) conductivity is a subordinate of mobility and hole concentration  
404 (number of ions). With an increment of AP content, the number of ions increases. On the  
405 contrary, the higher concentration AP because of the hydrophobic nature resulted in swelling  
406 ratio decrement which restricts the mobility of ions. Thus, the conductivity of samples should be  
407 optimized in accordance with ion mobility and number. Conductivity increase with raising the  
408 AP content until an optimum point and after that, it can be deteriorated because of the hindrance  
409 effect against water molecule diffusion. Autofi et al. observed that the aniline tetramer increment  
410 enhanced the conductivity because of the enhancement of ion and mobility. After adding more  
411 aniline tetramer ionic conductivity decreased because of the less mobility of ions [55]. The entire  
412 hole concentration is constant in samples with a specific AP concentration, and the increment in  
413 conductivity with temperature is due to mobility increment. On the other hand, in particular  
414 temperature, the mobility is constant. Therefore, the conductivity depends on the ion numbers  
415 [55, 56]. It has been reported that the conductive polymers affect the intracellular  $\text{Ca}^{2+}$  level of  
416 neural cells which affect their activities like proliferation and differentiation [78]. Wu et al.  
417 proposed that the CaSR-PCL pathway had more effective than voltage-gated calcium channel  
418 (VGCC) in regulating intracellular  $\text{Ca}^{2+}$  levels of neural cells on oligoaniline based conductive  
419 substrate [79]. Hence, an oligoaniline based conductive substrate can affect the signaling  
420 pathways and regulate the neural cell activities [43].

421 It is known that the lipid bilayer of nerve cells (the outer membrane of nerve cells with thickness  
422 around 8-10nm) performs as a leaky capacitor. Specific capacitance (capacitance per unit area of  
423 membrane) has fundamental factors in neural model which is related to signal propagation and  
424 synaptic integration. Capacitance and dendritic geometry affect the postsynaptic potential  
425 amplitude because of the depolarization of the dendritic tree at delivering the synaptic current to  
426 the membrane capacitance time interval. After that, the charge swiftly transfers from the synapse

427 which the speed of the signal propagation is related inversely to the capacitance. The neural cell  
 428 capacitance is reported around 0.9-1.1  $\mu\text{F}/\text{cm}^2$ . Electrochemical impedance spectroscopy (EIS)  
 429 is an outstanding method to study the cell feature and behavior using the Randles cell model  
 430 which consisted of two resistances and one capacitance (**Figure 3 (IV)**). Scaffold capacitance is  
 431 close to the neural capacitance which can be proper substrate for neural tissue engineering [53,  
 432 80]. Such resemblance can be declared that the scaffold can act as a bridge in the defected sites  
 433 and enhance the functionality. Such resemblance declared that the synthesized hydrogel can  
 434 mimic the impedance and electrochemical features of the neural tissues. Morphology, thermal  
 435 properties, and contact angle of hydrogels are discussed in supplementary information (**Figure**  
 436 **1S-I, Figure 1S(II-A), Figure 1S(II-B) and Figure1S III, respectively**).



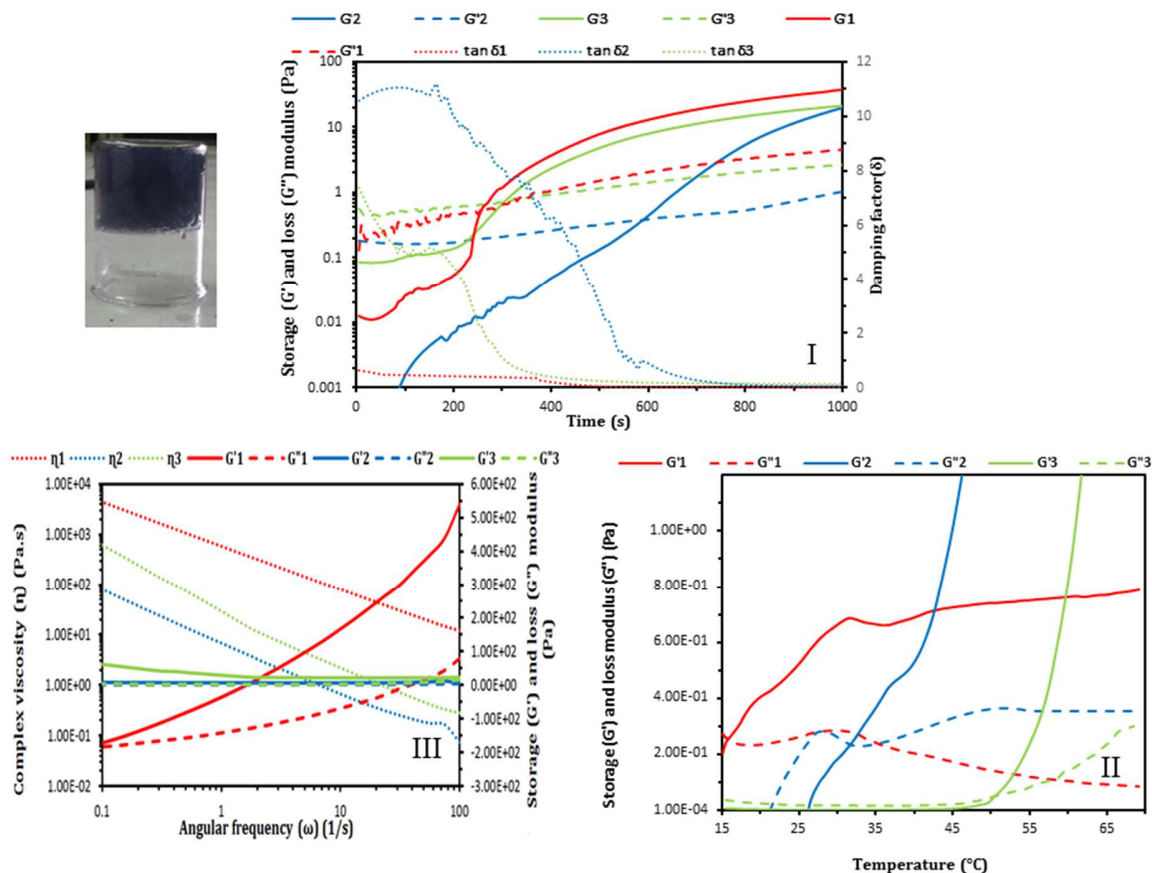
437  
 438 **Figure 3. (I)** FTIR spectra of CS, AP, CS/AP, and CS/AP/Pluronic. **(II) (A)** The scheme of aniline pentamer  
 439 transition in oxidation-reduction activities. **(B)** The UV spectra of hydrogels in deionized water and doped with HCl.  
 440 **(C)** Cyclic Voltammetry of hydrogels: the sample electroactivity is featured by three oxidation/reduction peaks. **(III)**  
 441 The ionic conductivity of hydrogels. Doping with camphor sulfonic acid produced emeraldine state and suitable

