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A polyphenolic biomacromolecule prepared from a flavonoid: Catechin as degradable microparticles

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Abstract

Catechin (CAT) was crosslinked with trimethylolpropane triglycidyl ether (TMPTGE) to obtain degradable poly(CAT) particles in a single step. Spherical p(CAT) particles with tens of micrometer size range and an isoelectronic point at pH 1.2 were obtained. The hydrolytic degradation of p(CAT) particles provided sustainable and extended release with 264 mg/g CAT release within 10 days at pH 7.4. The antioxidant capacity of 55.0 ± 0.9 μg/ml gallic acid equivalent in terms of total phenol content, and 0.88 ± 0.3 μmol/g trolox equivalent were estimated for p(CAT) particles displaying strong radical scavenging capability. Blood clotting and hemolysis assays demonstrated dose-dependent blood compatibility revealing higher blood compatibility for p(CAT) particles up to 10 μg/ml concentration. The cytotoxicity results show that p(CAT) particles have almost no toxicity for CCD841 normal colon cells at 250 μg/ml concentration in 24 h incubation time giving ~97% cell viability, whereas CAT molecules only provide ~34% cell viability.

KEYWORDS

biodegradable, biomaterials, biopolymers, catechin, renewable polymers

1 | INTRODUCTION

Flavonoids are phenolic compounds with many biological and pharmacological activities, such as, antioxidant/prooxidant, anti-inflammatory, antihypertensive, antiviral, antimicrobial, antihyperglycemic, antimutagenic, and also antitumor features.[1,2] These natural compounds have been extracted from a variety of plants to be used in medical, cosmetic, and food industries.[3,4] Flavonoids are generally used as supplements in daily diets due to their antiaging ability, prevention of neurodegeneration,[5] and mitigation of cardiovascular risk.[6] Catechin (CAT) and its derivatives are polyphenolic compound which exist in a variety of natural sources[1] and considered for many therapeutic use to treat or protect against a broad range of diseases.[7,8] As previously reported, CATs have significant chemo-preventive effects by protecting normal cells against genotoxic materials,[9] regulation of chemo-

preventive genes via activation of the antioxidant response element,[10] inducing apoptosis,[11] inhibiting transformation and proliferation of tumor cells and also deterrence of carcinogenesis in various types of cancer including skin, lung, intestinal, pancreas, colon, and so on.[12] Additionally, recent studies reported that CAT has the highest affinity against proteins and enzymes, it can directly interact with enzymes and inhibits the activity of α-amylase, α-glucosidase,[13] pepsin, trypsin, lipase, lipoxygenase as well as antioxidant enzymes.[1,14] For example, CAT has great potential as an antidiabetic agent by increasing insulin antidiabetic activity,[15] decreasing α-glucosidase, and α-amylase inhibitory activity.[16] Kim et al. reported that the polycondensate form of CAT has more preventive effects on proteolytic degradation of extracellular matrix by strongly limiting collagenase and human elastase enzyme activities.[17] Among these abilities, CAT shows more killing effect on gram positive bacteria than gram negative bacteria by generating damage in the membrane of microorganisms.[18] Another study reported that CAT provides great benefits for skin as an anti-aging agent[19] and also acts as an immune enhancer.[3]

In spite of their numerous benefits, flavonoids have not been used as a medicine directly in the pharmaceutical industry due to ineffective absorption, distribution, metabolism, excretion, and body reactions because of their pharmacokinetics and pharmacodynamics.[20] These stated shortcomings of flavonoids have been overcome by using biocompatible and biodegradable polymeric particles as carriers for flavonoids in treatments.[21] Recent studies revealed that these active compounds could be loaded into various types of carrier and polymeric structures via grafting,[16] entrapment,[21] encapsulation,[22] conjugation,[20] or adsorption[23] processes. However, these carrier materials could only be loaded and release a limited amount of flavonoid that may not be effective for treatment. To avoid these types problems, design, and fabrication of novel biomaterials from flavonoids directly have recently gained significant attention from us and others.[24–27] It is well-known that crosslinked polymeric flavonoid particles have more advantages than the monomer forms linked to being insoluble, slowly degradable, low toxicity, high surface area and tunable surface and chemistry. Also, the particle forms of flavonoids retain their inherent properties including antioxidant, antimicrobial, anticancer activities, and so on. Therefore, these combined properties make them original promising materials for a wide range of biological applications. Our group reported crosslinked macro, micro, and nanoparticle derivatives from tannic acid,[27] quercetin and rutin,[28] naringin,[29] and rosmarinic acid[30] to expand and increase the medical use of phenolic-based compounds by improving their bioactivity and biocompatibility as biomaterials.

Here, the aim was to design degradable polyphenolic microparticles of p(CAT) from a natural compound, CAT, with a biocompatible and degradable crosslinker, TMPTGE, using the water-in-oil microemulsion technique in a single step. The morphological and physicochemical characterization of p(CAT) particles was done by optical microscopy, scanning electron microscopy (SEM), FTIR spectroscopy, thermal gravimetric analyzer (TGA), dynamic light scattering (DLS), and zeta potential measurements. The hydrolytic degradation of p(CAT) particles was investigated at physiological conditions, for example, similar to stomach (pH 1), blood (pH 7.4), and intestinal region (pH 9.0) at 37.5°C. Antioxidant properties of CAT molecules and p(CAT) particles were determined by using the total phenol content and $ABTS^+$ scavenging assay against gallic acid and Trolox standards. Also, the blood compatibility and cytotoxicity of CAT molecules and p(CAT) particles were assessed and compared by employing hemocompatibility tests, blood clotting and hemolysis assays and the cytotoxicity was determined on CCD841 normal colon cell line.

2 | EXPERIMENTAL

2.1 | Materials

Catechin hydrate (CAT, 98%, Aldrich), trimethylolpropane triglycidyl ether (TMPTGE, technical grade, Aldrich), triethylamine (TEA, 99.5%, Sigma-Aldrich), L-alpha-Lecithin (granular, 98%, Acros Organic), gasoline (95 octane, local vender), ethyl alcohol (99%, Birkim), acetone (99%, BRK), sodium hydroxide (NaOH, Aldrich), Folin-Ciocalteau's phenol reagent (FC, Sigma-Aldrich), 2,2′-Azino-bis-(3-ethylbenzothioazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich), and potassium persulfate (KPS, 99%, Sigma-Aldrich), and gallic acid (GA, 97.5–102.5% [titration], Sigma-Aldrich) were used as purchased. All aqueous solutions were prepared using distilled water (DI) with 18.2 M.Ω.cm (Millipore-Direct Q3UV).

