



Full length article

## Development of tannin-inspired antimicrobial bioadhesives

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## ABSTRACT

Tissue adhesives play an important role in surgery to close wounds, seal tissues, and stop bleeding, but existing adhesives are costly, cytotoxic, or bond weakly to tissue. Inspired by the water-resistant adhesion of plant-derived tannins, we herein report a new family of bioadhesives derived from a facile, one-step Michael addition of tannic acid and gelatin under oxidizing conditions and crosslinked by silver nitrate. The oxidized polyphenol groups of tannic acid enable wet tissue adhesion through catecholamine-like chemistry, while both tannic acid and silver nanoparticles reduced from silver nitrate provide antimicrobial sources inherent within the polymeric network. These tannin-inspired gelatin bioadhesives are low-cost and readily scalable and eliminate the concerns of potential neurological effect brought by mussel-inspired strategy due to the inclusion of dopamine; variations in gelatin source (fish, bovine, or porcine) and tannic acid feeding ratios resulted in tunable gelation times (36 s–8 min), controllable degradation (up to 100% degradation within a month), considerable wet tissue adhesion strengths (up to 3.7 times to that of fibrin glue), excellent cytocompatibility, as well as antibacterial and antifungal properties. The innate properties of tannic acid as a natural phenolic crosslinker, molecular glue, and antimicrobial agent warrant a unique and significant approach to bioadhesive design.

## Statement of Significance

This manuscript describes the development of a new family of tannin-inspired antimicrobial bioadhesives derived from a facile, one-step Michael addition of tannic acid and gelatin under oxidizing conditions and crosslinked by silver nitrate. Our strategy is new and can be easily extended to other polymer systems, low-cost and readily scalable, and eliminate the concerns of potential neurological effect brought by mussel-inspired strategy due to the inclusion of dopamine.

The tannin-inspired gelatin bioadhesives hold great promise for a number of applications in wound closure, tissue sealant, hemostasis, antimicrobial and cell/drug delivery, and would be interested to the readers from biomaterials, tissue engineering, and drug delivery area.

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## 1. Introduction

Tissue adhesives have revolutionized surgical procedures, assuming multiple roles as wound closure, tissue sealants, and

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hemostatic agents to reduce blood loss and promote healing [1–7]. However, existing bioadhesives demonstrate weak adhesion strength to wet tissue, require harsh chemical reactions, or possess poor biocompatibility, thereby greatly limiting clinical applications [1–8]. For example, tissue adhesives based on cyanoacrylate (Super Glue) possess high mechanical strength but are cytotoxic and the curing process is exothermic [9], while adhesives based on poly(ethylene glycol) (PEG) swell too much and fragile in nature. Most commercially available polyurethane-based bioadhesives, such as TissuGlu, suffer from exothermic and harsh chemical reactions during adhesive curing [9,10]. Moreover, surgical site infections

are major concerns that can prolong wound healing or cause abscess formation, especially for the cases of wound care in infection prone areas such as diabetic foot ulcers, yet traditional bioadhesives lack innate antimicrobial properties.

Mussel-inspired bioadhesives have gained wide attention, mimicking the strong underwater adhesion of the blue mussel *Mytilus edulis* by employing catechol group containing compounds such as L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine. Our recent works have highlighted the major functionalities of catechol side groups in forming polymeric networks and tissue chemical bonding, revealing strategies for facile synthesis of catechol-functionalized biodegradable polymers with greatly improved wet adhesion strengths [11–15]. However, the prohibitive costs of such compounds [4–8] and neurological effects of dopamine pose concerns on the commercialization of such catechol-functionalized tissue adhesives [16].

To overcome such challenges in bioadhesive engineering, plant-derived polyphenol compounds such as tannins may serve as a remarkable source of catechol/pyrogallol groups that is both safe and low-cost [17–19]. Tannic acid, in particular, meets the following criteria as a keystone ingredient in bioadhesive synthesis: (i) diverse bonding functionalities owed to its five-arm polyphenol structure, enabling dense polymeric crosslinks through hydrogen and ionic bonding or hydrophobic interactions [19,20], (ii) covalent interactions with amino groups of peptides, leading to improved mechanical properties in gelatin-based adhesives [19,21], (iii) pyrogallol moieties which, when oxidized, can chemically bond with tissue to yield strong tissue adhesion [21], and (iv) innate antimicrobial capabilities owed to the astringency and membrane action of oxidized tannins [22–25]. Despite these advantages, tannic acid is rarely incorporated into bioadhesive formulations, as its multiple noncovalent interactions tend to drive hydrogels toward coacervation rather than network formation [21,26–29]. Thus, by taking advantage of the above unique combinatory features, we herein designed a new family of tannin-inspired bioadhesives produced by a facile, one-step Michael addition reaction of tannic acid and gelatin under oxidizing conditions (Scheme 1). Various sources of gelatin (fish, bovine, or porcine), tannic acid feeding ratios, and crosslink initiator content (% silver nitrate) were formulated systematically to produce a family of bioadhesives with fine tunable gelation times and degradation rates (Scheme 2). Overall, the tannin-inspired gelatin bioadhesives exhibited considerable wet tissue adhesion strength and intrinsic antibacterial and antifungal properties, promising a versatile platform for the design of surgical tissue adhesives.

## 2. Experimental section

### 2.1. Materials

Gelatin from cold water fish skin (FGel, G7041), gelatin from bovine skin (BGel, gel strength ~225 g Bloom, Type B, G9382), and tannic acid (TA, 403040) were all purchased from Sigma-Aldrich and used without further purification.

### 2.2. General measurements

<sup>1</sup>H-NMR spectra of modified and unmodified gelatin polymers were recorded on a 300 MHz Bruker DPX-300 FT-NMR spectrometer in DMSO-*d*<sub>6</sub>. Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectra were measured with a Nicolet 6700 FTIR spectrometer using polymer powder directly, with air used as background.

### 2.3. Synthesis of tannic acid modified gelatin (Gel-TA)

Gelatin was modified by tannic acid (TA) through the Michael Addition reaction between the amine groups of gelatin and the double bonds of catechol groups on oxidized TA, under basic condition. Briefly, gelatin (20 g) was stirred in DI water (200 mL) at 60 °C for 2 h to form a gelatin solution with a final concentration of 10 w/v%. The pH value of the solution was adjusted to 8.5 using 12 M NaOH. A solution of TA was then slowly added to the gelatin solution, while stirring, at defined TA/gelatin ratios (1, 2, 5, or 10%, w/w to gelatin), and the mixture was allowed to react for another 3 h at 60 °C. The pH value of the mixture during reaction was monitored with a pH meter to maintain a constant pH of 8.5 by dropwise addition of NaOH (1 M). Air was bubbled into the systems throughout the reaction. Following the reaction, the pH value of the solution was adjusted into 7.4, and the solution was dialyzed against DI water at room temperature (for TA modified FGel, or FGel-TA) or 37 °C (for TA modified BGel, or BGel-TA) using a dialysis tube with a molecular weight cut-off (MWCO) of 1000 Da. The dialyzed solution was then freeze-dried to obtain a TA modified gelatin with x wt% of TA (FGel-TAx or Fx; BGel-TAx or Bx). Gel-TAs with different gelatin sources and TA feeding ratios were synthesized, the names are listed in Table 1. <sup>1</sup>H NMR (300 MHz; DMSO-*d*<sub>6</sub>; δ, ppm) of FGel-TA: 0.86–5 (m, amino acid on gelatin), 6.78–6.9 (m, protons on the benzene rings of TA). FTIR of FGel-TA (cm<sup>-1</sup>): 1638 (strong,  $\text{—COO—}$  and  $\text{—CONH—}$ ), 1036 (weak, benzene rings from TA).

