Free Radical Research, March 2008; 42(3): 244–252

informa
healthcare

Scavenging of free radicals in gas-phase mainstream cigarette smoke by immobilized catalase at filter level

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Accepted by Professor J. Cadet

(Received 23 July 2007; in revised form 12 January 2008)

Abstract

Catalase is well known as capable of inducing the decomposition of H_2O_2. In this study, a kind of immobilized catalase (entrapped in cross-linked chitosan beads) was dispersed in conventional acetate filter as an antioxidant additive. Quantitative estimation of the free radicals in mainstream cigarette smoke (MCS) was performed to address the effect of this modified filter. It was found that the levels of PBN adduct and $NO^{\bullet}/NO_2^{\bullet}$ associated with the gas-phase mainstream cigarette smoke (GPCS) were efficiently decreased by $\sim$40% through catalase filtering. Besides, the modified filter was found to lower the MCS-induced adverse biological effects including lipid peroxidation and mutagenicity. This was proved to be substantially attributed to the catalase-dependent breakdown of $NO^{\bullet}$, which was stimulated by some of peroxides (most probably being H_2O_2), the dismutation products of tar particulate matters (TPM). These results highlighted a promising approach to reduce the smoking-associated health risks to passive smokers. Moreover, the mechanisms of catalase filtering may be helpful for the development of appropriate immobilized enzyme systems to be applied for reducing health risks associated with gaseous pollutants.

Keywords: *Free radical, immobilized catalase, $NO^{\bullet}/NO_2^{\bullet}$, cigarette filter*

Abbreviations: *EPR, electron paramagnetic resonance; GPCS, gas-phase mainstream cigarette smoke (separated by Cambridge filter pad); MCS, mainstream cigarette smoke; CSC, cigarette smoke condensate; TPM, tar particulate matters; PBN, N-tert-butyl-α-phenylnitrone; $NO^{\bullet}$, nitric oxide; $NO_2^{\bullet}$, nitrogen dioxide; H_2O_2, hydrogen peroxide; CA filter, conventional acetate filter; TBA, thiobarbituric acid; TMP, 1,1,3,3-tetramethoxypropane; BHT, butylated hydroxytoluene; TCA, trichloroacetic acid; MDA, malondialdehyde*

Introduction

Cigarette smoking has long been proved to induce various devastating effects on both passive and involuntary smokers. More than 5000 kinds of chemicals including hundreds of biological toxicants or mutagens [1,2] have been identified from MCS. Among these, free radicals were well accepted to play a central role in inducing most of the smoking-associated pathological conditions [3–7], triggers for a variety of respiratory and vascular diseases [8,9] and various kinds of cancer [10].

It has been well established that there are two distinctly different populations of free radicals in MCS. One, referredto as particulate phase free

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760801911631

radicals, is the conjugated quinones of mixed oxidative states [11,12] which could be observed by direct EPR spectroscopy. The other, GPCS free radicals, is mainly made up of short-lived alkoxyl and alkyl radicals [12,13] arisen from the chain reactions driven by $NO^{\bullet}$ through the interaction of its oxidation product $NO_2^{\bullet}$ with alkenes (e.g. isoprene). Being embedded in a very complex polymeric matrix, particulate phase free radicals may be efficiently decreased by lowering the level of TPM, but the change amplitude is limited by the desired nicotine delivery and natural tobacco flavour. Therefore, it appears to be a more practical way to reduce smoking-associated health risks through the scavenging of GPCS free radicals and their sources, $NO^{\bullet}$ and $NO_2^{\bullet}$, both also being free radicals.

Several studies have been conducted to scavenge free radicals in cigarette smoke [14–16] and demonstrated that haemoglobin and haem-containing compounds could partly decrease the contents of reactive species including CO, NO, etc. in MCS [14,17]. However, the 'bio-filter' (containing activated carbon impregnated with dry haemoglobin) was unfortunately proved to substantially aggravate the smoking-associated health risks due to the increased TPM yield [17]. However, Culcasi et al. [7] reported that TPM could contrarily lower the smoking-induced oxidative damage and suggested diminishing MCS toxicity by rendering the TPM composition more reductive to GPCS free radicals. As haem-containing redox enzyme is found in nearly all aerobic cells, catalase is the most important protective enzyme *in vivo* that serves to protect cells against oxidative stress by catalysing the decomposition of H_2O_2 into H_2O and molecular oxygen. Moreover, catalase was reported to readily form a reversible complex with $NO^{\bullet}$ and consume more through catalysing its breakdown in the presence of H_2O_2 [18,19]. Catalase showed attractive potential to lower the levels both of H_2O_2 and GPCS free radicals in MCS. Application of enzymes out of their natural environments, however, has been generally restricted due to their sensitivity both to process conditions other than the optimal ones, normally narrow ranged, and to trace levels of substances that can act as inhibitors. Several methods have been proposed to overcome these limitations, one of the most successful being enzyme immobilization and chitin-based materials [20–23] were considered as promising carriers.

