Nanozyme: a promising multifunctional antioxidant for skin and hair care

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Abstract (Maximum of 200 words)

Nanozymes, also known as catalytic antioxidants, are a class of nanomaterials with enzyme-like activity. Nanozymes have been extensively studied in biosensing, cancer therapeutics and drug delivery due to their high stability and versatility. The objective of this study is to prepare a multifunctional nanozyme and evaluate the activities of the nanozyme and explore its application in skin and hair care.

The nanozyme composed of copper and tannic acid (CuTA) was prepared using a simple and green technology. The structure of the coordinated nanomaterial was characterized by Fourier transform infrared spectroscopy (FTIR), UV-vis and X-ray photoelectron spectroscopy (XPS). The antioxidant and anti-inflammatory activities of the CuTA nanozyme were evaluated *in vitro*. In addition, the microbial inhibitory effect of the CuTA nanozyme on the representative skin microorganisms of *Cutibacterium acnes* and *Malassezia furfur* was further investigated. Furthermore, the CuTA nanozyme was also formulated into surfactant rinse-off and leave-on products to evaluate its potential application in skin care and hair care.

The multifunctional CuTA nanozyme not only has catalytic properties similar to those of superoxide dismutase (SOD), but also shows effective microbial inhibitory activity. It is indicated as a promising active ingredient in skin and hair care.

Keywords: Nanozyme；CuTA; Metal-phenol coordination; Antioxidant; Antimicrobial

Introduction.

Nanozymes, also known as catalytic antioxidants, are a class of nanomaterials with enzyme-like activity. [1, 2] The redox-active nanozymes have emerged as promising alternatives to natural enzymes due to their high stability and versatility properties. [3, 4] The nanozymes are attracting more attention and have made achieved progress in recent years.[5, 6] The natural polyphenol tannic acid (TA) and Fe III have been reported to form assembly of coordination complexes in a single step.[7] The components are readily available and inexpensive. They are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. The ease, low cost, and scalability of the assembly process, combined with the pH-responsiveness and negligible cytotoxicity, make these versatile complexes as potential candidates for biomedical, biosensor and environmental applications, etc. [8-12]

In recent years, different types of nanozymes have been developed and investigated for various potential applications. A novel copper-tannic acid coordination (CuTA) nanozyme is reported as a highly active and thermostable ROS scavenger with multiple antioxidant capabilities. It was demonstrated to be able to reduce the toxic effects of cigarette smoke when CuTA was loaded in commercial cigarettes using mouse model.[13] The antioxidant property of CuTA nanozyme was reported to be induced by phenolic ligand-metal charge transfer (LMCT) to eliminate reactive oxygen species (ROS) and facilitate the healing of chronic diabetic wounds.[14] In addition, the recent study has shown that CuTA nanoparticles overcame the drawback of Cu^{2+} inefficacy against Gram-positive bacteria, and exhibited robust antibacterial effects at extremely low doses against both Gram-positive and Gram-negative bacteria and reduced biofilm biomass. The results demonstrated that CuTA nanoparticles are extraordinarily effective against pathogenic bacterial infections and significantly contribute to collagen deposition and skin regeneration in the skin-infected model in vivo. [15]

However, the potential application of the versatile nanozymes in cosmetics and hair care has not been well explored. [16, 17] Therefore, we developed a multifunctional nanomaterial that is a coordination of copper and tannic acid (CuTA) and evaluated the properties of CuTA and potential to explore the potential application in skin and hair care.

Materials and Methods.

Chemicals and reagents:

Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and $FeSO_4 \cdot 7H_2O$ were reagent grade (purity > 99%) were purchased from Beijing Chemical Industry Group Co. Ltd. Tannic acid (TA, purity > 98%) was purchased from Meilun Biotechnology Co. Ltd. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Shanghai DiBo Co. Ltd. Other chemicals including salicylic acid, sodium hydroxide (NaOH), ethanol and hydrogen peroxide (H_2O_2, 30%), were of at least of analytical grade and were used directly without further purification.