442 conductivity. (A) Conductivity against temperature and (B) Arrhenius plots of samples & table indicates the  
443 activation energy derived from the figure (B). (IV) Bio-impedance evaluation Nyquist plot of hydrogel which was  
444 yielded from electrochemical impedance spectroscopy that it was used for gaining the Randles circuit model  
445 components.

446

### 447 ***3.3 Rheological behavior evaluation of hydrogels***

448 The most important factor in the injectable hydrogel is gelation time. For this, the gelation time  
449 of hydrogel was measured using the inversion method. The samples gelation was evaluated at  
450 37°C to simulate the body temperature (**Figure 4(I)**). Gelation time was around 4, 5, and 7  
451 minutes for CAP1, CAP2, and CAP3 samples, respectively. The gelation time increased with  
452 decreasing the Pluronic concentration in hydrogels. The amount of –CHO was increased with an  
453 increasing Pluronic concentration. Hence, CHO groups have more possibility to reaction with -  
454 NH<sub>2</sub> of CS to form Schiff base in the hydrogels. Moreover, Pluronic because of its nature  
455 exhibited thermosensitive gelation, decrement of Pluronic content decreased hydrogel sensitivity  
456 to temperature [81, 82]. Rheological properties are shown in **figure 4(II)**. The hydrogels with  
457 different amount of Pluronic were conducted by a time sweep experiment to evaluate the gelation  
458 process. Prompt dynamic Schiff base reaction between –CHO and –NH<sub>2</sub> groups resulted in rapid  
459 surpass of storage modulus from loss modulus in the initial stage [83]. The elastic modulus of  
460 hydrogels was reduced by increasing the percentage of AP because of the steric hindrance of the  
461 AP which disrupts the LCST behavior of Pluronic. The sol-gel transition of the samples can be  
462 observed in modulus versus temperature graph which indicates the thermosensitive behavior of  
463 the samples. At low temperature, G' was lower than G'', indicating a viscous sol state. During the  
464 sol-gel transition, a significant increase was observed. Also with reducing the percentage of AP  
465 in samples, the gelation temperature increased [72]. Furthermore, the storage and loss modulus  
466 versus angular frequency were plotted to analyze the stability of the hydrogels. When the angular  
467 frequency altered from 0.1 rad·s<sup>-1</sup> to 100 rad·s<sup>-1</sup>, storage and loss modulus of hydrogel  
468 demonstrated no noteworthy changes, representing proper durability of hydrogels (**Figure**  
469 **4(III)**).



470  
 471 **Figure 4.** Photograph of hydrogel after crosslinking. (I) Rheological behavior of hydrogels in time sweep test. (II)  
 472 storage ( $G'$ ) and loss ( $G''$ ) modulus of hydrogels in temperature sweep test. (III) Angular frequency sweep test.

### 473 **3.4 Swelling/deswelling and hydrophilicity behavior of the hydrogels**

474 The mechanical properties, diffusivity, and drug release of hydrogels are related to their swelling  
 475 and deswelling behavior. **Figure 5(I-A)** represents the swelling ratio of hydrogels versus time at  
 476 room temperature. It was obvious that the samples absorbed a high amount of water at initial  
 477 time intervals and then reached an equilibrium state. During the swelling process, water  
 478 molecules overcome the osmotic pressure inside the gel to permeate the gel. Elasticity force is  
 479 acted against osmotic force to balance the stretching of networks to prevent the network collapse.  
 480 The amount of absorbed water in the hydrogel resulted in higher elasticity and consequently  
 481 higher osmotic pressure. Therefore, water needs to overcome higher pressure to permeate inside  
 482 the gel. Thus, the swelling rate decreased gradually over time.

