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Synthesis and bioactivity of a conjugate composed of green tea catechin and hyaluronic acid†

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(-)-Epigallocatechin-3-gallate (EGCG) is a green tea polyphenol that has several biological activities, including anti-cancer and anti-inflammation. Hyaluronic acid (HA) is a naturally-occurring polysaccharide widely used as a biomaterial for drug delivery and tissue engineering due to its viscoelastic, biocompatible and biodegradable properties. By conjugating HA with EGCG, the resulting HA-EGCG conjugate is expected to exhibit not only the inherent properties of HA but also the bioactivities of EGCG. Toward this end, we report the synthesis of an amine-functionalized EGCG as an intermediate compound for conjugation to HA. EGCG was reacted with 2,2-diethoxyethylamine (DA) under acidic conditions, forming ethylamine-bridged EGCG dimers. The EGCG dimers comprised of four isomers which were characterized by HPLC, high-resolution mass spectrometry and NMR spectroscopy. The amine-functionalized EGCG dimers were conjugated to hyaluronic acid (HA) through the formation of amide bonds. HA-EGCG conjugates demonstrated several bioactivities which were not present in unmodified HA, including resistance to hyaluronidase-mediated degradation, inhibition of cell growth and scavenging of radicals. The potential applications of HA-EGCG conjugates are discussed.

Introduction

Tea (*Camellia sinensis*) is a beverage that is consumed worldwide. There are three types of tea, namely black tea, oolong tea and green tea. Green tea differs from the others in that the tea leaves do not undergo fermentation, or oxidation, but are steamed to inactivate the oxidative enzymes after harvest.[1, 2] Accumulated evidences over the past few decades strongly support the potential health benefits of green tea, particularly its anti-cancer activity.[3-5] Several epidemiological studies have revealed an inverse relationship between green tea consumption and cancer incidence.[2, 6] Moreover, topical application of green tea extracts has been shown to be effective in treating genital warts caused by human papilloma virus (HPV), a non-malignant squamous tumor.[7] In addition to its anti-cancer activity, studies have reported that green tea exhibits anti-bacterial and anti-inflammatory properties, as well as reducing the risk of cardiovascular diseases and obesity.[6]

The health benefits of green tea have been attributed to catechins; in particular, (-)-epigallocatechin-3-gallate (EGCG, **1**, Scheme 1), the most abundant catechin contained in green tea (more than 50% by mass of the total catechin content).[8] The mechanism through which EGCG exerts its health benefits is complex. However, research has revealed several key mechanisms of action, particularly with regards to its anti-cancer activity, including anti-oxidant and pro-oxidative properties, inhibition of enzymatic activities, and modulation of signal-transduction pathways through interactions with cell surface receptors or other molecular targets.[4, 5] Despite the growing interests in EGCG as a therapeutic agent, the potentials of polymer-EGCG conjugates for biomedical applications remained underexplored. Polymer-drug conjugates are known to enhance the therapeutic efficacy of small molecule drugs by improving their solubility and stability, and increasing plasma half-life when administered intravenously.[9] Furthermore, the drugs can be more precisely delivered to the diseased site by conjugation to a polymeric carrier containing active targeting moieties. To date, only chitosan-EGCG conjugates have been reported, which were synthesized by either tyrosinase-mediated[10] or free radical-induced grafting[11] of EGCG *via* the B ring.

It is known that the B and D rings of EGCG are crucial for its bioactivities such as enzyme inhibition,[12] induction of apoptosis[13] and scavenging of radicals.[14, 15] For instance, decreasing the number of hydroxyl groups in the B ring reduced its anti-cancer activity as a proteasome inhibitor.[16] Therefore, it is desirable to maintain the structure of B and D rings in order to preserve the bioactivity of EGCG. From this perspective, our group has previously reported a EGCG-terminated poly(ethylene glycol) synthesized *via* an aldehyde-mediated reaction that selectively reacts with the C-6 and C-8 positions of the A ring.[17] In this study, we introduced amine functional group, in the form of ethylamine, to the A ring of EGCG using the same aldehyde-mediated reaction, forming ethylamine-bridged EGCG dimers (Scheme 1). The amine-functionalized EGCG dimers are useful intermediate compounds which can be synthesized in gram-scale for conjugation to polymers.

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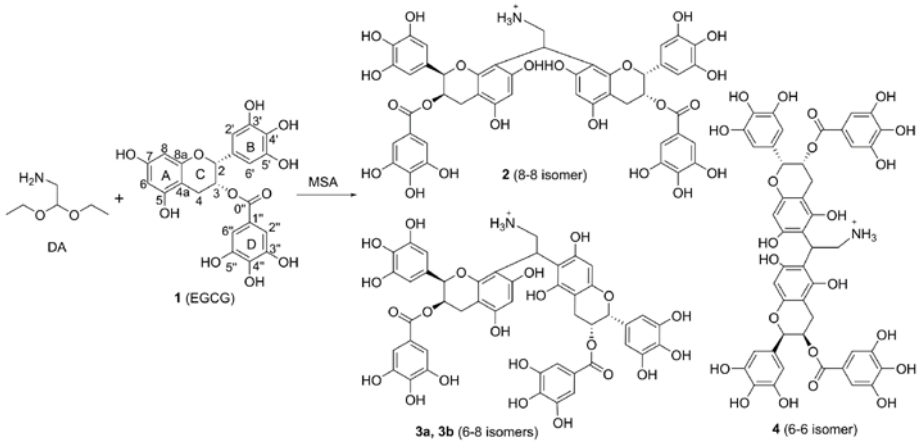
† Electronic Supplementary Information (ESI) available: HSQC spectrum, ^{1}H spectra and figures of radical scavenging activities. See DOI: 10.1039/x0xx00000x



Scheme 1. Synthesis of ethylamine-bridged EGCG dimers

We demonstrated the conjugation of amine-functionalized EGCG dimers to hyaluronic acid (HA) using conventional carbodiimide-mediated coupling chemistry. HA is a non-sulfated glycosaminoglycan found in the extracellular matrix (ECM). It exists as high molecular weight polymers under normal physiological conditions (10^6-10^7 Da), forming a viscoelastic mesh that is capable of retaining water and thereby maintaining tissue volume.[18] HA is the major constituent in the vitreous humor and synovial fluid, and is used in various medical[19, 20] and cosmetic applications. For instance, HA is used as a viscoelastic material in ophthalmological surgery to prevent the anterior chamber from collapsing and to protect the corneal endothelium from damage.[21] Intra-articular injections of HA provides lubrication and restores viscoelasticity in arthritic joints.[22] More recently, the interactions between HA and the cell surface receptors, such as CD44 and HA receptor for endocytosis (HARE), have been explored to achieve targeted drug delivery.[23] By conjugating EGCG to HA, the resulting HA-EGCG conjugate is expected to exhibit not only the inherent properties of HA but also the bioactivities of EGCG. Indeed, it was shown that HA-EGCG conjugates inhibited the enzymatic activity of hyaluronidase, decreased the growth rate of cells, and scavenged radicals more effectively compared with HA.