DMEM, FBS, PBS, LPS, penicillin/streptomycin reagents were purchased from Meilun Biotechnology Co. Ltd. RAW264.7 cell line was purchased from Wuhan Pricella Biotechnology Co., Ltd. IL-6 (lot: BMS603-2) and TNF-α (lot: BMS607-3TWO) Elisa kits were purchased from Invitrogen, Thermo Fisher Scientific. Malassezia furfur (lot: BNCC358921） and *Cutibacterium acnes* (*Propionibacterium acnes*, Lot: BNCC336649) were purchased from BeNa Culture Collection Co. Ltd.

Synthesis of CuTA nanozyme:

CuTA nanozyme was prepared by an oxidative coupling assembly strategy. In a typical synthesis, tannic acid and $CuSO_4 \cdot 5H_2O$ in a mass ratio of 1:35 were dissolved in deionized water to form a homogeneous solution. After adjusting the pH to 7.4 with NaOH (2 M), the solution was heated to 50 °C and kept for 3 h. The substance was synthesized and could

be separated by centrifugation and washed thoroughly with deionized water. The purified light green product was dried overnight at 60 °C under vacuum.

Characterization of CuTA nanozyme:

The microstructure of CuTA nanozyme was characterized by Fourier transform infrared spectroscopy (FTIR, VERTEX 70, Bruck, Germany) and UV–vis absorption spectroscopy (UV-vis, V-770, JASCO, Japan). X-ray photoelectron spectroscopy (XPS) measurement of CuTA nanozyme was performed using with an X-ray monochromator (XR5 Gun-500 μm, Thermo scientific, USA).

Antioxidant activity of CuTA nanozyme:

The antioxidant activity of CuTA nanozyme was evaluated by various *In vitro* assays, including DPPH[18] and OH radical scavenging assays[19]. DPPH radical scavenging assays were performed according to previously published literature. The CuTA nanozyme was prepared at concentrations of 100, 50, 25, 12.5 and 6.25 mg/ml, respectively. 100 μL of the solution was added to 100 μL of ethanol solution containing 200 μmol/L DPPH. L-ascorbic acid and deionized water were used as positive and control groups, respectively. After shaking, the plates were kept in the dark at room temperature for 30 minutes. The absorbance of the different groups was determined at 517 nm. The hydroxyl radical scavenging activity of CuTA nanozyme was further evaluated by a method based on the Fenton reaction, which is widely used for antioxidant screening. [20] The initiation of the OH radical reaction was generated by adding 0.2×10^{-3} mol/L H_2O_2 and 0.2×10^{-3} mol/L $FeSO_4$ and mixing for 3 minutes. CuTA nanozyme was then added to the solution and incubated for a further 3 minutes to eliminate OH radicals. Finally, 1.0×10^{-3} mol/L salicylic acid (SA) was added to detect remaining OH radicals. SA was oxidized by OH radicals to 2,3-dihydroxybenzoic acid with an absorption peak at 510 nm. The remaining OH radicals were quantified by UV absorption and the scavenging percentage of OH radicals by CuTA nanozyme was calculated.

Anti-inflammatory assay of CuTA nanozyme:

The cytotoxicity and anti-inflammatory tests of CuTA nanozyme were evaluated by MTT assay using RAW264.7 macrophage cells. 2.0×10^4 cells/well of RAW264.7 cells were cultured in DMEM containing 10% FBS with 1% penicillin/streptomycin and incubated for 24 hours in a 96-well tissue culture plate at 37°C in a CO_2 incubator. After cell attachment, CuTA nanozyme was added to the cells at concentrations ranging from 0.1 to 100 μg/mL. After 24 hours of incubation, the cells were washed and incubated for 1 hour at 37°C with fresh medium containing MTT reagent. Finally, the absorbance of formazan crystals dissolved in DMSO was measured at 490 nm using a microplate reader (INFINITE F50, TECAN, Switzerland).

To evaluate of the anti-inflammatory effect of CuTA nanozyme, RAW264.7 macrophage cells were seeded in 24-well tissue culture plates (5×10^5 cells/well). After incubation for 24 hours, the cells were stimulated with LPS (1 μg/mL) with or without CuTA nanozyme at a concentration of 2, 5, 10 μg/mL, respectively. Cells without any LPS treatment were used as control. After 48 hours of incubation, the expression levels of the inflammatory factors of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-*α*) in the medium supernatant of cells from different groups were determined using ELISA kits.

