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ARTICLE

Synthesis and tissue adhesiveness of temperature-sensitive hyperbranched poly(amino acid)s with functional side groups

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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The incorporation of L-3,4-dihydroxyphenylalanine (L-DOPA) residues in mussel-like biomimetic copolymers have drawn great attention due to their adhesive properties. In this work, a series of temperature-sensitive and hyperbranched poly(amino acid)s containing different functional side groups (catechol, guanidyl, mercapto and double bond) were designed and synthesized by ring-opening polymerization of N-carboxy-α-amino acid anhydride (NCA), using a temperature-sensitive and multi-hydroxyl end pluronic L-31 as initiator in this process. The tissue adhesive properties of these copolymers were evaluated by tensile strength tests on wet pork skin. We found that the topological structure, side groups of copolymers, adhesive temperature and cure time had influence on the wet adhesive strength, especially side groups. While only L-DOPA was incorporated into copolymeric chains, the wet adhesive strength was about 106 KPa. With guanidyl, mercapto and double bond incorporated into copolymeric chains, an obvious increase in wet adhesive strength was observed. Additionally, the wet adhesive strength improved about 20 KPa at the body temperature (37 °C) than at room temperature (25 °C). Meanwhile, we found that it has good biocompatibility and degradability through its cytotoxicity and degradation tests.

Introduction

Tissue adhesives could be used to close wound, stop bleeding and repair tissue.[1, 2] Nevertheless, it is always a big challenge for the scientists, because the adhesive materials should show not only appropriate physical characters (elasticity, tensile and adhesive strength), but also biocompatibility and biodegradability in contact with physiological fluids.[3-5] And they are divided into natural and synthetic materials.[3, 6] Some natural materials usually have high biocompatibility and the capability of degradation; however, high costs and mechanical properties often cause challenges.[7-9] On the contrary, synthetic polymers can be tailored with suitable mechanical properties and controllable biodegradability.[4,7,10] Presently, the scientists have been focused on catechol-containing tissue adhesives and the inspiration comes from a marine mollusk named blue mussels.[10-12] It was reported that blue mussels are small bivalves capable of attaching to a wide range of surfaces such as rocks, wood and ship hulls with high strength even at the intertidal line.[13,14] By analyzing their secreta, it was found that there is a repetition of the 3,4-dihydroxyphenyl-alanine (DOPA) residue.[15] It is responsible for both the speedy curing of the adhesive and for interfacial binding, so the catechol side chain of DOPA is a distinct and multipurpose adhesive molecule capable of binding to both inorganic and organic surfaces via covalent attachment or forceful reversible bonds.[16-18] Lee et al considered these polymers which linear or branched polymers functionalized with DOPA, DOPA peptides, or their catechol mimics through variety of chemical methods might be introduced, provided a simple platform for studying the role of DOPA in mussel adhesion as well as novel materials for wet adhesion, and they also reported a Lysine in the form of a DOPA-Lys copolypeptide adhesion.[19,20] Miaoer et al reported a range of different copolymers to probe the effects of functional group composition on adhesive and cross-linking behavior.[21] On the other hand, Kou et al reported that the guanidyl-containing stabilized the heterotropic conjugate of actin and myosin (actomyosin), thus acted out the strong adhesive capability.[22] Aurelie et al reported that wet adhesive strength of polysaccharide-based adhesive reached to only 10.3 kPa.[23] The maximum adhesion force of a mussel-inspired hyperbranched poly(amino ester) polymer reached a plateau value around 70 KPa after 1 day curing time.[24]

Herein, we sought to synthesize a series of temperature-sensitive hyperbranched poly(amino acid)s by ring opening polymerization of NCA, using a temperature-sensitive and multi-hydroxyl end pluronic L-31 as initiator. As is known to us all, biocompatibility, biodegradability and adhesive strength are all important characters for poly(amino acid)s used in bioadhesive, so we investigated the effect of topological structure, side groups of copolymers, adhesive temperature and cure time on the copolymeric adhesive properties. Then the changes of copolymeric

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Electronic Supplementary Information (ESI) available: [Additional information on ^{1}H NMR spectroscopy data]. See DOI: 10.1039/x0xx00000x

hydrophilicity and hydrophobicity were realized by measuring contact angle value at 25 °C and 37 °C, respectively. In addition, the cytotoxicity and degradability were evaluated by MTT assay and the test of enzymatic degradation. Finally, it turns out that these poly(amino acid)s can be used in bioadhesive successfully due to their superior biocompatibility, biodegradability and adhesive strength.