Results and discussion

Synthesis and characterization of ethylamine-bridged EGCG dimers

The bioactivity of EGCG is attributed mainly to the trihydroxyl groups in the B and D rings,[12-15] hence it is important to keep these sites intact during chemical modifications. Toward this goal, we utilized an aldehyde-mediated condensation reaction which selectively reacts with the A ring of EGCG.[17] The reaction, also known as Baeyer acid-catalyzed condensation, occurs during wine-aging in which flavanols react with aldehydes to form dimeric or oligomeric compounds.[24-26] The resulting chemical bridge between the flavanols depends on the aldehyde that is used for the reaction. In this study 2,2-diethoxyethylamine (DA), a protected aldehyde, was chosen to react with EGCG because it contains a primary amine which can form amide bond with the carboxyl groups of HA. Under acidic conditions the diethylacetal exists in equilibrium with aldehyde, which in turn accepts nucleophilic attack by the C-6 or C-8 of EGCG. The resulting intermediate further involves another EGCG to elaborate the EGCG dimers **2, 3a, 3b**, and **4** (Scheme 1).

It was reported that the aldehyde-mediated condensation reaction of flavanols would yield four isomers with 8-8, 6-6 and two 6-8 linkages, which stems from the linkage between the two reactive sites on the A ring (C-6 and C-8) and the asymmetric carbon.[25, 26] Indeed, four major peaks were observed in HPLC analysis in which two peaks in the middle overlapped with each other (Fig. 1). According to the previous studies the aldehyde-mediated condensation reaction was regioselective due to the sterically favorable substitution of C-8 over C-6, hence the 8-8 isomer should be generated the most while the 6-6 isomer the least.[25, 27] Based on this information, the largest peak observed in the HPLC, i.e., the first eluted peak, was assigned to **2**, the 8-8 isomer. The next two overlapping peaks accounted for **3a** and **3b**, the two 6-8 stereoisomers. The last eluted peak, which was also the smallest in area, came from

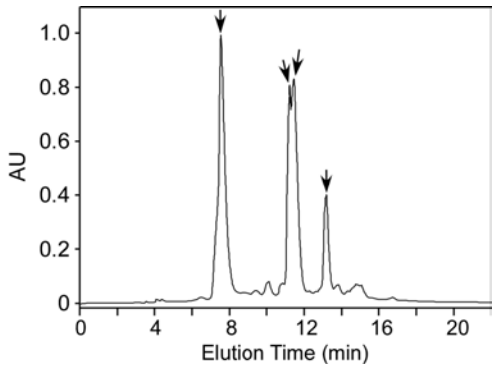


Fig. 1 HPLC chromatogram (UV absorbance at 280 nm) of ethylamine-bridged EGCG dimers. Arrows indicate the major peaks which correspond to the four isomers.

4, the 6-6 isomer. Each isomer was isolated by preparative HPLC for NMR analysis to elucidate the chemical structures. Only three fractions were collected because the two overlapping peaks, which contained **3a** and **3b** (6-8 stereoisomers), were

eluted together as one fraction. All three fractions had *m/z* of 958 as determined by LC/MS, which corresponded to $[M + H]^+$.

^{1}H NMR spectrum of the first eluted fraction, which contained **2** (8-8 isomer), revealed that the chemical shifts were paired, i.e., two peaks with equal intensity, particularly for H-2' and H-6' of the B ring and H-2'' and H-6'' of the D ring (Fig. 2a). Similarly, several of the ^{13}C chemical shifts were paired (Table 1). These results imply the presence of two sets of EGCG with different resonance, which was likely caused by the formation of an ethylamine bridge between two EGCG molecules. The broad peak centered at 3.89 ppm was assigned to the CH_2 protons of the ethylamine bridge. The assignment was supported by the chemical shift of the corresponding carbon determined by ^{1}H-^{13}C HSQC (Fig. S1, ESI†), which was negative (43.74 ppm, Fig. 2b), indicating either a secondary, as in this case, or a quaternary carbon. A triplet centered at 5.49 ppm was found to overlap with the chemical shifts of the H-3 proton of EGCG (Fig. 2a inset). This was assigned to the CH proton of the ethylamine linkage as coupling of the CH proton to the CH_2 protons of the ethylamine bridge would result in a triplet. Indeed, integration from 5.4 to 5.6 ppm revealed the presence of three protons, which corresponded to one CH proton and two H-3 protons. The chemical shift of the CH carbon as located by ^{1}H-^{13}C HSQC (Fig. S1a, ESI†) was positive (31.10 ppm, Fig. 2b), indicating either a primary or a tertiary carbon. The CH carbon is a tertiary carbon. The unambiguous assignment of the resonance of CH_2 and CH confirmed the formation of an ethylamine bridge between two EGCG molecules. Correlations between the protons of CH_2 and CH of the ethylamine bridge and C8 carbon of EGCG were observed (Fig. S1b, ESI†).

In order to assign the six carbon peaks of C-5, C-7 and C-8a which resonate closely between 154 to 156 ppm (Fig. 2b, inset), ^{1}H-^{13}C HMBC focusing on this region was performed to obtain a high resolution spectrum. The HMBC correlations were shown