In vitro antimicrobial activity of CuTA nanozyme:

The antibacterial abilities of CuTA nanozyme at different concentrations were determined using the optical density at 600 nm (OD 600). The absorbance at 600 nm was recorded and the bacteria without CuTA nanozyme were used as control and the culture medium without bacteria was considered as background. Subsequently, to determine the minimum inhibitory concentration (MIC), bacterial suspensions of *Cutibacterium acnes* and *Malassezia furfur* (10^6 CFU/mL) in Luria-Bertani (LB) broth solution were incubated with different concentrations of CuTA nanozyme (0.1-10 mg/ml) at 36±1°C for 24 hours, respectively. Then, to enumerate the bacterial colonies, we prepared agar plates by dissolving agar in LB broth medium (0.015

g/mL). The bacteria were then treated with MIC concentrations of CuTA nanozyme on the agar plates.

The antimicrobial activity of CuTA nanozyme-containing skin/hair care product was evaluated against *Cutibacterium acnes* and *Malassezia furfur* using the disc diffusion method. We used LB agar medium plates and then sample wetting filter papers were placed on the plates. After 24 hours of incubation at 36±1°C, the diameter of the CuTA nanozyme growth inhibition zone was measured. A clean zone surrounding the bacteria indicates that the antimicrobial treatment has effectively stopped or prevented microbial development.

Results.

Synthesis and characterization of CuTA nanozyme

CuTA nanozyme was synthesized by one-pot green synthetic method using oxidative coupling assembly of tannic acid and Cu^{2+}. The process was performed and screened by Cu^{2+} precursor concentrations under different pH. The CuTA nanozyme was obtained under optimal conditions and the structure of the CuTA nanozyme was confirmed by FTIR, UV-vis and XPS.

As shown in Figure 1a, the characteristic FTIR absorption spectra of TA, $CuSO_4$, and CuTA nanozyme were different, indicating different microstructures. The UV-vis spectra of TA and CuTA nanozyme were scanned and showed a coincident absorption wavelength between 300-350 nm as observed in Figure 1b. The Cu content and oxidation state were also determined by XPS. The XPS spectra of Cu 2*p* showed the main peaks of Cu 2*p*3/2 at 934 eV and Cu 2*p*1/2 at 955 eV accompanied by their characteristic shake-up satellite peaks at 942 eV and 961 eV, respectively, as shown in Figure 1c.