483 It was observed that the swelling ratio decreased with increasing the AP content in hydrogels  
 484 which such a phenomenon attributed to the decrement of free space within polymeric network

485 hindering the diffusion of water molecules inside the hydrogel; Moreover, the hydrophobic  
486 nature of AP resulted in repulsion of water molecules. Swelling rate can be determined using rate  
487 parameter ( $\tau$ ) which can be obtained by rearranging the Voigt equation. The lower value of  $\tau$   
488 declares a higher the swelling rate. With plotting  $\ln(1-S_t-S_e)$  versus time the slop which is  $-1/\tau$   
489 can be calculated. It was understood that the swelling ratio exhibited different behaviors during  
490 swelling time intervals which its behavior differed in the initial stage and final stage (**Figure 5(I-**  
491 **C,D)**). The swelling rate was high at the initial stage and became low at final stage because the  
492 hydrogel reached the equilibrium swelling weight. Based on **figure 5(I-C)**, hydrogel behavior at  
493 initial time intervals obeys Voigt equation properly; however, the data in the second section does  
494 not. In the initial minutes, as the content of AP increments in the hydrogels, the slope of the trend  
495 lines decreases and the  $\tau$  value increases which declared the decrement of the swelling rate. AP  
496 because of the hydrophilic nature repels the water molecules and the rate of the swelling is  
497 decreased.

498 The deswelling trend of hydrogels is demonstrated in **figure 5(I-B)** disclosing the fact that the  
499 amount of absorbed water by hydrogels decreases fast at the first minutes during the deswelling  
500 process, and then becomes slower until reaching the equilibrium state.

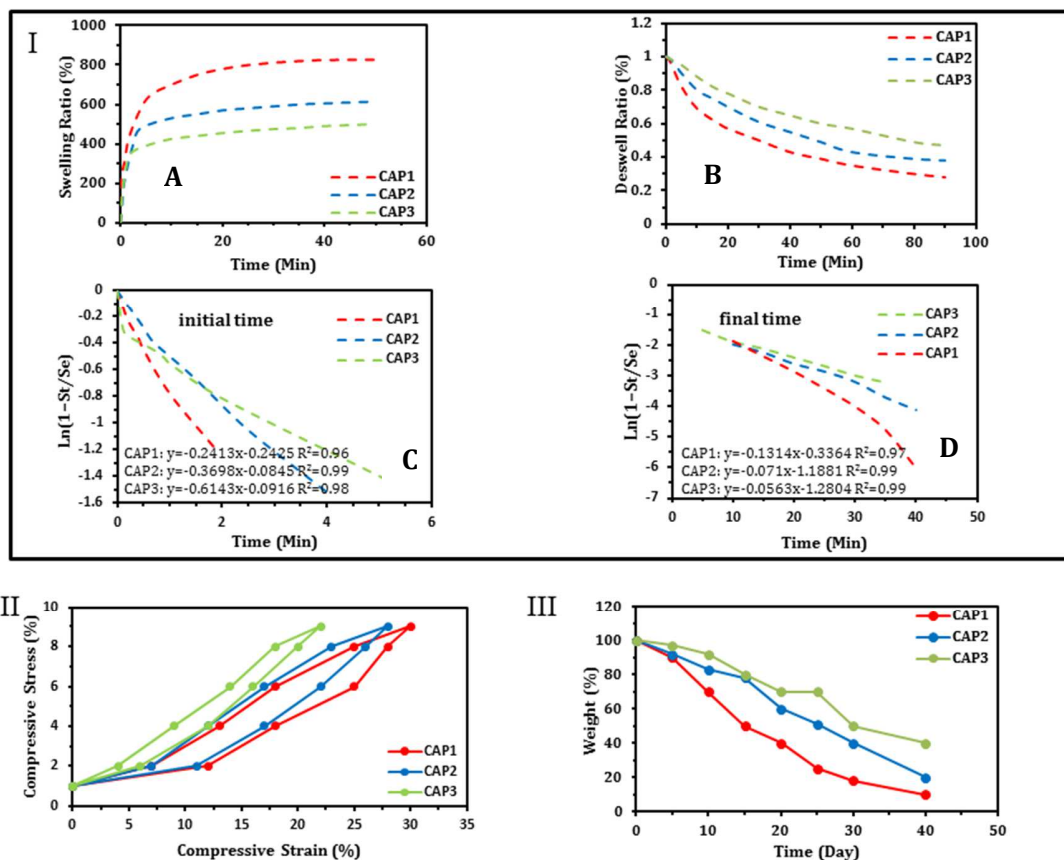
### 501 ***3.5 Shape memory***

502 The result of deformation and recovery properties of hydrogels was studied. All samples recover  
503 their initial shape after they have lost amount of their water under compression which can be in  
504 accordance with the hippocampus behavior under compression test [84]. As a result, these  
505 hydrogels are suitable for use in the body due to the ability to absorb extracellular fluid (ECF)  
506 and recovering primary shape. Samples with higher AP content exhibited less compression strain  
507 (**Figure 5-II**). By increasing the percentage of AP, the rigid segments of AP limited the  
508 segmental motion and hindered movement during compression. Also, when samples recover  
509 their dismissed water, hydrogel chains retained their mobility and hold on to their initial position  
510 [55]. Based on the aforementioned results, synthesized hydrogel mimics the hippocampus  
511 mechanical behavior.