Experimental section

Materials

N(α)-Benzyloxycarbonyl-arginine (N-Cbz-Arg), Glycidol, Cysteine (Cys), Acryloyl chloride, Lysine (Lys) hydrochloride, Levodopa (DOPA) and 8-hydroxyquinoline were purchased from Aladdin in Shanghai. Pluronic L-31(L-31) was purchased from Hai'an petrochemical plant in Jiangsu. Tetrahydrofuran (THF) was refluxed by calcium hydride (CaH_2) and got dry THF. Horseradish peroxidase was purchased from Xueman Biological Science and Technology limited company in Shanghai.

Methods

Synthesis of HPG-K initiator

Pluronic L-31 (2.0 g, 1.818 mmoL) and excess potassium were added to 50 mL flask and stirred at room temperature under a nitrogen atmosphere, until there was no air bubble. After taking out residual potassium, glycidol (1.89 g, 25.54 mmoL) was added to the mixture and stirred for 12 h at 95 °C under a nitrogen atmosphere. After the residue was dissolved in slight methyl alcohol, the solution was added to excess acetone, and then the transparent high viscous liquid was obtained with centrifuging. Hyperbranched polyglycerol (HPG) was dried for 15 h at 85 °C in vacuum. Excess potassium was added to HPG and stirred, until there was no air bubble. After taking out residual potassium, the HPG-K initiator was obtained.

Synthesis of NCA

Levodopa-N-Carboxyanhydride (DOPA-NCA), Cysteine-N-Carb-oxyanhy-dride (Cys-NCA), Arginine-N-Carboxyanhydride (Arg-NCA) and Lysine acrylamide-N-Carboxyanhydride (Lys acrylam- ide-NCA) were prepared according to the method reported.[25-27]

Synthesis of copolymers

DOPA-NCA (0.85 g, 3.78 mmoL) was dissolved in dry DMF (5 mL) in 50mL three-neck flask, then the HPG-K which was dissolved in dry DMF (3 mL) was added to the previous solution and stirred for three days at room temperature under a nitrogen atmosphere. After adding the mixture to ethyl alcohol, the faint yellow oily matter Hyperbranched poly[DOPA] (HPD) was obtained. The product was dried for 24 h at 60 °C in vacuum.

According to above method Arg-NCA, Arg-NCA and Cys-NCA, Arg-NCA and Lys acrylamide-NCA were separately dissolved in dry DMF (3 mL). Then, these solutions were respectively added to the DMF solution of HPD, Hyperbranched poly[DOPA-co-Arg] (HPDA), Hyperbran-ched poly[DOPA-co-Arg-co-Cys] (HPDAC) and Hyperbranched poly[DOPA-co-Arg-co-Lys acrylamide] (HPDAL) were prepared. And the linear copolymer poly[DOPA-co-Arg-co-Cys] (HPDAC) and poly[DOPA-co-Arg-co-Lys acryla-mide] (PDAL) which initiated by pluronic L-31 potassium were prepared according to previous method.

NMR spectra

NMR spectra were recorded on VARIAN JNM-ECP 600 MHz instruments using deuterium dimethyl sulfoxide (DMSO) as the solvents.

GPC testing

Molecular weight distributions of the resulting polymers were characterised by gel permeation chromatograph (GPC) in series using H_2O as the eluent.

Lower critical solution temperature (LCST)

The lower critical solution temperature (LCST) is one of the important characterizations of polymer temperature-sensitive performance parameters.[28] The temperature measured when the transmittance drops to 50% of its initial transmittance was defined as LCST. Transmittance was measured by UV-VIS spectrophotometer at 500 nm.

Contact angle measurement

Static contact angle values were acquired on a SL200KB apparatus at ambient temperature (20 °C) and the normal temperature of the human body (37 °C). The volumes of the individual water droplet in all the contact angle measurements were 5 μL. The average water contact angle was obtained by measuring the same sample in at least five different positions. All the contact angles were measured with about 5s of residence time of the water droplet on the surface.