2.2 | P(CAT) particle synthesis

To synthesize p(CAT) particles, 1.59 mmol of CAT solution in 5 ml of 0.5 M NaOH was dispersed into 150 ml 0.1 M lecithin-gasoline emulsion at 50°C under 1000 rpm mixing rate. After 10 min, 1.59, 3.18, and 4.77 mmol of TMPTGE at three different mole ratios, 100, 200, and 300% mole of CAT was added as a crosslinker into the separated reaction media, and 10 μl of TEA as an accelerator was also added immediately to each of the reaction

flasks. The reactor contents were stirred at 1000 rpm at 50°C for 12 more h. Then, the p(CAT) particles were precipitated using centrifugation at 10,000 rpm for 20 min, and washed twice with gasoline, and then with cyclohexane, ethanol, twice with ethanol-DI (50:50), and finally once with acetone to purify the particles and eliminate unreacted species. Then, the p(CAT) particles were dried with a heat gun and stored in a closed container for further use. The p(CAT) particles crosslinked with the same mole, two-fold, and three-fold of CAT mole numbers were numbered as A, B, and C, respectively.

2.3 | Characterization of p(CAT) particles

The prepared p(CAT) particles A were visualized by optical microscopy (BX54 Olympus) and scanning electron microscopy (SEM, Jeol JSM-5600 LV). To acquire SEM images, the p(CAT) particles were sprayed on carbon tape coated to a few nm thicknesses with gold under vacuum and the images were taken at an operating voltage of 20 kV. The functional groups of CAT and p(CAT) particles A were confirmed via FTIR spectroscopy (FT-IR, Nicolet iS10, Thermo) in the spectral range of 4000–650 at 4 cm^{-1} resolution using ATR technique. The size distribution of p(CAT) particles A was determined by dynamic light scattering (DLS, Brookhaven Instrument Nanobrook Omni) measurements with 35 mW solid-state laser detector at 658 nm wavelength. The p(CAT) particles were placed in 10 mM KNO_3 aqueous solution and sonicated for 30 s to disperse the particles. The isoelectronic point of p(CAT) particles A was determined by means of zeta potential measurement in the pH range 1–10 by using a Zeta Potential Analyzer (Brookhaven Instrument, BI-ZTU). Approximately, 50 mg of p(CAT) particles were suspended in 20 ml of 1 mM KNO_3 aqueous solution and the pH of the solution was adjusted with dropwise addition of 0.1 M HNO_3 and 0.1 M KOH solutions. Thermal stabilities of CAT and p(CAT) particles A, B, and C were compared by employing a thermogravimetric analyzer (TGA, Seiko, SII TG/DTA 6300) with heating to 1000°C at 10°C/min heating rate under nitrogen atmosphere with 100 ml min^{-1} flow rate. Hydrolytic degradation of p(CAT) particles A, B, and C prepared at different crosslinker ratios, 100, 200, and 300%mole of CAT, were determined in 0.1 M phosphate buffer solution (PBS) at pH 7.4. Additionally, the degradation amounts of p(CAT) particles A was also determined at three different pHs; pH 1 (0.1 M citrate buffer), pH 7.4 (0.1 M PBS) and pH 9.0 (0.1 M PBS). The weight loss% of p(CAT) particles was determined from the degradation solution of about 10 mg of p(CAT) particles suspended in 1 ml buffer solution within dialysis membrane (molecular weight cut off 12,000 Da, Aldrich). A dialysis sack containing these p(CAT) particles was placed in 30 ml of buffer solutions in a shaker bath at 100 rpm at 37°C. The degraded CAT amounts were calculated from a calibration curve, prepared at 280 nm wavelength, which is the maximum absorbance value of CAT in buffer solutions, via UV–vis spectroscopy (T80 UV/VIS Spectrometer, PG Ins. Ltd).

2.4 | Blood compatibility of CAT and p(CAT) particles

Hemolysis ratio and blood clotting index of CAT and p(CAT) particles A at different concentrations from 0.5 to 100 μg/ml were determined in accordance with a previously reported procedure.[27] These processes were described in detail in Appendix S1. In these tests, whole blood was donated by healthy volunteers with a procedure approved by the Human Research Ethics Committee of Canakkale Onsekiz Mart University (2011-KAEK-27/2020).

2.5 | Antioxidant activities of CAT and p(CAT) particles

Antioxidant capacity of CAT and p(CAT) particles A was determined by the Folin–Ciocalteu (FC) test and $ABTS^+$ scavenging assay in accordance with the earlier reported procedures.[29,30] These methods are also described in detail in the Appendix S1. Gallic acid, as a well-known phenolic compound, was used as standard material in these tests.

2.6 | Cell culture and cytotoxicity by cell Titer-Glo™ luminescent cell viability assay

The CCD 841 CoN human colon normal cells were provided by ATCC (CRL-1790). Cells were cultured in Minimal Essential Medium with Earle's (MEM/EBSS, HyClone) with 2 mM L-Glutamine and Earle's Balanced Salt supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin in a humidified incubator (Thermo Fisher) with a 5% CO_2 atmosphere at 37°C. The living CCD841 cells were stained with Nuc Blue Live Cell Stain Ready Probes Reagent (DAPI, Thermo Fisher) and the images were visualized by using Olympus BX51 fluorescence microscopy.

Cell proliferation of CAT and p(CAT) particles A was performed using luciferin luminescence agent via

CellTiter-Glo cell viability assay (Promega) as previously described in detail.[31] In short, CCD841 CoN human colon normal cells were seeded at a density of 5×10^3 for each well into 96-well plates and cultured in a humidified, 5% CO_2 atmosphere at 37°C for 24 h for adhesion of cells. Then, the culture medium was discarded and 100 µl of MEM/EBSS containing various concentrations (25, 50, 100, 250, 500, and 1000 µg/ml) of CAT or p(CAT) particles were added to the well plate and incubated at 37°C under 5% CO_2 for 24 and 72 h. Cells with culture medium were used as a control group. The culture medium was discarded, and the cells were washed with fresh MEM/EBSS slowly several times to remove CAT or p(CAT) particles on the cells to avoid interaction with the luciferin reagent. Luciferin reagent was diluted with the same amount of MEM/EBSS and 100 µl of this reagent solution was interacted with the cells under in darkness. After gently shaking for 25 min, 80 µl of reagent solution was taken and put into a white colored well plate and the absorbance of luminescence was read at 700 nm in a microplate reader (Bio-Tek Synergy H4).