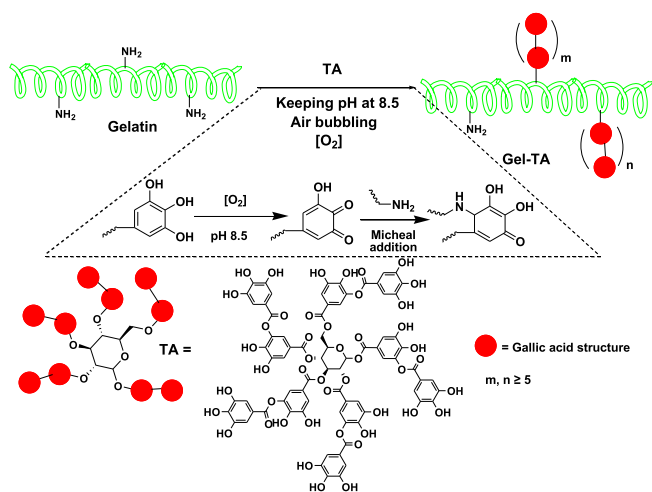
### 2.4. Assessment of gelation times

#### 2.4.1. Cross-linking of Gel-TA and measurement of set time

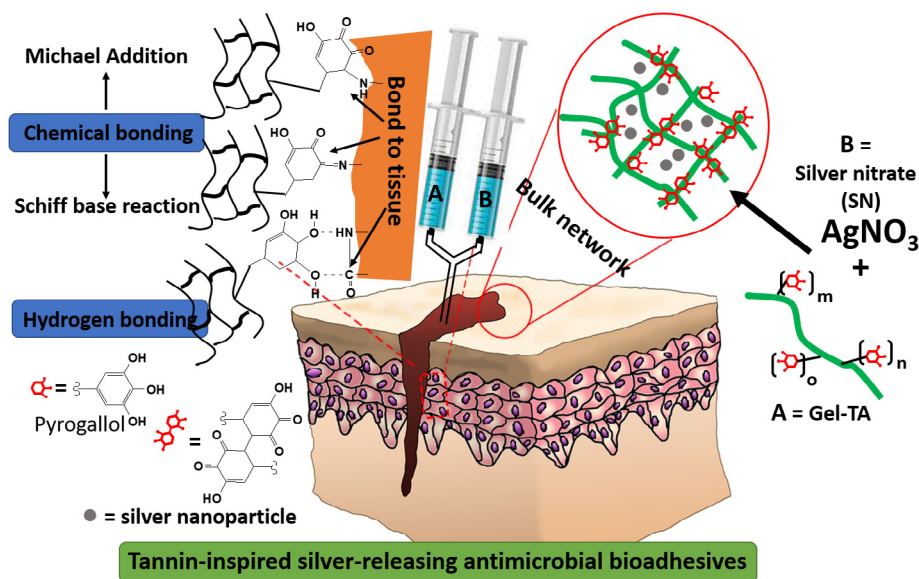
Gel-TA polymers, at 15 wt% in Tris-HNO<sub>3</sub> buffer (pH 8.5), were crosslinked using equal volume of silver nitrate (SN) solutions at either room (25 °C) or body (37 °C) temperature (Scheme 2). The gel times (or set times) of various formulations were determined by the tilt test, repeated in triplicates and averaged (Table 2). The names of SN crosslinked Gel-TA hydrogels are also listed in Table 2.

#### 2.4.2. Rheological evaluations

Rheological evaluations were conducted using Discovery Series Hybrid Rheometer (DHR-1, TA Instruments, USA) in a parallel plate configuration, employing sandblasted stainless steel 40 mm diameter plates throughout and a Peltier plate for temperature control. In a representative rheological test for gelling kinetics, 2 mL Gel-TA in Tris-HNO<sub>3</sub> (15 wt%, pH 8.5) was mixed with 2 mL of silver nitrate (SN) solution in DI water (0.075 g/mL or 0.1 g/mL). This mixture was applied to the lower plate of the rheometer, preheated



**Scheme 1.** Synthesis of tannic acid modified gelatin (Gel-TA).



**Scheme 2.** Tannin-inspired antimicrobial bioadhesives crosslinked by silver nitrate (SN). The bulk network and tissue bonding structures are also schemed.

**Table 1**  
Nomenclature and feeding ratio of TA modified gelatin (Gel-TA).

Polymer name	Gelatin used	TA feeding ratio to Gel (wt%)
FGel-TA1 (F1)	Gelatin from cold fish skin (FGel)	1
FGel-TA2 (F2)	FGel	2
FGel-TA5 (F5)	FGel	5
FGel-TA10 (F10)	FGel	10
BGel-TA2 (B2)	Gelatin from bovine skin (BGel)	2
BGel-TA10 (B10)	BGel	10
PGel-TA2 (P2)	Gelatin from porcine skin (PGel)	2

to a preset temperature. The upper plate was immediately brought down to a gap distance of 40  $\mu\text{m}$  to begin measurement. A low frequency of 1 Hz and 1% strain was applied to minimize interference with the gelation process and to keep the measurement within the linear viscoelastic region. The gelation kinetics was measured in a time sweep by monitoring the change of storage ( $G'$ ) and loss ( $G''$ ) moduli as a function of time. All measurements were repeated in triplicates.

**Table 2**  
Gel times of tannic acid modified gelatins at different pH values, with different tannic acid feeding ratios (1, 2, 5, or 10 wt%) or obtained from different sources (cold fish skin (F), bovine skin (B), or porcine skin (P)), crosslinked by  $\text{AgNO}_3$  solutions at different temperatures.

Polymer <sup>a</sup>	Polymer concentration <sup>a</sup> (wt%)	$\text{AgNO}_3$ concentration <sup>b</sup> (g/mL)	pH value <sup>c</sup>	Test temperature ( $^{\circ}\text{C}$ )	Measured gel time (s)	Names of crosslinked hydrogels
FGel-TA1	15	0.1	$\sim 7.4^d$	RT <sup>e</sup>	Uncross-linkable	–
FGel-TA2	15	0.1	$\sim 7.4^d$	RT <sup>e</sup>	235 $\pm$ 35	F2-S100 (F-100)
FGel-TA2	15	0.075	$\sim 7.4^d$	RT <sup>e</sup>	282 $\pm$ 23	F2-S75 (F-75)
FGel-TA2	15	0.05	$\sim 7.4^d$	RT <sup>e</sup>	480 $\pm$ 23	F2-S50
FGel-TA2	15	0.05	8.5	RT <sup>e</sup>	229 $\pm$ 5	–
FGel-TA2	15	0.05	$\sim 7.4^d$	37	246 $\pm$ 8	–
FGel-TA5	15	0.05	$\sim 7.4^d$	RT <sup>e</sup>	189 $\pm$ 42	F5-S50
FGel-TA10	15	0.05	$\sim 7.4^d$	RT <sup>e</sup>	36 $\pm$ 11	F10-S50
FGel-TA2	5	0.1	$\sim 7.4^d$	RT <sup>e</sup>	Uncross-linkable	–
BGel-TA2	15	0.075	$\sim 7.4^d$	RT <sup>e</sup>	410 $\pm$ 88	B2-S75 (B-75)
PGel-TA2	15	0.075	$\sim 7.4^d$	RT <sup>e</sup>	25 $\pm$ 7	P2-S75

<sup>a</sup> Polymers were dissolved in Tris- $\text{HNO}_3$  (pH 8.5) buffer solution.

<sup>b</sup> Polymer solution/ $\text{AgNO}_3$  solution ratio was kept at 1/1 g/mL.

<sup>c</sup> This is the pH value of polymer solution.

<sup>d</sup> The pH values of Gel-TAx in Tris- $\text{HNO}_3$  (pH 8.5) buffer solution were all around 7.4 for different polymers.

