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Enzyme-Mediated Assimilation of DNA-Functionalized Single-Walled Carbon Nanotubes

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When pyrimidine-functionalized carbon nanotubes were incubated with single-stranded DNA ligase, formations of macroscopic aggregates were observed. Wet-cell transmission electron microscopy imaging revealed that the nanotubes were radially bound to form a 3D latticelike structure. These structures were not observed in control reactions lacking ligase or adenosine triphosphate. Raman spectroscopy analysis revealed no spectra indicative of carbon nanotubes in ligase-unamended controls; however, spectra were observed in radial breathing mode and in the G and G' bands in reactions containing ligase. Furthermore, the addition of deoxyribonuclease to the ligated reactions dispersed the aggregates, and a reduction in Raman spectral intensity was observed.

I. Introduction

Since the relatively recent discovery of single-walled carbon nanotubes (SWNTs),¹ there has been great interest in exploiting the phenomenal physical and electrochemical properties of these molecules. Because of their unique mechanical, electrical, and thermal characteristics, SWNTs hold enormous potential for widespread use in a multitude of industries.² On the molecular level, carbon nanotubes are believed to be the strongest molecules in existence.³ Carbon nanotubes are predicted to be a 100 times stronger than high-strength steel at a tenth of the weight and to have a Young's modulus approximately 5 times that of hardened steel.⁴ Contingent on chirality, SWNTs can be either metallic or semiconducting in nature. In theory, metallic nanotubes can conduct electric current densities 1000 times greater than can copper.⁵ Additionally, carbon nanotubes are exceptional thermal conductors.⁶ These unique physiochemical properties make carbon nanotubes promising materials for structural, mechanical, chemical, and electrical technologies. However, very low aqueous solubility and the intrinsic difficulties in proficiently aligning carbon nanotubes in a controlled fashion have limited their applications to date.

Low solubility can be overcome by covalent chemical modifications;⁷ however, these processes can have adverse affects on the physical and electrochemical properties that render nanotubes novel.⁸

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Biological molecules that bond noncovalently offer a less invasive means of modification and subsequently can be used both to separate and purify carbon nanotubes. Zheng et al.⁹ have demonstrated the intrinsic ability of single-stranded DNA (ssDNA) to bind and effectively disperse SWNT bundles in aqueous solution. The authors hypothesized that π bonds are formed between the graphine surface and the hydrophobic base pairs of the DNA, resulting in a helical wrapping of ssDNA around the nanotube. The hydrophilic phosphate backbone of the DNA remains exposed, causing electrostatic stabilization in water. After being solubilized, SWNTs can be easily separated and purified on the basis of size and chirality by ion exchange chromatography or gradient centrifugation.^{9–13} Creating pure soluble SWNTs is the first step in extending their application potential in bioengineering and nanotechnology.

Although much progress has been made in recent years in the production, characterization, and purification of carbon nanotubes, limited studies have demonstrated methods to assemble nanotubes, particularly methodologies using biological means. The use of DNA to direct the assembly of nanotubes into nanodevices has attracted much attention because of the recognition specificity of the DNA molecule, which can provide increased fidelity over the assembly process. RecA-based motifs have been developed using DNA to localize nanotubes to form nanowires and transistors.^{14,15} Additionally, nucleic acid hybridizations

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have been shown to join DNA-functionalized nanotubes having complementary sequences.^{16,17} Exploiting such biologically based assembly motifs has the potential to serve as a highly selective method for building higher-order nanostructures with precise control. Here we report a novel method for assembling DNA-functionalized SWNTs by phosphodiester bonding catalyzed by ssDNA-ligase to form macroscopic carbon nanotube aggregates.

II. Experimental Section

Materials. High-purity SWNTs derived from catalytic chemical vapor deposition were purchased from Nanostructured & Amorphous Materials, Inc. (Houston, TX) consisting of 95% CNTs and 90% SWNT. Oligonucleotides were purchased from Invitrogen (Carlsbad, CA), ssDNA-ligase (CircLigase) was purchased from Epicentre Biotechnologies (Madison, WI), and DNase I was obtained from New England BioLabs (Beverly, MA). All other chemicals, reagents, and supplies were obtained from major chemical suppliers and were of the highest quality and purity obtainable.

