

Multi-walled carbon nanotubes: biodegradation by gastric agents *in vitro* and effect on murine intestinal system

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Abstract. One of the main questions limiting application of fibrous carbon nanomaterials (CNM) in medicine and food industry concerns presumptive degradation of CNM in living organisms. In this study, we have investigated biodegradation of multi-walled carbon nanotubes (MWCNTs) by gastric agents *in vitro* and influence of ingested MWCNTs on murine intestine. Using scanning, conventional transmission and analytical electron microscopy, we demonstrated that industrial MWCNTs treated *in vitro* by 0.1 M hydrochloric acid (pH=1) and gastric juice (pH=2-3) isolated from murine stomach, are subjected to incomplete degradation. After 30 days of oral administration to experimental mice, we did find MWCNTs in the cells of small intestine, and it may indicate that agglomerates of MWCNTs do not penetrate into colon epithelia and do not accumulate in enterocytes. However, we observed local areas of necrotic damages of intestinal villi. It seems likely, therefore, that MWCNTs end up leaving gastrointestinal tract by excretion with the feces. Our results suggest that MWCNTs do not undergo complete degradation in gastrointestinal tract of mice, and passing through non-degraded particles may negatively affect intestinal system.

1. Introduction

Nowadays multi-walled carbon nanotubes (MWCNTs) are widely used as components of filters for drinking water, various adsorbents, carriers of medical drugs for anti-cancer therapy and addressed delivery to the target organs [1-4] through intravenous or oral administration [5, 6]. In spite of growing perspectives of the MWCNTs' applications, there is not enough evidence on the consequences of their prolonged contact with living organisms.

Contemporary research data are controversial and reported effects of carbon nanotubes depend on model organism, types of nanotubes, exposure conditions, dispersion state and concentration. It was shown that MWCNTs are able to penetrate into plant cells and accumulate in tissues [7, 8]. At the same time, rodents excrete at least 75% of ingested MWCNTs with the feces [9]. *In vitro* studies demonstrated that MWCNTs affect genes expression [10] and may induce different types of cell death [11, 12]. It has been suggested that fibrous carbon nanotubes may show effects similar to asbestos and



cause inflammatory processes in lungs and blood cells [13]. Notably, uptake and accumulation of MWCNTs in the cells often results in production of reactive oxygen species (ROS) *in vitro* and *in vivo* [14]. At the same time, other studies demonstrated, that carbon nanotubes degrade by ROS-related pathways and via other biological oxidizers, which are produced by the cells of immune system [15, 16]. Thus, the aim of this study was to investigate biodegradation of MWCNTs *in vitro* and evaluate possible changes to the intestinal system caused by prolonged administration of MWCNTs with drinking water to mice.

2. Object of research

The object of the study was an industrial carbon nanomaterial Taunit (NanoTechCenter Ltd., Tambov, Russia). This material is a loose black powder, composed of grainy agglomerates with a size of several μm . Taunit is produced by chemical vapor deposition with the 98.5% purity. 1.5% of additions includes 0.3-0.5% of amorphous carbon and 1-1.2% of remaining metallic catalysts (Ni/Mg).

3. Gastric juice extraction

Three males of C57Bl/6 \times DBA/2 mice were fasted overnight with free access to water. The gastric juice (pH=2-3) was collected using pyloric ligation procedure: the abdomen was incised under anesthesia, and the pylorus was ligated for 5 hours. Then mice were sacrificed using cervical dislocation, and the gastric juice was collected.

4. Experimental biodegradation of MWCNTs *in vitro*

For investigation of MWCNTs biodegradation, we have used 0,1M hydrochloric acid (pH=1) and isolated from murine stomach gastric juice. Nanotubes were suspended (400 $\mu\text{g}/\text{ml}$) in gastric juice or hydrochloric acid and incubated at 38°C for 24 hours. Then samples were consecutively centrifuged at 5000 rpm and washed in distilled water. Suspended in water MWCNTs were placed on copper grid coated with Formvar film and dried at room temperature for 24 hours. Dried specimens were further used for TEM imaging.