<sup>e</sup> RT: room temperature.

## 2.5. Properties of the crosslinked Gel-TA family

Mechanical properties of dried Gel-TAs crosslinked by SN, including tensile strength, Young's modulus and elongation at break, were measured according to ASTM D412A on an Instron 5966 machine fitted with a 1 KN (for dry samples) or 10 N (for wet samples) load cell (Instron, Norwood, MA). Briefly, strip shaped samples (25 mm  $\times$  6 mm  $\times$  1.5 mm, length  $\times$  width  $\times$  thickness) were pulled at a rate of 500 mm/min and elongated to failure. The Young's modulus was obtained by calculating the gradient from 0 to 1% (for dry samples) or 10% (for wet samples) of elongation of the stress-strain curve. Eight specimens per sample were tested and averaged. In order to evaluate the effect of hydration on the mechanical properties, the mechanical tests were also conducted on wet samples with a water content of around 10 wt% or 50 wt%.

The sol/gel content, an indication of non-crosslinked/crosslinked fractions of the hydrogel, and swelling ratio were measured by the mass differential before and after incubation of the crosslinked polymer in 1, 4-dioxane (sol content) or water

(swelling ratio) as described previously [4–7]. The sol content and swelling ratio were then calculated using Eqs. (1) and (2), respectively.

$$\text{Sol content(\%)} = \frac{W_i - W_d}{W_i} \times 100 \quad (1)$$

$$\text{Swelling ratio(\%)} = \frac{W_s - W_d}{W_d} \times 100 \quad (2)$$

Here  $W_i$  represents the initial dry weight of crosslinked Gel-TA hydrogel disk,  $W_d$  represents the weight of freeze-dried sample after the un-crosslinked part was washed by 1, 4-dioxane for 48 h, and  $W_s$  represents the network weight after the leached and dried sample was suspended in water for 24 h. Six samples were tested for each study group ( $n = 6$ ).

Degradation studies were conducted in PBS (pH 7.4) and at 37 °C using cylindrical disc specimens (7 mm in diameter, 2 mm thick) as described previously [4–7]. The mass loss was calculated by comparing the initial mass ( $W_0$ ) with the mass measured at the pre-determined time points ( $W_t$ ) using Eq. (3). Six samples were tested for each study group ( $n = 6$ ).

$$\text{Mass loss(\%)} = \frac{W_0 - W_t}{W_0} \times 100 \quad (3)$$

## 2.6. Adhesion strength of SN crosslinked Gel-TAs

The adhesion strength of Gel-TAs crosslinked by SN was determined by the lap shear strength test adapted from ASTM F2255-05 standard and method used in previous literatures [30,31]. The detailed testing process was described in our previous study [4,6].

## 2.7. Cytocompatibility tests of Gel-TAs and crosslinked Gel-TA hydrogels

The cytocompatibility of Gel-TA polymers and crosslinked Gel-TA hydrogels were evaluated as described previously [6]. Briefly, human-derived mesenchymal stem cells (hMSCs, ATCC® PCS-500-012™) were purchased from ATCC and cultured in growth media (Dulbecco's modified eagle's medium (DMEM), 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic antimycotic solution (100×)) up to passages 5–10 prior to the cell cytotoxicity and proliferation studies. *In vitro* pre-polymer cytotoxicity was assessed by the MTT (methylthiazolyl-diphenyl-tetrazolium bromide) assay against hMSCs, with commercially available poly (ethylene glycol) diacrylate (PEGDA, Mn = 700 Da) and unmodified gelatins (from cold fish skin or bovine skin) serving as positive and negative controls respectively. Various Gel-TA polymer formulations, gelatin, and PEGDA were prepared at concentrations of 10, 1, and 0.1 mg/mL in growth media, with pH adjusted to 7.4. To each well of a 96-well cell culture plate, 200 μL of hMSCs in growth media, at a density of  $5 \times 10^4$  cells/mL, were added and incubated for 24 h at 37 °C, 5% CO<sub>2</sub> and 95% relative humidity. The medium was then completely replaced by 200 μL of the above polymer solutions (10, 1, and 0.1 mg/mL in growth media), and incubated for another 24 h prior to performing MTT assays. Viabilities of cells in Gel-TA polymer, gelatin, or PEGDA containing growth media were normalized to that of cells cultured in blank growth media.

The cytotoxicity of sol contents (or leachable fractions) and degradation products of SN crosslinked Gel-TA hydrogels were also studied using the MTT assay against hMSCs, while FDA approved poly (lactic-co-glycolic acid) (PLGA, LA/GA = 50/50, Mw ~60kDa, purchased from Polysciotech) served as control. The sol content solutions of Gel-TA hydrogels were obtained by incubating equal mass (0.5 g) hydrogel samples in 5 mL of PBS (pH 7.4) at 37 °C for 24 h. Next, three different dilutions were prepared for testing:

1×, 10× and 100×, where 1× was the solution of leached products with no dilution, while 10× and 100× were solutions with 10 times and 100 times dilution of the 1× solution in PBS, respectively. To each well of a 96-well cell culture plate, 200 μL of hMSCs in growth media at a density of  $5 \times 10^4$  cells/mL were added and incubated for 24 h. Next, 20 μL of the above sol content solutions were added and the cells were incubated for another 24 h prior to performing the MTT assays. Six samples were tested for each study group ( $n = 6$ ).

Following, cytotoxicity of the hydrogels' degradation products were evaluated by fully degrading equal weight (1 g) of Gel-TA hydrogel samples as well as PLGA in 10 mL of 0.2 M NaOH solution. After adjusting pH to 7.4, the resultant solutions were diluted to three concentrations (1×, 10× and 100× as above) using PBS (pH 7.4), and used for MTT analysis as described above. Six samples were tested for each study group ( $n = 6$ ).

All of the above solutions were pH-neutralized and passed through a sterilized 0.2 μm filter prior to use for cell culture. The cell viability results were normalized to the viability of cells in blank growth media.

As a further assessment of film cytotoxicity, cell adhesion and proliferation of hMSCs were studied on BGel-TA2 SN 0.075 g/mL (B2-S75, or B-75) as a representative film using Live/Dead staining. Briefly, 20 μL of BGel-TA2 (B2) and 20 μL of silver nitrate solution (0.075 g/mL) were mixed together and uniformly coated onto the surface of a glass slide, forming roughly 15 mm diameter thin films. The crosslinked B75 films were then sterilized by incubation in 70% ethanol for 24 h followed by exposure to UV light for 3 h. The samples were then placed in 24-well plates and seeded with 500 μL hMSC solutions at 5000 cells/cm<sup>2</sup>, followed by growth media replacement the next day. At each time point (1, 3 and 7 days post cell seeding), the constructs were removed from the well plate, rinsed by PBS, and stained by Live/Dead Viability/Cytotoxicity Kit (Invitrogen, molecular probes, Eugene, OR) to observe cell morphology using an inverted light microscope (Nikon Eclipse Ti-U) equipped with a ANDOR DL-604M-#VP camera and Prior Lumen 200.

## 2.8. Antimicrobial performance of crosslinked Gel-TA hydrogels

The anti-bacterial and anti-fungal potencies of SN crosslinked Gel-TA hydrogels were evaluated using *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) as positive and negative bacteria models, and *Candida albicans* (*C. albicans*) as a fungi model.