Study on wet tissue adhesive using universal testing machine

Wet adhesive strength measurements were performed on porcine tissue following the procedures described in ASTM standard F2255-05. The experimental setup for measuring the adhesive strength of copolymers was shown as following picture (Fig. 1). Pork skin was prepared by cutting into rectangular pieces (5 cm long × 1cm wide),[29,30] and they were

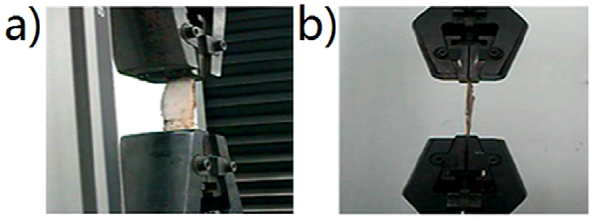
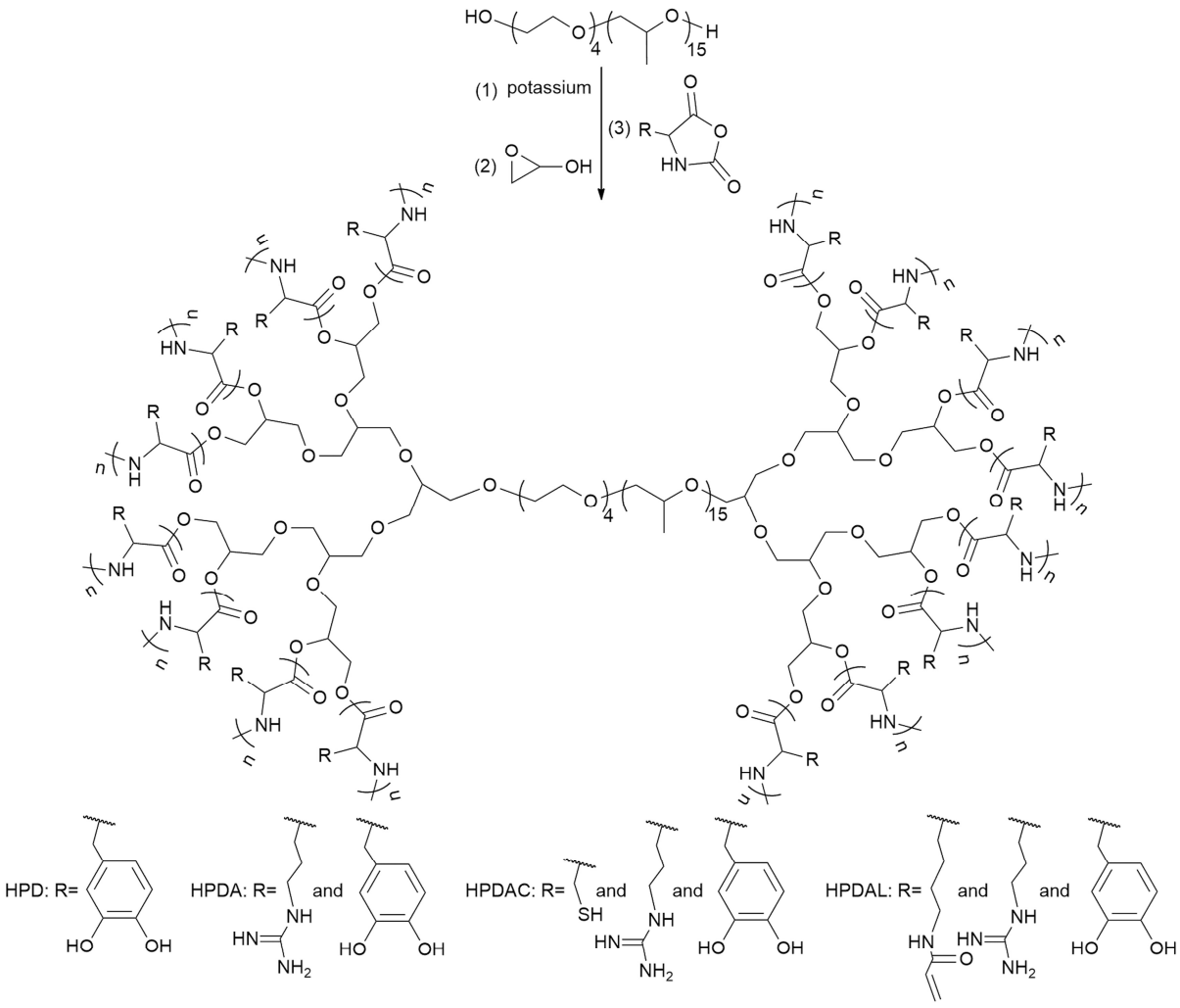