5. Experiments *in vivo*

Detection of MWCNTs in cells and tissues was carried on mice C57Bl/6 \times DBA/2 (9 males in each group, approximately 10 weeks old). The average weight at the beginning of experiment was $29 \pm 1.7\text{g}$ and $31.8 \pm 1.8\text{g}$ at the end of experiment. Control group of animals was getting pure drinking water. Experimental group was exposed for 30 days to suspension of industrially produced nanomaterial «TAUNIT» composed of MWCNTs, added to drinking water (30 mg/kg). Suspension of MWCNTs in water was prepared by sonication.

6. Electron microscopy analysis

For transmission (TEM) and analytical electron microscopy, segments of small intestine were cut out and washed in fixative (2.5% glutaraldehyde and 2% neutral formalin mixture in 0.1 $\text{mol}\cdot\text{L}^{-1}$ Na-K-phosphate buffer, pH 7.2), cut into fragments of 1 \times 1 mm and again plunged in 2.5% glutaraldehyde with 2% neutral formalin in 0.1 $\text{mol}\cdot\text{L}^{-1}$ Na-K-phosphate buffer (pH 7.2) for 2 h at room temperature. Afterwards the samples were washed in PBS for 30 min and post-fixed in 1% osmium tetroxide for 2 h at 4°C. Then samples were dehydrated and embedded in epon resin according to standard procedure. Ultrathin sections (60-80 nm) were made with a diamond knife and mounted on copper grids, coated with Formvar film. Ultrathin sections were additionally stained with uranyl acetate and lead citrate (Reynolds stain) and used for TEM imaging («JEM 1011», Jeol, Japan)). Detection of MWCNTs was made without additional staining. Electron diffraction patterns of MWCNTs were observed by analytical electron microscope «JEM 2100» (Jeol, Japan). For scanning electron microscopy, one drop of aqueous suspension of MWCNTs was placed on glass cover slip and dried at room temperature for 24 hours. Dried specimens were sputter-coated with a thin film of gold and examined under scanning electron microscope «JSM-6380 LA» (Jeol, Japan).

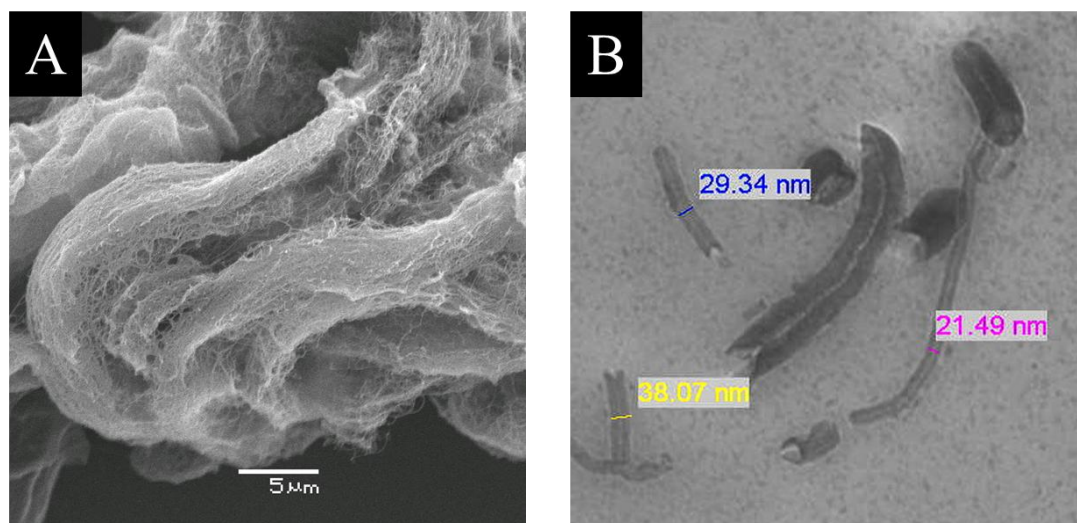


Figure 1. Industrial carbon nanomaterial «Taunit». a) Scanning electron microscopy. Aggregates of the MWCNTs; b) Transmission electron microscopy. Variety of shapes and forms of MWCNTs.

7. Results and discussion

Intact nanomaterial is a black hydrophobic powder with low ability to suspend in water. MWCNTs from nanomaterial «Taunit» usually form aggregates up to 40 mkμm (figure 1a). Single MWCNTs are seen as hollow cylinders with length from 100 nm up to few micrometers, with inner diameter 5-10 nm and outer diameter 15-100 nm (figure 1b). The diameter and length of the MWCNTs were determined from $n=100$ random observations.

