Development of nitroxide radicals–containing polymer for scavenging reactive oxygen species from cigarette smoke

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Abstract

We developed a nitroxide radicals–containing polymer (NRP), which is composed of poly(4-methylstyrene) possessing nitroxide radicals as a side chain via amine linkage, to scavenge reactive oxygen species (ROS) from cigarette smoke. In this study, the NRP was coated onto cigarette filters and its ROS-scavenging activity from streaming cigarette smoke was evaluated. The intensity of electron spin resonance signals of the NRP in the filter decreased after exposure to cigarette smoke, indicating consumption of nitroxide radicals. To evaluate the ROS-scavenging activity of the NRP-coated filter, the amount of peroxy radicals in an extract of cigarette smoke was measured using UV–visible spectrophotometry and 1,1-diphenyl-2-picrylhydrazyl (DPPH). The absorbance of DPPH at 517 nm decreased with exposure to cigarette smoke. When NRP-coated filters were used, the decrease in the absorbance of DPPH was prevented. In contrast, both poly[4-(cyclohexylamino)methylstyrene]- and poly(acrylic acid)-coated filters, which have no nitroxide radical, did not show any effect, indicating that the nitroxide radicals in the NRP scavenge the ROS in cigarette smoke. As a result, the extract of cigarette smoke passed through the NRP-coated filter has a lower cellular toxicity than smoke passed through poly[4-(cyclohexylamino)methylstyrene]- and poly(acrylic acid)-coated filters. Accordingly, NRP is a promising material for ROS scavenging from cigarette smoke.

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Keywords: anti-oxidative stress, cigarette smoke, cigarette filter, nitroxide radical-containing polymers, reactive oxygen species

1. Introduction

Many epidemiological studies have linked cigarette smoking to numerous diseases, including lung cancer, cardiovascular diseases, stroke, chronic bronchitis, chronic obstructive
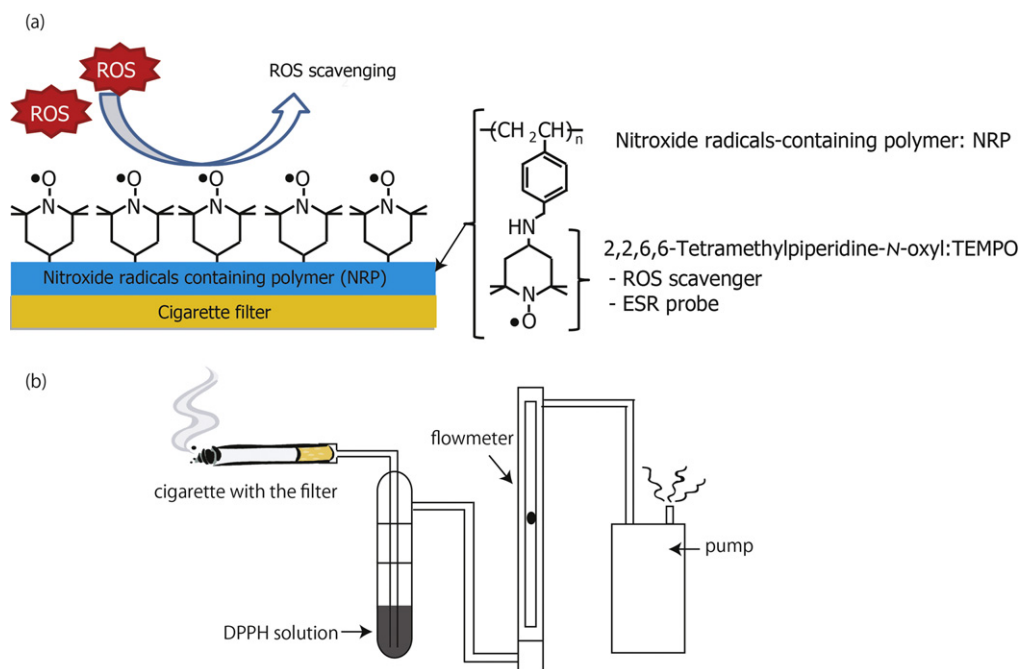
Figure 1. Schematic illustrations of the NRP and smoking device. (a) The NRP is composed of poly(4-methylstyrene) possessing nitroxide radicals, which acts as a ROS scavenger, as a side chain via an amine linkage; for this study, it was coated on to a commercially available cigarette filter. (b) Cigarette smoke was suctioned from a cigarette using a smoking device at a rate of $500 \, \text{mL min}^{-1}$.