SWNT DNA Functionalization. SWNTs were functionalized with single-stranded 30-mer polythymine oligonucleotides ($d(T)_{30}$) by ultrasonication. Briefly, 0.3 mg of SWNTs and 0.15 mg of desalated $d(T)_{30}$ were combined in 30 mM NaCl (total volume 0.5 mL). The ssDNA SWNT mixture was sonicated continually with a CPX 130 Ultrasonic Processor (Cole Parmer, Vernon Hills, IL) at 75 W for 3 h in a 1 L water bath. Ice was added periodically to the water bath to maintain a continuous temperature of 4 °C. After sonication, samples were centrifuged at 14 000g for 5 h to remove insoluble nanotubes. The degree of functionalization was estimated by subtracting the dried pellet weight from the original weight. After centrifugation, the supernatant was decanted and the absorbance was read at 730 nm as a measurement of nanotube dispersal. Ten separate 0.5 mL DNA-functionalization reactions were performed and centrifuged as described above. After centrifugation, supernatants were pooled into one fraction, which was used in all subsequent experiments. Potentially unbound ssDNA was removed using a 7 kDa molecular weight cutoff Slide-A-Lyzer Dialysis Cassette (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's specifications. Free DNA within the diffused fractions was quantified using an Invitrogen Quant-iT ssDNA assay kit per the manufacturer's instructions.

Enzymatic SWNT Assimilation. Ligation reactions were performed using adenosine triphosphate (ATP)-dependent, thermostable ssDNA-ligase (CircLigase). CircLigase was chosen for this study for its unique ability to proficiently link ssDNA having free 5'-phosphate and 3'-hydroxyl groups by intermolecular phosphodiester bonding. Ligation reactions were performed with the following parameters: 5 μ L of purified DNA-functionalized SWNTs, 2 μ L of 10 \times ligase buffer (Epicentre Biotechnologies), 2 μ L of 1 mM ATP, and 2 μ L of CircLigase (200 U). The final reaction volume was 20 μ L in molecular-grade water. Reactions were initiated by incubation in a 60 °C water bath. After 1 h, reactions were terminated by cooling to room temperature. Controls included ligase-unamended reactions and ATP-unamended reactions. Five reactions were performed under each condition and pooled for subsequent analysis. The carbon nanotube aggregate size and distribution were estimated using a Nikon Eclipse E400 light microscope (Melville, NY) equipped with a Diagnostic Instruments, Inc. Insight digital camera and Spot imaging software (Sterling Heights, MI).

Temperature-Dependent DNA Dissociation. To test the degree of DNA dissociation due to the enzymatic reaction temperature, 150 μ L of the purified DNA-functionalized SWNTs was added to 150 μ L of molecular-grade water and incubated at 40, 60, 80, and 100 °C for 60 min. The samples were allowed to

cool to room temperature and were centrifuged at 14 000g for 30 min. Absorbance readings at 730 nm were taken prior to incubation and on the supernatant after centrifugation as a measurement of DNA dissociation.

Imaging. Nonmagnified bulk ligation reactions were imaged using a Canon PowerShot G5 (Canon USA, Lake Success, NY) digital camera. Magnified images were obtained during Raman analysis as described below and with wet-cell transmission electron microscopy (TEM). Wet-cell TEM was performed using a JEOL 2010 LaB6 TEM (Tokyo, Japan) with a beam acceleration voltage of 200 keV as previously described.¹⁸

Raman Spectroscopy. Raman spectra and associated images were recorded using a Jobin Yvon LabRam HR 800 micro-Raman with 633 nm laser excitation and air objectives (both 10 \times and 50 \times), providing a spot size of 7–36 μ m in this work. Images were captured with a digital charge-coupled device (CCD) camera. Wavenumber calibration was performed using the 521 cm^{-1} emission of silica slides used for analysis. Approximately 10 μ L of each experimental sample was spotted onto a silica substrate, and the laser was focused at both 10 \times and 50 \times long lenses using a laser power of 10 mW and spectra were collected from 100 to 3000 cm^{-1} . Raman spectra obtained were for qualitative purposes only; further study is necessary to infer structural relationships of aggregate formation.

III. Results and Discussion

On the basis of a literature review, polythymine ($d(T)_{30}$, $d(T)_{60}$, and $d(T)_{90}$) and polyguanine thymine ($d(GT)_{30}$, $d(GT)_{60}$, and $d(GT)_{90}$) oligonucleotide sequences were evaluated for their ability to disperse SWNTs in aqueous solution. Consistent with published reports, $d(T)_{30}$ oligonucleotides were routinely found to disperse the maximum number of nanotubes on the basis of the spectroscopic absorbance at 730 nm.⁹ Postcentrifugation weight measurements established that approximately 30–40% of the carbon nanotubes were dispersed into solution in the presence of oligonucleotides, which was also consistent with published data.⁹ In an attempt to increase ssDNA ligase activity toward bonding only free 5'- and 3'-oligonucleotides on functionalized nanotubes, dialysis was performed to remove unbound ssDNA and concentrate the samples 2-fold. However, nucleotides were not detected in the diffused fractions, suggesting that all oligonucleotides were bound to the nanotubes.

When the DNA-functionalized SWNTs were incubated in the presence of ATP and ssDNA-ligase, macroscopic aggregates, clearly visible without magnification, were readily formed (Figure 1A). The aggregates were random in size with the majority measuring several millimeters in length and diameter. When observed under 10 \times magnification, most of the larger aggregates appeared to be interconnected to one another by smaller, less-dense aggregates (Figure 1B). In contrast, no macroscopic aggregates were observed in reactions lacking ligase (Figure 1C) or ATP (data not shown). On the basis of microscopic analysis, most of the control reaction contained no visible SWNT aggregates when viewed under 50 \times magnification (Figure 1D). A small number of random SWNT bundles measuring less than 2 μ m in width were observed (inset in Figure 1D); however, these were estimated to comprise less than 5% of the aggregate formation in the ligase-amended reactions (Figures 1A). The functionalization of nanotubes with DNA is exceptionally sensitive to physical parameters such as temperature and nanotube radius.^{19,20} Thus, it

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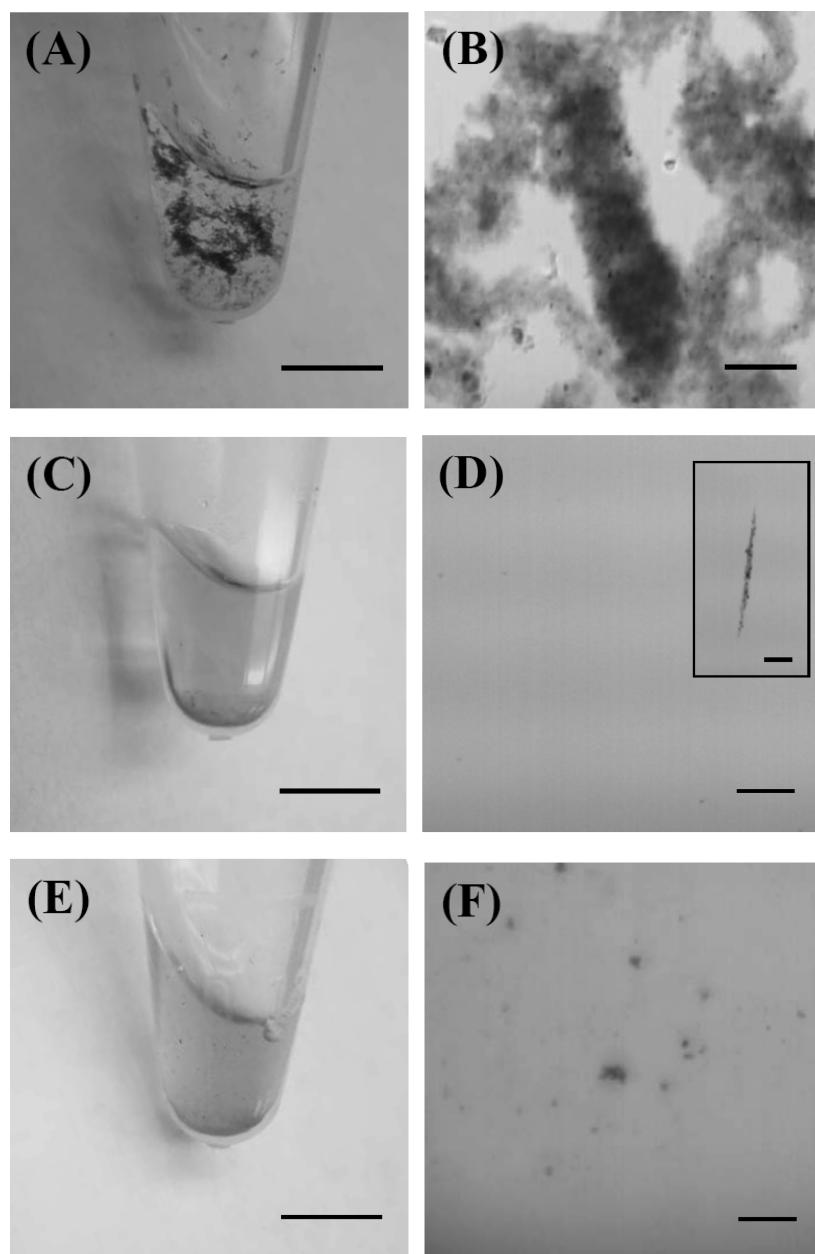