In the present study, a kind of immobilized catalase was prepared and applied as an antioxidant addictive in conventional acetate filter tips. It was recently reported that MCS-induced H_2O_2 was generally formed in the aqueous cigarette smoke condensate [24]. In that respect, the positive impact of catalase filtering could have been mostly attributed to the decrease in the levels of GPCS free radicals and $NO^{\bullet}$/$NO_2^{\bullet}$. A linear nitrone, N-tert-butyl-α-phenylnitrone (PBN) was dissolved in benzene and used as the spin trapping system [25] for the quantitative estimation of GPCS free radicals. Computer simulations, which were widely applied for the identification and quantitation of multiple spin-trap EPR spectra [26–28], were also performed. Contents of $NO^{\bullet}$ and $NO_2^{\bullet}$ were quantitated according to the Griess-Saltzman procedure [29,30]. Modified simulation schemes were applied to gain mechanistic insights into the interactions between the immobilized catalase and MCS components at filter level. MCS-induced adverse biological effects including lipid peroxidation and mutagenicity were assessed for a reliable evaluation of the positive impact of catalase filtering.

Materials and methods

Materials

Catalase was produced by submerged fermentation of a modified *Bacillus* organism and concentrated to be 1.0×10^4 CIU/mL by ultra-filtration before use. Chitosan (medium molecular weight) was used without further treatment. PBN, N-(1-Naphtyl)-ethylenediamine dihydrochloride, lecithin (from egg yolk), thiobarbituric acid (TBA) and 1, 1, 3, 3-tetramethoxypropane (TMP) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Hydrogen peroxide (30%, v/v), glyoxal hydrate, butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), para-aminobenzenesulphonic acid and all other chemicals were of analytical grade purity.

Preparation of immobilized catalase

Free catalase was entrapped in chemically cross-linked chitosan beads according to the methods described in the literature of Çetinus and Öztop [21] with slight modifications. Cryogenic grinding was applied to minimize the enzyme activity loss in the processes of dehydration and particle size reduction. Besides, pure cross-linked chitosan without catalase was prepared as the control for the carrier of immobilized catalase.

Enzyme activity assay

The catalytic activity of immobilized catalase was determined by the molar extinction coefficient of H_2O_2 which was measured at 240 nm for 1 min with a Shimadzu UV-2450 spectrophotometer (Tokyo, Japan). Ten milligrams of immobilized catalase was added to a cuvette containing 4.0 mL phosphate buffer solution (pH 7.0, 35°C) and 1.0 mL H_2O_2 (0.5 mM). One unit of the catalytic activity is defined as the ability to catalyse the decomposition of one mole H_2O_2 in 1 min and is expressed as units per g immobilized catalase (U/g); the results are presented in a normalized form.

Preparation of research cigarettes and routine analysis

All research cigarettes were manufactured by Shanghai Tobacco (Group) Cooperation with the same tobacco blend. Conventional acetate filter (CA filter) and the modified ones impregnated with immobilized catalase or cross-linked chitosan (referred as CAT or CTS filter) were prepared on a rod forming machine; subsequently attached to cigarette rods on a cigarette making and tipping machine combination. As prepared, the research cigarettes (CA, CTS and CAT corresponding to the filter tip) were stored at 4°C in sealed packages.

Smoking simulation for routine analysis was performed at ambient temperature using a SM-450 20-port smoking machine (Cerulean, Milton Keynes, UK). Prior to the smoking simulation, the research cigarettes were unpacked and kept in a constant humidity environment (20°C, 60% relative humidity) using a saturated NaBr solution for a minimum of 2 days. The research cigarettes were smoked according to the smoking condition (puff volume, 35 mL; duration, 2 s; frequency, 60 s) certified by International Standards Organization (ISO). Besides the puff number and draw resistance, weight difference of the Cambridge filter pad before and after the smoking procedure was recorded as the TPM yield. Contents of nicotine and moisture in TPM were determined by gas chromatography according to the standard procedure of ISO 10315 and ISO 10362-1, respectively.

EPR spectroscopy and spectral analysis

Smoking simulation for the quantitative estimation of GPCS free radicals was performed in conjunction with the spin trapping system in an intense smoking condition. Eight intense puffs (35 mL/puff) were taken for each single research cigarette. GPCS was separated from TPM by passing MCS through a Cambridge filter pad and introduced into spin trapping solution (0.1 M PBN in benzene, 5.0 mL). After the end of the last puff, the bubbled trapping solution was readjusted to its initial volume (5.0 mL) with the same benzene and a 400 μL aliquot was transferred into the EPR cell (standard X-band suprasil high purity quartz cell, o.d. 4 mm, Wilmad, Buena, NJ) for spectral measurement.

EPR spectroscopy was performed on a Bruker EMX spectrometer (Bruker UK Ltd., Coventry, UK) operating at X band (9.736 GHz) and using a modulation frequency of 100 KHz. Spectrometer settings were: modulation amplitude, 0.1 G; microwave power, 19.92 mW; time constant, 40.96 ms; receiver gain, 5.02×10^5; and scan rate, 8.0 G/s for a sweep width of 200 G. The spectra, displayed as the first derivative of EPR signals, were recorded and digitized using the microprocessor and commercial software supplied by Bruker (UK). Computer simulation of the experimental EPR spectra was performed with the program of WinSim 0.98 (http://www.niehs.nih.gov/). The hfsc (hyperfine splitting constants) applied throughout in the analysis of PBN adducts were determined from the simulated spectrum of the best-resolved signals.

$NO^{\bullet}/NO_2^{\bullet}$ assay

In quantitative analysis of $NO^{\bullet}$ and $NO_2^{\bullet}$ in MCS by the Saltzman procedure, $NO_2^{\bullet}$ (including the oxidation product of $NO^{\bullet}$) were allowed to react stoichiometrically with the Saltzman reagent (mixture of glacial acetic acid, 50 mL/L; para-aminobenzenesulphonic acid, 5.0 g/L; N-(1-Naphtyl)-ethylenediamine and dihydrochloride 0.05 g/L). After the end of the last puff, the absorbance of the treated Saltzman reagent was measured at 517 nm using the Shimadzu UV-2450 spectrophotometer. Contents of $NO_2^{\bullet}$ and $NO^{\bullet}$ were calculated by the calibration measurements performed with the reference solution of sodium nitrite.

CS-induced lipid peroxidation, MDA-TBA assay

CS-induced lipid peroxidation was studied in lecithin-formed liposome membrane by the modified MDA-TBA assay [31], as described below. Whole MCS was introduced directly into the reaction mixture containing 10 mg/mL lecithin in 50 mM Tris-HCl buffer solution (pH 7.4) in a total volume of 5.0 mL. After the smoking procedure, a 0.5 mL aliquot of the treated mixture was transferred into the test-tube containing 2.0 mL of TBA reagent (0.37% TBA, 15% TCA and 0.04% BHT (w/v) in 0.2 M HCl solution) and subsequently incubated in a boiling water bath for 20 min. After rapid cooling in an ice-water bath, the reaction mixture was extracted by shaking vigorously with 2.5 mL methanol-butanol (15:85, v/v) subsequently centrifuging at 3000 rpm for 10 min. The reaction mixture for negative control was treated in the same way without being exposed to the MCS. Absorbance of the supernatant was measured at 532 nm using the Shimadzu UV-2450 spectrometer. The content of MDA-TBA complex was calculated with TMP as standard.

Mutagenicity and mutagenic potency of MCS condensate (CSC), Ames fluctuation test

CSC was obtained by passing whole MCS through plenty of cold acetone. Following evaporation of the acetone after the smoking procedure, the residual condensate was redissolved in 5.0 mL DMSO. A commercial kit (Muta-ChromoPlate kit, BEAK International Inc., Ontario, Canada) was used to evaluating the mutagenicity of CSC and its mutagenic potency according to the scheme shown in Table I. Briefly, the sterile filtered sample was mixed with reaction mixture and test-strain (*salmonella*

Table I. Set of Muta-chromate plate assay with and without S-9 activation and the number of positive wells scored in a 96 well microplate.

Treatment	Volume added (mL/tube)				Bacterial test strain (TA-100)	Number of positive wells
	Standard	Sample	H_2O	S-9 Mix[c]		
00. Blank (sterile control)	–	–	17.5	–	–	0
01. Background-1	–	0.5 (DMSO)	17.0	–	+	4
02. Background-2	–	0.5 (DMSO)	15.0	2.0	+	7
03. Standard mutagen[a]-1	0.1	–	17.4	–	+	96
04. Standard mutagen-2	0.1	–	15.4	2.0	+	96
05. Sample[b]-1	–	0.5	17.0	–	+	31
06. Sample-1	–	0.5	15.0	2.0	+	57
07. Sample-2	–	0.5	17.0	–	+	18
08. Sample-2	–	0.5	15.0	2.0	+	41
09. Sample-3	–	0.5	17.0	–	+	27
10. Sample-3	–	0.5	15.0	2.0	+	55

[a]Standard mutagen supplied with the kit for positive control, 1: direct-acting mutagen, sodium azide (NaN_3, 0.5 μg in 100 μL distilled water); 2: indirect-acting mutagen, 2-Amino-Anthracen (AA, 10 μg in 100 μL distilled water).
[b]CS condensate from five research cigarettes redissolved in 5.0 mL DMSO, 1: CA cigarette, 2: CAT cigarette, 3: CTS cigarettes.
[c]S-9 activation mix reagents supplied with the kit.

typhimurium TA-100) broth culture supplied with the kit; 200 μL aliquots of the mixture were dispensed into each well of a 96-well microtitration plate and incubated at 37°C for 5 days. The number of positive wells for each plate was scored virtually to determine the statistical significance as described in the literature of Rao and Lifshitz [32].

Statistical analysis

Data were expressed as mean ± SD unless otherwise noted. Differences were analysed by one way ANOVA (analysis of variance) followed by one-sample *t*-test using the statistical analysis program of OriginPro 7.5 software (http://www.originlab.com/). The differences between groups were considered significant when $p < 0.05$.