### 2.8.1. Anti-bacterial performance of SN crosslinked Gel-TA hydrogels

**2.8.1.1. Bacterial incubation.** *Staphylococcus aureus* (*S. aureus*, ATCC® 6538™) and *Escherichia coli* (*E. coli*, ATCC® 25922™) were purchased from ATCC (American Type Culture Collection) and used following established safety protocols. Tryptic soy broth (Cat. #: C7141) and tryptic soy agar (Cat. #: C7121) used for *S. aureus* culture were purchased from Criterion (via VWR). Luria Broth Base (LB broth, Cat. #: 12795-027) and Select Agar (Cat. #: 30391-023) used for *E. coli* culture were purchased from Invitrogen. *S. aureus* and *E. coli* were cultured at 37 °C in sterilized tryptic soy broth and LB broth respectively under 150 rpm in a rotary shaker overnight, and the obtained bacteria suspensions were diluted into desired concentrations before use.

**2.8.1.2. In-vitro evaluation of bacterial inhibition.** The bacterial inhibition ratios of SN crosslinked Gel-TA hydrogels against *S. aureus* and *E. coli* were evaluated using F2-S75 (F-75), F2-S100 (F-100), B2-S75 (B-75), and B-2-S100 (B-100) as the representative experimental samples and PEGDA/HEMA (w/w = 1/1) as negative control [5,6,32]. Briefly, 0.2 g of freeze-dried hydrogels were immersed in 20 mL of germ containing broth with bacterial concentrations of



around  $1 \times 10^6$  CFUs/mL (CFU: colony forming unit) [32]. The samples were then incubated at 37 °C with oscillation at a frequency of 150 rpm. Broth containing only bacteria served as another negative control. After 24 h incubation, the bacterial inhibition ratios were determined. To eliminate the effect of silver chloride (formed by the reaction between residual SN in crosslinked Gel-TA hydrogels and NaCl in broth) dispersion on optical density (OD) of bacteria suspension, instead of OD value test, an agar-casting and colony-counting method was used to determine the bacterial inhibition ratio. Briefly, the broth of each sample was removed after 24 h incubation, diluted and casted onto an agar plate for colony counting after another 24 h incubation. For each hydrogel, at least 3 specimens were used and 2 agar-casting and colony-counting were conducted for each specimen. The bacterial inhibition ratios of hydrogels were calculated by equation (4):

$$\text{Inhibition ratio(\%)} = 100 - 100 \times \frac{N_t - N_0}{N_{con} - N_0} \quad (4)$$

where  $N_0$  is the seeding bacteria concentration (CFUs/mL) in broth medium,  $N_t$  and  $N_{con}$  are bacteria concentrations following 24 h incubation of the hydrogel samples and pure broth control respectively.

**2.8.1.3. Antibacterial evaluation by zone of inhibition.** F-75, F-100, B-75 and B-100, as well as PEGDA/HEMA hydrogel (as control), were used to test anti-bacterial inhibition halos by a modified Kirby Bauer technique [6,33,34]. Briefly, 10 mL of *S. aureus* or *E. coli* in broth (at OD<sub>600nm</sub> values around 0.07) were respectively dispersed onto a tryptic soy or LB agar plate ( $\Phi 100 \times 7$  mm). Next, hydrogel disks ( $\Phi 5$  mm) were placed onto the agar plate and incubated for 24 h at 37 °C. After incubation, the bacterial inhibition halos around the hydrogel samples were observed and their diameters were measured.

## 2.8.2. Anti-fungal performance of SN crosslinked Gel-TA hydrogels

**2.8.2.1. Fungi incubation.** Fungi (*Candida albicans*, *C. albicans*) was purchased from ATCC (ATCC<sup>®</sup> 10231<sup>™</sup>), and used following established safety protocols. YM medium broth (Lot #: 1964C030) and YM agar (Lot #: 1964C030) used for fungi (*C. albicans*) culture were respectively obtained from Amresco and Acumedia. Tween 20, for stabilizing fungi suspensions, was added to YM broth medium to obtain a final concentration of 0.5 wt% and then sterilized. Throughout the experiments, *C. albicans* was maintained on YM agar plates. To obtain a working concentration of fungi, *C. albicans* was scraped from YM agar plates, dispersed in Tween 20 containing YM broth medium, counted with a hemocytometer, and then diluted into a final fungi concentration of  $0.5\text{--}1 \times 10^7$  cells/mL [6,35]. Quantitative evaluation of fungal survival was obtained using a colony counting method, described below in Section 2.8.2.2.

**2.8.2.2. Anti-fungal effect of direct exposure to hydrogels.** The anti-fungal effect of direct exposure to SN crosslinked Gel-TA hydrogels was tested using F-75, F-100, B-75, and B-100 as the representative experimental groups and PEGDA/HEMA as control. Briefly, 20 mg freeze-dried hydrogel disks were placed in the wells of a 24-well tissue culture plate, and 2 mL of *C. albicans* suspension in Tween-20 containing YM broth medium ( $0.5\text{--}1 \times 10^7$  cells/mL) was added to each well. Samples without hydrogel were used as blank control. The 24-well plates were incubated for 3 h at 37 °C with a shaking speed of 100 rpm. Then the fungi containing medium was diluted 300 times, and 0.3 mL diluted medium was removed and cast on YM agar plates ( $\Phi 6 \times 2$  mm). After incubation at 37 °C for 24 h, fungi colonies on the YM agar plates were counted, and the fungi survival ratios were calculated according to equation (5). For each

sample, at least 6 plates were cast ( $n = 6$ ), and the numbers were averaged.

$$\text{Fungal survival ratio(\%)} = \frac{N_s}{N_{con}} \times 100 \quad (5)$$

Here,  $N_s$  is the number of fungal colonies for sample, and  $N_{con}$  stands for the number of fungal colonies for YM broth blank control.

**2.8.2.3. Halo test.** The anti-fungal performance of SN crosslinked Gel-TA was also evaluated using the halo test method with PEGDA/HEMA as control. Briefly, 4 mL of YM broth medium containing  $0.5\text{--}1 \times 10^7$  cells/mL *C. albicans* was evenly cast onto YM agar plates ( $\Phi 100 \times 7$  mm). The hydrogel discs (around  $\Phi 5$  mm) were placed on the agar plate and the constructs were incubated at 37 °C for 24 h under dark before being examined for a “halo” or “zone of inhibition” surrounding the gel disc.

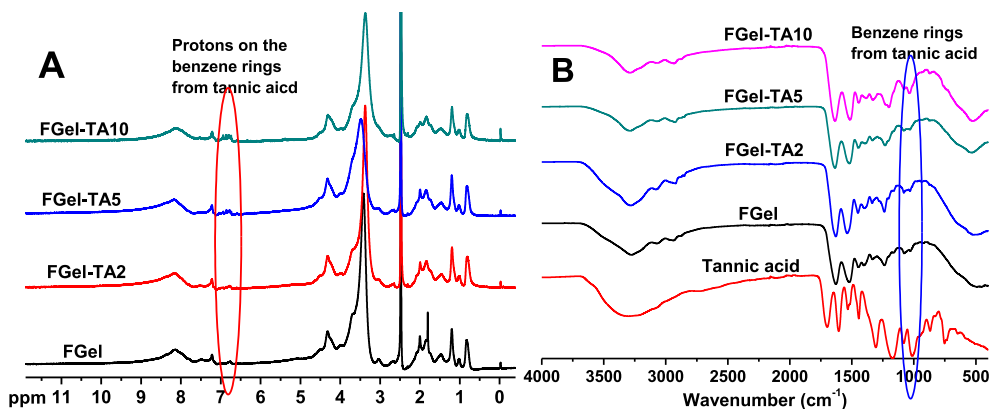
## 2.9. Statistical analysis

All statistical data were expressed as mean  $\pm$  standard deviation. Statistical analysis was performed using Student's *t*-test. Data were considered to be significant, when  $p < 0.05$ .