2.7 | Statistics

All experiments were repeated at least 3 times and the results are presented as means ± standard deviations. Statistical analyses were determined with Student's t tests by using the GraphPad Prism software. All data were compared with control groups, and *p*-value of less than 0.05 was considered indicative of significant differences.

3 | RESULTS AND DISCUSSION

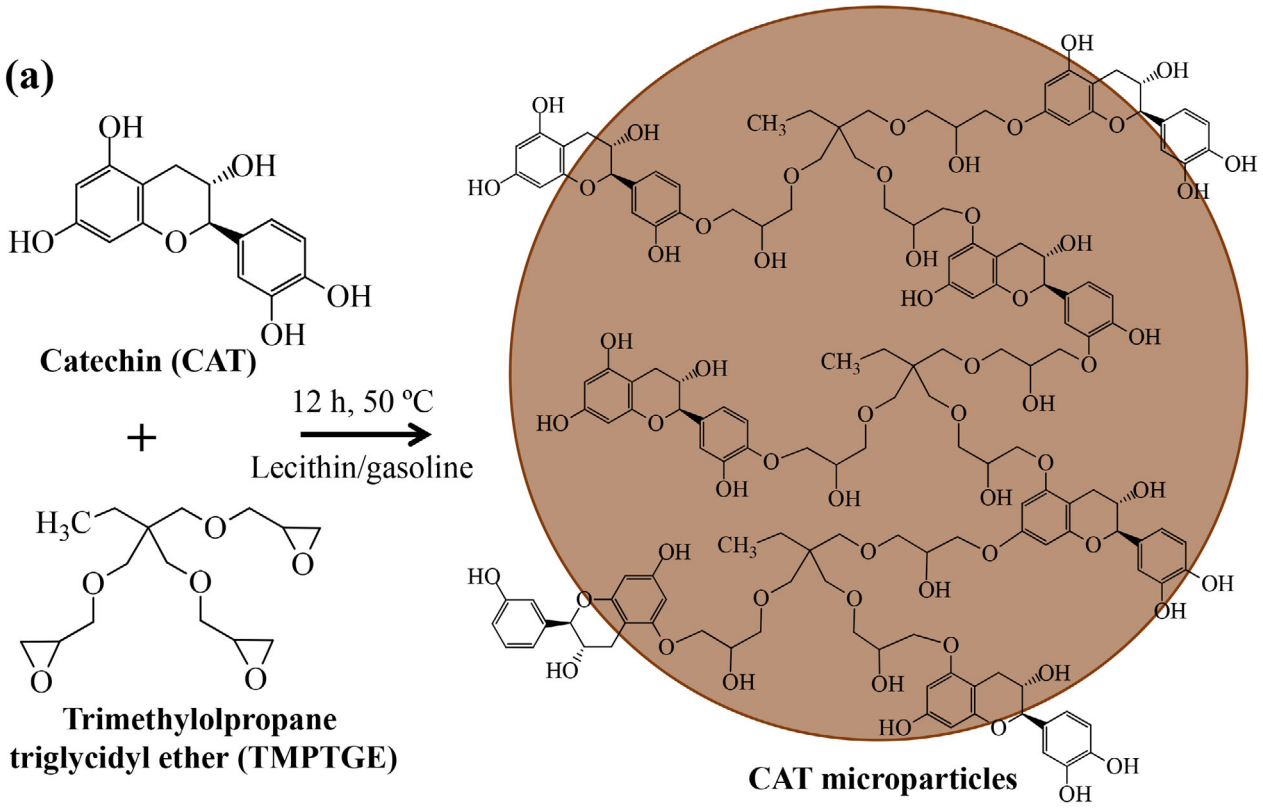
Compounds naturally possessing antioxidant,[32] anti-inflammatory, anticancer,[33] antimicrobial, and antifibrotic properties, such as, CAT, attract a great deal of interest for biomedical use.[7,34] To improve biocompatibility and bioactivity of CAT,[2] different natural polymers, such as, inulin,[16] dextran,[35] and hyaluronic acid[36] were widely investigated for grafting CAT or conjugation onto natural polymers or their derivatives. However, there is no report about the use of crosslinked particle forms of CAT as macromolecular material in the form of p(CAT) particles for potential biomedical use. In this study, p(CAT) particles were prepared by directly crosslinking them using a benign epoxide-based crosslinker, TMPTGE, in a microemulsion system. The p(CAT) particle formation chemical reaction pathway is illustrated in Figure 1(a). The reaction between the hydroxyl groups (—OH) that exist in phenolic molecules, such as, CAT and epoxide groups of TMPTGE are well known as base catalyzed ether linkage.[29] P(CAT) particles could not be obtained at ≤100% mole ratio of crosslinkers. Therefore, the crosslinker, TMPTGE ≥100% mole ratio of CAT molecules found to be the limit amount to render p(CAT) particles. Different molar amounts of TMPTGE, for example, the same mole, two-fold, and three-fold based on CAT molecule, were tested for the synthesis of p(CAT) particles as A, B, and C, respectively to determine their effect on hydrolytic degradation.