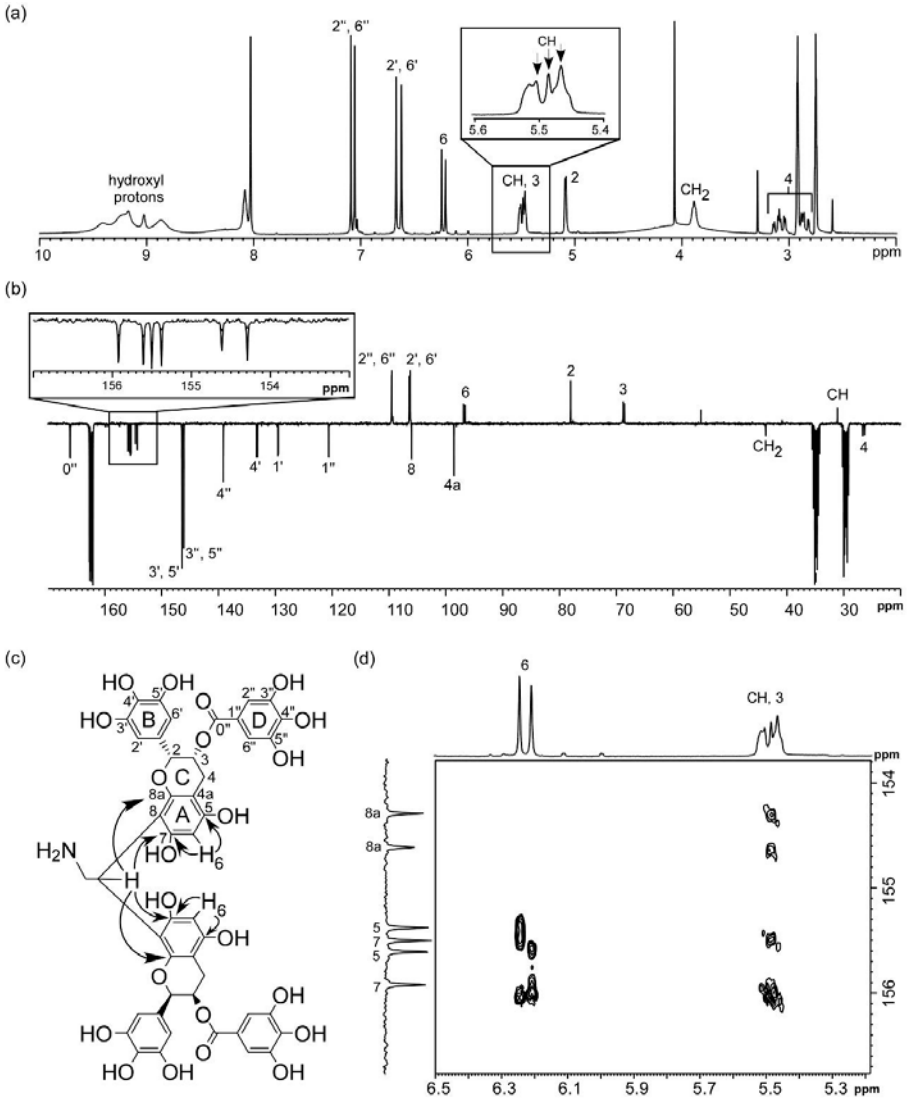


Fig. 2 NMR spectra of **2** (8-8 isomer): (a) ^{1}H spectrum, the CH protons were buried within the resonance of H-3 as indicated by arrows (inset); (b) ^{13}C spectrum; (c) ^{1}H-^{13}C HMBC correlations; (d) ^{1}H-^{13}C HMBC spectrum.

Table 1 ^{1}H and ^{13}C assignments of **2, 3a, 3b** and **4** in DMF-d_7 (δ in ppm, J in Hz)

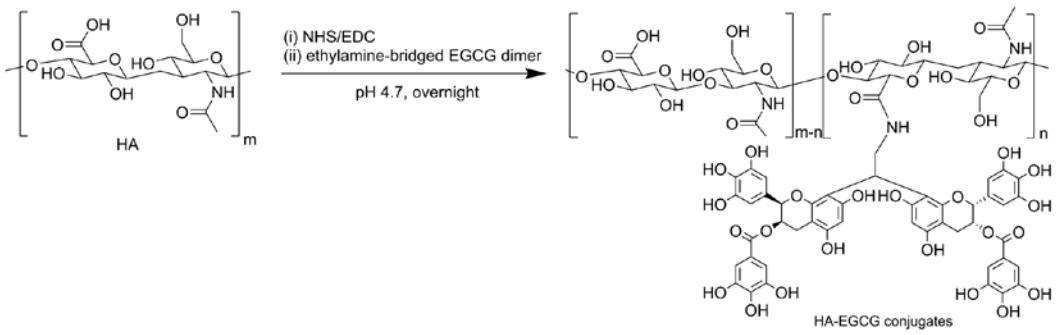
No.	**2** (8-8 isomer)		**3a, 3b** (6-8 isomers)		**4** (6-6 isomer)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
CH_2	3.89	43.73	Buried[a]	42.80, 43.57	3.90	42.15
CH	5.45-5.52	31.10	5.35-5.45, 5.50-5.60	30.73, 30.96	5.19 (t, J = 7.5)	31.10
2	5.08, 5.09	78.05	5.00-5.20	77.60, 78.10	5.0-5.1	77.77
3	5.40-5.55	68.57, 68.80	5.47	68.66, 68.82, 69.09, 69.21	5.48	69.06
4	2.80-3.20	26.31, 26.66	2.80-3.20	26.29, 26.90	2.80-3.20	26.89
4a	-	98.58	-	99.33, 99.38, 99.69, 100.08	-	100.98
5	-	155.38, 155.61	-	N.A.[c]	-	154.82, 154.88
6	6.21, 6.24	96.62, 96.90	6.25, 6.28[b]	107.95[b]	-	107.20, 107.37
7	-	155.51, 155.92	-	N.A.[c]	-	154.11
8	-	106.04	6.18, 6.21[b]	104.74, 105.26[b]	6.24, 6.25	96.21, 96.38
8a	-	154.30, 154.62	-	N.A.[c]	-	155.28, 155.33
1'	-	129.46, 129.55	-	129.28, 129.58, 129.63, 129.31	-	129.36
2', 6'	6.62, 6.67	106.28, 106.47	6.60, 6.62, 6.67, 6.68	106.18, 106.27, 106.37, 106.20	6.60	106.16
3', 5'	-	146.44	-	146.36, 146.38, 146.43	-	146.46
4'	-	133.14, 133.28	-	133.15, 133.21	-	133.27, 133.29
0"	-	166.08, 166.16	-	165.98, 166.01, 166.06	-	166.02, 166.06
1"	-	120.61, 120.64	-	120.52, 120.57	-	120.45
2", 6"	7.06, 7.09	109.50, 109.56	7.00, 7.03, 7.06, 7.09	109.31, 109.49	7.03	109.31
3", 5"	-	146.10	-	146.07, 146.12	-	146.17
4"	-	139.14	-	139.10	-	139.14

[a]Buried in the H_2O peak. [b] Values may be switched. [c] Unable to assign accurately because there are two stereoisomers in the sample.

