Mitigation of bactericidal effect of carbon nanotubes by cell entrapment

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ABSTRACT

This study investigated the effects of the alginate and polyvinyl alcohol (PVA) entrapment on the viability of *Escherichia coli* cells exposed to single wall carbon nanotubes (SWCNTs) with a diameter of 1-2 nm. Viability was examined using a galactosidase enzyme assay, LIVE/DEAD *BacLight* assay, and total ribonucleic acid quantity. Variables studied included SWCNT concentration (5, 10, 20, 50, 100, 200, 500, and 1000 µg/ml), SWCNT length (0.5-2 µm for short SWCNTs and 5-30 µm for long SWCNTs), and initial bacterial concentration (6.5 log₁₀ CFU and 9 log₁₀ CFU per test). Results showed that both alginate and PVA entrapments mitigate the bactericidal effect of SWCNTs. At the highest SWCNT concentration tested (1000 µg/ml), the viability of the cells relative to controls (systems with only *E. coli*, no SWCNTs), was 0-60% for free cells and 60-90% for alginate and PVA entrapped cells. The bactericidal effect depended on SWCNT type and concentration, and bacterial concentration. In general, long SWCNTs (5-30 µm) caused significantly greater reductions in the viability of entrapped cells than the short SWCNTs except for the two highest SWCNT concentrations studied, 500 and 1000 µg/ml. Microscopy showed that the entrapment matrices prevented SWCNTs from entering the beads. This study shows that bacterial entrapment is effective at limiting the bactericidal effect of SWCNTs.

Keywords: Carbon nanotubes; adverse impact; bacteria; cell entrapment; cell viability

1. Introduction

Carbon nanotubes (CNT) are one of the most widely used nanomaterials in consumer products due to their large surface area to volume ratio, electrical properties, high tensile strength, and ultra light weight. Common consumer products such as bicycle frames (MBC Swissland) and rims (ENVE composites and Zyvex technologies), tennis rackets (Babolat), hockey sticks (Easton), and plastic films (Unidym) incorporate CNTs as key components. Consequently, novel materials containing CNTs are entering the environment at an alarming rate, and research to understand the environmental and public health impact is struggling to keep pace. However, studies have shown that CNTs can negatively impact organisms, organs, cells, and biomacromolecules (Al-Hakami et al., 2013; Kang et al., 2007; Kang et al., 2008; Le et al., 2015; Li et al., 2013; Rodrigues et al., 2013; Zhao and Liu, 2012).

CNTs can cause dermal toxicity (following skin contact) and possibly lung cancer (following inhalational exposure) in humans. The pathology is due to oxidative stress and immune-mediated responses including inflammation, granuloma formation, fibrosis, and apoptosis (Crouzier et al., 2010; Shvedova et al., 2008; Wang et al., 2010). CNTs also negatively impact non-mammal animal species such as zebrafish (Cheng et al., 2009), earthworms (Li et al., 2013), plant species (Stampoulis et al., 2009), and bacteria (Al-Hakami et al., 2013; Kang et al., 2008; Le et al., 2015; Rodrigues et al., 2013; Vecitis et al., 2010).

The antibacterial effect of CNTs is dependent on factors such as solution buffering capacity, CNT concentration and length, species and concentration of bacteria, and exposure time (Le et al., 2015; Zarubina et al., 2010). Although the precise mechanism of CNT toxicity for bacteria is unclear, direct contact appears to be important. Both gram-negative and gram-positive species show altered cell morphology, membrane damage, cell wall disruption, and cytoplasm leakage

following exposure to CNTs (Kang et al., 2007; Le et al., 2015; Liu et al., 2010; Obratsova et al., 2009). It appears that damage to bacterial cells is caused by a mesh of interacting CNTs rather than individual CNTs (Liu et al., 2010).

The well-documented antibacterial properties of CNTs (Al-Hakami et al., 2013; Brady-Estevez et al., 2010; Kang et al., 2008; