Cell-penetrating CNTs for delivery of therapeutics

The use of carbon-based nanostructures, such as carbon nanotubes, in biomedicine is increasingly attracting attention. One key advantage of carbon nanotubes is their ability to translocate through plasma membranes, allowing their use for the delivery of therapeutically active molecules in a manner that resembles cell-penetrating peptides. Moreover, exploitation of their unique electrical, optical, thermal, and spectroscopic properties in a biological context is hoped to yield great advances in the detection, monitoring, and therapy of disease. Here we offer a speculative overview of the general principles behind the mechanism of carbon nanotube penetration of the plasma membrane and a snapshot of the different therapeutic modalities based on these fascinating nanostructures that are currently being investigated.

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In the last few years, a number of groups have shown that single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs) (Fig. 1) can be internalized by a variety of cell types[1–4] to deliver therapeutic and diagnostic small molecules and macromolecules to cells. However, the exact mechanisms of cellular binding, internalization, and intracellular trafficking remain elusive. These initial biological investigations using carbon nanotubes (CNTs) have stirred up a vibrant and interesting debate in the rapidly developing field of CNT biotechnology[5]. Moreover, identification of the critical factors determining CNT cell internalization will help determine the advantages they offer compared with spherical nanoparticles or any potential hazards they may entail (Table 1).

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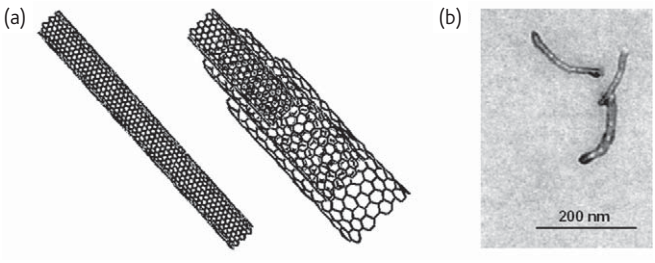

Fig. 1 (a) Schematic of a SWNT (left) and MWNT (right). (b) Transmission electron micrograph (TEM) of MWNT-NH_3^+.

Can CNTs act as 'nanoneedles' across the plasma membrane?

Determination of the exact mechanism leading to cellular internalization of CNTs is considered of great importance in their continued development as components of biomedical devices and therapeutics intended for implantation or administration to patients. The most important parameter in all such studies is the type of carbon nanotubes used, which is determined by: (i) the preparation and manufacturing process followed; (ii) the structural characteristics of the CNTs; and (iii) the surface characteristics of the CNTs and the characteristics of the functional groups at the surface of CNTs. Interactions with cells have to be performed using biocompatible CNTs, achieved by either covalent or noncovalent surface functionalization to produce water-soluble CNTs. A variety of different functionalization strategies for CNTs have been reported by various groups, therefore direct comparisons are often hampered by the inability to correlate experimental conditions.

The covalent functionalization of CNTs can generally be achieved by two main approaches: (i) esterification or amidation of oxidized tubes; and (ii) sidewall covalent attachment of functional groups[6]. Regarding the first approach, the oxidation process is conducted under strong acid conditions, which induce the opening of CNT end-caps, generating carboxylic groups suitable for further derivatization. In addition, carboxylic functions are created where defects in the nanotube sidewalls are present. On the other hand, the direct sidewall functionalization of CNTs with organic groups is possible using reactive species such as nitrenes, carbenes, and radicals. To this end, alkyl azides, dipyridyl imidazolium salts, and perfluoroalkyl radicals have been employed as efficient reagents. The high water dispersibility of the resulting modified CNTs can be explained by the debundling/unroping that occurs via mutual electrostatic repulsion of the CNT surfaces and their ability to accommodate water molecules.

Other strategies to achieve sidewall functionalization of CNTs use a variety of chemical routes, such as the 1,3-dipolar cycloaddition of azomethine ylides[7]. The easiest way to prepare such derivatives is the decarboxylation of immonium salts obtained by the reaction of an α-amino acid with an aldehyde. In particular, a glycine modified at the N-terminus with a mono N-Boc-protected diaminotriethylene glycol has been proposed because of its high solubilizing capacity after removal of the Boc group. The free amino function dramatically increases the solvent compatibility of both SWNTs and MWNTs, which are well dispersed in aqueous solvents and available for further derivatization. In Fig. 2, several functionalized derivates of CNTs that can be adequately and individually dispersed in water are shown.

Only since the issue of CNT compatibility with the aqueous biological environment was adequately overcome has it become possible to explore their interaction with living cells. We have experimentally observed that CNTs are able to interact with plasma membranes and cross into the cytoplasm without the apparent need of engulfment into a cellular compartment to facilitate intracellular transport. In these initial studies, functionalized CNTs are able to facilitate transport of plasmid DNA (pDNA) intracellularly[8]. Interestingly, model nanotube structures have also been proposed to interact with lipid bilayers via a diffusion process directly through the biomembrane, as illustrated in Fig. 3a[9,10]. Spontaneous transmembrane penetration via the flipping of membrane lipid molecules is, contrary to endocytosis, an energy-independent process, not dependent on receptor, coat, or lipid raft interactions, and is, therefore, potentially

Table 1 Pros and cons of using CNTs for biomedical applications.

Pros	Cons
• Unique mechanical properties offer *in vivo* stability	• Nonbiodegradable
• Extremely large aspect ratio, offers template for development of multimodal devices	• Large available surface area for protein opsonization
• Capacity to readily cross biological barriers; novel delivery systems	• As-produced material insoluble in most solvents; need to surface treat preferably by covalent functionalization chemistries to confer aqueous solubility (i.e. biocompatibility)
• Unique electrical and semiconducting properties; constitute advanced components for *in vivo* devices	• Bundling; large structures with less than optimum biological behavior
• Hollow, fibrous, light structure with different flow dynamics properties; advantageous *in vivo* transport kinetics	• Healthy tissue tolerance and accumulation; unknown parameters that require toxicological profiling of material
• Mass production – low cost; attractive for drug development	• Great variety of CNT types; makes standardization and toxicological evaluation cumbersome

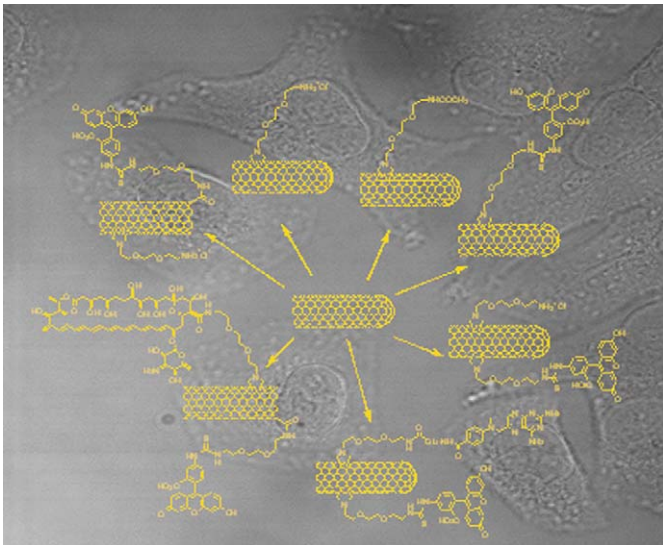

Fig. 2 Several types of functionalized CNTs that can be adequately and individually dispersed in biological environments. Background image: differential interference contrast image of epithelial lung carcinoma (A549) cell culture.

relevant to all cell types. Lopez *et al.*[9] have described the spontaneous diffusion of nanotubes functionalized with hydrophilic termini through lipid bilayer membranes using molecular dynamics simulations. Such theoretical determinations exemplify the importance of charge interactions on the internalization process and agree with the experimental electron microscopy imaging data obtained for ammonium (NH_3^+)-functionalized CNTs[8].

In Fig. 3, two high-resolution images of NH_3^+-functionalized MWNTs are shown during initial contact, interaction (Fig. 3b), and penetration (Fig. 3c) of mammalian cells. Consistently, nanotubes have been found in an orientation perpendicular to the plasma membrane of the cells through the process of cellular internalization[8]. However, the selection of cell types for these investigations is considered important, as Gao *et al.*[11] have indicated, since nonprofessional phagocytosis can also contribute to the cellular internalization of CNTs. There are various reports of CNT internalization using multiple cell types (fibroblasts, epithelial, and cancer cells; phagocytes, bacteria, and fungi) under different experimental conditions[1,4,12]. Furthermore, the effect of functional group type at the surface of the functionalized CNT (Fig. 2) has also been investigated using techniques such as confocal microscopy, fluorescence-activated cell sorting (FACS), and protocols that inhibit energy-dependent internalization mechanisms (incubation at 4°C and addition of sodium azide or 2,4-dinitrophenol to the cell culture media).

Interestingly, initial observations of the ability of CNTs to pierce or penetrate the plasma membrane, to a large extent by a process independent of energy, have been confirmed, regardless of cell type or characteristics (e.g. surface charge) of the functional group attached onto the CNT[1]. In addition, very recently, the hypothesis of CNTs acting as 'nanoneedles' with regard to the plasma membrane has been experimentally reproduced for two different types of CNT: (i) block copolymer-coated noncovalently functionalized MWNT binding studies using microglia cells[13]; and (ii) oxidized, water-soluble CNTs interacting with *E. coli* under the application of microwaves[12]. The gradually accumulating work is confirming that novel and interesting mechanisms other than 'classical' endocytosis are contributing to the high levels of cellular internalization of CNTs.

These interesting properties of water-dispersible, individualized CNTs can be used in biomedical applications, for example as novel carrier systems for therapeutics and diagnostics because (i) CNTs can be internalized by wide range of cell types; and (ii) their high surface area can potentially act as a template for cargo molecules such as peptides, proteins, nucleic acids, and drugs. CNTs have been described as delivery systems in mainly proof-of-principle studies for a variety of different biomedical applications, some of which are depicted in Fig. 4. A short overview of each of these biomedical applications where CNTs are used to deliver different classes of therapeutic agents is now given.

CNTs for vaccine delivery

The cellular uptake of free peptides and oligodeoxynucleotides is extremely poor, therefore conjugation of these molecules onto CNT surfaces may allow improvements in the delivery of such biological molecules[14–16]. In particular, the conjugation of a peptide sequence from the VP1 protein of the foot-and-mouth disease virus (FMDV) has been linked to SWNT–NH_3^+ via a stable covalent bond[15]. In these initial studies, the peptide linked to the CNT displays the necessary and correct secondary conformation and shows immunological reactivity to specific polyclonal and monoclonal antibodies. In order to evaluate the antigenicity and immunogenicity properties, as well as the influence of the number of peptides covalently linked to CNTs, mono- and bis-peptide derivatized CNTs have been prepared[14]. Specific antipeptide antibody recognition has been obtained by enzyme-linked immunosorbent assays (ELISA) and surface plasmon resonance for both conjugates. In addition, immunization of mice with these conjugates elicits higher antibody responses compared with the peptide alone and no anti-CNT antibodies have been detected, suggesting that CNTs do not have intrinsic immunogenicity properties. However, only the monoderivatized CNT conjugates induce high levels of virus-neutralizing antibodies. Increasing the number of peptide units around the CNT surface enhances the immunogenicity, but does not improve the neutralizing capacity. This finding can be attributed to a reduced specificity of the antibodies generated using the bis-conjugate, likely to be the result of a conformation adopted *in vivo* by the peptide on the CNT different from the native protein. This result underlines the critical role that the carrier system may play in the presentation of the linked peptide to the immune system.

In a subsequent study[16], negatively charged oligodeoxynucleotides containing CpG motifs (ODN CpGs) were noncovalently complexed

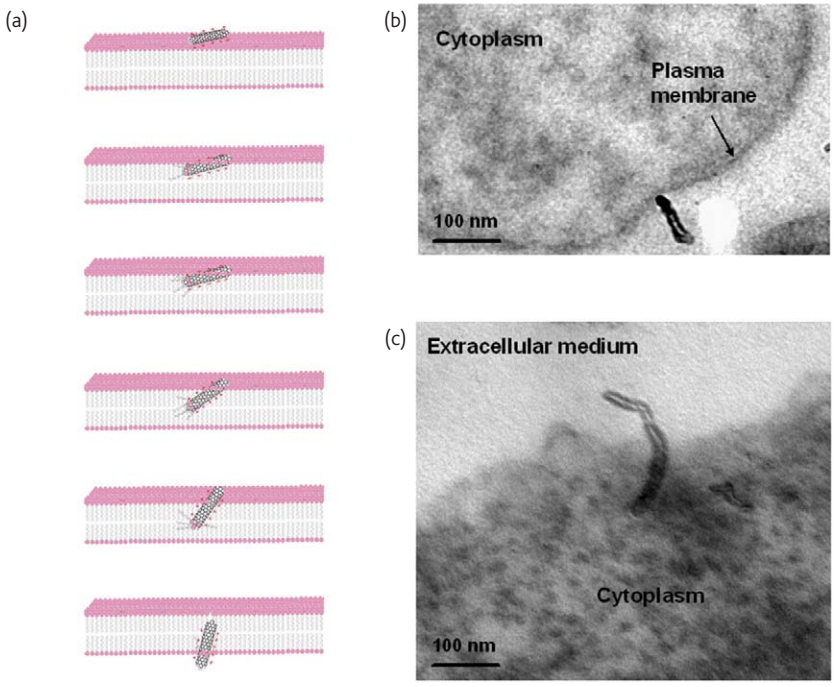

Fig. 3 CNTs acting as nanoneedles. (a) Schematic of a CNT crossing the plasma membrane; (b) TEM image of MWNT-NH_3^+ interacting with the plasma membrane of A549 cells; and (c) a TEM image of MWNT-NH_3^+ crossing the plasma membrane of HeLa cells.

with cationic CNT–NH_3^+. The ODN CpGs confer nonspecific protection against various intracellular pathogens and enhance antigen-specific immune responses. After incubation of the complexes with mouse splenocytes, an increase of the immunostimulatory activity of the ODN CpG that is not associated with the enhancement of proinflammatory cytokine IL-6 secretion is observed. Moreover, cationic CNTs do not induce any mitogenic or toxic effect on the lymphocytes. In conclusion, such studies show that functionalized, water-dispersible CNTs can be considered as scaffolds for the cellular delivery and receptor presentation of immunologically active molecules with the ultimate aim of achieving novel tools for effective vaccination.

CNTs for gene delivery

One of the most promising concepts to correct genetic defects or exogenously alter the cellular genetic makeup is gene therapy. The main aim is to efficiently, specifically, and safely introduce nucleic acid molecules into cells. However, a major drawback of this methodology is the rapid degradation of exogenous nucleic acids. To overcome this problem, one strategy is to use a vector system able to associate with DNA, RNA, or another type of nucleic acid by self-assembly and assist its intracellular translocation. Some effective delivery systems that now constitute components of various nonviral gene transfer systems include liposomes, cationic lipids, polymers, and nanoparticles[17]. These systems offer several advantages, including easy upscaling, flexibility in terms of the size of nucleic acid to be delivered, and reduced immunogenicity compared with viruses.

We have demonstrated CNT-mediated gene delivery and expression leading to the production of marker proteins encoded in double-stranded pDNA[8,18]. In these studies, we observe that pDNA is able to associate in a condensed globular conformation through electrostatic interactions at the surface of CNTs covalently functionalized with NH_3^+ groups. We have also shown that using cationic CNTs to condense DNA has several advantages, such as an enhancement of cell membrane interactions arising from electrostatic forces and increased cellular uptake. The delivery of pDNA and expression of β-galactosidase (marker gene) in Chinese hamster ovary (CHO) cells is five to ten times higher than naked pDNA alone. Furthermore, it has been found that the charge ratio (+:–) CNT–NH_3^+:pDNA is an important factor in determining the level of gene expression[8].

The concept of gene delivery systems based on CNTs has also been reported by Liu *et al.*[19] using polyethylenimine (PEI)-functionalized CNTs. They report a noncovalent association of pDNA with PEI–CNTs and have tested CNT–PEI:pDNA complexes at different charge ratios in different cell lines. The levels of expression of luciferase (marker gene) are much higher for the complexes incorporating CNTs than pDNA alone and about three times higher than PEI alone. Subsequently, Gao *et al.*[20] have demonstrated that the CNT surface functionalization group also plays an important role in the formation of the CNT–pDNA complex. By studying the gene delivery of pDNA with CNTs functionalized with different chemical (amino, carboxyl, hydroxyl, and alkyl) groups, they have found that only positively charged, CNT–NH_3^+ is able to complex and deliver pDNA.

A very different gene delivery approach has been adopted by Cai *et al.*[21], who propose the use of gene delivery systems formed by CNTs containing Ni particles enclosed in their tips and pDNA immobilized on the surface. Using a creative magnetic 'spearing' technique (exposure to an external magnetic field followed by centrifugation), the researchers have shown that the CNT–pDNA conjugates enter mammalian cells and achieve gene expression in 80–100% of the cell population. An alternative approach involving the application of microwaves has been developed by Rojas-Chapana *et al.*[12], who demonstrate that oxidized, water-dispersible CNTs can deliver pDNA into *E. coli* by opening up temporary nanochannels across the cell envelope.

Recently, CNTs have also been conjugated with siRNA and some promising initial results have been reported in siRNA-mediated gene silencing. Kam *et al.*[22] have linked siRNA through disulfide bonds to polyethylene glycosylated (PEG) lipids, which coat the CNT surface. The siRNA-CNT conjugates are internalized by mammalian cells and the siRNA is delivered intracellularly, leading to gene silencing. This work also compares the gene silencing efficiency of siRNA-CNT conjugates with the more conventional siRNA:Lipofectamine complexes finding that the use of CNTs improves the degree of gene silencing. However, the mechanism underlying this effect has not been clearly elucidated. The delivery of siRNA by CNTs will certainly become more widespread and it is expected that therapeutic applications will also diversify.

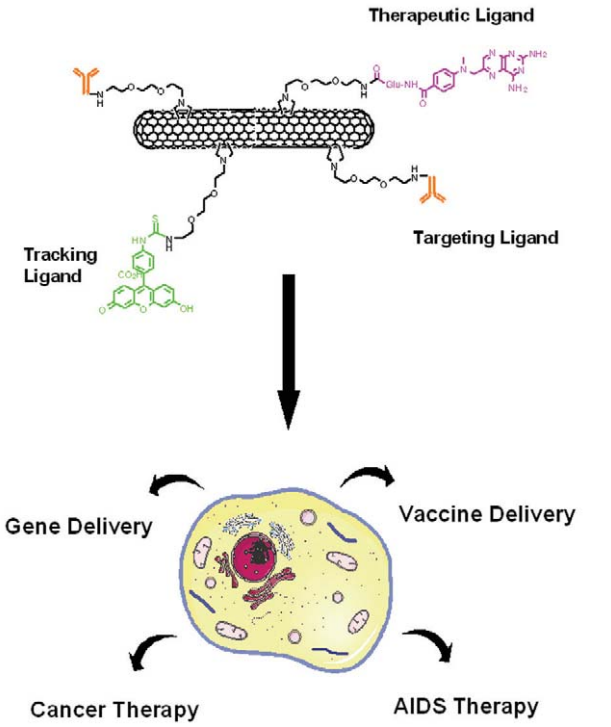

Fig. 4 Some specific biomedical applications of CNTs being explored by various groups as novel delivery systems.

CNTs for cancer therapy

The development of smart delivery systems that will target and be internalized by specific tumor cell populations without secondary consequences and toxic effects for healthy tissues is still a challenge for cancer treatment. CNTs can be used as platforms for multiple derivatization by loading their surface with therapeutic agents (treatment), fluorescent, magnetic, or radionuclide probes (tracking), and active recognition moieties (targeting).

One strategy to achieve this is via the functionalization of CNTs with two different molecules using the 1,3-dipolar cycloaddition of azomethine ylides[23]. This methodology allows the covalent attachment of a fluorescent probe (FITC) and an anticancer drug with limited cellular uptake (methotrexate) around the sidewalls of a CNT. A similar approach has been reported by Feazell *et al.*[24], who have used CNTs covalently bound to Pt(IV) to deliver a lethal dose of an anticancer drug and to a noncovalently bound (via a lipid coating of the CNTs) fluorescein to track the system. Here the toxic effect of the anticancer drug is dependent upon its release and reduction inside the cell, only possible at lower pH environments such as endocytic vesicles, which was exemplified using a testicular carcinoma cell line NTera-2.