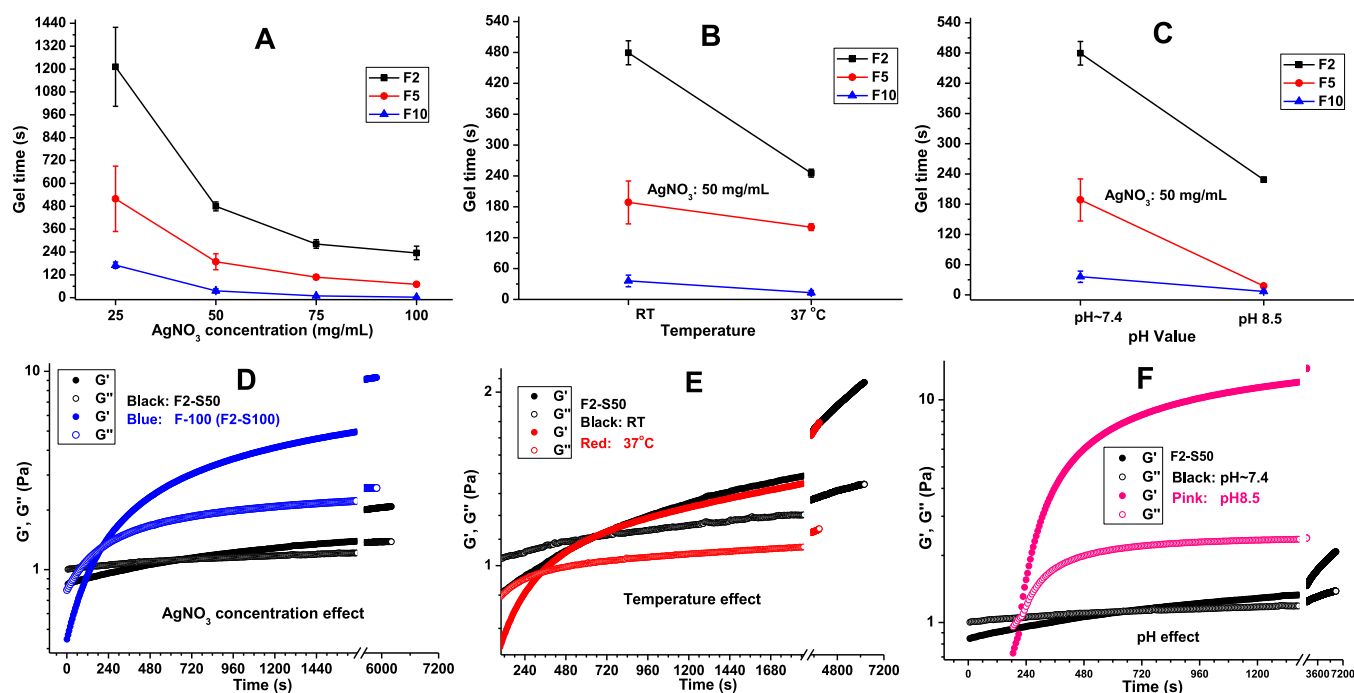
## 3. Results and discussion

We chose animal-derived gelatins as our starting material due to their wide availability, favorable biocompatibility, and high wet mechanical strengths [6]. Typically, the modification of gelatin for adhesives employs 1-Ethyl-3-(3-dimethylamino propyl) carbodiimide (EDC) coupling or vinyl groups (e.g. methacrylated gelatins), but former method can self-crosslink gelatin [36–39], while the latter requires a large excess of methacrylic anhydride and subsequent dialysis [40]. Here, we demonstrate that gelatin modification can be performed with tannic acid (TA) through a one-step Michael addition reaction that is low-cost, convenient, and scalable. Additionally, tannic acid is a widely used food additive that is categorized as GRAS (generally recognized as safe) [29]. Since tannic acid (TA) and gelatin (Gel) are both multifunctional molecules, the conjugation of TA onto Gel may lead to self-crosslinking, especially at high gelatin concentrations and high tannic acid feeding ratios, especially with porcine derived gelatin (PGel) that possesses high molecular weights and low water solubility. By adjusting gelatin concentrations and tannic acid feeding ratios, a family of tannic acid modified gelatin (Gel-TA, Scheme 1) was synthesized using low gelatin concentrations (up to 10 wt%), 1–10% tannic acid (wt % of gelatin), and various gelatin sources (fish, bovine, or porcine) in slightly basic water (pH 8.5). In this paper, both TA modified fish gelatin (derived from cold water fish skin) (FGel-TA) and TA modified gelatins from bovine skin (BGel-TA) were synthesized. NMR (Fig. 1A) and FTIR (Fig. 1B) spectra of FGel-TA polymers revealed characteristic peaks corresponding to the C—H protons (NMR) or C—H vibration (FTIR) on the benzene ring near the phenol —OH groups of TA ( $\sim 6.8$  ppm in NMR and  $\sim 1000$  cm<sup>-1</sup> in FTIR) that increased with higher TA feeding ratios ranging from 0, 1, 2, 5 to 10 wt%.

Following, we studied the gelation times of Gel-TA varying temperature, pH conditions, and crosslink initiator content. We chose silver nitrate (SN) as the crosslink initiator based on its compatibility with pyrogallol-functionalized polymers [41] and as an additional source of antimicrobial agent (Scheme 2). Overall, the gelation times ranged from 36 s to 8 min, as tabulated in Table 2. Increasing SN content resulted in a decrease in gelation times among FGel-TA2 (F2), FGel-TA5 (F5), and FGel-TA10 (F10)



**Fig. 1.**  $^1\text{H-NMR}$  (A) and FTIR (B) spectra of gelatin (from cold fish skin) modified by tannic acid. FGel: gelatin from cold fish; FGel-TAx (Fx) refers to gelatin (from cold fish) modified by x wt% (to gelatin) of tannic acid (TA).



**Fig. 2.** Gel times (obtained by tilting tests) of Fx (x = 2, 5, or 10) crosslinked by  $\text{AgNO}_3$  solutions with different concentrations (A), at different temperatures (B), or at different pH values (C, pH values mean that of Fx solutions, pH value of  $\text{AgNO}_3$  solution was used without adjusting); Rheology test results of F2 crosslinked by  $\text{AgNO}_3$  (SN) solutions with different concentration (D), at different temperatures (E), or at different pH values (F).

**Table 3**  
Mechanical properties of different gelatin-TA crosslinked by silver nitrate (SN) in dry and fully hydrated (swollen) states.

Sample	Tensile strength (MPa)		Elongation at break (%)		Modulus (MPa)	
	Dry	Swollen	Dry	Swollen	Dry	Swollen
F-75	33 ± 8	3 ± 0.5 <sup>a</sup>	1.1 ± 0.1	391 ± 19 <sup>a</sup>	3000 ± 540	76 ± 13 <sup>a</sup>
F-75		0.50 ± 0.0 <sup>b</sup>		713 ± 54 <sup>b</sup>		0.12 ± 0.0 <sup>b</sup>
F-100	30 ± 3	–	2.3 ± 1.0	–	1600 ± 500	–
F5-S75	20 ± 6	3.2 ± 0.7 <sup>a</sup>	1.5 ± 0.1	280 ± 49 <sup>a</sup>	1400 ± 360	102 ± 44 <sup>a</sup>
B-75	59 ± 7	3.3 ± 0.7 <sup>a</sup>	1.0 ± 0.1	185 ± 34 <sup>a</sup>	3700 ± 730	48 ± 10 <sup>a</sup>
B-75		0.7 ± 0.1 <sup>b</sup>		348 ± 39 <sup>b</sup>		0.5 ± 0.1 <sup>b</sup>
B5-S75	–	6.6 ± 0.8 <sup>a</sup>	–	111 ± 11 <sup>a</sup>	–	173 ± 14 <sup>a</sup>

<sup>a</sup> Film samples with water content around 10 wt% (for F2-S75 (F-75), 9.9 wt%, for F5-S75, 11.66 wt%, for B2-S75 (B-75), 12.0 wt%, for B5-S75, 11.55 wt%).