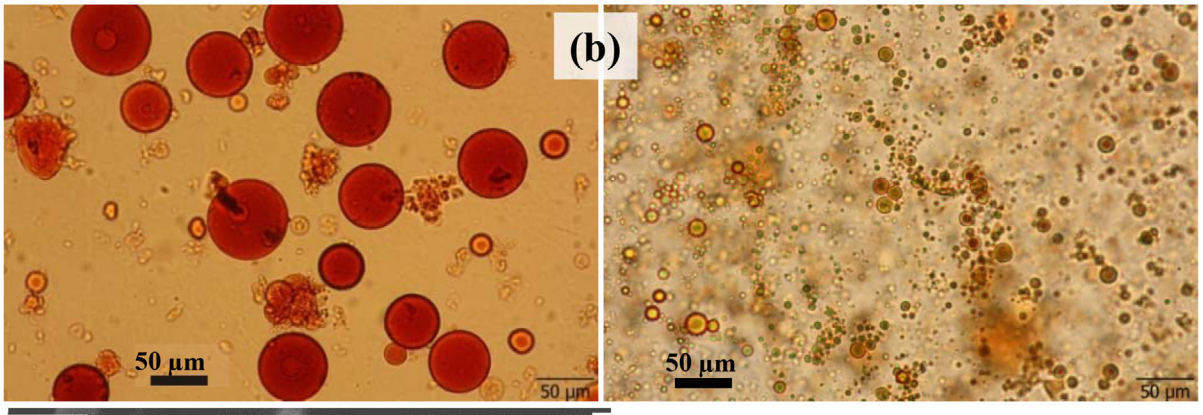
The optical microscope and SEM images as well as the hydrolytic size distributions shown in Figure 1(b)-(d) clearly show that the prepared p(CAT) particles A have spherical shape with broad size distribution ranging between a few tens of micrometers to few hundred nanometers. The hydrodynamic size distribution of the p(CAT) particles A measured after filtering the synthesized p(CAT) particles with a filter with pore size of 10 µm resulted in separation of submicron sizes with the average size of 1080 ± 16 nm from DLS measurements as shown in Figure 1(d).

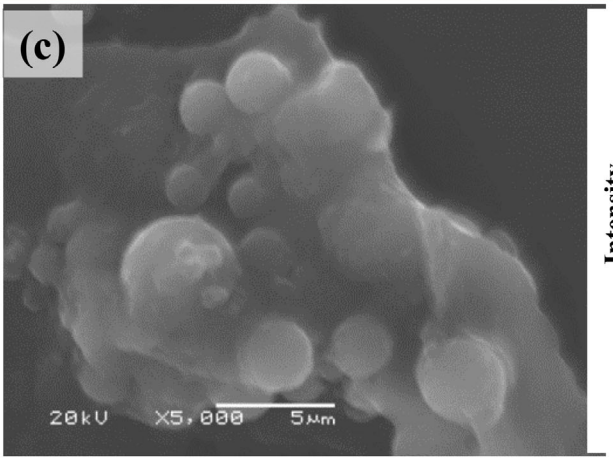
To confirm p(CAT) particle A formation via new functional group generation between the reaction of CAT and TMPTGE, the FTIR spectra of CAT molecule and p(CAT) particles A were taken and are presented in Figure 2(a).

The intensity of the broad band in the 3000–3600 cm^{-1} range for hydroxyl is significantly increased in the spectrum of p(CAT) particles due to the generation of new mobile —OH groups coming from the epoxide groups of the crosslinker, TMPTGE, that are generated upon reaction of TMPTGE with —OH groups of CAT molecules. As these —OH groups replace phenolic —OH groups of CAT molecules as shown in Figure 1(a), the intensity of —OH vibrations are expected to increase. Additionally, the peak at about 2930 cm^{-1} belonging to C—H stretching is also slightly increased because of the newly-generated methylene groups coming from the crosslinker into the particles. Also, the specific peaks of CAT as monomer were observed at 1613, 1518, and 1460 cm^{-1} stretching vibrations attributed to the substituted phenyl ring, and the peak at1144 cm^{-1} corresponding to —OH groups on aromatic compounds. The stretching vibrations at 1034 cm^{-1} belong to —C—O alcohol groups.[32] In the FTIR analysis of p(CAT) particles, the new peak at about 1060 cm^{-1} can be attributed to the stretching of C—O groups disclosing the presence of ether bonds between hydroxyl groups of CAT and epoxy groups of TMPTGE after the crosslinking reaction. FTIR analysis clearly confirms that CAT molecules were successfully crosslinked with TMPTGE into macromolecular p(CAT) particle forms.

To determine the surface charge and isoelectronic point of p(CAT) particles A, zeta potential values were







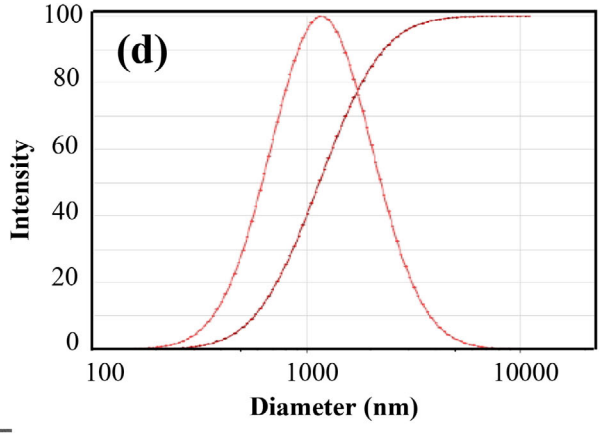
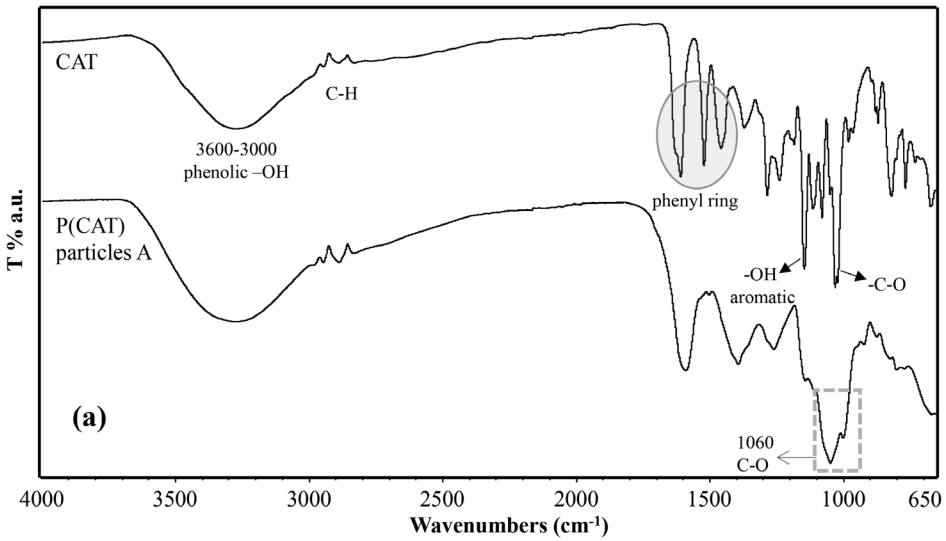