Recently, McDevitt *et al.*[25] have reported a successful multiple derivatization of CNTs with a monoclonal antibody used as a targeting ligand. The team constructed a CNT-antibody conjugate specifically to target the CD20 epitope on Human Burkitt lymphoma cells and simultaneously deliver a radionuclide. The complex system uses water-dispersible CNT–NH_3^+ as a platform to which the targeting antibody and radionuclide chelating agent are bound by covalent linkages. They observe *in vitro* that the delivery system attaches to and is internalized by cell lines expressing the relevant epitope at the plasma membrane. They were also able to target lymphoma cells specifically *in vivo*, but not to a greater extent than using antibody alone. Another *in vivo* study[26] has also reported the preferential absorption or retention of substituted C_2B_{10} carborane cage-modified CNTs in tumor cells, suggesting that CNTs can be used as a boron delivery vehicle. Despite the fact that the mechanism of the accumulation of carborane-modified CNTs in tumors is not known, this work illustrates the potential of using CNTs in boron neutron capture therapy for the treatment of cancer.

In an alternative approach, the unique intrinsic properties of CNTs have been used to develop novel cancer therapeutics. Exploring the strong optical absorbance of CNTs in the near-infrared (NIR) region, a proof-of-principle study has shown that CNTs can cause cell death by localized hyperthermia[27]. In the protocol described, PEGylated lipid molecules with folic acid appendages at their distal ends are used to coat the surface of CNTs noncovalently to target cancer cells overexpressing folate receptors. Cell cultures incubated with the CNTs and then irradiated with NIR light for 2 mins show dramatic changes in cell morphology and extensive cell death, while cells lacking folate receptors remain intact and proliferate as normal. Nevertheless, it has also been shown that extensive NIR radiation

displaces the functional groups from CNTs and induces aggregation of the nanotubes within the cytoplasm. Therefore, this approach indicates the important considerations of the potential toxic effects that exposure to surface untreated, bundled CNTs may entail.

Finally, CNTs have also been shown to deliver nucleic acids specific for cancer therapy. The work reported by Zhang *et al.*[28] illustrates the use of CNTs as siRNA delivery systems. The team employed CNT–NH_3^+ to mediate the delivery of TERT siRNA into tumor cells (murine and human) and silence the TERT gene, which is critical for the development and growth of tumors. They observe that the treatment of different tumor cells with the CNT:TERT siRNA complexes leads to the suppression of cell growth.

Overall, research to date shows how CNTs can offer a promising new technology for the development of advanced cancer treatments. However, much more work is needed and will surely appear in order to explore and carefully define the opportunities and limitations of CNTs as delivery systems for anticancer therapy.

CNTs for HIV/AIDS therapy

Recently, Liu *et al.*[29] have shown the delivery of siRNA molecules conjugated to CNT to human T cells and peripheral blood mononuclear cells. The siRNA sequences used in these studies are able to silence the expression of the cell-surface receptors CD4 and coreceptors CXCR4 necessary for HIV entry and infection of T cells. This work demonstrates that siRNA linked through cleavable disulfide bonds to lipid molecules coating CNTs can be efficiently delivered, leading to knockdown (about 60%) of the CD4 and CXCR4 expression. Furthermore, the siRNA–S–S–lipid coated CNT conjugates greatly improve the silencing in T cells compared with Lipofectamine 2000 and other liposome-based transfection agents. Even though preliminary at this stage, these results indicate the potential use of CNTs for the treatment of HIV.

Conclusion

In conclusion, different types of CNT of adequate aqueous dispersibility have been shown independently to interact with various cell types leading to cellular internalization. This has been explored in various biomedical applications.

Since currently available CNT preparations exhibit an inherently wide distribution in length and diameter, multiple mechanisms responsible for CNT cellular internalization are possible depending on the effect of length, hydrophobicity, functional grafting density, degree of bundling, and radius of the nanotubes coming into contact with cells. The data reported to date indicates that no single mechanism can be solely or predominantly responsible for the cellular uptake of CNTs and that different or combinations of mechanisms may be contributing to their observed cellular internalization. This effect is primarily dependent on the cell type and the chemical nature and characteristics (e.g. molecular weight) of the molecules used to functionalize the nanotube surface. Consideration of all possible mechanisms leading to CNT uptake by cells is essential in our efforts to transform one of the most promising types of novel nanomaterial into a useful and clinically relevant biotechnological and biomedical tool. nt

Acknowledgments

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