Figure 1. Enzyme-mediated aggregate-formation SWNT in 1.5 mL of microcentrifuge tubes: (A) photograph of the ligation reaction with ligase added; (B) light microscopy image of a ligase-amended sample at 10 \times magnification; (C) photograph of the ligation reaction without the addition of ligase; (D) light microscopy image of the ligase-unamended control at 50 \times magnification (inset is of a random aggregate formed during incubation); (E) photograph of a ligated sample after the addition of deoxyribonuclease (DNase I); and (F) light microscopy image of ligated samples after endonuclease amendment at 50 \times magnification. Scale bars in images A, C, and E represent 5 mm. The scale bar in image B equals 40 μ m, and those in images D and F represent 8 μ m.

was hypothesized that the formation of the small nanotube bundles observed in the ligase-unamended control reactions was likely due to a loss of DNA functionalization. This theory was confirmed by incubating functionalized SWNTs at various temperatures. Incubation at 60 °C reduced the absorbance at 730 nm by $5.0 \pm 0.4\%$, which was the amount estimated to have dropped out of solution in the control reactions lacking ligase. Furthermore, increasing the incubation temperature to 80 and 100 °C reduced the absorbance by 38.2 ± 0.1 and $76.1 \pm 0.1\%$, respectively. This suggested that the small aggregates formed within the control reactions were likely due to the loss of functionality.

In the presence of DNase I, the size of the ligase-mediated aggregates was reduced significantly (Figure 1E). Microscopic analysis revealed, after a 10 min treatment period, that the

average aggregate size was reduced roughly 100- to 200-fold and the junctions between larger aggregates were completely eliminated (Figure 1F). Small aggregates ranging from 2 to 4 μ m remained. The enzymatic specificity of DNase I toward phosphodiester bond cleavage within polynucleotide chains demonstrated that the SWNT aggregates were due to DNA linkages.

In addition to macroscopic and microscopic analysis, reactions were also characterized by wet-cell TEM on the nanoscale. Enzymatic-mediated aggregates were found to consist of a lattice-like structure where nanotubes appeared to be radially bound to themselves and adjacent nanotubes (Figure 2A). Many of the nanotubes formed small loop structures, and others seemed to be bound to other nanotubes, forming a 3D network. Neither