Fig. 1 (a) Positive of pork skin in the test process. (b) Side of pork skin in the test process.

washed with sodium chloride(0.9%) aqueous solution, then soaked for 12h with PBS buffer solution(pH=7.4). The copolymer (0.3 g) which dissolved in PBS buffer solution (pH=7.4, 3 mL) was averagely added to horseradish peroxidase(0.06 g/mL, 3 mL) and hydrogen peroxide solution (0.06%, 3 mL), then respectively spread on two pieces(1cm long × 1cm wide) of pork skin surface. After they were pressed together for different time, adherends were pulled apart at a rate of 1 mm/min. The data, which measured parallel to the three sets, was recorded. The adhesive strength was obtained by dividing the maximum load (N) observed by the area of the adhesive overlap (m^2), giving the lap-shear adhesive in Pascals (Pa = N/m^2).[31]

Cytotoxicity evaluation

Cell viability was quantified by the MTT assay. L929 cells (normal healthy mammalian cell line) were cultured in 96-well plates at a density of 1 ×10^5 cells per well in 100 uL of growth medium (RPMI 1640) containing 10% (v/v) FBS (fetal bovine serum), 100 ug/mL of penicillin, and 100 ug/mL of streptomycin.[32] Those cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 overnight and then the copolymer of varying concentration (10 uL, in PBS) was added, after 24, 48, 72 h of incubation, MTT (20 uL, 5 mg/mL^{-1} in PBS) was added. After a further 4 h of incubation, the culture medium was removed and DMSO (100 uL) was added, and the absorbance was measured by a microplate reader after 10 min at 37 °C and the experiment was repeated three times.[33]



Scheme 1 Preparation process of the initiator and copolymers.

Results and discussion

Synthesis and characterization of hyperbranched copolymers The copolymers were synthesized by ring opening polymerization of NCA using multi-hydroxyl end pluronic L-31 as initiator according to Scheme 1. The structures of the copolymers were also verified by the ^{1}H NMR spectroscopy (Fig. S1). Calculation by the ratio of proton signals at 1.2-1.5 ppm (corresponding to methyl in pluronic L-31) to those at 2.6-2.8 ppm (due to α-methylene of guanidyl in Arginine), 6.0-6.5 ppm (corresponding to ethenyl in Lys acrylamide) and 6.5-7.0 ppm (due to aryl protons of DOPA) revealed the conjugation percentage.

Table.1 The GPC data and grafting ratio of copolymers.

	Mn ($\times 10^{-4}$)	Mw ($\times 10^{-4}$)	PDI	D:A:C:L
HPD	3.56	5.21	1.46	12:0:0:0
HPDA	4.30	6.57	1.53	8:9:0:0
HPDAC	6.49	11.6	1.79	7:10:9:0
HPDAL	8.93	14.9	1.67	11:9:0:10

D, A, C, L show the numbers of DOPA, Arginine, Cysteine, Lys acrylamide which were grafted in each hydroxide radical.

As monomer types were increased, the molecular weight of copolymers became larger and their polydispersity indexs (PDI) were also wider (Table 1). It was normal absolutely from the statistical law. The degree of polymerization (DP) was figured out according to previous percentage and number-average molecular weight (Mn).

According to Kricheldorf et al. reported that alcohol was grafted from polymers after alcoholate ions initiated ring opening polymerization of the NCA.[34] So the HPG would be grafted from polymers after the HPG-K initiated ring opening polymerization of the NCA. It is well known that the pluronic L-31 is one of the important temperature-sensitive materials, therefore the copolymers could have temperature-sensitive property.