FIGURE 1 (a) Schematic representation of the reaction between TMPTGE and CAT to attain p(CAT) microparticles, and their (b) optical microscopy, and (c) SEM images, and (d) hydrolytic size distribution of p(CAT) particles a [Color figure can be viewed at wileyonlinelibrary.com]



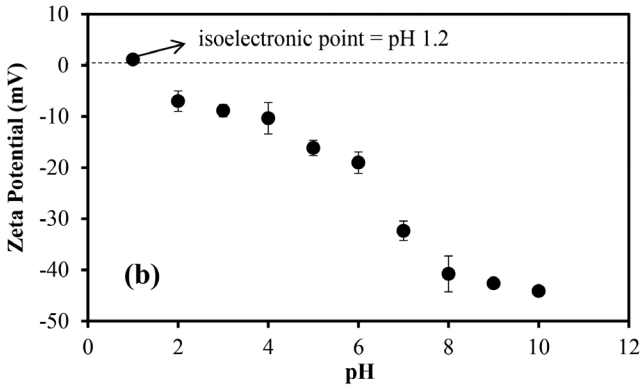


FIGURE 2 (a) FTIR spectra of CAT and p(CAT) microparticles a and (b) the zeta potential of p(CAT) microparticles a at different pH solutions (pH range 1–10)

measured in pH 1–10 solutions and results are shown in Figure 2(b). The zeta potential is positive at 1.2 ± 0.6 mV at pH 1, whereas at pH 2≥negative charge values ranging between -7.0 ± 2.1 mV to -44.1 ± 0.7 mV were obtained. Particle suspensions are considered to have generally stable dispersion above ±30.0 mV zeta potential values due to the strong repulsion forces preventing the aggregation of particles.[37] According to the pH versus zeta potential results, p(CAT) particles were well dispersed and more stable in the pH 7–10 range as their zeta potential values are larger than −32.35 mV. The isoelectronic point where zero zeta potential values are assumed for p(CAT) particles was found to be pH 1.2.

The comparison of thermal stabilities of CAT and p(CAT) particles A, B, and C prepared at 100%, 200%, and 300% mole ratios of crosslinker are illustrated in Figure 3(a) and their main decomposition steps are given in Table 1. These results indicate that although p(CAT) particles are thermally less stable than CAT in the 200–550°C range, they are thermally more stable above 600°C due to the crosslinked network structure. The more degradable structure of p(CAT) particles up to 550°C could be due to the more degradable nature of the crosslinker than CAT molecules within the particle network. Also, p(CAT) particles A and B show similar thermal decomposition steps with nearly 82 and 80 wt% losses; on the other hand, thermal stability of p(CAT) particles C was notably improved with about 60 wt% loss at 600°C observed due the higher concentration of crosslinker than the other particles. Thermal stability depends on the chemical structure of each material and their components.

Therefore, crosslinked polymeric particles can be thermally less stable and more degradable than each one constituent of that material, for example, monomer and crosslinker can be thermally more stable than the polymeric structure and at different temperature materials degrade differently depending on the material chemical structures.

The degradation of p(CAT) particles is governed by amounts of crosslinker used and the pH conditions of the medium. Therefore, the hydrolytic degradation of p(CAT) particles A, B, and C was investigated at pH 7.4 for particles prepared by using 100%, 200%, and 300% molar ratios of TMPTGE at different pH conditions that are close to physiological conditions, for example, pH 1.0