### 512 ***3.6 In vitro degradation assessment of hydrogels***

513 Degradation time of hydrogels is a vital factor in tissue engineering because of the chronic  
514 inflammation happen due to the prolonged presence of the material within the body [85].  
515 Therefore, the degradation rate of the hydrogels should be synchronized with tissue regeneration.

516 AP reduced swelling capacity due to its intrinsic hydrophobic properties. Hence, as the  
 517 percentage of the AP in hydrogel increased, water swelling and degradation decreased (**Figure 5-**  
 518 **III**).



519 **Figure 5.** (I) (A) The swelling behavior of samples with various amounts of Pluronic. (B) The deswelling trend of  
 520 hydrogels with various amounts of Pluronic.  $\ln(1-St/Se)$  vs.  $t$  for initial (C) and final (D) minutes of swelling. (II)  
 521 Deformation and recovery of hydrogels. (III) Degradation profile of the hydrogels with different concentrations in  
 522 PBS with pH 7.4 at 37 °C.  
 523

### 524 3.7 *In vitro* drug release properties of the hydrogel

525 Conductive hydrogels are proper platforms for the drug delivery system with on-demand electro-  
 526 responsive features in which the voltage just use to promote the release of drugs [20, 86]. AP and  
 527 CS can captivate drugs and interact with them by the carboxyl and amine groups via ionic  
 528 interaction bonds. Besides, conductive hydrogels interact with drugs using hydrophobic, acid-  
 529 base,  $\pi$ - $\pi$  interaction, ion exchange, H-bond, and polar function. Applying electrical voltage to  
 530 hydrogel generated an oxidation/reduction process and can release the drug with the desired

531 pattern [87, 88]. Dexamethasone in aqueous solution due to its hydrophobic nature encounters  
532 repulsion driving forces and has a tendency to be released rapidly with a high initial burst  
533 release. **Figure 6(I-A)** depicts the stimulated and passive release behavior by applying an  
534 electrical potential to the hydrogel. Conductive hydrogels were sensitive to voltage in which  
535 voltage applying resulted in increasing the drug release content due to the variation of AP's  
536 redox state. Variation causes bond cleavage in hydrogels and enhances drug release amount [89].  
537 The first burst release of the drug-loaded CAP2 hydrogel is attributed to the drug molecules  
538 located near the surface or those loosely bond with the hydrogel. In the passive drug release,  
539 there is a direct relationship between drug release pattern and swelling/deswelling pattern, where  
540 more swelling ratio yields more drug release ratio. CAP1 because of the higher content of AP  
541 exhibited more hydrophobicity and low swelling ratio, the lower drug release from CAP1 due to  
542 the low diffusion of water molecules [90]. In electro-stimulated drug release, the release  
543 behavior of dexamethasone was determined using applying electrical stimulation to the hydrogel.  
544 Drug release mechanism in passive system was just diffusion-driven force but the electro-  
545 stimulated drug release of conductive hydrogels was attributed to the diffusion-driven force,  
546 charged molecules movement, and total net charge changing by oxidation/reduction. Oxidation is  
547 a possible mechanism for the release of positive charge drugs, conversely, drugs with negative  
548 charge are released by reduction [91]. Korsmeyer-Peppas equation is proper for evaluating drug  
549 release from CAP hydrogels with high enough correlation ( $r^2 > 0.97$ ). It has been supposed that  
550 when the diffusion exponent (n) equal to 0.5 indicates that the release mechanism obeyed the  
551 Fickian diffusion model, and the dexamethasone was released by the Fickian diffusion [92]. The  
552 fitted data in **figure 6(I-B)** designated that CAP2 hydrogel obeys the Fickian diffusion systems  
553 and dexamethasone released by diffusion [59].

554

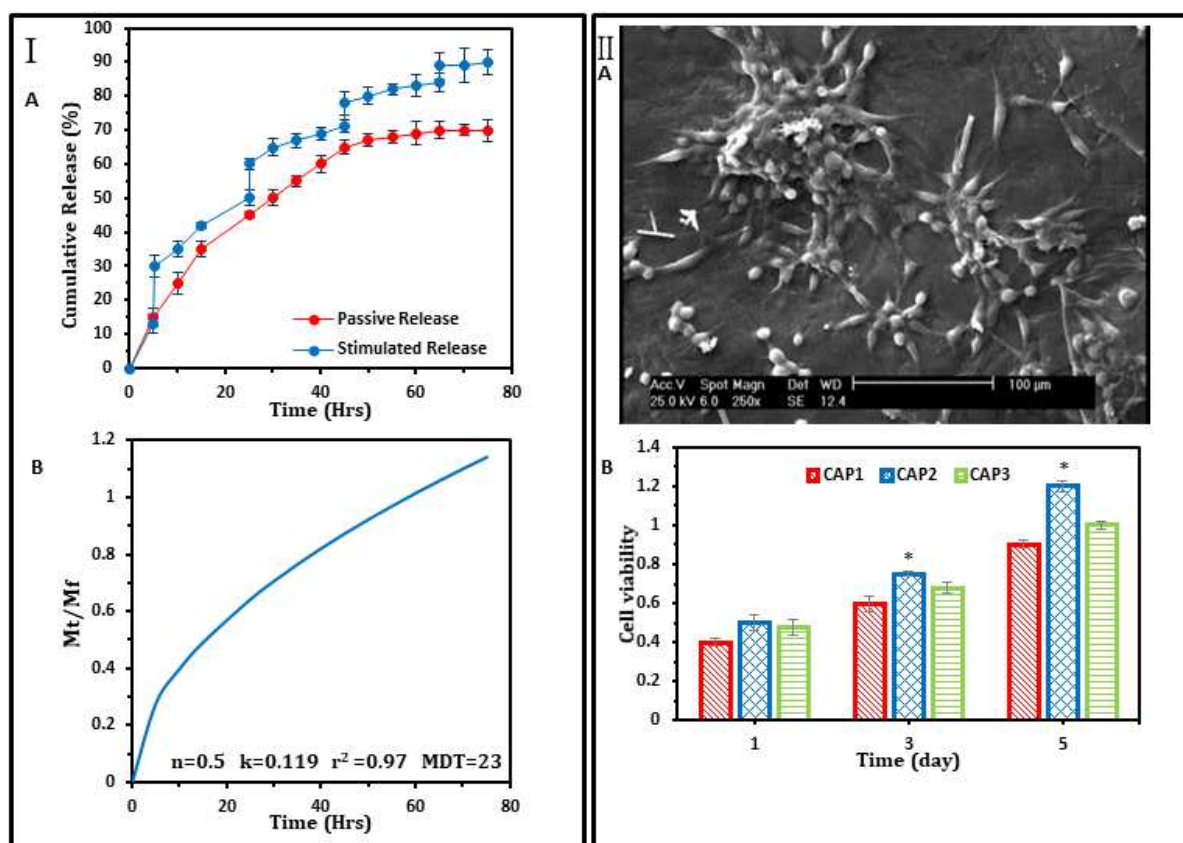
### 555 **3.8 Antibacterial assay and In vitro biocompatibility**

556 The antibacterial analysis results confirmed the successful antibacterial activity of CAP2  
557 hydrogel against *Escherichia coli* and *Staphylococcus aureus* (**Figure 2S(I)**, Supporting  
558 Information).

559 The biocompatibility of hydrogel plays a key role in supporting basic cellular activities such as  
560 proliferation and adhesion. Biocompatibility and cytotoxicity were evaluated by MTT  
561 assessment with PC12 cells in various time intervals (1, 3, and 5 days). SEM images of PC12



562 cells seeded on CAP2 exhibited in **figure 6(II-A)** which reveals a good adhesion of cells on the  
 563 scaffold. Therefore, the SEM image and MTT assessment confirmed the biocompatibility of  
 564 hydrogels. Moreover, cell viability increase with time and the highest cell viability of the cell  
 565 was observed after 5 days (**Figure 6(II-B)**). These properties could be attributed to the  
 566 biocompatibility of CS, pluronic, and AP. On the other hand, AP has toxicity in higher  
 567 concentration and affect the cellular activities inversely, therefore there is an optimum percent of  
 568 AP should be determined for maximum cell proliferation. In terms of various percentages of AP,  
 569 CAP2 showed the highest cell viability during the culture days which is ascribed by its swelling  
 570 ratio and proper electrical/ionic conductivity.  
 571



572  
 573  
 574 **Figure 6.** (I) Relationship between cumulative release (%) and time (hours). Passive and stimulated drug release of  
 575 CAP2. (A) Electrical stimulation with various voltages results in releasing dexamethasone with the desired pattern.  
 576 (B) The release profiles of CAP2 hydrogel in PBS with pH 7.4 were fitted well using Eqs. (9) and (10). (II) (A)  
 577 SEM image of PC12 cells adhesion on CAP2, it shows that the hydrogel has the cell attachment. (B) Cell viability



578 test: colloidal hydrogel biocompatibility was evaluated using MTT assay with neural-like PC12 cells which the  
579 CAP2 exhibit the best cell compatibility (\*P < 0.05).

580

#### 581 **4. In vivo studies**

##### 582 **4.1 Injectable electroactive hydrogel containing VEGF decreased the infarct volume** 583 **of hippocampal ischemia area**

584 Infarct volume was assessed 5 days after drug injection using triphenyltetrazolium chloride  
585 (TTC) staining procedure. Based on **figure 7-I**, the brain infarct volume in VEGF or VEGF plus  
586 hydrogel groups was smaller in comparison with the control group (**Figure 7 (I-A)**). Quantitative  
587 data of the infarct volume was calculated using standard formula which is modified to post-  
588 ischemic edema and presented as a percentage of left hemisphere volume in **figure 7 (I-B)**. The  
589 infarcted volume in rats treated with VEGF or VEGF plus hydrogel was decreased in comparison  
590 with the control group (\*\*\* p < 0.001). Interestingly, animals treated with VEGF plus hydrogel  
591 showed less ischemic area compared with those treated with VEGF alone (# p < 0.05). There was  
592 no infarct lesion in the sham group.

##### 593 **4.2 Passive avoidance memory improvement by using injectable electroactive** 594 **hydrogel containing VEGF**

595 In the passive avoidance test, step-through latency (STL) intensely declined and the elapsed time  
596 in the dark compartment (TDC) intensely enhanced in the control group compared with the sham  
597 group in the retention test day (**Figure 7 (II-A)**). VEGF as a bolus injection and VEGF hydrogel  
598 both inhibited memory impairment following hippocampal ischemia as they increased the STL in  
599 passive avoidance test (\* p < 0.05). Furthermore, **figure 7 (II-B)** indicates that treatment with  
600 bolus injection of VEGF (\* p < 0.05) and VEGF hydrogel (\*\* p < 0.01) decreased time spent in a  
601 dark compartment indicating an improvement of passive avoidance memory. Interestingly, TDC  
602 in VEGF gel was significantly lower than that in animals treated with VEGF alone (# p < 0.05).

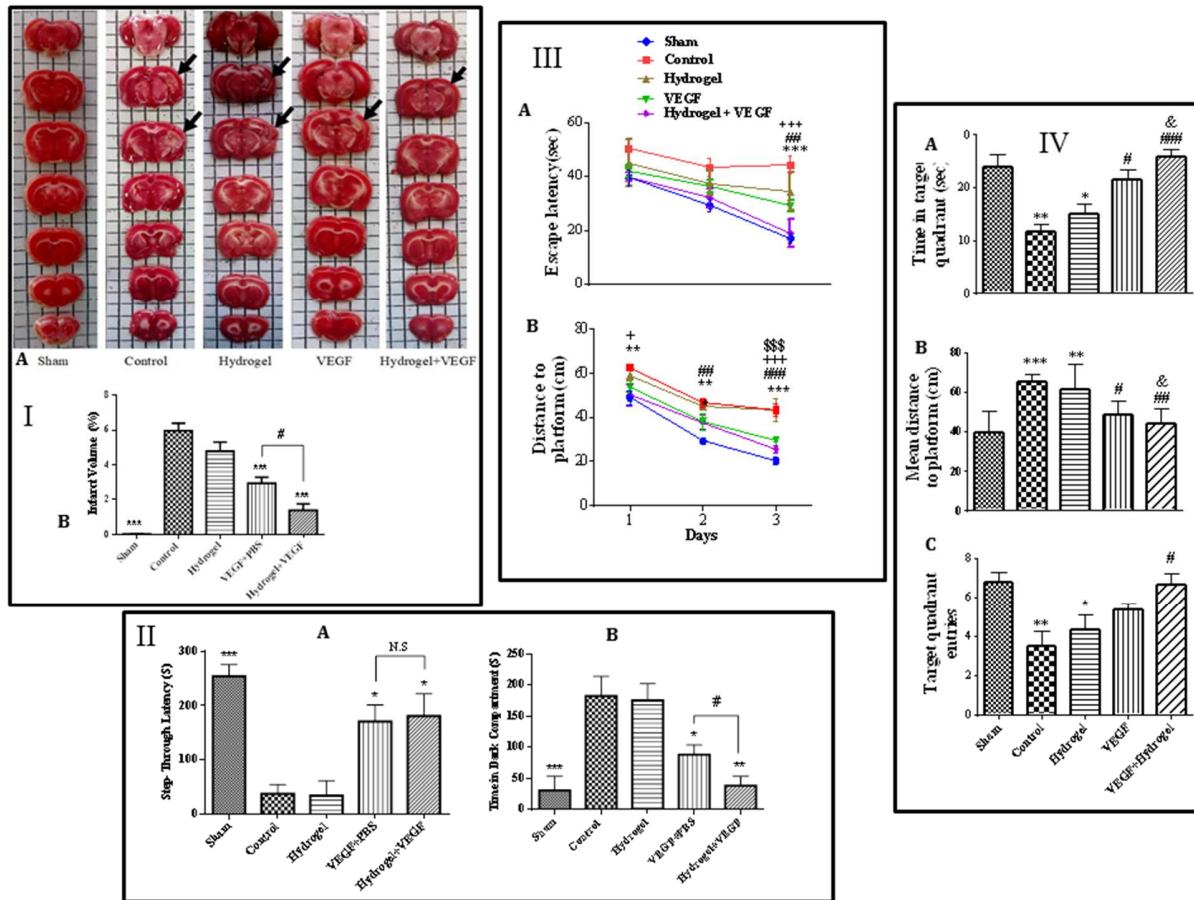
##### 603 **4.3 Injectable electroactive hydrogel containing VEGF improved spatial learning** 604 **and memory in animals suffering from selective hippocampal ischemia**

605 Two-way ANOVA revealed a significant influence of treatment and time (F (2, 63) = 21.03, P <  
606 0.001 and F (4, 63) = 14.17, P < 0.001, respectively) for the latency to locate the platform  
607 (escape latency) during four trials each day. Further analysis using Bonferroni posttest indicated  
608 a significant difference among sham and control (ischemic) groups (p < 0.05) on the second day.