Results

The extended activity of immobilized catalase

As prepared, the activity of immobilized catalase was determined as $4.21 \pm 0.15 \times 10^4$ CIU/g. Enzyme activity assays were carried out to assess the improved resistance of the immobilized catalase to the unfavourable environmental conditions including pH, temperature, etc. when applied in cigarette filter. The impact of environmental pH on the enzyme activity was studied at various pHs ranging from 2–9. Enzyme activity assay was performed in acetate and phosphate buffer solutions at 35°C and the activity-pH profiles are shown in Figure 1. The immobilized catalase reserved high relative activities in a broader range of pH and thus showed an improved resistance to the changing of environmental pH. The nature of catalase was not changed in the process of immobilization, dehydration and particle size reduction since both free and immobilized catalase shared a same optimum pH of 7.0. Taking into account the impact of environmental temperature on the enzyme activity, both catalases were exposed at 37°C to access their storage stability. Figure 2 shows their time-dependent deactivation in a period of 7 weeks. The immobilized catalase showed satisfactory storage stability; over 90% of its initial activity was reversed after being directly exposed at 37°C for 7 weeks. While in the case of free catalase, over 80% was lost after being kept in buffer solution for 7 weeks. The immobilized catalase was further assessed for its thermal resistance by being exposed at 60°C. Figure 3 shows its time-depended deactivation in the first 60 min. Over 90% of its initial activity could be reversed for at least 20 min and it was believed to be durable enough to finish smoking a single cigarette.

Routine analysis of research cigarettes

In this study, 10.3 ± 0.5 mg immobilized catalase and 10.1 ± 0.8 mg pure cross-linked chitosan (from $n = 20$ determination) were homogeneously dispersed

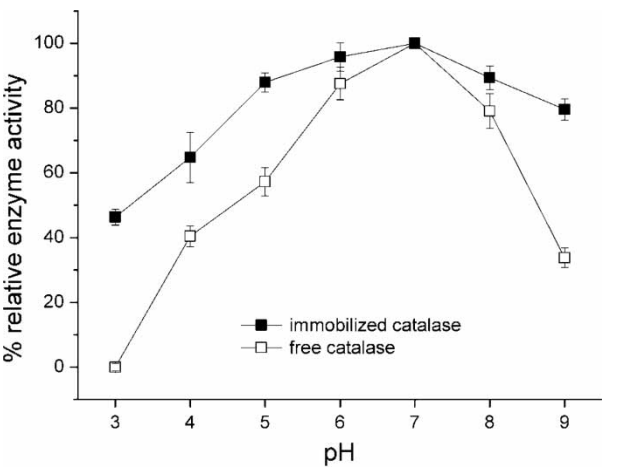

Figure 1. pH-Activity profiles of free and immobilized catalase. Enzyme activity assay was performed at 35°C in acetate and phosphate buffer solutions of various pH ranging from 3.0–9.0.

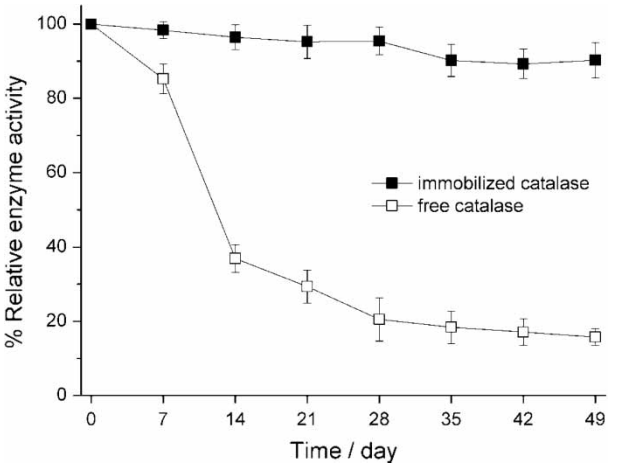

Figure 2. Time-dependent deactivation of free catalase (incubated in phosphate buffer solution, pH = 7.0) and immobilized catalase when exposed at 37°C. Enzyme activity assay was performed at 35°C in phosphate buffers solution (pH = 7.0).

into the matrix of cellulose fibres on a rod forming machine. Table II shows the descriptive data of the research cigarettes. By comparison with the CA cigarette, both CTS and CAT cigarettes exhibited slightly increased draw resistance and their machine-yielded levels of tar and moisture were apparently decreased. However, the change amplitude in draw resistance was successfully restricted within the acceptable range (< 5%); the puff number and nicotine delivery were not apparently affected.