in Fig. 2c and the six carbons peaks were assigned as follows. The carbons at 154.30 and 154.62 ppm, which showed correlations with the CH proton of the ethylamine bridge but not the H-6 protons of the A ring, were assigned as C-8a (Fig. 2d). On the contrary, the carbons at 155.38 and 155.61 ppm, which correlated with the H-6 protons but not with the CH proton of the ethylamine bridge, were assigned as C-5. The remaining two carbons at 155.51 and 155.92 ppm, which exhibited correlations with both the CH proton and the H-6 protons, were assigned as C-7. Similar procedure was applied to assign the ^{1}H and ^{13}C peaks for the other isomers (Table 1). However, we could not assign the ^{13}C resonance for C-5, C-7 and C-8a of **3a** and **3b** as they were eluted together in one fraction. It is worth mentioning that the ^{1}H NMR spectrum of the fraction containing **3a** and **3b** showed four peaks for each of the protons of the B ring and D ring (Fig. S2a, ESI†). On the other hand, ^{1}H NMR of **4** showed a single peak for each of the protons of the B and D rings (Fig. S2b, ESI†).

Synthesis and characterization of HA-EGCG conjugates

Ethylamine-bridged EGCG dimers were conjugated to HA by a typical carbodiimide-mediated coupling reaction to obtain HA-EGCG conjugate (Scheme 2). Removal of unreacted EGCG dimers from reaction mixture by dialysis against water was



Scheme 2. Synthesis of HA-EGCG conjugates.

attempted but found to be inefficient. Dialysis against a mixture of DMF:H_2O (10:90, v/v) could remove the unreacted EGCG dimers, however the process took several days. On the contrary, by precipitating the HA-EGCG conjugates with ethanol in the presence of NaCl, HA-EGCG conjugates formed slurry precipitates which could be easily collected by centrifugation. The unreacted EGCG dimers remained in the supernatant and were decanted. After lyophilization the purified HA-EGCG conjugate was dissolved in D_2O and analyzed by ^1H NMR (Fig. 3a). Although the peaks corresponding to the protons of the B and D rings could be observed (Fig. 3a inset), the signal to noise ratio of these peaks was too low for accurate integration. Hence absorbance measurement was employed to determine the degree of substitution. HA-EGCG conjugate showed a peak at around 273 nm (Fig. 3b, solid line), which corresponds to the π-π* transitions of the aromatic rings of EGCG. Ethylamine-bridged EGCG dimers also showed a peak at 273 nm (dashed line) while HA did not (dotted line). The degree of substitution (DS, the number of EGCG dimers conjugated for every 100 disaccharide units) was calculated by comparing the absorbance at 273 nm to a set of EGCG standards. The DS of HA-EGCG conjugates made from 90 kDa and 800 kDa of HA were 0.8 and 1, respectively.

Hyaluronidase inhibition by HA-EGCG conjugates

Tea polyphenols are known to bind to proteins through hydrophobic interaction and hydrogen bonding, thereby inhibiting the activity of enzymes.[28, 29] Mammalian hyaluronidase cleaves the β-1,4-glycosidic bonds of HA, generating HA fragments with *N*-acetyl-D-glucosamine reducing ends.[30] It is expected that the EGCG moieties of HA-EGCG conjugates would inhibit hyaluronidase and reduce the rate of enzymatic degradation of the HA backbone. HA-EGCG conjugates and HA (2 mg mL^{-1}) were incubated with hyaluronidase solution for 20 h and the amount of reducing ends and the dynamic viscosity (η) of the degraded samples were measured. In the case of HA-EGCG conjugates, the reducing end concentration remained below 0.1 mM as the concentration of hyaluronidase increased from 0 to 12.5 U ml^{-1} (Figure 4a). On the contrary, the reducing ends increased to more than 0.7 mM in the case of HA. Moreover, the dynamic viscosities of HA-EGCG conjugates were higher than HA after incubation with hyaluronidase (Figure 4b). The results suggested that HA-EGCG conjugates are less susceptable to degradation by hyaluronidase compared with HA, likely due the inhibition of hyaluronidase activity by the EGCG moieties.

Chemical modification of HA is known to disrupt hyaluronidase binding which in turn hinders degradation by the enzyme and can potentially prolong HA residence time *in vivo*.[31] In one study, the resistance to enzymatic degradation of serine-grafted HA increased as the degree of substitution increased from 40 to 100.[32] Extensive modification of HA, however, would lead to changes in its physical and biological properties. In

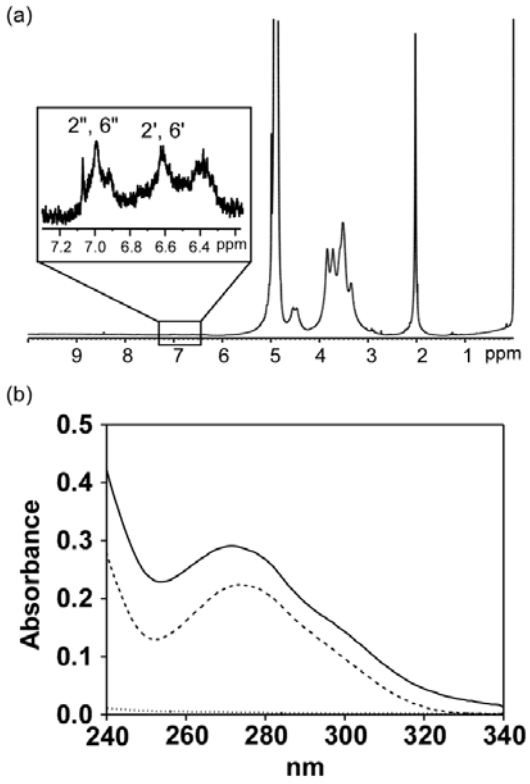


Fig. 3 Characterization of HA-EGCG conjugates (800 kDa). (a) ^1H NMR of 10 mg mL^{-1} of HA-EGCG conjugates in D_2O. Chemical shifts of H-2′ and H-6′ of B ring and H-2′′ and H-6′′ of D ring of EGCG were visible (inset). (b) Absorbance spectra of ethylamine-bridged EGCG dimer (dashed line, 10 μg mL^{-1}), HA-EGCG conjugate (solid line, 0.5 mg mL^{-1}) and unmodified HA (dotted line, 0.5 mg mL^{-1}) in H_2O.