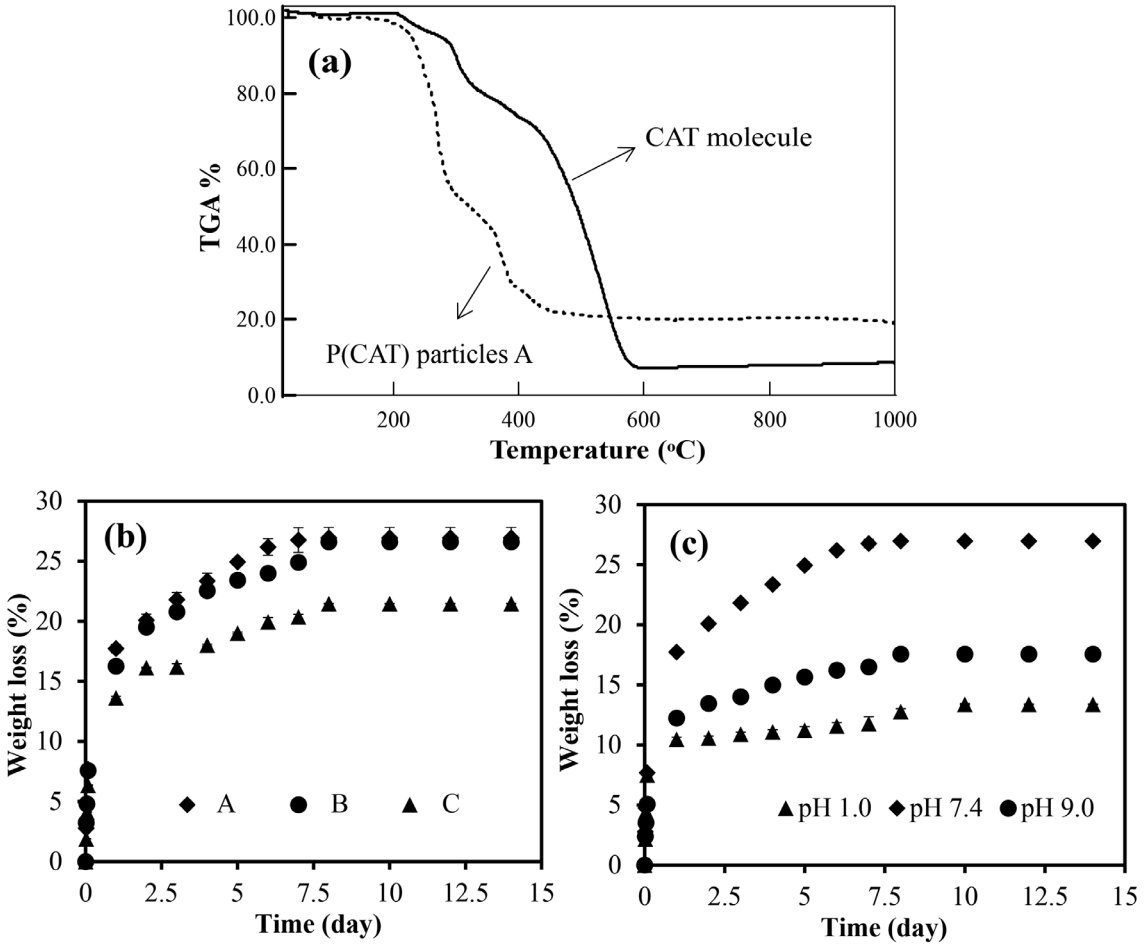


FIGURE 3 (a) Thermal degradation of CAT molecule and p(CAT) microparticles a. (b) Hydrolytic degradation of p(CAT) microparticles A, B, and C prepared at 100%, 200%, and 300% molar ratio of CAT to TMPTGE at pH 7.4, and (c) hydrolytic degradation of p(CAT) microparticles a at pH 1, 7.4, and 9

TABLE 1 Thermal degradation steps of CAT and p(CAT) microparticles A, B, and C

	Main thermal degradation steps		
Materials	**1**	**2**	**3**
CAT	220–286°C 6 wt%	290–365°C 22 wt%	420–585°C 92 wt%
P(CAT) particle A	210–350°C 56 wt%	350–460°C 82 wt%	—
P(CAT) particle B	210–298°C 56 wt%	300–460°C 80 wt%	—
P(CAT) particle C	130–532°C 43 wt%	639–750°C 61 wt%	833–912°C 80 wt%

for stomach, pH 7.4 blood plasma, and pH 9.0 close to intestinal conditions for p(CAT) particles A as illustrated in Figure 3(b), (c), respectively. It is apparent from the hydrolytic degradation curves as shown in Figure 3(b) that the degradations initially start fast and almost linearly, but slowly continue up to 10 day at pH 7.4 regardless of the amount of TMPTGE used. P(CAT) particles swell rapidly upon contact with water in buffer solution due to hydrogen bonding because of higher numbers of —OH groups that are hydrophilic and degradable ether linkages in the p(CAT) network. As the amount of crosslinker is increased in p(CAT) particles, the network becomes tighter and the swelling is reduced so the hydrolytic degradation also reduces. Therefore, the degradation of p(CAT) particles can be controlled by the amounts of crosslinkers used to a certain degree.

Also, there is no significant change observed for p(CAT) particles A and B at pH 7.4 solution conditions over a degradation time of 15 days. Additionally, p(CAT) particles A show pH responsive degradation profile with $13.4 \pm 0.2\%$, $26.9 \pm 0.9\%$, and $17.6 \pm 0.1\%$ weight losses at pH 1.0, 7.4, and 9.0, respectively. Therefore, it is obvious that p(CAT) particles A can be hydrolytically degraded at pH 7.4 up to about 27% of weight releasing CAT molecules over 10 days and can be very beneficial in

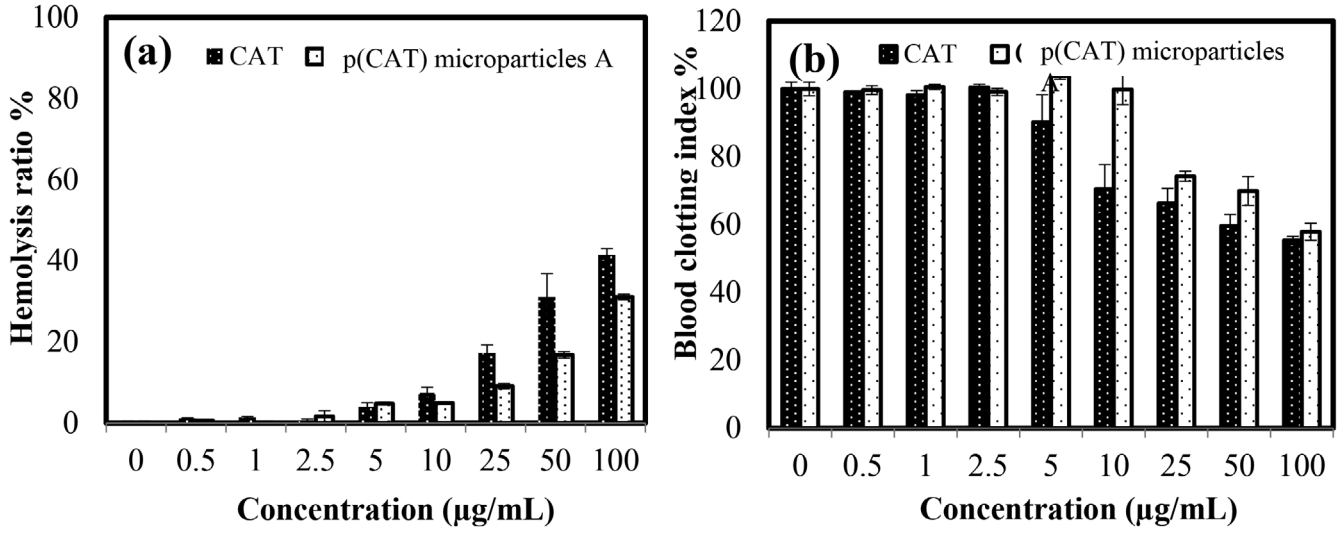


FIGURE 4 (a) Hemolysis ratio%, and (b) blood clotting index% of CAT molecule and p(CAT) microparticles a at different concentrations

biomedicine as antioxidant and antibacterial material. Zemljic et al reported that CAT molecules have about 2.5 and 10 mg/ml minimum inhibition concentration (MIC) values against *Staphylococcus aureus* and *Escherichia coli* bacteria strains.[38] According to the degradation results of p(CAT), about 50 mg/ml p(CAT) particles could inhibit gram-negative and gram-positive bacteria within 1 day and prevent re-growth by protecting for up to 10 days with sustainable CAT release capability for biological applications.