The impact of catalase filtering on PBN adduct level arisen from GPCS

Bubbling GPCS of a single research cigarette through 5.0 mL trapping solution yielded an EPR spectrum as the represented one shown in Figure 4. In the case of nitrone-type spin trapped radicals, the spectrum was expected to be comprised of a triple of doublets arising from the interactions of the unpaired electron

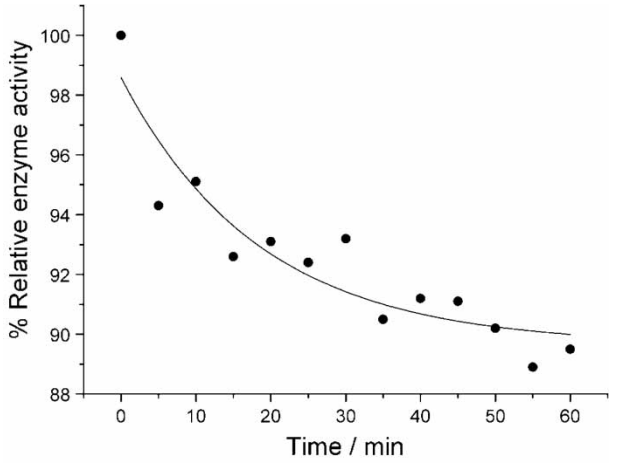

Figure 3. Time-dependent deactivation of immobilized catalase when exposed at 60°C. Enzyme activity assay was performed at 35°C in phosphate buffer solution (pH = 7.0).

with the ^{14}N nucleus ($I = 1$) and $^{\beta}H$ proton ($I = 0.5$). Judging from the EPR parameters of the simulated spectrum, the main spin adducts responsible for Figure 4 were verified to be alkoxyl radical ($\alpha_N =$ 13.40 G, $\alpha_{H=}$ 1.71 G, 66.67%) and alkyl radical ($\alpha_N = 13.91$ G, $\alpha_{H=}$ 1.62 G, 31.25%) with a secondary radical species identified as the oxidation product of PBN ($\alpha_N = 7.88$ G, 2.08%). Signal amplitudes of the corresponding simulated spectra were measured as the relative levels of spin adduct and the results are shown in Figure 5. The CAT cigarette gave the lowest spin adduct level (− 38.84% vs CA cigarette, − 35.52% vs CTS cigarette, $p < 0.05$), while the difference between that of CA and CTS cigarettes was not significant. The immobilized catalase played a central role in lowering the PBN adduct level by the CAT filter. This may be explained by the decrease in the level of GPCS free radicals which are believed to be involved in the formation of PBN adduct.

TPM is a trigger for catalase filtering

Catalase filtering was proved to decrease the level of GPCS-induced PBN adduct. In actual smoking condition, however, the immobilized catalase has been exposed to MCS including both gaseous components and TPM. A modified simulation scheme (Figure 6) was thus applied to address the impact of TPM on the catalase-dependent decrease in the GPCS-induced PBN adduct level. Filter tips were separated from research cigarettes and secured right after the Cambridge filter pad. Only GPCS was then allowed to be drawn through filter tips. The profiles of EPR signal amplitudes that account for the levels of GPCS-induced PBN adduct are shown in Figure 5. By comparison with the results of simulation scheme A, the catalase-dependent decrease in the PBN adduct level was significantly reduced. The level of PBN adduct induced by GPCS of CA cigarette increased by 37.42% in CAT cigarette in simulation scheme B. While in the case of CA and CTS cigarettes, the PBN adduct levels were not apparently affected. TPM appeared to be a trigger for the interactions of the immobilized catalase with the reactive species in GPCS, probably being $NO^{\bullet}$/ $NO_2^{\bullet}$ and the alkyl/alkoxyl radicals.

Evidence for the catalase-dependent decrease in the $NO^{\bullet}$ level

In order to gain mechanistic insights into the catalase filtering and the contribution of TPM, quantitative analysis of $NO^{\bullet}$ and $NO_2^{\bullet}$ was performed in conjunction with the modified simulation schemes. Results of the $NO^{\bullet}/NO_2^{\bullet}$ measurements are shown in Figure 7 correspondingly. The levels of $NO^{\bullet}$ and $NO_2^{\bullet}$ were efficiently lowered by 48.22% and 39.13%, respectively, in CAT cigarette vs CA cigarette in simulation scheme A. The decreases were

Table II. Descriptive data of the research cigarettes in this study.

Cigarette type	TPM (mg/cig)			Puff number	Draw resistance (Pa)
	Tar	Nicotine	Moisture		
CA	14.4	1.3	2.6	9.3	1225
CAT	11.7*	1.2	2.3*	9.5	1261*
CTS	11.4*	1.2	1.8**	9.8	1287*

$n = 20$, data accuracy are within 5%, $*p < 0.05$ and $**p < 0.01$ vs CA cigarette.

reduced to 9.19% and 8.02% with the removal of TPM in simulation scheme B. As expected, TPM exerted a similar impact on the catalase-dependent decrease in the levels of $NO^{\bullet}/NO_2^{\bullet}$ as that of PBN adduct level. The TPM-stimulated decrease in $NO^{\bullet}/NO_2^{\bullet}$ levels by the CAT filter may be attributed to the catalytic breakdown of $NO^{\bullet}$ in the presence of H_2O_2 [18,24].