Analytical study of intact nanotubes with TEM is demonstrated in figure 2. Electronograms of single nanotubes show diffraction pattern typical for MWCNTs (obtained from $n=100$ independent observations).

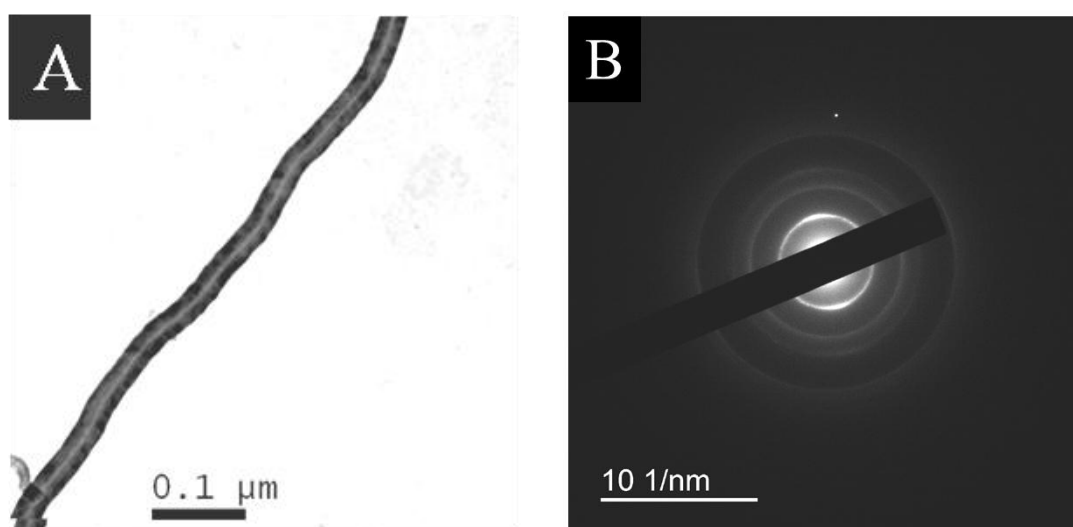


Figure 2. a) Transmission electron microscopy. Multiwalled carbon nanotube. Inner and outer diameters are clearly seen. c) Electron diffraction pattern.

For assessment of possible degradation of MWCNTs in gastrointestinal tract, we have studied the effect of murine gastric juice on the structural characteristics of this nanomaterial. The data on structural changes of MWCNTs exposed to gastric juice are shown on figure 3. Thus after gastric juice treatment, we detected few nanotubes without significant structural changes (figure 3a), yet numerous nanotubes had distorted structure. Some nanotubes had disruptions in the inner channel and intact outer walls (figure 3b). However, most MWCNTs had significantly altered structure, were ruptured or malformed, and often lost their tubular appearance (figure 3c). Such distorted nanotubes had rough surface and heterogeneous electron density throughout the length, probably due to the coating with organic material.