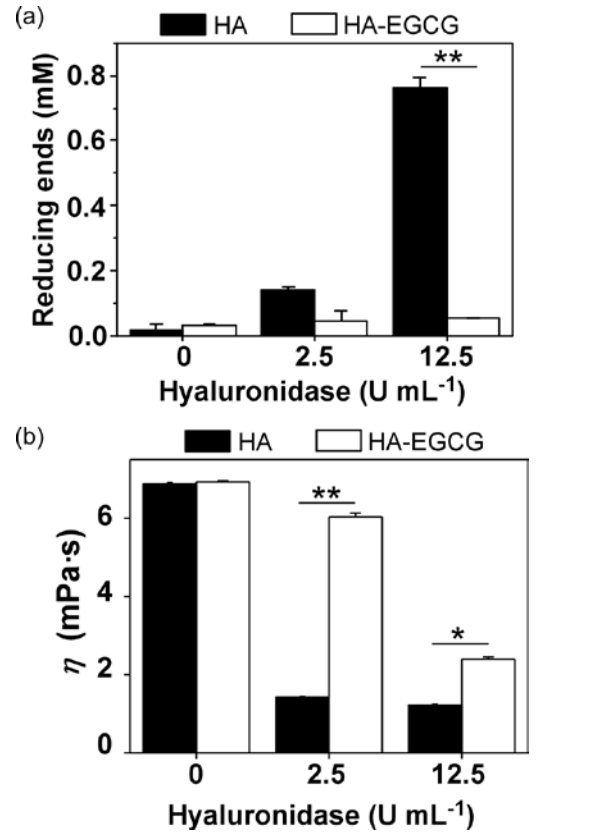


Fig. 4 Inhibition of hyaluronidase activity. (a) Concentration of reducing end generated and (b) dynamic viscosity (η) of HA or HA-EGCG conjugates (2 mg mL^{-1}) after treatment with hyaluronidase for 20 h at 37 °C (n = 3, mean ± s.d.). *P < 0.05 and **P < 0.005.

comparison, only 1% of the carboxyl groups of HA were substituted with EGCG dimers in the HA-EGCG conjugate, hence it was unlikely that the observed resistance to enzymatic degradation was caused by modification of HA. Rather it was the interaction between the EGCG moieties and hyaluronidase that inactivated the enzyme.

Anti-proliferative activity of HA-EGCG conjugates

EGCG is well-known for its anti-proliferative property. We examined the anti-proliferative property of HA-EGCG conjugates using an *in vitro* model of rheumatoid arthritis. Rheumatoid arthritis is an autoimmune joint disease characterized by hyperplasia of the synovial lining, which behaves like a local tumor that invades the cartilage and underlying bone, leading to joint destruction.[33] Human fibroblast-like synoviocytes (HFLS) derived from rheumatoid arthritis patient were treated with HA, HA-EGCG conjugates or EGCG while under stimulation by TNFα to simulate the inflammatory environment *in vivo*. After 3 days incubation, cells treated with HA retained healthy morphology across all the concentrations (Fig. 5a). Cells treated with HA-EGCG conjugates displayed normal morphology; with exception at the highest concentration of HA-EGCG conjugates, i.e., 2.5 mg mL^{-1} (containing 100 µM of EGCG). At this concentration, cells lost their elongated morphology but remained attached to the well. Cells treated with EGCG appeared apoptotic at EGCG concentrations above 50 µM. The cell number, measured in terms of the amount of DNA and expressed as a percentage of the control (no treatment), of samples treated with HA was similar to the control across the different concentrations (Fig. 5b). In contrast, DNA amount decreased with increasing concentrations of HA-EGCG conjugates or EGCG, suggesting the inhibition of cell growth (Fig. 5c). Remarkably, HA-EGCG conjugates inhibited cell growth as effectively as EGCG. The observed growth inhibition is in agreement with a previous study which reported that EGCG can downregulate uncontrolled growth and induce apoptosis in HFLS.[34]

Radical scavenging activity of HA-EGCG conjugate

HA is known to have antioxidant activity, which is attributed to reactive oxygen species (ROS)-induced chain depolymerization and the formation of a viscous meshwork that restricts ROS movement.[35, 36] However, only high molecular weight HA (3000-6000 kDa) showed significant radical scavenging activity and little effect was observed for low molecular weight HA.[35] Indeed, we found that HA (800 kDa) showed negligible scavenging activities towards $^{\bullet}NO$, $^{\bullet}OH$ and $^{\bullet}O_2^-$ (Fig. S3, ESI†). On the other hand, HA-EGCG conjugates showed concentration-dependent radical scavenging activities (Fig. S3, ESI†). The concentrations at which 50% of the radicals were scavenged (SC_{50}) were listed in Table 2. The radical scavenging activity of HA-EGCG conjugates was attributed to the EGCG moieties. EGCG is one of the most potent radical scavengers among the different green tea catechins due to the high number of hydroxyl groups contained in the B ring and the galloyl moiety (D ring).[14, 37] In addition to direct scavenging of radicals, EGCG can inhibit the generation of $^{\bullet}OH$ radicals by chelating metal