Influence of copolymeric composition for wet adhesive strength

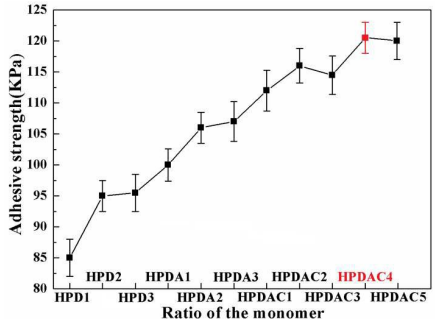


Fig. 2 Ratio of copolymeric monomer-Adhesive strength curves after 24h at 37 °C.

These copolymers which had different ratio of the monomers were synthesised, for example, HPD1 (pluronic L-31: DOPA =1:6), HPD2 (1:12), HPD3 (1:24); HPDA1 (DOPA:Arginine=1:1), HPDA2 (1:1.5), HPDA3 (1:2); HPDAC1 (DOPA:Arginine:Cysteine =1:1:1), HPDAC2 (1:1.5:1), HPDAC3 (1:1:1.5), HPDAC4 (1:1.5: 1.5), HPDAC5 (1:2:2). Fig 2 shows that with increasing each component of copolymers, the wet adhesive strength presented a rising trend, and it eventually remained stable. Therefore HPDAC was adopted from the following mole percents of amino-acid residues, for example, DOPA (about 25 mol%), Arginine (about 37.5 mol%), Cysteine

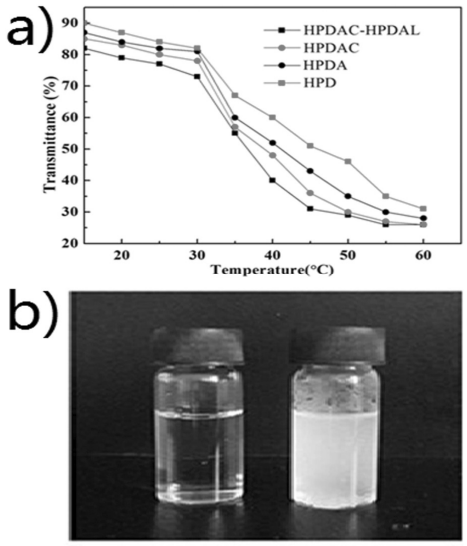


Fig. 3 Temperature-sensitive test results (a) Temperature-Light transmittance curves of HPD, HPDA, HPDAC, HPDAC-HPDAL. (b) HPD aqueous solution (0.3 mg/mL) respectively under 20 °C and 37 °C.

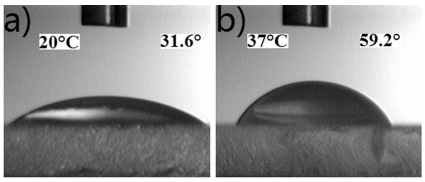


Fig. 4 Photographs of water droplet shape and contact angle on the film surface of polymer at 20 °C and 37 °C.

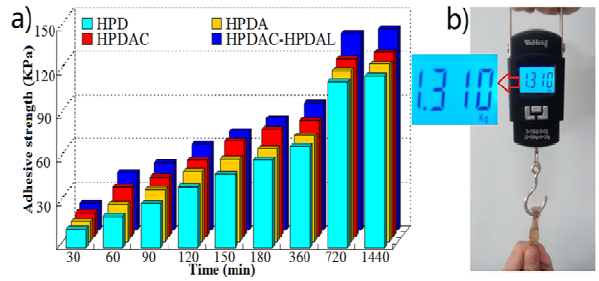


Fig. 5 (a) Time-Adhesive strength relation of the different copolymers under 37 °C.

(about 37.5 mol%).[35]

Temperature-sensitivity and hydrophilicity of copolymers

We have given the temperature-transmittance curves of HPD, HPDA, HPDAC, HPDAC-HPDAL aqueous solution (Fig. 2). The copolymers containing thermosensitive group pluronic L-31 could form thermally sensitive composite adhesive, leading to their transmittance decrease with temperature becoming high. It is clear that the transmittance reduced by about 50% when the temperature was varied from 20 °C to 37 °C, indicating the temperature of the aqueous solution has giant effect on the solubility. The copolymers of hemorrhaging site could be adhered onto the surrounding tissue and rapidly solidified to serve a bleeding-arrest barrier. This result is very important for the application of the polymers in the arresting bleeding. Moreover, their transmittance is not absolutely identical, because of catechol, guanidyl, mercapto, cross-linked and so on.