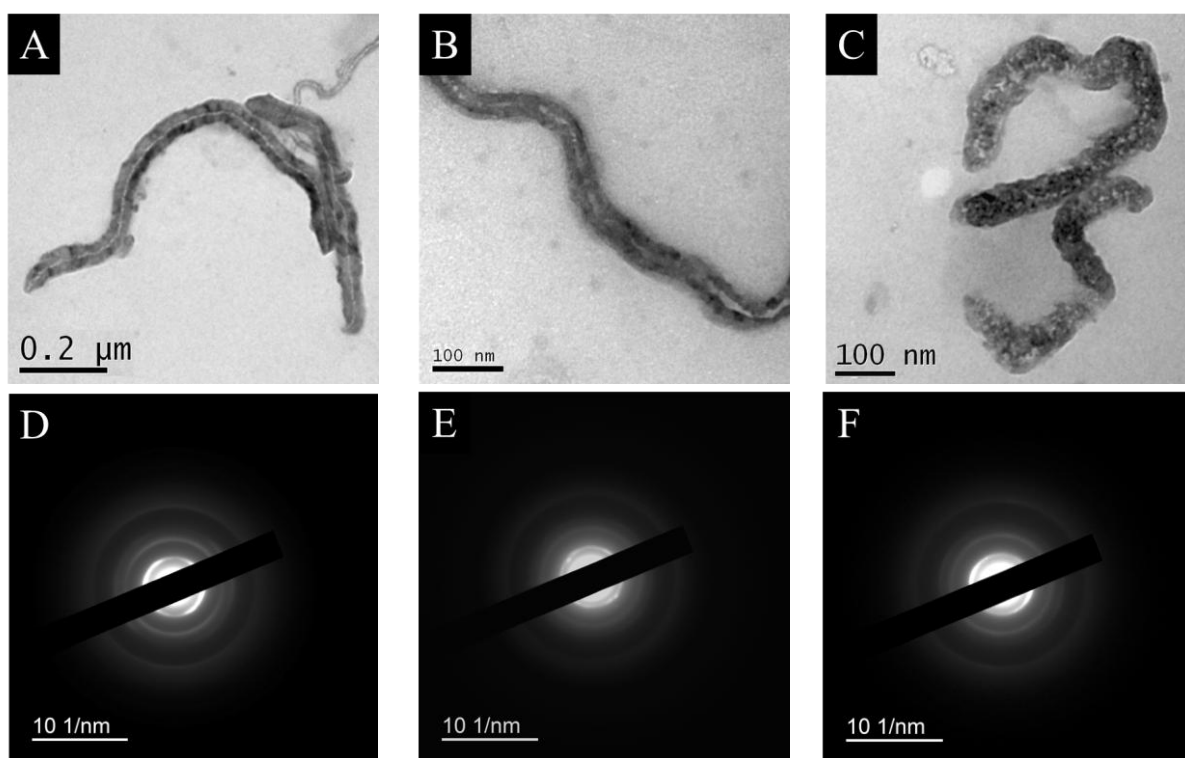


Figure 3. Gastric juice-induced distortion of MWCNTs (24 hours) a) Intact MWCNT. Walls and inner channel are well seen; b) MWCNT with ruptured inner channel; c) MWCNT with severely distorted structural appearance. No signs of inner channel can be observed.

Since the content of gastric juice is highly heterogeneous, we tested the main active component, which may affect the structural integrity of MWCNTs. For this purpose, nanomaterial was treated with 0.1M hydrochloric acid. TEM analysis showed that hydrochloric acid caused structural changes of MWCNTs, inducing rupture of the surface and/or complete distortion of the appearance of individual nanotubes within agglomerates (figure 4a), although some MWCNTs retained almost intact structure (figure 4b). In contrast to gastric juice treatment, MWCNTs treated with hydrochloric acid did not have heterogeneity in electron density throughout their length, probably, due to the absence of mucus and proteins (figure 4c). It is worthwhile to note, that distorted nanotubes appeared to be more curved than intact ones. Thus, the inconsistent distortion of MWCNTs exposed to gastric juice or hydrochloric acid may be explained by the fact that during industrial manufacturing, MWCNTs have different structural imperfections, which determine their ability to undergo degradation during the treatment.