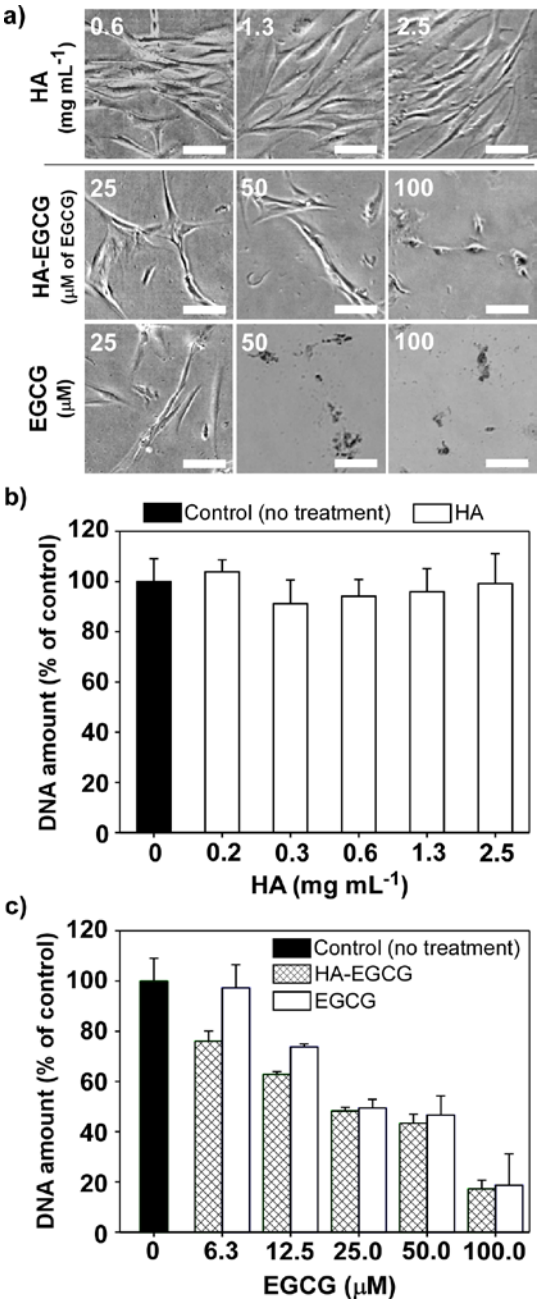


Fig. 5. HFLS were treated with HA, HA-EGCG conjugates or EGCG for 3 days. (a) Representative microscope images of the treated cells. Scale bar = 50 µm. (b-c) Amount of DNA, expressed in terms of percentage of the control (no treatment), for samples treated with (b) HA, (c) HA-EGCG conjugates or EGCG. HA-EGCG conjugates at 0.2, 0.3, 0.6, 1.3 and 2.5 mg mL^{-1} contained 6.3, 12.5, 25, 50 and 100 µM of EGCG, respectively (n = 4, mean ± s.d.).

s.d.)

Sample	SC_{50} (µM)		
	$^{\bullet}NO$	$^{\bullet}OH$	$^{\bullet}O_2^-$
HA-EGCG conjugate[a]	32.3 ± 3.8	36.3 ± 4.3	20.4 ± 3.8
EGCG	30.4 ± 1.4	18.0 ± 0.5	5.7 ± 1.3

[a] Concentration of HA-EGCG conjugates was expressed in terms of the amount of EGCG contained in the conjugates

ions and preventing them from participating in Fenton reaction.[38] EGCG can also bind to enzymes such as xanthine oxidase (XO) and inhibit its enzymatic activity, which in turn prevents the generation of $^{\bullet}O_2^-$.[39]

Hyaff®-11p75 (benzyl esters of HA with an approximate molecular weight of 300 kDa), a commercially available HA-derived product used in wound healing applications, was shown to scavenge $^{\bullet}O_2^-$ more effectively than high molecular weight HA (3000-6000 kDa) despite having a lower molecular weight.[35] The superior scavenging activity is attributed to the high degree of benzyl groups which offer alternative sites for $^{\bullet}O_2^-$ attack. Though Hyaff®-11p75 can scavenge $^{\bullet}O_2^-$ effectively, its physio-chemical property is quite different from that of native HA because 75% of the carboxyl groups are esterified with benzyl alcohol. As a result, Hyaff®-11p75 requires DMSO to be dissolved due to its high hydrophobicity.[35] In contrast, HA-EGCG conjugates could not only scavenge $^{\bullet}O_2^-$ but also remain soluble in water. Furthermore, HA-EGCG conjugates could scavenge $^{\bullet}OH$ whereas Hyaff®-11p75 was incapable of doing so.

ROS and reactive nitrogen species are implicated in the pathogenesis and progression of various diseases, including osteoarthritis and rheumatoid arthritis. For instance, the presence of nitrotyrosine in osteoarthritis cartilages and the elevation of nitrated type II collagen peptides in the sera of osteoarthritis patients suggest oxidative damage caused by the highly reactive peroxynitrite ($ONOO^-$), which is a derivative of $^{\bullet}O_2^-$ and $^{\bullet}NO$.[40, 41] Intra-articular injection of HA, also known as viscosupplementation, is a treatment mode that aims to alleviate pain in osteoarthritis patients through restoration of HA concentration in the synovial fluid. As HA-EGCG conjugate could inhibit HFLS proliferation and scavenge radicals, it is a potential candidate for arthritis treatment. Moreover, EGCG has been shown to inhibit interleukin-1β (IL-1β)-induced gene and protein expressions of metalloproteinases (MMPs) in human chondrocytes, including MMP-1 and MMP-13.[42] Elevated levels of MMP-13 in the joint can lead to increased cleavage of collagen type II in the cartilage, resulting in cartilage destruction. Therefore the EGCG moieties in HA-EGCG conjugates could potentially inhibit IL-1β-induced MMP-13 expression.

Experimental

Materials

TEAVIGO™ green tea extract (EGCG, 1, minimum 90%) was purchased from DMS Nutritional Products Ltd. (Switzerland). Hyaluronic acid (HA, 90 kDa and 800 kDa) was kindly donated by JNC Corporation (Tokyo, Japan). Methanesulfonic acid (MSA), 2,2-diethoxyethylamine (DA), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl), xanthine, sodium nitroprusside, Greiss reagent, nitrotetrazolium blue chloride (NBT), thiobarbituric acid, 2-deoxy-D-ribose, ascorbic acid, hydrogen peroxide (H_2O_2), trichloroacetic acid and hyaluronidase from bovine testes (439 U mg^{-1}) were all purchased from Sigma-Aldrich (Singapore). 2-(*N*-Morpholino)ethanesulfonic acid (MES), iron (II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) and *N*-acetyl-D-glucosamine were obtained from Merck (Singapore). Xanthine oxidase (XO, 0.34 U mg^{-1}) from buttermilk was purchased from Oriental Yeast Co., Ltd. (Japan). *p*-Dimethylaminobenzaldehyde (DMAB) was purchased from Wako Pure Chemical Industries (Japan). *N,N*-Dimethylfomamide-d_7 (DMF-d_7) was purchased from Cambridge Isotope Laboratories, Inc (USA). Human fibroblast-like synoviocytes (HFLS) derived from rheumatoid arthritis patient were obtained from Cell Applications, Inc. (USA). Fetal bovine serum (FBS), penicillin-streptomycin and PicoGreen® dsDNA quantitation assay kit were purchased from Life Technologies (Singapore). Phosphate buffer saline (PBS, pH 7.3) and RPMI 1640 were supplied by media preparation facility in Biopolis (Singapore). MilliQ H_2O was used in all experiments.