Fig. 4 shows transition between hydrophilicity and hydrophobicity of copolymer. If the film of copolymer was heated until the surface temperature risen up to 37°C, it would switch from hydrophilic to hydrophobic. A droplet on the copolymer film surface had a low contact angle value (about 31.6°) at 20 °C, however, a high contact angle value (about 59.2°) can be observed at 37 °C. This was because the hydrophilicity of pluronic L-31 could change with the temperature. The reversible hydrophobicity to hydrophilicity can be easily carried out by adjusting the temperature. These results demonstrate that the adhesive is a viscous state solution at room temperature but became a solid state preventing body fluid leakage when applied on wound.[36]

Influence of functional groups for wet adhesive strength

We measured the wet adhesive strength of the different polymers under 37°C. For the picture (Fig. 5a), the wet adhesive strength of HPD is 106 KPa because the hydroxyl groups of L-DOPA are deprotonated under oxidative conditions, which turned dopamine into dopaquinone via Michael-type addition reaction.[6,36] The wet adhesive strength of HPDA is 113 KPa due to turning dopamine into dopaquinone and the guanidinium forming a salt bridge with oxyanions by electrostatic and hydrogen bonding interactions, which can inhibit for the ATP-driven sliding motion of actomyosin.[37] As a result, the wet adhesive strength of HPDA is better than HPD. According to some studies, cross-linking of the polymers was triggered by chemical or enzymatic oxidation and remarkably increased cohesion.[38] This mechanism can improve the adhesive strength of HPDAC because the sulfydryl could become disulfide bond and covalent cross-link between the catechol and thiol groups.[16] In addition, the wet adhesive strength is further increased when HPDAC and HPDAL click on the cross-linked.[39] The wet adhesive strength of polymer HPDAC-HPDAL is larger than other copolymeric, which can be up to 138 KPa. Fig. 5b also demonstrated the maximum tension of

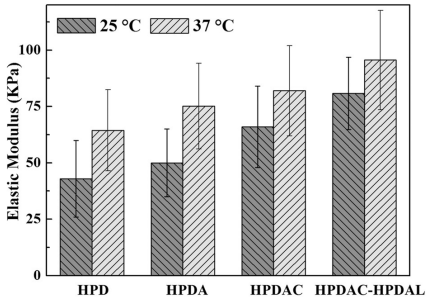


Fig. 6 The elastic modulus value of copolymer was also evaluated at 25 °C and 37 °C after 12 hours of reaction.

HPDAC-HPDAL. The weight shown by spring balance is 1.310 Kg, so we could obtain that tension was about 12.839 N (128.39KPa).

The elastic modulus value of copolymer was also evaluated at 20 °C and 37 °C (Fig. 6), and it is higher at 37 °C than those of 20 °C. After 12 hours of reaction at 37 °C, the elastic modulus value of copolymer showed a gradual increase, indicating that the significant covalent bonds were formed slowly between catechols and amines or catechols and thiols. Those values emphasize the potential utility of this polymer in soft tissue-contacting applications.

Influence of temperature and cure times for wet adhesive

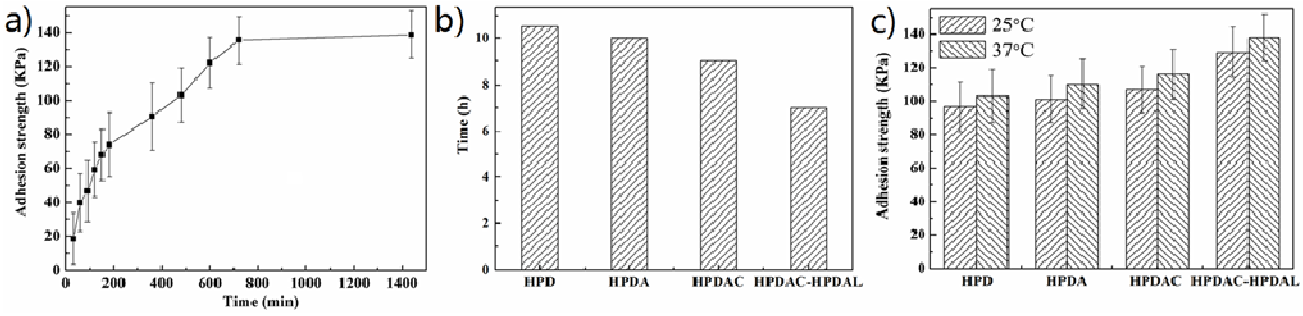


Fig. 7 (a) Time-Adhesion strength curves of HPDAC-HPDAL. (b) Respectively the time of copolymers when adhesion strength was 100 KPa at 37 °C. (c) The adhesion strength of copolymers respectively at 20 °C and 37 °C after 12 h.