Further experiments were performed to verify the contribution of H_2O_2 to the catalase-dependent decrease in the $NO^{\bullet}/NO_2^{\bullet}$ levels; 400 μL H_2O (distilled water) or H_2O_2 solution (30%, v/v) was injected into the separated filters. Smoking simulation for $NO^{\bullet}/NO_2^{\bullet}$ assay was performed according to the simulation scheme B and the results are shown in Figure 7. In the case of H_2O addition, $NO^{\bullet}/NO_2^{\bullet}$ levels were not apparently affected except that the $NO^{\bullet}$ level of CAT cigarette was decreased by 14.88% ($p < 0.05$ vs the results of simulation scheme B). It may be explained by the improved affinity of the moistened catalase to the gaseous components, e.g. NO and CO through its haem group [18,33–35]. This decrease rose dramatically to 87.52% ($p < 0.01$) in the case of H_2O_2 addition and the $NO_2^{\bullet}$ level was significantly decreased by 65.21% ($p < 0.01$). It was observed that H_2O_2 addition may also help to decrease the $NO^{\bullet}/NO_2^{\bullet}$ levels in CA and CTS cigarettes to a certain extend. However, the change amplitude was far smaller than that in CAT cigarettes.

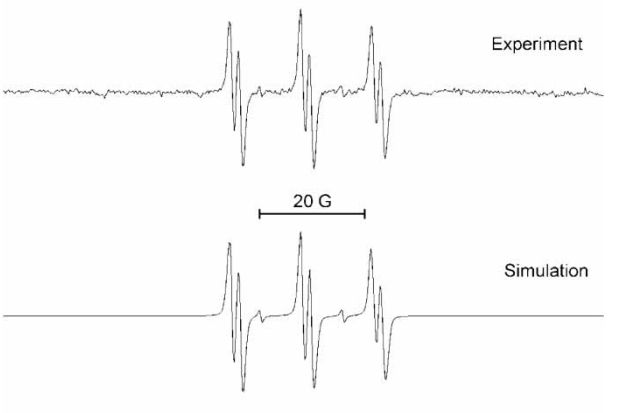

Figure 4. EPR spectrum of the spin trapping solution (0.1 M PBN in benzene solution, 5.0 mL) bubbled with the machine-yielded GPCS of a single research cigarette. Research cigarettes were smoked in an intense smoking condition and EPR spectra were recorded right after the last puff and the computer simulation was performed with the WinSim 0.98 software. Spin adducts responsible for the spectrum were defined as alkoxyl ($\alpha_N = 13.40$ G, $\alpha_{H=} 1.71$ G, 66.67%), alkyl radical ($\alpha_N = 13.91$ G, $\alpha_{H=} 1.62$ G, 31.25%) and the oxidation product of PBN (POBN, $\alpha_N = 7.88$ G, 2.08%).

The effect of catalase filtering on the MCS-induced adverse biological effects: Lipid peroxidation and mutagenicity

In the actual smoking condition, it is the MCS to which smokers are directly exposed. Moreover, MCS-induced oxidative damage (e.g. lipid peroxidation and oxidative modification of low density lipoprotein) and mutagenicity were well accepted as direct triggers for a variety of smoking-associated diseases [8–10]. In that respect, biological toxicity induced by MCS and its condensate, as evidenced among CA, CTS and CAT cigarettes, were assessed for a reliable evaluation of the positive impact of catalase filtering. Research cigarette were smoked in the ISO smoking condition to imitate the natural conditions as much as possible.

First, MDA-TBA assay was performed for the assessment of MCS-induced peroxidation of lecithin-formed liposome membrane and the impact of catalase filtering. The profile of MDA-TBA complex formation, an index of peroxidation of liposome

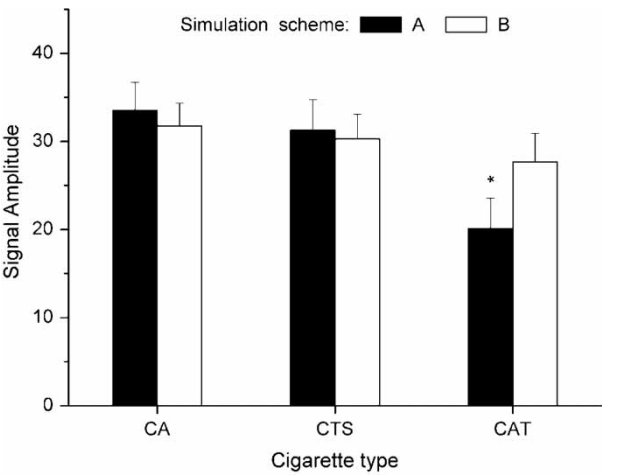

Figure 5. Signal amplitudes recovered from the simulated EPR spectra of the spin trapping solution (0.1 M PBN in benzene) bubbled with the machine-yielded (intense smoking condition) GPCS of the CA, CTS and CAT cigarettes corresponding to the applied simulation scheme. $*p < 0.01$ vs CA and CTS cigarettes.