Synthesis of ethylamine-bridged EGCG dimers

In a glass vial containing 1.2 mL of cold MSA:THF (1:5, v/v) mixture, 145 μL of DA (1.0 mmol) was added while stirring. The mixture was transferred dropwise to EGCG (2.29 g, 5 mmol), which was dissolved in 3.8 mL of THF and 1.7 μL of MSA, and stirred overnight in the dark at ambient temperature. The resulting mixture was concentrated under reduced pressure and further dried under vacuum overnight at ambient temperature. The products were dissolved in 10 mL of H_2O and the unreacted EGCG were removed by extraction with 10 mL of ethyl acetate using a separatory funnel. Unreacted EGCG moved to the organic layer while the ethylamine-bridged EGCG dimers remained in the aqueous layer. The extraction was repeated until no free EGCG was detected in the aqueous layer using a Waters Acquity UPLC-MS system. The concentration of the purified ethylamine-bridged EGCG dimer in the aqueous phase was determined by absorbance at 273 nm and was found to be 84 mg mL^{-1} (yield = 88%). HRMS-ESI calcd for $C_{46}H_{40}NO_{22}$ $[M + H]^+$ 958.2036, found 958.2062. The dimers were stored at -80 °C.

High-performance liquid chromatography (HPLC)

HPLC was performed by Waters 2695 Separation Module equipped with a Waters 2996 Photodiode Array Detector. A Spirit HPLC C_{18} column (5 μm, 25 × 0.46 cm; AAPPTec) was used. The mobile phase consisted of H_2O and acetonitrile with 1% acetic acid. Sample concentration was 5 mg mL^{-1} and elution was carried out at 1 mL min^{-1} flow rate, 23 °C, with a linear gradient of acetonitrile from 10 to 35% in 30 min.

Nuclear magnetic resonance (NMR)

The purified EGCG dimers, which contained different isomers, were separated by a Gilson preparative HPLC system equipped with a Gilson UV/VIS-156 detector. A C_{18} reversed phase preparative column (21.2 mm x 250 mm, Kromasil®) was used. The mobile phase consisted of H_2O and acetonitrile with 0.1% trifluoroacetic acid. Samples were eluted at 15 mL min^{-1}, 25 °C, with 10% acetonitrile for 3 min and then gradient to 60% over 40 min. For NMR analysis, 10 mg of sample from each fraction was dissolved in 0.7 mL of DMF-d_7 and transferred to a Wilmad J-Young low pressure/vacuum NMR tube. Oxygen in the sample was removed by three cycles of freeze-pump-thaw. NMR

experiments were performed with a Bruker Avance II (400 MHz) NMR equipped with a 5 mm PABBO probe. A series of NMR experiments were performed for each compound, including 1D ^{1}H and ^{13}C APT spectra as well as 2D ^{1}H-^{13}C HSQC and HMBC spectra using standard pulse programs. All chemical shifts are given in ppm values relative to DMF-d_7 (^{1}H: δ 2.75 ppm; ^{13}C: δ 34.89).

Synthesis of HA-EGCG conjugates

Ethylamine-bridged EGCG dimers were conjugated to HA by a typical carbodiimide-mediated coupling reaction.[43] HA (250 mg, 0.62 mmol) was dissolved by stirring in 20.2 mL of 0.4 M MES buffer (pH 5.2) with 2.5 mL of DMF. Next, NHS (89 mg, 0.78 mmol) and ethylamine-bridged dimers (0.205 mmol in 2.33 mL of H_2O) were added to the reaction mixture. Then, EDC·HCl (150 mg, 0.78 mmol) was added and the pH of the reaction was adjusted to 4.7. The reaction mixture was purged vigorously with N_2 for 10 min and then incubated in the dark overnight under N_2 at ambient temperature. The HA-EGCG conjugates were purified by precipitation using a previously established method with some modifications.[43, 44] Briefly, 125 mL of H_2O and 16.7 mL of 5 M NaCl solution were added to the reaction mixture and the pH was lowered to 3 with HCl solution. Then, 310 mL of ethanol was added while stirring. Under these conditions the HA-EGCG conjugates formed slurry precipitates which were collected by centrifugation (6000 rcf, 5 min). After decanting the supernatant, the precipitates were re-dissolved in 250 mL of water. Then, 33 mL of 5 M NaCl solution was added, the pH was adjusted to 3, and 620 mL of ethanol was added. The precipitates were collected by centrifugation and re-dissolved in 500 mL of H_2O. After adding 67 mL of 5 M NaCl solution and lowering the pH to 3, 1.24 L of ethanol was added. The precipitates were again collected by centrifugation and re-dissolved in 300 mL of H_2O. The conjugates were then dialyzed (Spectra/Por 7, MWCO = 3500 Da) against H_2O in N_2 atmosphere overnight. Finally, the purified HA-EGCG conjugates were lyophilized. The yield was 185 mg (74%). For NMR analysis, the lyophilized conjugates were dissolved in D_2O at 10 mg mL^{-1}. To determine the degree of substitution (DS, the number of EGCG dimers conjugated for every 100 disaccharide units), the conjugates were dissolved at 0.5 mg mL^{-1} in water and UV-Vis spectrum was recorded with a Hitachi U-2810 spectrometer. The amount of EGCG contained in the conjugate was determined by comparing the absorbance at 273 nm with a set of EGCG standards. HA-EGCG conjugates made from 800 kDa HA were used for hyaluronidase inhibition and radical scavenging assays. HA-EGCG conjugates made from 90 kDa HA were filtered through a 0.2 μm syringe filter before lyophilization; the lyophilized products were dissolved in sterile H_2O at 50 mg mL^{-1} and used for cell growth experiments.

Hyaluronidase inhibition study

HA and HA-EGCG conjugates (2 mg mL^{-1}) were treated with 0, 2.5 and 12.5 U mL^{-1} of hyaluronidase in 0.1 M sodium phosphate buffer (pH 5.7) for 20 h at 37 °C. The amount of reducing ends generated was determined by Morgan-Elson assay.[45] DMAB stock solution was prepared by dissolving 5 g of DMAB in 6.25 mL of 10 M HCl and then topped up to 50 mL with acetic acid. The DMAB reagent was diluted 10-fold with acetic acid immediately before use. The hyaluronidase-treated samples were first diluted 4-fold with H_2O, then 32 μL of which was mixed with 8 μL of borate solution (0.741 g of boric acid and 0.297 g of potassium hydroxide in 15 mL of H_2O, pH 9). The mixture was heated at 99 °C for 5 min. After cooling down to room temperature, 160 μL of DMAB was added and the mixture was then incubated at 37 °C for 20 min. 150 μL of the solution was transferred to a 96-well plate and the absorbance at 585 nm was determined by a Tecan Infinite 200 microplate reader. The amount of reducing ends was determined by comparing to a set of *N*-acetyl-D-glucosamine standards. The dynamic viscosity was measured using a HAAKE Rheoscope 1 rheometer equipped with a cone sensor (3.5 cm in diameter and 1.029° cone angle) at a shear rate of 400 s^{-1} and 25 °C.