In the blood compatibility assays, CAT molecule and p(CAT) particles A were interacted with fresh human blood at concentrations of 0.5–100 µg/ml to assess hemolytic behavior with results shown in Figure 4(a), (b). Hemolysis shows the ratio of lysed erythrocyte cells upon contact with the potentially hemolytic material. The hemolysis ratios at 0–2%, 2–5%, and 5–100% are stated to be non-hemolytic, slightly hemolytic, and hemolytic behaviors, respectively.[39] From the tests, CAT molecule was considered to be slightly hemocompatible up to 5 µg/ml concentration with $4.7 \pm 0.3\%$ hemolysis ratio, whereas p(CAT) particles were found to be much safer at 10 µg/ml with $3.9 \pm 1.0\%$ hemolysis ratio. The possible red blood cell destroying mechanism of polyphenolics was speculated. The small molecules of polyphenolics can readily interact with the cells by means of intermolecular hydrogen bonds and may cause to cells rupture when adsorbed onto the surface of the cell membrane with hydrophilic phenolic hydroxyl groups.[40] So, it is clear that the hemolytic effect of CAT can be diminished in particle formulation, and can be attributed to the larger size as well as structural changes due to the existence of crosslink units.

The other important parameter is the coagulation of blood when in contract with biomaterials. The blood clotting index shows the effects of the materials on the blood coagulation mechanism. As illustrated in Figure 4 (b), CAT molecules started coagulating blood at 10 µg/ml concentration with $70.5 \pm 7.2\%$ clotting index, whereas there is no significant clotting index observed for p(CAT) particles, and a $99.8 \pm 4.5\%$ clotting index was obtained at the same concentration. The clotting mechanism is assumed to be triggered in two different ways, (a) interaction with negatively-charged materials such as polyphenols, kaolin, and silica, which is called endogenous coagulation and (b) interaction with materials containing tissue thromboplastins, which is called exogenous coagulation. Deng et al indicated that polyphenolics seriously affect the exogenous coagulation mechanism at low concentrations, but endogenous coagulation can be triggered at high concentration of phenolics in the blood.[40] Our results show that p(CAT) particles lead to endogenous coagulation at ≥25 µg/ml of particle concentrations because of the highly negatively charged nature of p(CAT) particles. These blood compatibility tests indicate that p(CAT) particles can be safely used up to 10 µg/ml concentration in the vascular system.

Among flavonoids, CAT has low toxicity and no significant mutagenic activity.[41] There are also a few reports about the toxic effects of CAT on normal cell lines. Alshatwi et al identified that cell death was observed at only 24.2% at 100 µM concentration of CAT molecule on human peripheral blood lymphocytes.[9] Also, CAT molecules were also reported to possess weak cytotoxicity for human umbilical vein endothelial cells at 40 µg/ml concentration with $80 \pm 18\%$ cell proliferation.[42] Moreover,

Babich et al. reported that CAT molecule showed slight cytotoxicity on normal human gingival HGF-2 fibroblast up to 500 μM concentration in 3 days exposure.[43] In order to evaluate the cytotoxicity of CAT molecule and p(CAT) particles A, cell viability% on CDC841 human normal colon cells was assessed by using CellTiter-Glo Luminescence cell viability assay. Dose and time dependent cytotoxicity results for CAT molecule and p(CAT) particles A are shown in Figure 5(a), (b), respectively.

According to these results, CAT molecules show no toxicity up to 50 μg/ml concentration, but importantly prohibits cell growth at ≥100 μg/ml concentration with about 64 ± 17% cell viability at 24 h incubation. P(CAT) particles, on the other hand, are more biocompatible than CAT molecules as high cell viability obtained, 95 ± 5% at 250 μg/ml p(CAT) particle concentration. As the incubation time increased, e.g., 72 h, noticeable cytotoxicity of CAT molecules occurred even at lower concentrations, e.g., at 25 μg/ml concentration 70 ± 8% cell viability was obtained. Interestingly, the p(CAT) particles show moderate cell viability, for example, 81 ± 3% viability even at 100 μg/ml concentration. To examine the visibility of cell proliferation, CCD841 cells interacted with 100 and 1000 μg/ml of CAT molecule and p(CAT) particles were treated with NucBlue dye and the images were taken under DAPI filter. Figure 5(c) illustrates the cell proliferation images for these materials after 72 h incubation time compared with the control group. NucBlue dyes