pulmonary disease, and emphysema [1, 2]. More than 5000 kinds of chemicals, including hundreds of biological toxicants and mutagens, have been identified in cigarette smoke. Among them is benzo[*a*]pyrene, a well-known carcinogen [3]. However, because cigarette smoking induces systemic inflammation, such as cardiovascular diseases and stroke, the causative substances are still controversial.

Excessive generation of reactive oxygen species (ROS) induces oxidative damage to cellular components, which is referred to as an oxidative stress injury [4]. Cigarette smoke contains and generates excessive amounts of ROS [5, 6], including peroxy radicals, superoxide, hydrogen peroxide, hydroxyl radicals, and peroxynitrite, which continuously amplify inflammation, thereby increasing the risk of various systemic diseases [7, 8]. To reduce cigarette-smoke-induced oxidative stress in the body, scavenging ROS from cigarette smoke using a filter is crucial. Various studies have focused on scavenging ROS from a stream of cigarette smoke through a cigarette filter using natural plant extracts, which have ROS-scavenging activity [9, 10]. However, because natural plant extracts are hard to immobilize on the filter via covalent linkage, they can possibly be inhaled during cigarette smoking, leading to adverse effects and changes in the cigarette taste.

To solve this issue, we designed and developed a nitroxide radical-containing polymer (NRP), which comprises hydrophobic poly(4-methylstyrene) chains possessing nitroxide radicals as a side chain via amine linkage (see figure 1(a)), as a coating material for cigarette filters. Nitroxide radicals are chemically synthesized organic compounds that possess an unpaired electron and are known to catalytically scavenge ROS, such as superoxide, hydroxyl radicals,

peroxy radicals, and so on [11]. Because they show bioactivity in the body, the covalent linkage of nitroxide radicals to the polymer backbone is important to avoid inhalation of the nitroxide radicals. Thus far, we have developed polymeric micelles [12–21] and injectable hydrogels [22] possessing nitroxide radicals, 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), and demonstrated their *in vitro* and *in vivo* ROS-scavenging abilities. However, it is not clear whether nitroxide radicals scavenge ROS in the gaseous phase. In this paper, we prepared NRP-coated cigarette filters and evaluated their ability to scavenge ROS from cigarette smoke.

2. Materials and methods

2.1. Materials

The substance 2,2′-azobisisobutyronitrile (AIBN), provided by Kanto Chemical Co., Inc., (Tokyo, Japan) was purified via recrystallization from methanol. Chloromethylstyrene (CMS; > 95%), provided by Seimi Chemical Co., Ltd (Kanagawa, Japan), was washed three times with a 20% NaOH aqueous solution to remove inhibitors, washed three times with water, dried using sodium sulfate, and then vacuum distilled under a nitrogen atmosphere (2.0 mmHg, 56 °C). Tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), methanol, benzene, 1,4-dioxane (Kanto Chemical Co., Inc., Tokyo, Japan), 4-amino-TEMPO, cyclohexylamine, 1,1-diphenyl-2-picrylhydrazyl (DPPH) provided by TCI (Tokyo, Japan), and poly(acrylic acid) (PAAc) with a molecular weight (MW) of 5000 provided by Wako Pure Chemical

Industries, Ltd, (Osaka, Japan) were used without further purification.

2.2. Preparation of NRP

After 1 mmol of AIBN was weighed into a flask, the inside of the reactor was degassed and filled with nitrogen gas. The degassing–N_2 purge cycle was repeated three times. CMS (100 mmol; 14.2 mL) and 1,4-dioxane (50 mL) were then added to the flask under nitrogen. Polymerization was conducted for 24 h at 65 °C in an oil bath. After the reaction, the resultant poly(chloromethylstyrene) (PCMS) was recovered by precipitation from 1 L of methanol three times, followed by freeze-drying with benzene. The yield of the obtained polymer was 79.0% (12.0 g). To obtain the NRP, the chloromethyl groups in PCMS were reacted with 4-amino-TEMPO via amination in DMSO. The reaction mixture was purified by dialysis (molecular weight cutoff of the membrane tube = 3500) for 48 h against methanol, which was replaced after 2, 5, 8, 24, and 32 h to remove any unreacted 4-amino-TEMPO, followed by freeze drying with benzene. The yield of the product was > 99.0%.