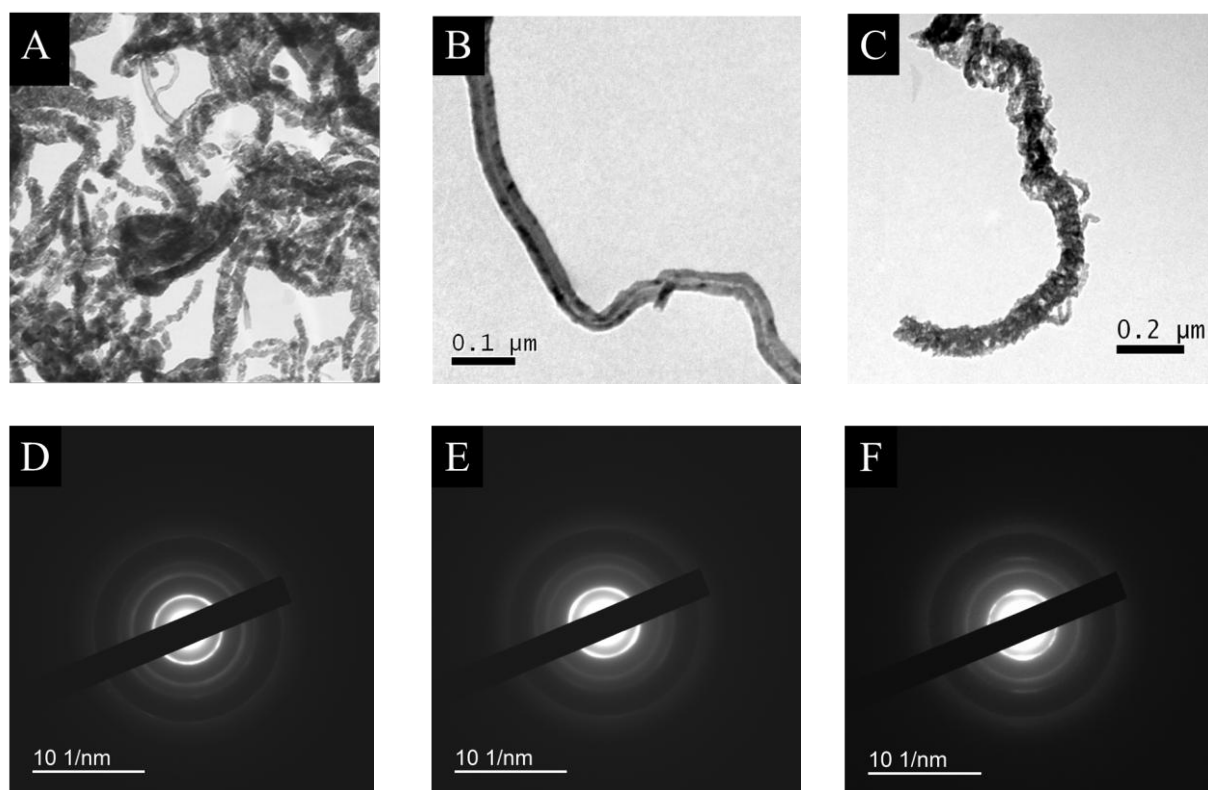


Figure 4. Hydrochloric acid-induced distortion of MWCNTs (24 hours) a) Aggregated MWCNTs with damaged surface. b) MWCNT with normal structure. Inner/outer diameters and walls are intact. c) MWCNT with distorted structure. No signs of inner channel or walls; d, e, f – electron diffraction patterns obtained for a, b, c, respectively.

We believe that distortion of MWCNT structure in the presence of gastric agents reflects the steps in progressive degradation of MWCNTs. Nanotubes with smooth surface and straight form (probably the most stable and chemically inert) seem to be less degradable, then rough and twisted ones. Analysis of electron diffraction patterns of intact and treated MWCNTs showed that crystal structure of degrading nanotubes remains unchanged. It seems that during biodegradation, MWCNTs decompose into smaller particles without significant changes in chemical bonds. Therefore degrading MWCNTs can be detected in biological material by conventional and analytical electron microscopy. According to some authors, smaller and shorter impurity-free carbon nanotubes show more tendencies to penetrate through the cell membranes and accumulate within the cells [17].

Since only partial degradation of MWCNTs treated with gastric ingredients *in vitro* was confirmed, we investigated whether MWCNTs could be absorbed passing through the small intestine of mice and accumulate in the enterocytes. TEM studies showed that small intestine epithelium cells maintain their normal structure, intact organelles (figure 5a) and microvilli (figure 5b). However, we found local areas of necrosis in small intestine of experimental mice (figure 5c). These areas were visualized as disturbances of epithelial layer of intestinal villi. Ultrastructural analysis has shown that groups of enterocytes in epithelium layer were undergoing different stages of cell death by necrosis.