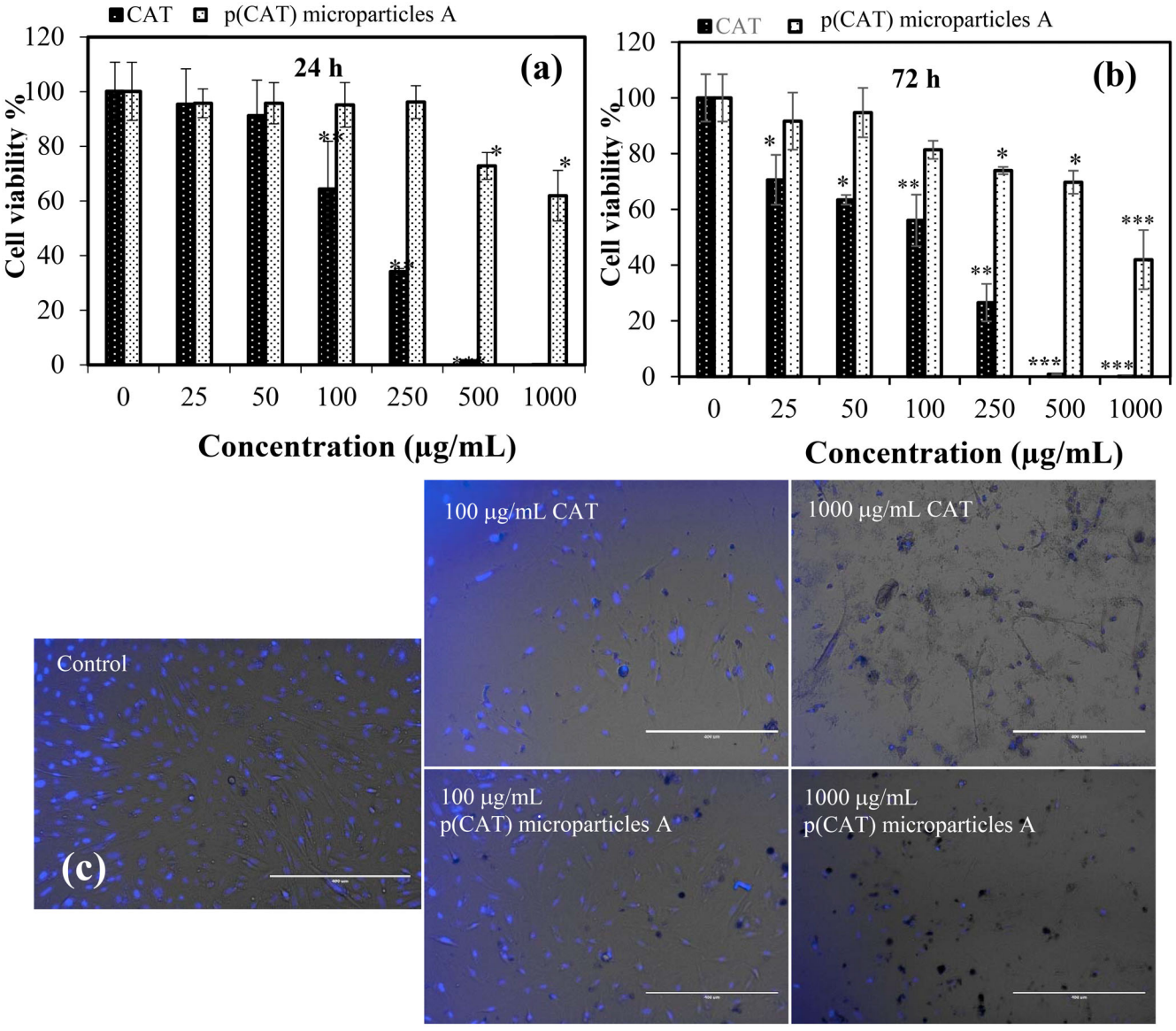


FIGURE 5 Cytotoxicity of CAT molecule and p(CAT) microparticles a on CCD841 CoN cell line at different concentrations, (a) in 24 h and (b) 72 h incubation time. (c) DAPI images of the CCD841 CoN cells for 72 h incubation time at 10× magnification [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Total phenol content and Trolox equivalent antioxidant capacity of CAT molecule and p(CAT) microparticles A

Antioxidant materials	Total phenol content in terms of gallic acid equivalency[a] (μg/ml) for FC test	TEAC values (μmol trolox equivalent/g) for ABTS test
Gallic acid	170.0 ± 0.0	9.15 ± 0.4
CAT	81.5 ± 3.1	2.94 ± 0.2
P(CAT) particles A	55.0 ± 0.9	0.88 ± 0.3

[a]170 μg/ml CAT and p(CAT) microparticles A.

the nucleus of living cells and is seen as brilliant blue color. The images indicate that about half of living cells died when treated with 100 μg/ml of CAT molecules for 72 h, whereas 100 μg/ml of p(CAT) particles resulted in no significant effect on cell proliferation. As the concentration of material increased, for example, at 1000 μg/ml, cell proliferation was significantly decreased for p(CAT) particles while there is destruction of cell structures with CAT molecule as seen in the image. These results support the view that in particle form the toxicity of CAT molecule is considerably reduced and suggests viable potential in biomedical applications even at moderately high concentrations, that is, at about 100 μg/ml.

Free radicals are commonly generated by reactive oxygen and nitrogen species (ROS and RNS) which cause damage to cells, proteins, and DNA in human body. CAT is a main component in green tea polyphenols, which attracts considerable attention due to its high radical scavenging ability for ROS and RNS species as an effective antioxidant material. Therefore, the antioxidant capacity of CAT molecule and p(CAT) particles A were investigated by FC phenol capacity test and ABTS scavenging assay. Table 2 shows the results of total phenol content in terms of gallic acid equivalent and trolox equivalent antioxidant capacity values for CAT molecule and p(CAT) particles.

Table 2 clearly shows that CAT monomer antioxidant capacity is almost half of the reference materials, in terms of gallic acid equivalent, 81.5 ± 3.1 μg/ml in terms of total phenol content and this value is slightly reduced to 55.0 ± 0.9 μg/ml gallic acid equivalent for crosslinked particle forms, such as, p(CAT) particles. Also, the TEAC values were measured as 2.94 ± 0.2 and 0.88 ± 0.3 μmol trolox equivalent/g antioxidant capacities for CAT molecule and p(CAT) particles, respectively. Both tests show that the high antioxidant capability of CAT molecule can still be retained in p(CAT) particles. Keeping in mind the hydrolytically degradable nature of p(CAT) particles, the antioxidant material with longer duration antioxidant properties can be useful to protect cells from a variety of biological reactions and hazardous reactive species and can play a significant role in the prevention of many ROS-related diseases including cancer, Alzheimer, Parkinson, ulcer, and cardiovascular diseases.

4 | CONCLUSION

CAT as a natural compound was prepared as degradable p(CAT) microparticles that can improve the bioavailability and biocompatibility of CAT molecules. It was demonstrated that p(CAT) microparticles A, B, and C can be readily prepared using TMPTGE as degradable crosslinker at 100%, 200%, and 300% molar ratios in a single step in a microemulsion system. It was further shown that hydrolytic degradation of p(CAT) particles depends on the pH of the medium and crosslinker ratio, and about 27% linear degradation profile was attained for 100% crosslinked p(CAT) particles A at pH 7.4 at 37.5°C for about 10 day. Hemocompatibility and blood clotting effects of CAT molecules were remarkably improved in particle form as p(CAT) particles afford safe to use for the vascular system up to 10 μg/ml concentration. Similarly, the cytotoxicity results revealed significant improvements in biocompatibility of p(CAT) particles for CCD841 normal colon cells in comparison to CAT molecules, for example, no cell inhibition up to 250 μg/ml concentrations for 24 h incubation. Therefore, the biodegradable p(CAT) particles along with their natural origin offers great promise in numerous biomedical fields because of sustainable and long-term CAT molecule release capability providing longer antioxidant behavior with less toxicity.

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