Poly[4-(cyclohexylamino)methylstyrene] (PCHMS) was similarly synthesized by amination of the chloromethyl groups in PCMS with cyclohexylamine in DMSO.

2.3. Preparation of NRP-coated cigarette filters

Commercial cigarettes (Seven Star; Japan Tobacco Inc., Tokyo, Japan) that yielded 14 mg tar and 1.2 mg nicotine were used in this study. The NRP was dissolved in aqueous acid at pH 4.0 at concentrations of 5, 20, 30, and 40 mg mL^{-1}. Cigarette filters were soaked in 1 mL of the NRP solution, followed by freeze-drying. To determine the amount of NRP in the filter, the filter was dissolved in DMF and analyzed via electron spin resonance (ESR) spectroscopy. PAAc and PCHMS were similarly coated on cigarette filters as controls.

2.4. Suction of cigarette smoke

For suction of cigarette smoke, the smoking device illustrated in figure 1(b) was used; the rate of gas suction was 500 mL min^{-1}.

2.5. Time profile of ESR signal intensity of the nitroxide radicals of NRP in a cigarette filter after exposure to cigarette smoke

The cigarette filter with 40 mg of the NRP was used in this experiment. After 0, 15, 30, 45, and 55 s of exposure to cigarette smoke, the cigarette was extinguished. Then, the filter was collected and dissolved in DMF. The ESR signals of the NRP in the DMF solution were measured. The normalized ESR intensity (%) is defined as obtained values relative to those obtained without cigarette burning.

2.6. ROS scavenging activity of NRP in cigarette smoke

Each filter was set in the smoking device as illustrated in figure 1(b). DPPH (1.5 mM; 2 mL) dissolved in ethanol was

exposed to cigarette smoke at a rate of 500 mL min^{-1}. After the burnt cigarette reached a length of 2.5 cm, the cigarette was extinguished. Then, DPPH solutions were diluted to 10 mL using ethanol, followed by a measurement of the absorbance of DPPH at 517 nm. The normalized absorbance of DPPH (%) is expressed as the obtained value relative to the initial absorbance of DPPH.

2.7. Preparation of cigarette smoke extract

An aqueous extract of cigarette smoke was prepared according to a previously described method [23], with slight modifications. To obtain the cigarette smoke extract, a smoking device was used for suction of the cigarette smoke, as shown in figure 1(b), using MilliQ water instead of the DPPH solution. The cigarette filter with 40 mg of the NRP was used in this experiment. The extract of cigarette smoke was prepared from three cigarettes with burnt lengths of 4 cm. The aqueous extract of cigarette smoke was diluted using culture medium before use.

2.8. In vitro exposure to cigarette smoke

Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics, Tokyo, Japan) and the human intestinal Caco-2 cell line. Caco-2 cells were obtained from the RIKEN cell bank (RIKEN RCB0988; Saitama, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, Missouri, USA) containing 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin/neomycin). To evaluate the cell viability, 10^4 cells were plated onto 96-well cell culture dishes and grown for 24 h in the medium previously described. The dishes were rinsed three times with phosphate-buffered saline and incubated in DMEM containing 0.25% aqueous cigarette smoke extract. At 48 h after treatment, 5 μL of MTT solution was added to each well and the wells were incubated for 4 h, followed by adding 100 μL of solubilization solution that included 10% sodium dodecyl sulfate in 0.01 M HCl. After overnight incubation, the viability of the cells in each well was evaluated by measuring the absorbance at 562 nm.

2.9. Measurements

UV–visible spectra were recorded using a UV-2550 spectrometer (Shimadzu, Kyoto, Japan). The ESR signals were recorded at room temperature using a Bruker EMX-T ESR spectrometer. ESR spectrometer settings were as follows: field center, 3500 G; field sweep width, 200 G; microwave frequency, 9.8 GHz; microwave power, 0.2 mW; magnetic field modulation frequency, 100 kHz; modulation amplitude, 2 G; conversion time, 40 ms; time constant, 20.48 ms.