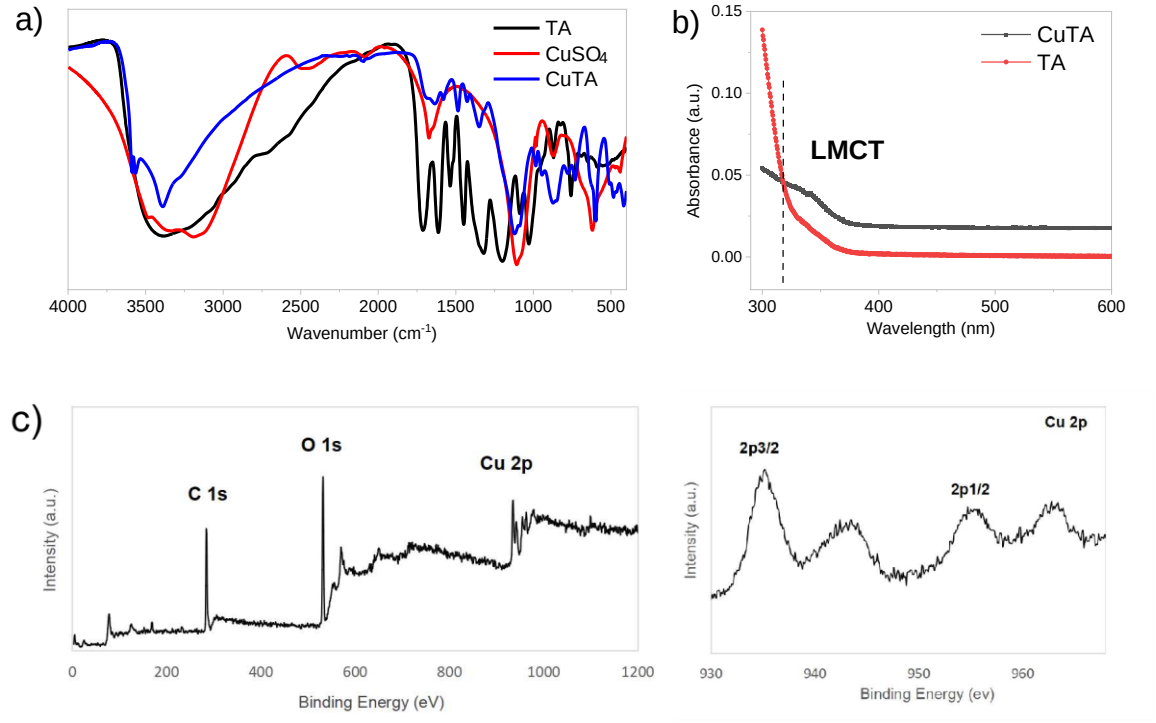

Figure 1. a) FTIR spectra of TA (black line), $CuSO_4$ (red line) and CuTA nanozyme (blue line). b) UV-vis spectra of TA (red line) and CuTA nanozyme (black line). c) XPS spectra of CuTA nanozyme and Cu 2p, respectively. FTIR, Fourier transform infrared spectroscopy; UV-vis, UV–vis absorption spectroscopy; XPS, X-ray photoelectron spectroscopy.

SOD-like antioxidant activity of CuTA nanozyme

To further evaluate the SOD-like activity of CuTA nanozyme, we evaluated the antioxidant activities of CuTA nanozyme using in vitro DPPH and OH radical scavenging assays. As shown in Figure 2, the scavenging rate of DPPH radical was 81.54% of CuTA nanozyme at 2 mg/ml, with an IC50 value of 0.77 mg/ml. Similarly, the scavenging rate of hydroxyl radical was 80.28% of CuTA nanozyme at 20 mg/ml, with an IC50 value of 10.29 mg/ml. The results showed good antioxidant efficacy of CuTA nanozyme at low concentrations compared to Vc, a known active ingredient, as a positive control group.

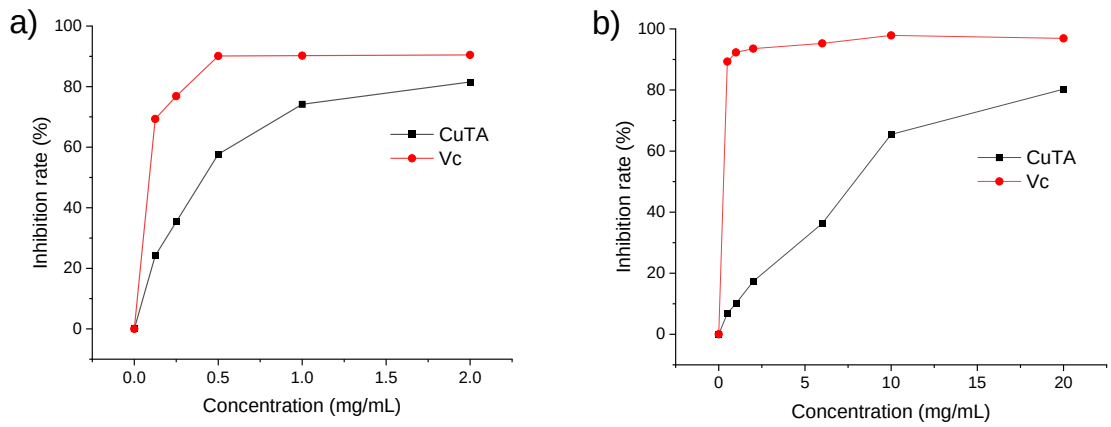

Figure 2. DPPH and OH radical scavenging abilities of CuTA nanozyme. a) DPPH radical scavenging rate and b) OH radical scavenging rates of CuTA nanozyme (black line) at different concentrations and positive group of Vc (red line).

Immunomodulatory effect of CuTA nanozyme by alleviating inflammatory oxidative stress

The cytotoxicity of CuTA nanozyme was tested and the anti-inflammatory activity was evaluated on RAW264.7 cells. The expression levels of inflammatory factors in LPS-induced RAW264.7 cells after treatment with CuTA nanozyme were determined. As shown in Figure 3, the levels of IL-6 and TNF-*α* were significantly increased in the LPS group compared with the blank control group ($P < 0.01$). The CuTA nanozyme group could significantly reduce the levels of IL-6 and TNF-*α* at 3~10 μg/ml.