<sup>b</sup> Film samples with water content around 50 wt% (for F-75, 51 wt%, for B-75, 45 wt%).

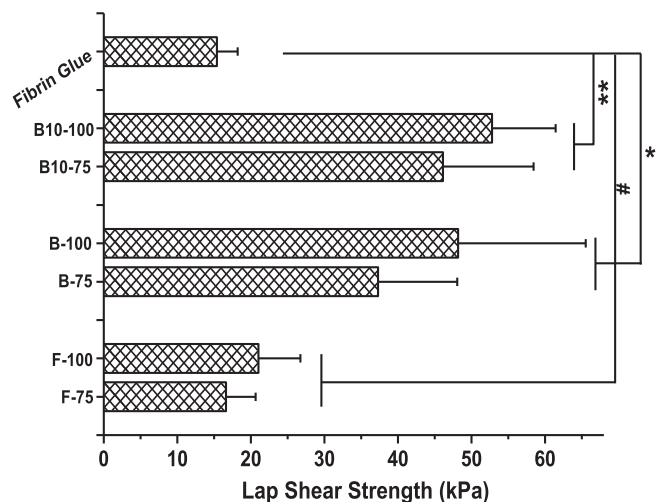
(Fig. 2A), as supported by rheological studies (Fig. 2D). Increase in temperature from room to body temperature also decreased gelation times (Fig. 2B and E). Finally, increasing the pH conditions of

gelatin solution from ~7.4 to 8.5 resulted in decreased gelation times for F2, F5, and F10, with F2 showing the most noticeable reduction in gelation time (Fig. 2C and F).

### 3.1. Mechanical characterization

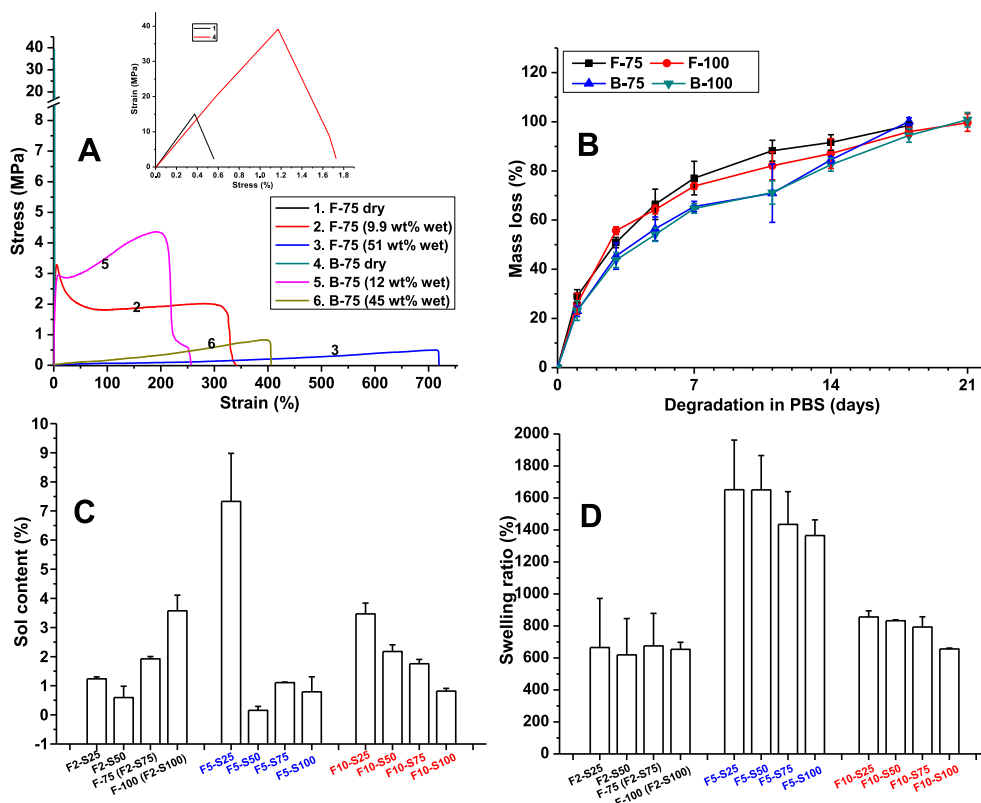
We then performed mechanical studies of dry and wet Gel-TA hydrogels crosslinked by silver nitrate (SN) (Gel-TA-SN) (Table 3 and Fig. 3A). Gelatins from different sources have different mechanical strengths; BGel itself possesses superior mechanical property compared to FGel. When Gel-TAs were crosslinked by 0.075 g/mL SN and at dry state, B-75 possessed much higher tensile strength (~40 MPa) than that of F-75 (~1.5 MPa). Both dry B-75 and F-75 were brittle with an elongation at break < 2%. However, upon absorbing 9.9% (of dry polymer weight) of water, F-75 became soft and elastic, with a tensile strength of  $2.97 \pm 0.487$  MPa and an elongation of  $391 \pm 19\%$ . Increasing water absorption to 51% resulted in a significant improvement in elasticity with an elongation higher than  $713 \pm 54\%$ , but with reduced tensile strength ( $0.5 \pm 0.0$  MPa) (Fig. 3A and Table 3). Likewise, B-75 exhibited improved elongation upon absorbing water (11.55 wt%), with even higher tensile strength ( $6.6 \pm 0.8$  MPa) than its fish-derived counterpart (Fig. 3A and Table 3). The above mechanical studies demonstrate the potential of tannin-derived gelatin hydrogels for applications ranging from topical use to tissue adhesives, as Gel-TA preserves sufficient mechanical strength upon water uptake while resembling the elasticity of soft tissue [42].

Subsequently, we sought to optimize the wet adhesion strength of our bioadhesive formulations. As shown in Fig. 4, the wet lap shear strengths of various Gel-TA-SN formulations ranged from  $16.6 \pm 4.0$  kPa (for F-75) and  $52.8 \pm 8.7$  kPa (for B-100). The lap shear strengths of FGel-TA-SN formulations to wet decellularized porcine small intestine submucosa (OASIS, Healthpoint Ltd. Fort Worth, TX) were close to that of the gold standard, fibrin glue [4,5,30], while the bovine-derived counterpart yielded even higher wet adhesion strengths, reflecting the stronger dry/wet mechanical properties above. Increasing of TA content also



**Fig. 4.** Adhesion strength of fibrin glue and gelatin-TA polymers crosslinked by silver nitrate (SN) to wet porcine small intestine submucosa, measured through lap shear strength test. B-100 (B2-S100): BGel-TA2-SN 0.1 g/mL; B10-75: BGel-TA10-SN 0.075 g/mL; B10-100: BGel-TA10-SN 0.1 g/mL (\*\*p < 0.01, \*p < 0.05, #p > 0.05).

improved the adhesion strength of the crosslinked bioadhesives. The lap shear strengths of BGel-TA-SN formulations were between 2.5 and 3.7 times that of fibrin glue ( $15 \pm 3$  kPa), with the greatest adhesion strength from B-100 (around 55 kPa). Although the lap shear strength tests using decellularized small intestine submucosa tissues can give a direct comparison between the Gel-TA bioadhesives and the fibrin glue under the same test conditions, using real tissues for lap shear strength tests will be conducted in our future studies to better mimic *in vivo* tissue adhesion situations.



**Fig. 3.** Mechanical properties (stress–strain curves, A), degradation profiles (B) of gelatin-TA (including FGel-TA and BGel-TA) crosslinked by silver nitrate (SN); sol contents (leachable fractions) (C), and swelling ratios (D) of FGel-TA crosslinked by silver nitrate (SN).