609 On the third day, ischemic animals spent more time to find the platform in contrast to the sham  
610 group. No significant difference was detected between the control group and ischemic animals  
611 treated with hydrogel alone. However, the treatment of ischemic animals with VEGF alone ( $p <$   
612  $0.05$ ) and VEGF loaded hydrogel ( $p < 0.001$ ) resulted in improved escape latency compared to  
613 the control group (**Figure. 7(II-A)**). Distance to the platform is another parameter to analyze the  
614 animal's behavior to know how far they have swum from the platform. Two-way ANOVA  
615 followed by Bonferroni posttest revealed significant difference between sham and control group  
616 in the consecutive days (1st, 2<sup>nd</sup> and 3<sup>rd</sup> days,  $p < 0.01$ ,  $p < 0.001$  and  $p < 0.001$ , respectively),  
617 indicating that the control group swam farther away from the platform and showed impaired  
618 acquisition of the task. No difference was found between the control group and animals treated  
619 with hydrogel alone. However, treatment ischemic animals with VEGF hydrogel led to swim  
620 closer to the platform in the first and third days ( $p < 0.05$  and  $p < 0.001$ , respectively) in  
621 comparison with the control group. VEGF alone improved the performance on the third day ( $p <$   
622  $0.05$ ) in contrast to the control group (**Figure 7 (III-A and B)**).

623 Twenty-four h after training, the platform was taken out and animals were permitted to swim for  
624 60 s. The statistical analysis declared that the control group elapsed less time in the target  
625 quadrant compared to the sham group ( $p < 0.001$ ), indicating that hippocampal ischemia caused  
626 to impaired spatial memory performance (**Figure 7 (IV-A)**). Treatment with hydrogel could not  
627 rescue the spatial memory function. However, treatment with VEGF alone and VEGF loaded  
628 hydrogel reversed impaired spatial memory toward the sham level. Intriguingly, animals treated  
629 with VEGF hydrogel showed better performance compared with those treated with VEGF alone  
630 (**Figure 7 (IV-A)**,  $p < 0.05$ ).

631 Animals with hippocampal ischemia have also swum farther from the platform position,  
632 indicating that they failed to recall the task comparing to the sham group (**Figure 7 (IV-B)**,  $p <$   
633  $0.001$ ). Also, they showed less number of entries into the target quadrant in the test day  
634 compared to the sham group (**Figure 7 (IV-C)**). While treatment with the scaffold alone did not  
635 change the performance, VEGF containing hydrogel decreased the distance to the platform and  
636 increased the number of the entrance to the target quadrant in comparison with the control group  
637 ( $p < 0.01$  and  $p < 0.05$ , respectively). VEGF hydrogel group swam closer to the platform  
638 compared to the animals treated with VEGF alone ( $p < 0.05$ ).



639  
640

641 **Figure 7. (I)** VEGF loaded hydrogel decreased the infarct size in animals suffering from selective hippocampal  
 642 ischemia. Five days after drug microinjection, coronal brain slices were prepared and stained with TTC. The  
 643 unstained areas of the brain slice were defined as ischemic lesions. (B) The percentage of actual infarct volume in  
 644 the left hemisphere volume. The infarct volume was significantly increased in the control group compared with the  
 645 sham group ( $***p < 0.001$ ). However, treatment with VEGF and hydrogel containing VEGF treated groups  
 646 decreased the infarct size in contrast to the control group ( $*** p < 0.001$ ). No infarction was found in the  
 647 hippocampus of sham group. Animals treated with VEGF hydrogel showed lesser infarct volume compared with the  
 648 VEGF treated group ( $\#p < 0.05$ ). All values were expressed as mean  $\pm$  SEM ( $n=6$ ). (II) VEGF hydrogel rescued  
 649 animals with hippocampal ischemia more efficiently from avoidance memory impairment. (A) Step-Through  
 650 Latency (STL) at the retention test day in passive avoidance test. Animals in the sham, VEGF and hydrogel  
 651 containing VEGF treated groups had more latency to enter the dark compartment compared with the control group  
 652 ( $* p < 0.05$  and  $*** p < 0.001$ ). (B) Time in the dark compartment (TDC) at the retention test day in passive  
 653 avoidance test. Animals in the sham, VEGF and hydrogel containing VEGF treated groups spend less time in the  
 654 dark compartment compared with control group ( $* p < 0.05$ ,  $** p < 0.01$  and  $*** p < 0.001$ ). VEGF treated animals  
 655 spent less time in the dark compartment compared with the VEGF group ( $\# p < 0.05$ ). All values were expressed as  
 656 mean  $\pm$  SEM ( $n=6-7$ ). (III) Microinjection of VEGF hydrogel into the hippocampus improves acquisition of spatial  
 657 tasks in animals suffering from the hippocampal ischemia. All animals were trained in three consecutive days. (A)  
 658 Animals with hippocampal ischemia spent more time to locate platform in the tank compared to the sham group  
 659 ( $***p < 0.001$ ). Microinjection of scaffold hydrogel did not improve the performance. However, treatment with  
 660 hydrogel containing VEGF decrease the time to find the platform compared to the control group ( $+++p < 0.001$ ).