2.10. Statistical analysis

All values are expressed as mean ± standard error of mean (SEM). Differences between two groups were examined for
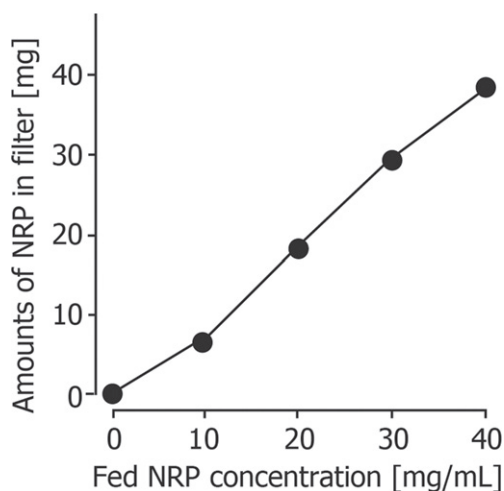
Figure 2. Amount of NRP in the cigarette filter. Increasing the concentration of the NRP solution increases the NRP in the filter.

statistical significance using Student's *t*-test. A P value of < 0.05 is considered significant for these statistical analyses.

3. Results

3.1. Preparation of NRP-coated cigarette filters

The PCMS homopolymer was synthesized via classical free-radical polymerization of CMS, according to our previous report [24]. Figures S1 and S2 (see supplementary information at stacks.iop.org/STAM/15/035002/mmedia) show the size-exclusion chromatography (SEC) diagram and ^{1}H nuclear magnetic resonance (NMR) spectrum of the PCMS obtained, respectively. The SEC diagram shows that the number-average MW and MW distributions are 13000 and 2.2, respectively. Nitroxide radical moieties were introduced as side chains of the homopolymer via amination with 4-amino-TEMPO. After amination, the number-average MW and MW distributions were 18000 and 1.9, respectively. The TEMPO content in the NRP was 83.3%, as determined by ESR spectrometry. Generally, the ESR spectra of low molecular weight (LMW) TEMPO derivatives feature a clear triplet signal, due to an interaction between the ^{14}N nuclei and unpaired electron in dilute solution, as shown in figure S3(a) (see supplementary information at stacks.iop.org/STAM/15/035002/mmedia). On the contrary, the ESR signal of the NRP became broad even in a good solvent, such as THF, owing to exchange interactions of the stable radicals in neighboring side chains (figure S3(b), see supplementary information at stacks.iop.org/STAM/15/035002/mmedia). Based on these results, it was confirmed that the TEMPO moieties were successfully introduced into the polymer as side chains.

Commercially available cigarette filters were soaked in 1 mL of the NRP solution at pH 4.0, followed by freeze-drying. The amount of polymer immobilized in the filter was determined using ESR spectrometry (figure 2) and increased with increasing concentration of NRP solution, suggesting that the NRP was successfully coated onto the cigarette filters.
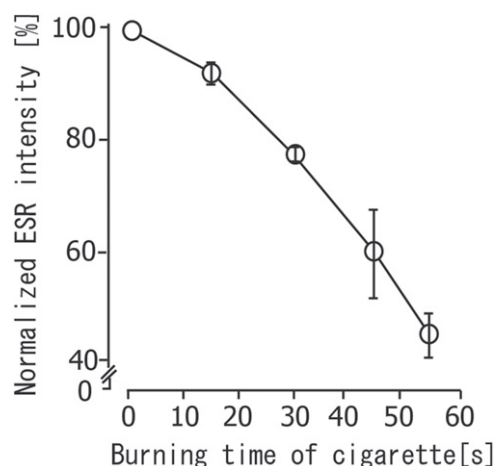

Figure 3. Decreased intensity of the ESR signal for nitroxide radicals in NRP after exposure to cigarette smoke. The cigarette filter was coated with 40 mg of the NRP. The normalized ESR intensity (%) is expressed as the value relative to that obtained under the same experiment without cigarette burning.