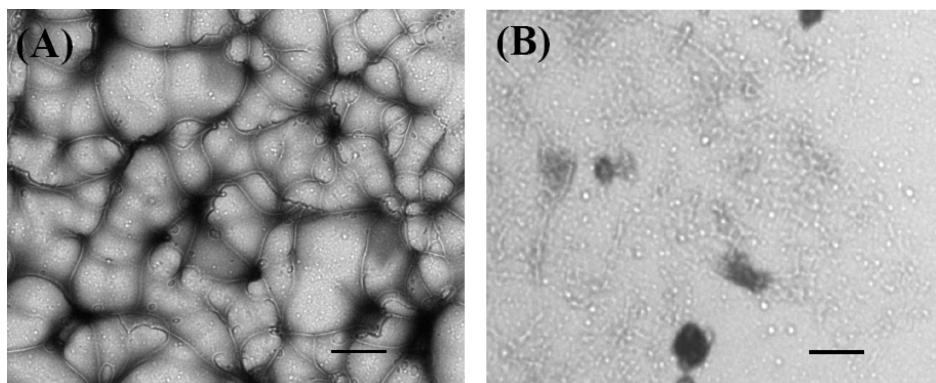


Figure 2. Wet-cell TEM of ligated ssDNA-functionalized SWNT: (A) ligated ssDNA-functionalized SWNT and (B) control reaction without the addition of ligase. The scale bars represent $0.4\text{ }\mu\text{m}$.