661 ##p<0.01 the sham group compared to the hydrogel group. (B) Induction of hippocampal infarction resulted in  
662 swimming farther from platform compared to the sham group. However, microinjection of hydrogel loaded VEGF  
663 into the infarcted hippocampus decreased the path swimming distance to the platform. All values were expressed as  
664 mean  $\pm$  SEM (n=6-8). \*\* p < 0.01 and \*\*\* p < 0.001 comparing the sham and control groups. ##p < 0.01 and ###p  
665 < 0.001 comparing sham and hydrogel groups. +p < 0.05 and +++p < 0.001 comparing control and VEGF loaded  
666 hydrogel groups. \$\$\$p < 0.001 comparing hydrogel and hydrogel containing VEGF groups. (IV) Treatment with  
667 VEGF hydrogel improved spatial memory function in animals with hippocampal infarction. (A) Animals  
668 hippocampal ischemia spent less time in the target quadrant compared with the sham group. However, treatment of  
669 ischemic animals with VEGF alone and hydrogel containing VEGF reverse the spatial memory toward sham group.  
670 (B) Induction of hippocampal ischemia resulted in swimming farther from the platform in control group compared  
671 to the sham group. Treatment with VEGF and hydrogel containing VEGF both decreased the distance to the  
672 platform. However, VEGF hydrogel group showed less swimming distance to the platform compared to the VEGF  
673 group. (C) The number of entrance into the target quadrant was significantly decreased in the control group  
674 compared to the sham group and treatment with hydrogel containing VEGF restored it toward the sham group. \* p <  
675 0.05, \*\* p < 0.01 and \*\*\* p < 0.001 comparing with sham group. All values were expressed as mean  $\pm$  SEM (n=6-  
676 8). # p < 0.05, ## p < 0.01 and ### p < 0.001 comparing with control group. & p < 0.05 comparing to VEGF group.

## 677 5. Conclusion

678 In the present study we designed a brain compatible hydrogel which mimics the electrical,  
679 electrochemical, and mechanical features of the hippocampus. Hydrogel was synthesized based  
680 on CS-aniline pentamer/Pluronic in which Pluronic endows the injectability feature to the  
681 hydrogel. Moreover, aniline pentamers endow the electroactivity along with antibacterial  
682 properties. CS has been selected due to its antibacterial and ECM-like properties. FTIR approved  
683 the hydrogel formation. Cyclic voltammetry and four probe method have been utilized to  
684 measure the electroactivity and conductivity, respectively. The conductivity of samples was  
685 similar to the brain tissue which can mimic the electrical properties of brain tissue. Mechanical  
686 properties of samples were evaluated by rheometry measurement which was accommodated with  
687 brain tissue. Moreover, the rheometry test proved the injectability properties. The AP enhanced  
688 the hydrogel thermal stability as confirmed by using DSC and TGA. We have shown that  
689 sustained release of VEGF in electroconductive hydrogel delivering system restored histological  
690 and behavioral consequences of focal cerebral ischemia. The results revealed that the infarcted  
691 size in VEGF hydrogel-treated rats was decreased more than 70 % compared to the ischemic  
692 (control) group. Behavioral tests confirmed the comparable beneficial effect of sustained  
693 delivery of VEGF on hippocampal dependent learning and memory performance. In almost all  
694 tests, sustained release of VEGF showed superior beneficial effects rather than VEGF alone. The  
695 hippocampal injuries resulted in different dysfunctions ranging from cognitive deficit to  
696 epilepsy, and multiple methods have been suggested for the regeneration of ischemic

697 hippocampus. Miscellaneous materials have been tested for regeneration of hippocampal neurons  
698 following ischemia. In the present study, a novel biocompatible conductive injectable hydrogel  
699 has been designed and examined on the rat model of ischemia induced in the hippocampus.  
700 VEGF is the main regulator of angiogenesis which has been reported that is strongly up-  
701 regulated within the penumbra (ischemic border). In addition, VEGF receptors have been shown  
702 to up-regulated at the border after two days and later in the ischemic core [93], indicating its  
703 important action on restoring tissue homeostasis after injuries. In consistent with our data  
704 multiple studies showed that VEGF may have potential therapeutic role in neuronal surveillance  
705 in brain injuries [93-96]. However, its short half-life and poor accessibility to the desired cerebral  
706 regions after peripheral administration limit its potential. Our injectable VEGF delivery system  
707 may provide continuous, dose-controlled delivery for long lasting time, controllable dosage, and  
708 capability of direct administration of VEGF at the site of injury. We found the protective effect  
709 of VEGF in young rat model of cerebral ischemia. Attention should be taken that stroke is  
710 usually occur in elderly individuals in which poor resilience impede the recovery of neural  
711 structure and function following injury [97, 98]. They also have different microenvironment and  
712 neuroinflammatory state rather than young individuals [99, 100]. Further study in old animal  
713 models may be required to address this issue.

#### 714 **Disclosure statement**

715 No potential conflict of interest was reported by the authors.

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