3.2. Scavenging of ROS from cigarette smoke by NRP

Nitroxide radicals are susceptible to ESR because they possess the unpaired electron. Conversely, the ESR signal of the nitroxide radicals disappears after loss of the unpaired electron. Using this character of nitroxide radicals, the consumption of nitroxide radicals can be monitored using ESR measurement. To confirm the decrease in intensity of the ESR signals of the immobilized nitroxide radicals in the cigarette filters by exposure to cigarette smoke, the ESR signals of the NRP in the cigarette filters were measured after exposure to cigarette smoke. As shown in figure 3, a decrease in the ESR signal for the NRP in the cigarette filter was observed and was dependent on the burning time; this indicates that the nitroxide radicals of the NRP in the cigarette filters were consumed by the passing cigarette smoke. To confirm that the NRP scavenged ROS from the cigarette smoke, the amount of peroxy radicals, which are one of main ROS components in cigarette smoke [9], was determined using the DPPH colorimetric method after extraction of the cigarette smoke by water. The strong absorption of DPPH at 517 nm decreases quantitatively upon reaction with peroxy radicals. As shown in figure S4 (see supplementary information at stacks.iop.org/STAM/15/035002/mmedia), the absorbance at 517 nm decreased with increased burning time of the cigarette, indicating that certain amounts of peroxy radicals were trapped in the extract of cigarette smoke. When cigarette smoke was passed through an NRP-coated filter, the decrease in the absorbance of DPPH at 517 nm was suppressed as an NRP amount--dependent manner, as shown in figure 4. In contrast, both PAAc- and PCHMS-coated filters, which have no nitroxide radical, did not show this tendency. These results indicate that the nitroxide radicals in the NRP could effectively scavenge peroxyl radicals from the mainstream of cigarette smoke.
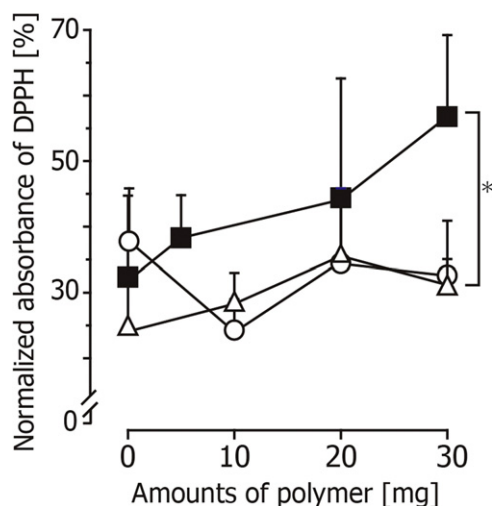
Figure 4. ROS-scavenging ability of (closed square) NRP-coated filter, (open triangle) PCHMS-coated filter and (open circle) PAAc-coated filter from cigarette smoke. The normalized absorbance of DPPH (%) is expressed as the value relative to the initial absorbance of DPPH. The data is represented as mean ± SEM for three independent experiments. *$P < 0.05$, Student's *t*-test.
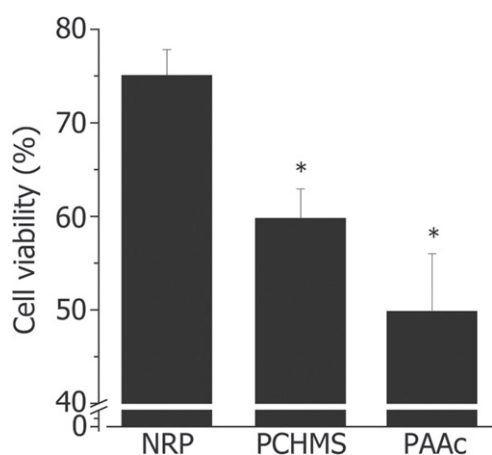

Figure 5. Cellular toxicity of cigarette smoke extract passed through NRP-coated filter, PCHMS-coated filter, and PAAc-coated filter toward Caco-2 cells, as determined by MTT assay. Bar graphs represent mean ± SEM for six experiments. *$P < 0.05$, Student's *t*-test.