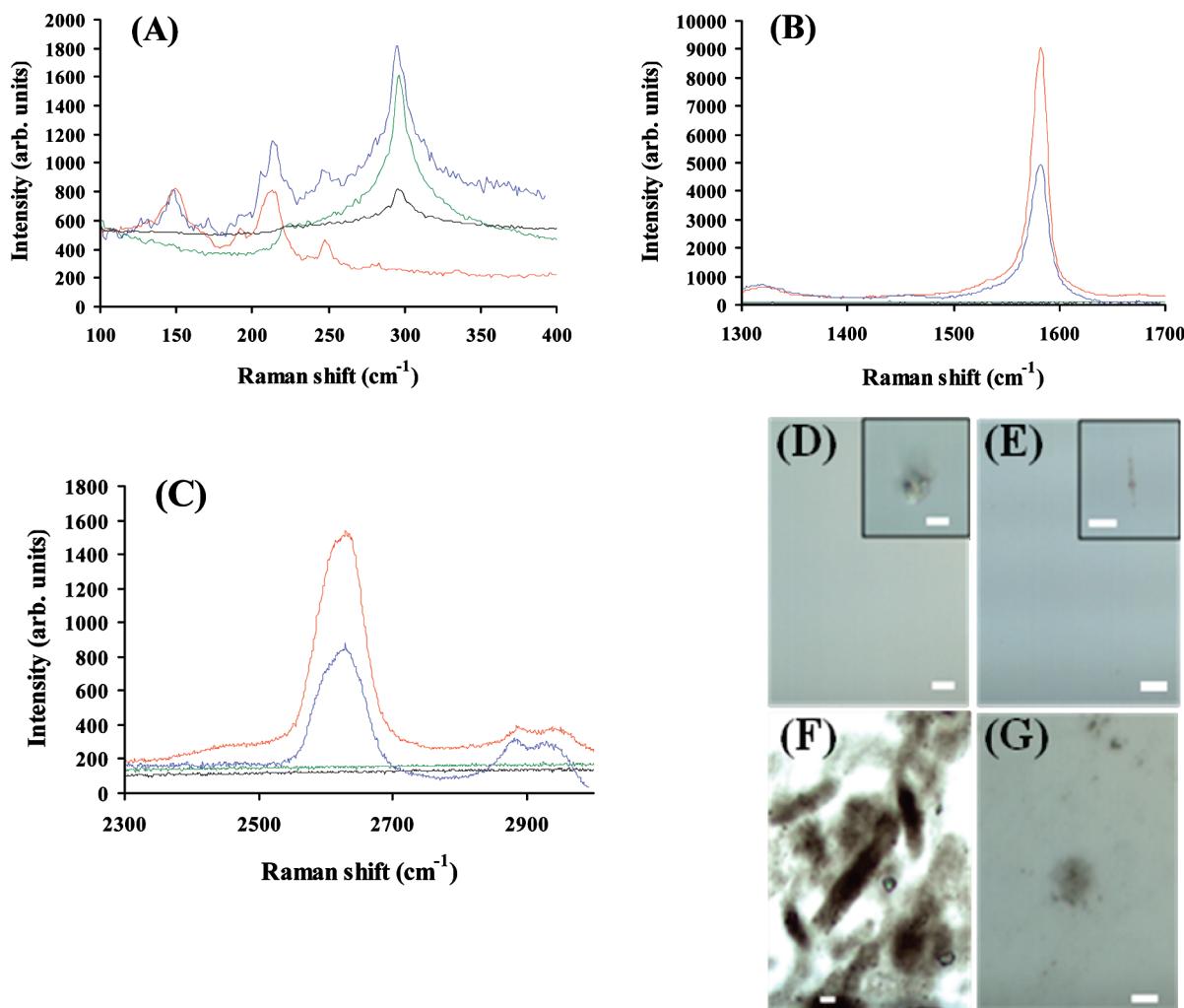


Figure 3. Raman spectroscopic analysis of ligated DNA-functionalized SWNT at a 633 nm excitation wavelength: (A) radial breathing mode (RBM), (B) G band, and (C) G' band. (D–G) Light microscope images for the samples represented in black, green, red, and blue from the graphs in sequence. (Insets of D and E) A few ($< 5\%$) small aggregates. All of the scale bars are $8\text{ }\mu\text{m}$ long, except for the bar in F that is $20\text{ }\mu\text{m}$ long. (Legend) Black, DNA-functionalized SWNT; green, DNA ligase-unamended; red, DNA ligase-amended; and blue, DNA ligase-amended after deoxyribonuclease treatment.

structure was detected in reactions lacking ligase (Figure 2B). Most of the control reaction was composed of randomly dispersed SWNTs; however, some small, tightly bound nanotube aggregates were observed. Again, these were hypothesized to be formed via a loss of ssDNA functionality incurred during incubation and/or the imaging process.

Reactions were further characterized by Raman spectroscopy analysis for qualitative purposes. Spectroscopy was performed on DNA-functionalized SWNTs, ligase-amended reactions, ligase-unamended controls, and endonuclease-treated reactions (Figure 3). No Raman spectra were observed in the DNA-functionalized SWNT or ligase-unamended controls; however,

prominent spectra indicative of carbon nanotubes were observed in radial breathing mode (RBM) (Figure 3A), the G band (Figure 3B), and the G' band (Figure 3C) in reactions containing ligase.² Corresponding light microscope images are shown in Figure 3D–G. The laser was focused on the centers of images D–F and on the darkest region in image G. The lack of spectra in the DNA-functionalized SWNTs and ligase-unamended controls was likely due to the uniform dispersion of the SWNTs, resulting in low nanotube concentrations at the point of laser excitation. However, intense Raman spectra were observed in RBM and in G and G' bands in the reactions containing ligase because of large aggregate formation. Furthermore, deoxyribonuclease treatment lowered the Raman spectral intensities significantly in both the G band (Figure 3B) and G' band (Figure 3C). Intensities decreased roughly 2-fold, which was associated with a reduction in SWNT aggregate size as determined by light microscopy. Further study is needed to determine if correlations exist between the Raman intensity in these areas of the spectrum and carbon nanotube aggregation densities and to determine if frequency shifts are indicative of nanotube bundling.

Collectively, these data strongly suggested that SWNT aggregation is indeed due to ATP-dependent 5',3'-phosphodiester enzymatic activity. The lack of significant aggregation in the absence of ligase and ATP confirms that, by and large, aggregate formation is not due to physical means such as the loss of DNA functionality, protein binding, or ionic changes caused by reaction buffer constituents. Additionally, the ability to easily disperse aggregates with the use of DNase I further demonstrates that the structures observed were formed by DNA linkage.

IV. Conclusions

When DNA-functionalized carbon nanotubes were incubated with ssDNA-ligase, the formation of macroscopic aggregates was easily observed and confirmed by multiple methods. Significant aggregate formation was not observed in reactions lacking enzyme or ATP, which indicated that the structures were enzymatically mediated. Furthermore, the addition of deoxyribonuclease to the ligated reactions resulted in a visible reduction in aggregate dimensions as well as a reduction in Raman intensity in the G and G' bands. These data strongly suggests that the assimilation of the DNA-functionalized SWNTs was due to 5',3'-enzymatic phosphodiester bonding. Exploiting biological means such as these to direct assembly carbon nanotube-based nanostructures has the potential to form the basis of manufacturing methods that can be scaled to a quantitative extent and extended in size.

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