### 3.2. Assessment of biodegradability

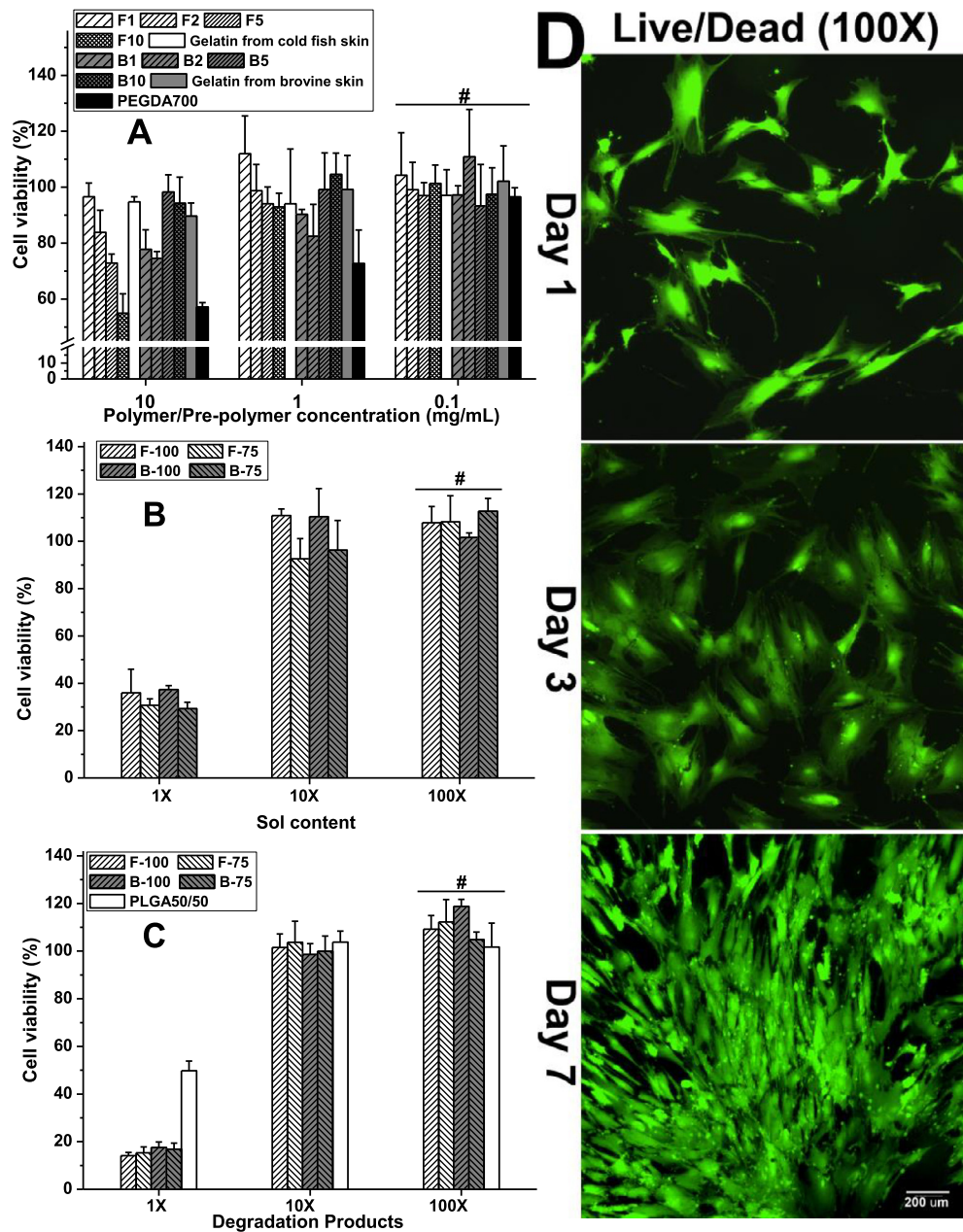
All of the tannin-inspired gelatin adhesives showed favorable degradation, with full degradation within 3 weeks for crosslinked FGel-TA-SN and BGel-TA-SN (Fig. 3B). As with dopamine-derived polyphenol polymers in our previous studies [6], silver nitrate appears to impart faster degradation compared to other crosslink initiators such as sodium periodate. We hypothesize that the oxidized catechol groups of Gel-TAs chelate silver nanoparticles (reduced from silver nitrate), limiting the extent of intermolecular crosslinks and contributing to faster degradation.

Afterwards, we studied sol content and swelling behaviors of our crosslinked bioadhesives. The sol contents of FGel-TA cross-linked by different concentrations of silver nitrate (SN) were between 0 and 5%, with the exception of FGel-TA5-SN 0.025 g/mL (F5-S25) at 10% (Fig. 3C). As expected, higher crosslinking density

led to lower sol content. The swelling ratios of the crosslinked FGel-TA-SN were in the range of 600% to 1600% (Fig. 3D). Compared to the FGel-TA2-SN formulations, FGel-TA5-SN formulations possessed higher swelling ratios regardless of SN content, likely because the ortho-quinone groups of oxidized tannic acid contributes to increased hydrophilicity. However, increasing TA content to 10% (e.g. FGel-TA10-SN) decreased swelling ratios, likely due to higher crosslinking densities.

### 3.3. Cytocompatibility of bioadhesive formulations

We next investigated the cytocompatibility of our Gel-TA-SN tissue adhesives, using FGel, FGel-TA, BGel, and BGel-TA, along with PEGDA as control (Fig. 5A). At 10 mg/mL concentrations, the hMSC viabilities of all Gel-TA polymers and gelatins were between  $55 \pm 7$  and  $98 \pm 6\%$ , comparable to that of control (at  $57 \pm 2\%$ ).



**Fig. 5.** Cell cytotoxicity evaluations of the gelatin-TA family: MTT cell viability assays against human-derived mesenchymal stem cells (hMSCs) for: gelatin (from cold fish (FGel) or bovine skin (BGel)), gelatin-TA pre-polymers and PEGDA (as control) (A), leachable (sol) content (B) and degradation products (C) of silver nitrate (SN) crosslinked gelatin-TA. Cell proliferation (D), observed by Live/Dead assay of hMSCs seeded onto SN (0.1 g/mL) crosslinked BGel-TA2 (B2) at 1, 3, and 7 days post cell seeding. # $p > 0.05$ .



Higher TA content led to lower cell viability. At 1.0 and 0.1 mg/mL concentrations, Gel-TA and gelatin performed even better than blank medium and PEGDA control in cell viability. The cytocompatibility of our bioadhesives was confirmed by Live/Dead assays. hMSCs seeded onto crosslinked Gel-TA-SN films showed excellent morphology, cell attachment and proliferation at 1, 3, and 7 day time points (Fig. 5D).

We also studied the cytotoxicity of leachable (sol) contents and degradation products of our crosslinked Gel-TA-SN hydrogels. At 1× dilution, Gel-TA-SN sol content resulted in roughly 35% cell viability, which improved to 90% at 10× dilution and ~100% at 100× dilution (Fig. 5B). The formation of silver nanoparticles likely contribute to the cytotoxicity of crosslinked Gel-TA-SN sol content. Likewise, Gel-TA-SN degradation products exhibited ~100% cell viability only upon 10× and 100× dilution (Fig. 5C).