3.3. Protection against cellular toxicity of cigarette smoke by NRP-coated filter

To confirm whether cigarette-smoke-induced toxicity is suppressed by passing the cigarette smoke through an NRP-coated filter, cell toxicity after exposure to the extract of cigarette smoke was evaluated. As shown in figure 5, when Caco-2 cells were exposed to the extracts of cigarette smoke that passed through PCHMS-and PAAc-coated filters, the cell viabilities were decreased by 59.7% and 49.8%, respectively. In contrast, when Caco-2 cells were exposed to the extract of cigarette smoke that passed through the NRP-coated filter, the cell viability was 75.0%. These results indicate that cigarette-smoke-induced cellular toxicity was suppressed by ROS scavenging of the NRP-coated filter.

4. Discussion

Many studies have thus far revealed that cigarette smoking-related diseases are closely related to increased oxidative stress. In fact, extracts of cigarette smoke contain various ROS, such as hydrogen peroxide [25], superoxide [9, 26], hydroxyl radicals [9], peroxy radicals [9], and nitric oxide [9]. In addition, numerous papers have reported that cigarette smoke induces not only ROS production, anti-oxidant depletion, neutrophil infiltration, and inflammation in lung [23], but also increased levels of oxidative stress markers, such as inflammatory cytokines, in the bloodstream [6]. This increased oxidative stress likely increases the risk of the various systemic inflammations caused by cigarette smoking.

To scavenge ROS from cigarette smoke using a filter, an NRP was developed in this study. Because cellulose acetate is used as a commercially available cigarette filter, which dissolves in organic solvents, an aqueous coating solution is desirable. One of the features of the NRP is its easy dissolution in acidic solutions due to protonation of the amino groups in the side chains. As shown in figure S5 (see supplementary information at stacks.iop.org/STAM/15/035002/mmedia), the transmittance of the NRP solution dramatically increases at pH below 5.5, indicating that the NRP is dissolved. With simple immersion of the cigarette filter in the acidic solution of the NRP, the NRP is easily coated onto the cigarette filter without denaturation of the filter. Conversely, the NRP does not dissolve in neutral solutions because of deprotonation of the amino groups; therefore, the NRP does not dissolve in saliva because the pH value of saliva is typically pH 6.2–7.4.

Inhalation of LMW nitroxide radicals is a potentially severe issue because they cause adverse effects, such as mitochondrial dysfunction and anti-hypertension effects [27, 28]. Because the polymer does not dissolve and the nitroxide radicals are covalently linked to the polymer backbone, inhalation of nitroxide radicals from the NRP is avoided and the safety and stability of the NRP-coated filter is ensured.

In this study, we evaluated the ability of nitroxide radicals to scavenge ROS from cigarette smoke. From the results shown in figure 3, it is evident that the nitroxide radicals in the NRP were consumed by the cigarette smoke, due to the reaction with ROS in the cigarette smoke. To obtain direct evidence of ROS scavenging by the NRP-coated filter, the colorimetric method using DPPH was carried out. Figure 4 shows that, by passing cigarette smoke through the NRP-coated filter, the level of peroxy radicals in the extract of cigarette smoke was reduced. It should be noted that PHCMS-coated filter had no ROS-scavenging ability, indicating that the ROS elimination is attributed to the nitroxide radicals and not to the phenyl ring or other parts of the coated polymer. Moreover, after passing through the NRP-coated filter, the cellular toxicity of the extract of cigarette smoke was remarkably reduced, compared to both the PCHMS- and PAAc-coated filters; this is due to the scavenging of ROS from cigarette smoke. Based on these results, an NRP is a promising material for scavenging ROS in the gas phase.

Recently, ROS in the atmosphere have been reported to play an important role in the adverse health effects of air pollution [29]. We believe that the polymeric material containing nitroxide radicals will be useful both as a filter for cigarette smoke and to remove air pollutants.

5. Conclusions

This study showed that an NRP was successfully synthesized and coated onto a cigarette filter. Nitroxide radicals in the NRP on a cigarette filter effectively scavenge peroxy radicals from a stream of cigarette smoke, resulting in a reduction of cigarette-smoke-induced cellular toxicity. Based on these results, NRP is a promising ROS-scavenging material for cigarette filters.

Acknowledgments

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