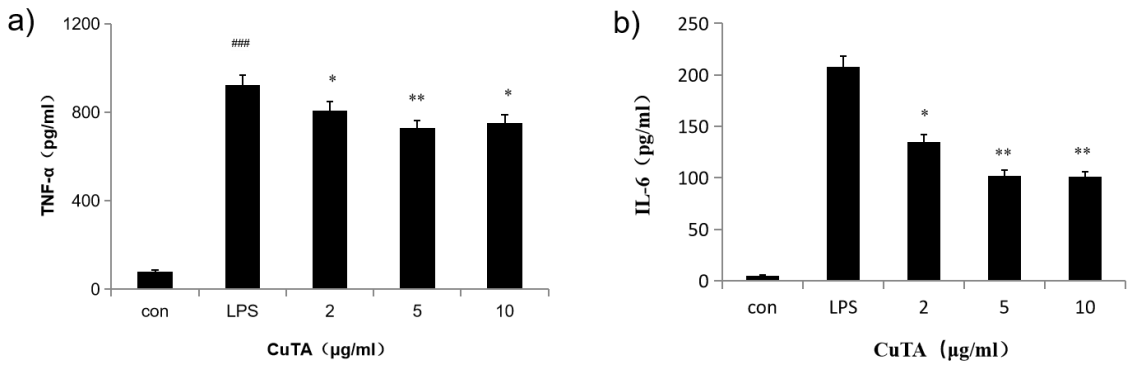

Figure 3. Anti-inflammatory effect of CuTA nanozyme in RAW264.7 cell model. A) TNF-*α* and IL-6 release levels after treatment with LPS and CuTA nanozyme at different concentrations (control vs. positive, ###, $P < 0.01\%$; LPS group vs. CuTA nanozyme, *, $P < 0.05\%$; **, $P < 0.01\%$).

Evaluation of antibacterial effect of CuTA nanozyme

In addition to the SOD-like and anti-inflammatory activities, we further investigated the microbial inhibitory effect of CuTA nanozyme. The CuTA nanozyme showed antimicrobial properties with a minimum inhibitory concentration (MIC) of 0.78 mg/mL and 1.56 mg/mL on the models of the representative skin microorganisms of *Cutibacterium acnes* and *Malassezia furfur*, respectively.

After formulation into a cosmetic shampoo, the bacteriostatic effect of the CuTA nanozyme shampoo was determined using the circle of inhibition method, which showed an inhibitory effect with a circle of inhibition of 15 mm compared to the blank substrate.

Discussion.

In this work, we successfully prepared CuTA nanozyme by oxidative coupling assembly of tannic acid and Cu^{2+} by one-pot green synthetic method, which is easy to scale up, low cost and environmentally friendly.[21] The process of CuTA nanozyme preparation was optimized by different ratios of TA and Cu^{2+}. The microstructure of CuTA nanozyme was characterized by FTIR, UV-vis and XPS.

As shown in Figure 1a, the FTIR spectrum of CuTA nanozyme is characterized by the splitting of the absorption centered at 3376 cm^{-1}. Due to the oxidation of tannic acid and subsequent coordination with Cu^{2+}, the CuTA nanozyme showed splitting and absorption shift in the FTIR spectrum as previously reported.[13] In specifical, the absorption shift in the fingerprint region indicated the perturbation of the HO-C vibration of TA after coordination with Cu^{2+} by the phenolic groups. The absorption bands at 1711 and 1612 cm^{-1} were related to the coupled vibrations of the ring ν(C-C) and the carbonyl ν(C=O) of TA, which decreased and shifted in the spectrum of CuTA nanozyme. In addition, the bands at 1320, 1448, and 1536 cm^{-1} had a large contribution from the ν(C-O), δ(C-OH), and ν(C-C) vibrations and underwent shifting and breaking, inferring an interaction of Cu^{2+} with the -OH groups of the phenolic group, in

agreement with other published results. [14] In addition, the CuTA nanozyme coupling assembly of various phenolic sources with Cu^{2+} was also attempted and the characteristics of the spectra were coincidentally illustrated by FTIR (data not shown).