### 3.4. Antimicrobial evaluation

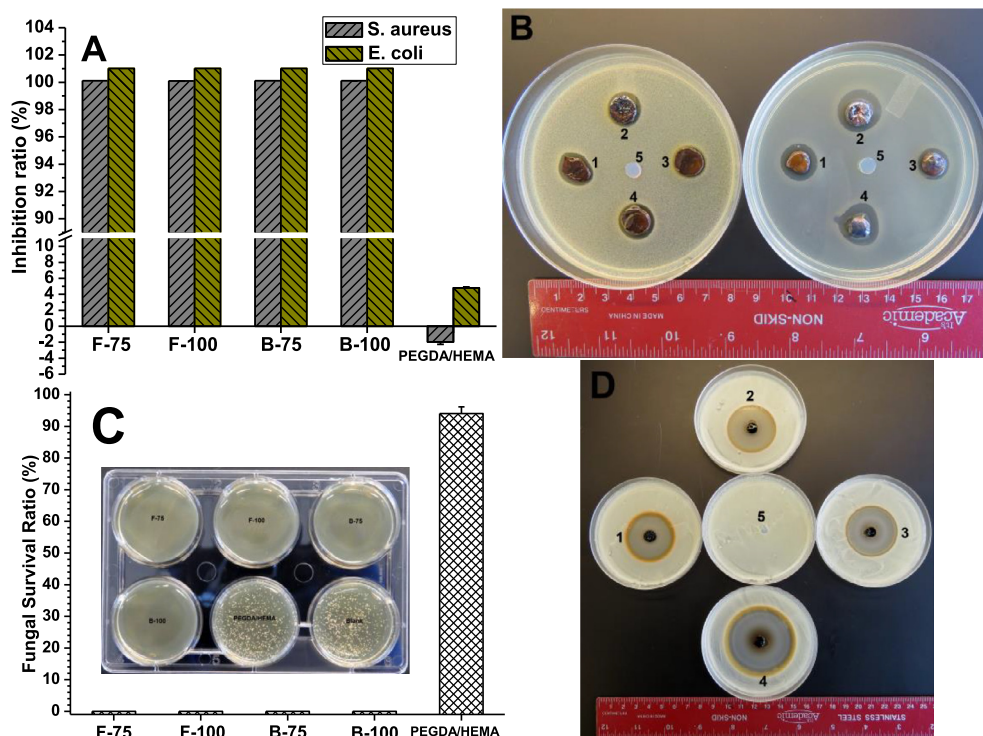
In fruits and vegetables, tannins can act as natural antimicrobial agents to provide resistance against microorganisms [23]. The potential antibacterial activity of tannins against one of the most antibiotic drug-resistant “superbugs”, methicillin-resistant *Staphylococcus aureus* (MRSA) strain has been previously reported [28]. One possible antibacterial mechanisms of tannic acid was reported as the interactions of tannic acid with bacterial cell wall leading to the complexation with cell wall protein and membrane disruption metal ions [29]. Silver nanoparticles resulted from the redox reaction between oxidative of tannic acid with reductive silver nitrate (SN) could also serve as antimicrobial agents [5,6,35].

In chronic wounds such as diabetic foot ulcers, *Streptococcus* and *S. aureus* typically colonize and lead to clinical infections. Moreover, long-term chronic wounds tend to contain more anaerobes (such as *Proteus* and *E. coli*) than aerobes (such as *Pseudomonas*, *Acinetobacter* and *Stenotrophomonas*). Infected chronic wounds are also prone

to fungal infections. Therefore, to study appropriate infection models for clinical treatment, we chose *S. aureus* and *E. coli* as representative Gram-positive and Gram-negative bacteria respectively, along with *C. albicans* as representative fungi, to evaluate the antimicrobial capabilities of our bioadhesives for the potential treatment of infected chronic wounds. Gel-TA-SN tissue adhesives were challenged with  $1 \times 10^6$  CFU/mL of bacteria while PEGDA/HEMA served as control, and bacterial viabilities were assessed upon 24 h incubation by the colony counting method (Fig. 6). The bacterial inhibition ratios of all Gel-TA-SN formulations (F75, F100, B75, and B100) were all close to 100% against both *S. aureus* and *E. coli* (Fig. 6A), indicating strong antibacterial performance of our bioadhesives. In contrast, the bacterial inhibition ratio of PEGDA/HEMA were close to 0 against both *S. aureus* and *E. coli*, indicating minimal inhibitory effects (Fig. 6A). We then performed the inhibition halo test as an additional assessment of microbial inhibition. The zones of bacterial inhibition around F-75, F-100, B-75, and B-100 hydrogels were all roughly 10 mm against *S. aureus* and between 12 and 20 mm against *E. coli*, while PEGDA/HEMA displayed no inhibition against either bacteria (Fig. 6B).

Similarly, fungal survival ratios upon direct exposure to Gel-TA-SN hydrogels were all 0% after 24 h incubation of *C. albicans*, indicating strong fungal inhibition (Fig. 6C). Fungal survival ratio of PEGDA/HEMA as positive control was 98%. The zones of inhibition of F-75, F-100, B-75, and B-100 hydrogels against *C. albicans* were 36.6, 37.2, 38.9, and 51.6 mm respectively, while PEGDA/HEMA showed no zone of inhibition (Fig. 6D).

Although antimicrobial agents may be doped into a biomaterial to combat infections, these materials often suffer from burst release and poorly sustained action that can exacerbate surgical complications. Similar to the antimicrobial and antifungal iCMBAs that exhibited sustainable antimicrobial ability [6], the Gel-TA-SN system herein shows potential for short-term, highly effective antimicrobial activity from the fast-releasing silver nanoparticles,



**Fig. 6.** Antimicrobial and antifungal performance of gelatin-TA family: Bacterial inhibition ratios (A, 0.5 g hydrogel in 5 mL bacteria containing broth) and inhibition halo (B) after 24 h' incubation of gelatin-TA hydrogels against *S. aureus* and *E. coli*; Fungal survival ratios after direct exposure to gelatin-TA hydrogels at 37 °C for 3 h against *C. albicans* (C); Fungal inhibition halos of gelatin-TA hydrogels against *C. albicans* after incubation at 37°C for 24 h (D). 1. F-75 (F2-S75): FGel-TA2 SN0.075 g/mL; 2. F-100 (F2-S100): FGel-TA2 SN0.1 g/mL; 3. B-75 (B2-S75): BGel-TA2 SN0.075 g/mL; 4. B-100 (B2-S100): BGel-TA2 SN0.1 g/mL; 5. PEGDA/HEMA (w/w = 1/1, as control).

as well as for long-term, sustained action from tannic acid molecules incorporated into the gelatin network. Therefore, the dual integration of silver nanoparticles and tannic acid into a cross-linked polymeric network could be a powerful strategy for bioadhesive design with innate antimicrobial properties.

#### 4. Conclusion

In conclusion, tannic acid (TA), as a representative of plant-derived polyphenol compound, was introduced into animal derived gelatin (gelatin from cold fish, FGel, or gelatin from bovine skin, BGel) through a facile one-step Michael addition reaction in aqueous solution at mild conditions to obtain gelatin-tannic acid (Gel-TA). Similar to mussel-inspired, dopamine containing polymers, Gel-TA can be crosslinked by oxidation, and the oxidized Gallic acid moieties on TA can also chemically bond to tissue by chemically reacting with nucleophilic groups such as  $-NH_2$  or  $-SH$  on tissue surface to serve as tissue adhesives. Upon crosslinking with silver nitrate (SN), the resulting tissue adhesives exhibited adjustable gel times, fast degradation, considerable wet tissue adhesion strengths, and favorable cytocompatibility. Gel-TA-SN tissue adhesives also demonstrated impressive antimicrobial properties. Compared to mussel-inspired bioadhesives that use expensive dopamine or DOPA as functional moieties, our polyphenol based Gel-TA tissue adhesives utilize tannic acid as an abundant and low-cost raw material, and eliminate the concerns of potential neurological effect brought by dopamine. Therefore, the studies herein suggest potential applications of the tannin-inspired gelatin bioadhesives toward wound closure, tissue sealant, hemostasis, antimicrobial, and cell/drug delivery. Further research will be necessary to evaluate these materials in various animal models toward clinical translation.

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