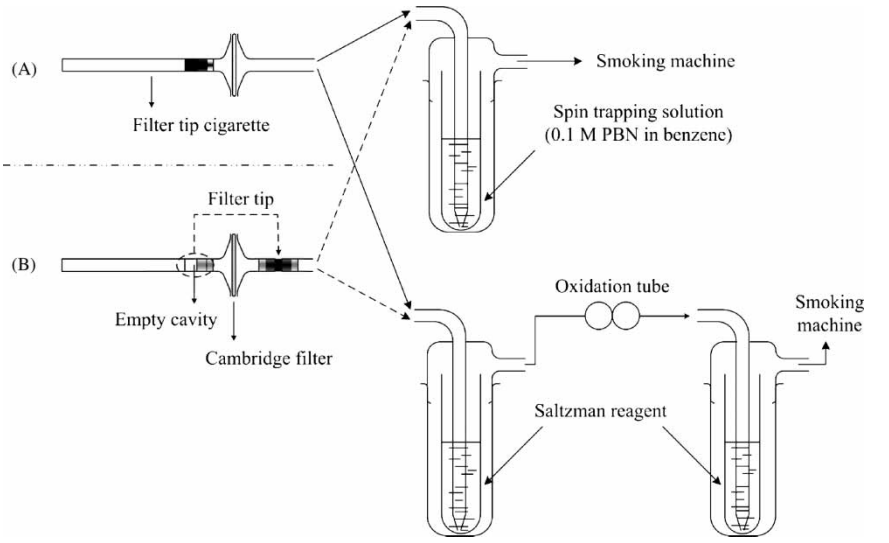

Figure 6. Schematic drawings of smoking simulation and the spin trapping and $NO^{\bullet}/NO_2^{\bullet}$ assay systems attached behind. (A) Whole MCS was drawn through filter tips. (B) Only GPCS separated from MSC was drawn through filter tips.

membranes exposed to MCS is shown in Figure 8. As expected from the above results of EPR spectroscopy, MCS-induced lipid peroxidation was significantly lowered by catalase filtering (−43.72% vs CA cigarette, −37.32% vs CTS cigarette, $p < 0.01$). Secondly, the mutagenicity of CSC was assessed through the Ames fluctuation test (Table I). By comparison with the background control and among research samples, CSC appeared to be mutagenic regardless of cigarette type. However, the mutagenic potency was significantly alleviated by catalase filtering ($p < 0.01$ vs CA and CTS cigarettes).

Discussion

In this paper, a new immobilized enzyme system (filter tip impregnated with the immobilized catalase) was proposed to reduce the smoking associated health risks by the scavenging of free radicals in MCS. There is an abundant literature reporting on various immobilized enzyme systems for multiplicity of applications, as reviewed by Krajewska [22]. So far, however, immobilized enzyme systems for gas-phase chemical transformation have been rarely studied. A kind of immobilized catalase was prepared and impregnated in the matrix of cellulose fibre of the conventional acetate filter to make an immobilized enzyme system for gaseous filtration.

First of all, the modified filter was assessed for its acceptability. Descriptive data obtained from the routine analysis (see Table II) confirmed a satisfactory tar reduction by this modified filter. While the parameters associated with natural smoking behaviour, e.g. draw resistance and puff number, and nicotine delivery were not apparently affected. It is

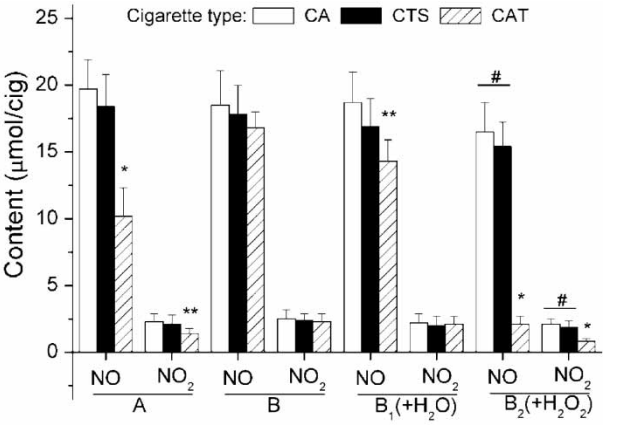

Figure 7. Contents of $NO^{\bullet}/NO_2^{\bullet}$ recovered from the machine-yielded (intense smoking condition) GPCS of CA, CTS and CAT cigarettes corresponding to the applied simulation schemes. $^{\star}p < 0.01$ and $^{\star\star}p < 0.05$ vs CA and CTS cigarettes; $^{\#}p < 0.05$ vs the corresponding $NO^{\bullet}/NO_2^{\bullet}$ levels in simulation scheme B and B_1.