Cell culture

Human fibroblast-like synoviocytes (HFLS) were grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were cultured in T75 tissue culture flasks and incubated at 37 °C and with 5% CO_2. Cells below passage 10 were used.

Cell growth assay

To determine the effect of HA, HA-EGCG conjugates and EGCG on cell growth, 2.5 x 10^3 HFLS in 100 μL of complete growth medium were seeded on the wells of a 96-well plate. After overnight incubation, the spent medium was replaced with 50 μL of fresh complete growth medium containing 40 ng mL^{-1} of tumor necrosis factor alpha (TNFα). Next, 50 μL of HA, HA-EGCG conjugates or EGCG was added. HA/HA-EGCG conjugates and EGCG were first dissolved in water at 50 mg mL^{-1} and 2 mM, respectively, before they were diluted in RPMI 1640 and added to the wells. The final concentration of TNFα in the wells was 20 ng mL^{-1}. The final concentrations of HA or HA-EGCG conjugates were 0.2, 0.3, 0.6, 1.3 or 2.5 mg mL^{-1}. The final concentrations of EGCG were 6.3, 12.5, 25, 50 or 100 μM, which corresponded to the concentrations of EGCG contained in the HA-EGCG conjugates. The plate was incubated at 37 °C for 3 days, after which images of the cells were taken using a microscope, and the number of cells in the wells were determined by DNA quantification using PicoGreen® dsDNA assay kit. Briefly, the spent medium was removed and the cells were lysed with 20 μL of 0.2% Triton X-100 in PBS for 30 min on a shaker. Then 180 μL of PicoGreen® reagent, prepared according the manufacturer's protocol, was added to each well. The plate was placed for 5 min in the dark and then the fluorescence was measured using a microplate reader (λ_{ex} = 480 nm and λ_{em} = 520 nm). The amount of DNA in each well was expressed in terms of the percentage of the controls (no treatment).

Radical scavenging assays

•NO was generated by sodium nitroprusside and measured according to a previously described method.[46] Briefly, 50 μL of HA, HA-EGCG conjugates or EGCG dissolved in H_2O at various concentrations was added to the wells of a 96-well plate. Then,

50 μL of sodium nitroprusside (10 mM in 0.2 M sodium phosphate buffer, pH 7.4) was added. After incubation at room temperature for 2.5 h under light, 100 μL of Greiss reagent was added. After 2 min incubation, the absorbance at 570 nm was recorded using a microplate reader. A reaction without any test sample (H_2O only) was performed as control. The percentage of •NO scavenged were calculated using the following equation, where $A_{control}$ and A_{sample} were the absorbance values of the control and sample, respectively:

$$\text{Radicals scavenged (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \quad (1)$$

•OH was generated by Fenton reaction using $FeSO_4$ and H_2O_2. The amount of •OH was determined by the thiobarbituric acid reaction in which the •OH degrades 2-deoxy-D-ribose into a 3-carbon compound, malondialdehyde, which then forms a chromogen in the presence of thiobarbituric acid.[47, 48] 100 μL of HA or HA-EGCG conjugates dissolved in H_2O at various concentrations was added to microcentrifuge tubes. Then, a solution containing 2-deoxy-D-ribose (20 mM), ascorbic acid (80 μM) and $FeSO_4$ (80 μM) in 50 μL of buffer (0.2 M sodium phosphate buffer with 0.3 M NaCl, pH 7.4) was added. Next, 50 μL of H_2O_2 (882 μM) prepared in buffer was added to initiate Fenton reaction. After incubating at 37 °C for 1.5 h, 50 μL of the reaction mixture was mixed with 50 μL each of trichloroacetic acid (2.8%, w/v) and thiobarbituric acid (1% in 50 mM NaOH, w/v). The mixture was heated at 99 °C for 15 min. After cooling down to room temperature, 100 μL was transferred to the wells of a 96-well plate and the absorbance at 532 nm was measured using a microplate reader. The percentage of •OH scavenged was determined by equation (1).

$•O_2^-$ was generated by XO and xanthine and measured by NBT reduction assay based on a previously established protocol with some modifications.[49] Stock solution of xanthine was prepared at 2.28 mg mL^{-1} in 1 M NaOH and diluted 10-fold in 0.2 M sodium phosphate buffer (pH 7.4) immediately before use. 50 μL of HA or HA-EGCG conjugates dissolved in H_2O was added to the wells of a 96-well plate. Then, 15 μL of XO (0.27 units mL^{-1}), 15 μL of NBT (0.2 mM) and 20 μL of xanthine (1.5 mM), all prepared in 0.2 M sodium phosphate buffer (pH 7.4), were added sequentially. Production of $•O_2^-$ was monitored by the formation of formazan dye at 560 nm using a microplate reader. Absorbance measurement was started immediately after adding xanthine and was recorded for 2 min during which the absorbance of the control (no test sample) increased linearly, suggesting a linear production of $•O_2^-$. The slope of the absorbance measurement during the 2 min period was determined by linear-fitting the data points using Microsoft Excel. The percentage of $•O_2^-$ scavenged was then determined by the following equation, where $S_{control}$ and S_{sample} were the slopes of the control and sample, respectively:

$$•O_2^- \text{ scavenged (\%)} = \left(\frac{S_{control} - S_{sample}}{S_{control}}\right) \quad (2)$$

The radical scavenging activities were expressed as 50% scavenging concentration (SC_{50}), which was the sample concentration required to decrease in the signal intensity (•NO and •OH) or the slope ($•O_2^-$) by 50% relative to the control.

Statistics

Results were expressed as the mean ± standard deviation (s.d.). The difference between the values was assessed using Student's *t*-test in Microsoft Excel.

Conclusions

Ethylamine-bridged EGCG dimers were synthesized *via* aldehyde-mediated reaction with the A ring of EGCG. Four isomers were formed due to the two reactive sites on the A ring, i.e., C-6 and C-8, and the asymmetric carbon at the ethylamine linkage. The EGCG dimers were conjugated to HA using carbodiimide-mediated coupling reaction, forming HA-EGCG conjugates. We demonstrated that the resulting HA-EGCG conjugates possess bioactivities such as resistance to hyaluronidase-mediated degradation, inhibition of cell growth and scavenging of radicals, whereas unmodified HA showed no or limited activities. The HA-EGCG conjugate would be a promising material for biomedical applications, such as arthritis treatment.

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