strength

Short (0.5 hour, 1 hour, 1.5 hours, 2 hours, 2.5 hours, 3 hours) and long (6 hours, 8 hours, 10 hours, 12 hours, 24 hours) cure times were researched at room temperature (25 °C) and the body temperature (37 °C), respectively. Fig. 7a show the rate of wet adhesive strength increasing was very rapid during the time period from 30-200 min. However, From 200 to 720 min, the wet adhesive strength increasing process slowed down and remained almost constant after 720 min. Those are because the concentration of DOPA and non-crosslinking copolymers is large at the start, and the oxidative and non-crosslinking reactions are genesic easily, they are gradually depressed after 200 min. On the other hand, the wet adhesive strength is about 72 KPa before 200 min, after 12 hours the wet adhesive strength will be reach to the maximum value. The optimal adhesive strength cannot be achieved at short time most possibly due to the remaining solvent (boiling point of water = 100 °C). Those residual solvent might cause the copolymer chains to be ambulatory, allowing them to slip past one another easily.[40] Fig. 7b shows that with different functional groups were incorporated into, the cure times get shorter gradually. When adhesive strength reached to 100 KPa, the cure time of HPD, HPDA, HPDAC，HPDAC-HPDAL was tested to be 10.5 h, 10 h, 9 h, 7 h respectively. We found that all of the wet adhesive strength of copolymers was stronger at 37 °C than 20 °C (Fig. 7c). It is possibly due to the physical cross-linking between the Pluronic L-31 molecules and forming closely packed micelles.[36] Cure times are faster at 37°C than 25°C for the identical copolymer. This is possibly because the DOPA can be easily deprotonated under oxidative conditions, which turned dopamine into dopaquinone and increased cross-linking rate at 37 °C, leading to the polymer solidifying quickly. Therefore, not only do the copolymers hold high potential in arresting bleeding but also have high adhesive strength.

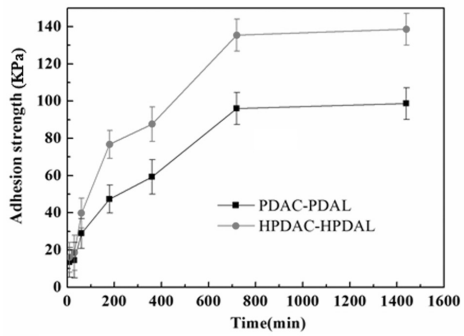


Fig. 8 Time-Adhesion strength curves of hyperbranched and linear polymers under 37 °C.

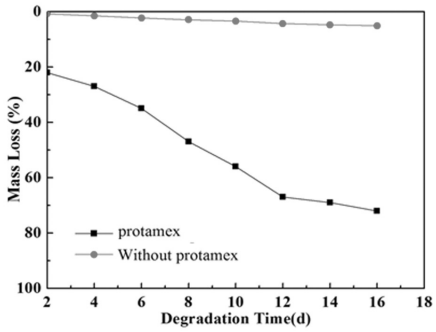


Fig. 9 Time-Mass Loss curves of HPDAC-HPDAL under 37 °C.

Influence of topological structure for wet adhesive strength

The wet adhesive strength of hyperbranched polymers are stronger compared with linear polymers (Fig. 8). Generally speaking, some properties of linear polymers functional group could not be absolutely expressed because their molecular weights are high and chain entanglements can easily occur. In contrast, hyperbranched polymers have globular structure and the properties of functional group can be well expressed. Therefore the adhesive strength of hyperbranched polymers is stronger than linear polymers.