The UV-vis spectra of TA and CuTA nanozyme showed a coincident absorption wavelength between 300-400nm was observed in Figure 1b, which indicated that the phenolic ligand-metal charge transfer (LMCT) occurred between TA and Cu^{2+}, as discussed previously.[14] According to the XPS results, the atomic ratio of Cu in the CuTA nanozyme was 5.94%. The XPS spectrum of Cu 2*p* showed the main peaks of Cu 2*p*3/2 at 934 eV and Cu 2*p*1/2 at 955 eV, which may be related to the thermally stable microstructure of CuTA nanozyme.[13]

In addition to the microstructure, the antioxidant activities of CuTA nanozyme have attracted much attention and have been demonstrated previously. The DPPH and OH radical scavenging assays are the most commonly used and well-known for antioxidant screening. [20, 22] To evaluate the SOD-like activity of CuTA nanozyme, in vitro DPPH and OH radical scavenging assays were performed to evaluate the antioxidant activities of CuTA nanozyme. The antioxidant results showed that the DPPH radical scavenging activity of CuTA nanozyme was 81.54% at 2 mg/mL and the hydroxyl radical scavenging ability was 80.28% at 20 mg/mL, indicating good antioxidant efficacy in various actions.

Moreover, as the development of new antimicrobial agents is on urgent need, nanozyme has recently been studied in antimicrobial due to its safety and low bacterial resistance. Based on the recent achievements, three mainstream mechanisms regarding the antibacterial activities of nanozymes are summarized, namely, ROS regulation, HOBr/Cl generation, and extracellular DNA clearance. [23] It has been recognized that nanozymes which can mimic the catalysis of oxidases and peroxidases to generate ROS, are considered as novel antimicrobial agents with promising applications. According to the previous studies, CuTA coupling nanoparticles could overcome the drawback of Cu^{2+} inefficacy against Gram-positive bacteria and exhibit robust antibacterial effects at extremely low doses against both Gram-positive and Gram-negative

bacteria in vitro. Due to the high redox potential, copper (Cu^{+}) is highly efficient in the catalyzing the trace H_2O_2 in the extracellular environment of bacterial cells to generate ROS, and this may be another choice to remedy the defect of Cu^{2+}. Moreover, because of the robust penetration ability of CuTA nanoparticles, it could significantly reduce the intracellular bacterial burden and decrease the biofilm biomass. And the assembled CuTA nanoparticle is extraordinarily effective against pathogenic bacterial infections and contributes significantly to collagen deposition and skin regeneration. [15]

However, the antimicrobial activity of CuTA nanozyme against *Cutibacterium acnes* and *Malassezia furfur* has not been extensively investigated. Therefore, we selected the representative microbes and evaluated the antimicrobial activity of CuTA nanozyme using antimicrobial assays. The results showed a minimum inhibitory concentration (MIC) of 0.78 mg/mL and 1.56 mg/mL of CuTA nanozyme against *Cutibacterium acnes* and *Malassezia furfur*, respectively. After formulation into a cosmetic shampoo, the bacteriostatic effect of the CuTA nanozyme shampoo was determined using the circle of inhibition method, which showed an inhibitory effect with a circle of inhibition of 15 mm compared to the blank substrate. Interestingly, the CuTA nanozyme-containing product showed good stability, bacteriostatic efficacy and attractive appearance with a light color. Further work has been carried out on CuTA nanozyme and it shows good potential for cosmetic applications.

Conclusion.

In this work, we have synthesized a novel CuTA nanozyme, which was characterized and evaluated by various methods, including FTIR, UV-vis, XPS. By coupling assembly with tannic acid and copper under appropriate conditions, the multifunctional CuTA nanozyme showed good antioxidant, anti-inflammatory, antimicrobial activities and stability by in vitro assays. It was demonstrated that the CuTA nanozyme has the potential to be explored as a promising active in skin care and hair care. Further work is in progress and further mechanisms and applications will be explored in the near future.

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Conflict of Interest Statement.

The authors declare no conflict of interest regarding the publication of this paper.

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