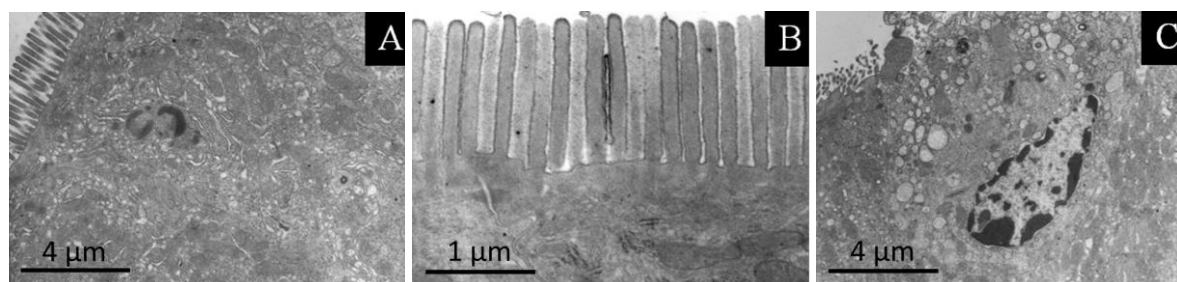


Figure 5. Transmission electron microscopy. Ultrastructure of enterocyte layer in small intestine of mice exposed to MWCNTs. a) Intact enterocytes with no pathological changes. b) Intact zone of microvilli c) Zone of necrosis. Enterocyte is vacuolated, apical membrane is destroyed.

Epithelium cells of small intestine were also analyzed for the presence of single or aggregated nanotubes. We did not find detectable clusters of MWCNTs in intact enterocytes, while tubular nano-sized structures, morphologically similar to MWCNTs (40-60 nm in diameter and 200-500 nm in length) were found at the areas of necrosis. These nanoparticles had inner cavity, distinct walls, outer and inner diameters (figure 6a, b) similar to MWCNTs, and noticeable layered structure (figure 6c). As MWCNTs without structural imperfections may be less prone to degrade within murine stomach, small fragments of stable nanotubes may be able to penetrate into epithelium cells, causing necrosis.

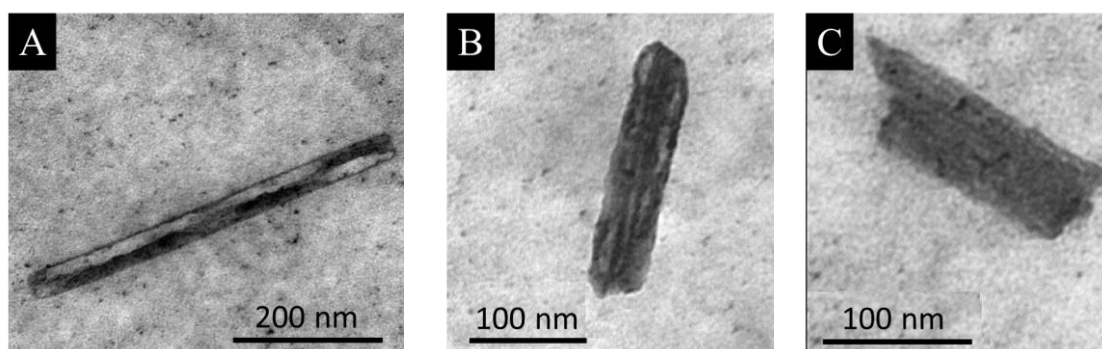


Figure 6. Nanoparticles detected in the cytoplasm of enterocytes. Transmission electron microscope. a,b) Tubular particles. Walls and lumen are seen. c) Nano-sized particle with layered structure.

8. Conclusions

We demonstrated that treatment of MWCNTs with gastric digestion reagents (gastric juice and hydrochloric acid) induced distortion of MWCNT structure, which may reflect the stages of their degradation. However, in our experiments, MWCNTs do not undergo complete destruction during the gastric stage of the murine digestive cycle. It is known that complete digestive cycle of mice lasts up to 3-24 hours [18], and gastric phase lasts from 1 till 6 hours [19]. Interestingly, MWCNTs can facilitate gastric functioning, including excessive production of gastric juice components, including hydrochloric acid. This may increase the reactivity of gastric juice and enhance the degradation of MWCNTs [20]. We believe that upon oral administration, MWCNTs only partially degrade in the murine stomach, and the remnants of distorted nanomaterial transit further to small intestine and then excrete with the feces. Moreover, the direct contact of epithelial cells with partially distorted carbon nanotubes may induce severe damage of cellular layers and tissues, resulting in necrotic changes of the intestinal villi. We also detected individual tubular nanoparticles, similar to carbon nanotubes, located in the cells at the areas of necrosis. However, the identity of these particles needs to be confirmed. We assume that MWCNTs in the presence of digestive agents undergo distortion and consecutive

disruption, followed by degradation, which is not fast enough to be completed during digestive cycle. Thus, nanomaterial composed of MWCNTs may cause negative effect on digestive system and should not be considered as harmless to living organisms.

Acknowledgments

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