Enzymatic degradation

The enzyme degradation reaction of polymer was used to measure the degradation property of polymers. We did the degradation process of HPDAC-HPDAL by dialysis at 37 °C, the specification of the dialysis-membrane was 3000. The polymer was split into two groups and dissolved in 50 mL PBS buffer solution. Protamex was added to only one group. At different time points, the dialysis bags were taken out and washed with H_2O. After lyophilization, the dry weights were measured. We can see that the mass loss of polymers was greater than 70% (Fig. 9). That is to say, those copolymers have excellent biodegradable.

Cytotoxicity

The cytotoxicity is a significant factor for the application of materials in the biomedicine field. Of note, DOPA, Arginine, Cysteine and Lysine acrylamide had no prominent influence on the cells of growth and proliferation. Fig. 10a shows the optical density (OD) of HPDAC-HPDAL, positive and negative control. Time-dependent changes in viability of L929 cells were detected for HPDAC-HPDAL at 600 μg/mL after 24, 48, 72 h (Fig. 10b). There was no significant difference between the three viability of cells viability for HPDAC. No qualitative differences in the attached cells were observed by microscopy (Fig. 10c). This indicates that HPDAC-HPDAL does not have any toxic effects on attached cells and haves excellent biocompatibility. And the cytotoxicities of other copolymers is demonstrated in support information.

Conclusions

In this study, a series of novel medical adhesives were synthesized based on hyperbranched polyglycerol and some amino acids (L-DOPA, L-arginine, L-cysteine, L-Lys acrylamide). These thermosensitive tissue adhesive showed excellent mechanical properties. In addition, the wet adhesive strength became better with grafting from various monomers. And the adhesive strength of HPDAC-HPDAL reached the largest due to multifarious cross-linking reactions. The cytotoxicity and degradation test showed that those adhesives were non-cytotoxic and biodegradable. Therefore, these copolymers have great potential for use in a range of biomedical applications including adhesives.

Disclosures

We declare that all experiments were performed in compliance with the relevant laws and institutional guidelines, and the institutional committee(s) that have approved the experiments. At the same time , The cells that used for experimentation were purchased in Shanghai Institutes for Biological Sciences(SIBS).

Acknowledgements

This work was supported in part by the National Natural Science Foundation of China (No. 51103118) and the Research Project of Universities of Gansu Province (2015A-005). We also thank Key Laboratory of Eco-environment-related Polymer Materials Ministry of Education, Key Lab of Polymer Materials of Gansu Province, and Northwest Normal University, for financial support.

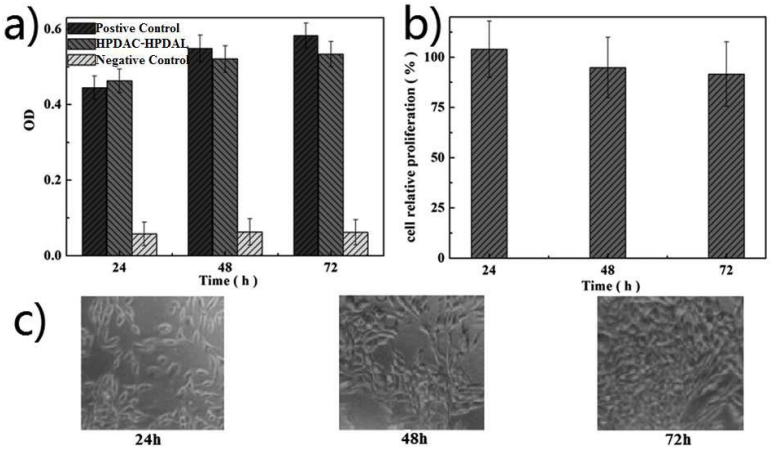


Fig. 10 (a) MTT assay OD value of L929 cells which were cultured with the extraction media from HPDAC-HPDAL. (b) Cell relative proliferation. (c) Microscopic pictures (10 ×) of L929 cells after incubation with HPDAC-HPDAL.

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Table of Contents Graphic and Synopsis

Synthesis and tissue adhesiveness of temperature-sensitive hyperbranched poly(amino acid)s with functional side groups

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The adhesive strength of temperature-sensitive hyperbranched poly(amino acid) could be improved by clicking cross-linked, forming disulfide bond and so on. In addition, the adhesion strength becomes better with grafting from different monomers at 37 °C. And the adhesive strength of polymer HPDAC-HPDAL is larger than other copolymeric, which can be up to 138 KPa.