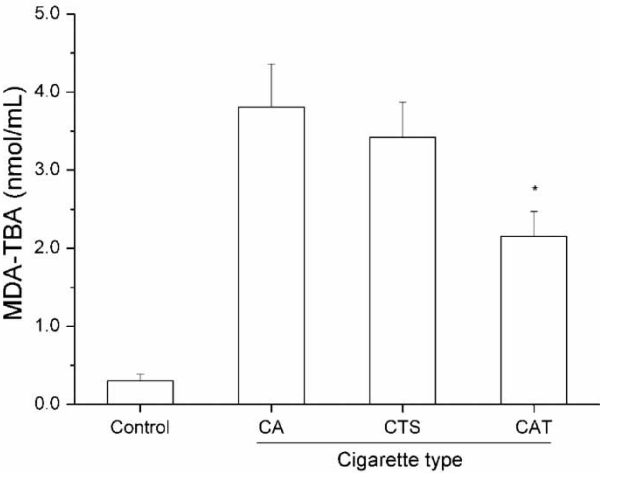

Figure 8. Contents of MDA-TBA compounds recovered from the peroxidation of lecithin-formed liposome membrane exposed to the machine yielded (ISO smoking condition) whole MCS of CA, CTS and CAT cigarettes. $^{\star}p < 0.01$ vs CA and CTS cigarettes.

believed to be helpful to make this modified filter more acceptable for both tobacco industries and ultimate consumers. Secondly, EPR spin trap method and Griess-Saltzman procedure were performed to access its impact on the levels of GPCS free radials and $NO^{\bullet}/NO_2^{\bullet}$. Being one of the major components in MCS [36], $NO^{\bullet}$ was well accepted to initiate the chain reactions that gave rise to the steady state of GPCS free radicals [12,13]. Our data confirmed that the levels of GPCS-induced PBN adduct and $NO^{\bullet}/NO_2^{\bullet}$, both being closely relevant to the GPCS free radicals, were efficiently decreased by the immobilized catalase at filter level (see Figures 5 and 7). It is shown in the MDA-TBA assay and Ames fluctuation tests (see Figure 8 and Table I) that MCS-induced lipid peroxidation and the mutagenic potency of its condensate are significantly lowered by catalase filtering. It is probably because the formation of smoking-generated mutagens (e.g. aromatic amines, benzo[α]pyrene and the tobacco specific nitrosamines [37]) may be lowered by the decrease in the levels of GPCS reactive species including H_2O_2. It was recently reported that a rosemary extract containing filter was found to decrease the levels of benzo[α]pyrene-guanine adducts in the DNA of human cells exposed to smoke [38]. This was explained by the decomposition of reactive oxygen species which are involved in the activation of the second step of the benzo[α]pyrene metabolic pathway. The CAT filter shows promising potential to reduce smoking-associated health risks to passive smokers. However, it shall be seriously pointed out that advanced filter technologies may only partly reduce, but could never eliminate the adverse biological effects of cigarette smoking.

Besides the positive impact of catalase filtering discussed above, in further experiments that the catalase-dependent breakdown of $NO^{\bullet}$ at filter level was found interestingly to depend on the presence of TPM. It has been previously demonstrated that tar fractions of MCS could partly alleviate GPCS-induced oxidation damage to biomolecules, most probably attributed to the antioxidant power [39,40] of the reductive components in its aqueous extract. Culcasi et al. [7] recently reported a temporary inhibition of GPCS-induced cytotoxicity by TPM. These studies naturally underline the protective effects of TPM and its aqueous extract as lowering the oxidants level (e.g. GPCS free radicals and some of the unsaturated aldehydes) by some of reductive components. In the present study, however, TPM was paradoxically proved to exert its contribution to the catalase-dependent breakdown of $NO^{\bullet}$ by some peroxides (most probably being H_2O_2) derived from its dismutation. Catalase readily forms reversible complex with $NO^{\bullet}$ in bulk solutions. Brown [18] showed that catalase could consume more $NO^{\bullet}$ through catalysing its breakdown in the presence of H_2O_2. It was confirmed by the work of Brunelli et al. [19], which further suggested the oxidation product of $NO^{\bullet}$ being nitrite. For the first time, this catalase-dependent breakdown of $NO^{\bullet}$ in the presence of H_2O_2 was evidenced out of bulk solutions in this study. In the aid of appropriate control experiments, exogenous H_2O_2 added in the CAT filter was proved to recover the catalase-dependent decrease in the levels of $NO^{\bullet}/NO_2^{\bullet}$, which was reduced by the removal of TPM (see Figures 6 and 7). Two more likely alternative mechanisms may account for the contribution of TPM to the catalase filtering: (1) H_2O_2 was generated through the TPM dismutation in the presence of moisture, both being trapped by the immobilized catalase in the CAT filter and (2) some other peroxides contained in MCS or derived from TPM dismutation could also provoke the catalase-dependent breakdown of $NO^{\bullet}$ like H_2O_2.

In conclusion, the results of the present study proposed a new immobilized enzyme system for gaseous filtration to be applied in cigarette filter, which is proved to lower the MCS-induced adverse biological effects by decreasing the level of $NO^{\bullet}$. Before a smoke-free society could be achieved, this kind of modified filter is supposed to help to reduce the smoking-associated health risks to passive smokers. Moreover, it is believed that these findings could be helpful in developing appropriate immobilized enzyme systems for reducing health risks associated with even more gaseous pollutants arisen from such as mobile exhaust, incineration treatment of waste materials, etc.

Acknowledgements

This work was supported by the Fund of State Tobacco Monopoly Administration, China Tobacco Company (No. 110200302031). Thanks to Professor Yunxia Sui at the Modern Analysis Center of Nanjing University for her kind help with free radicals analysis.

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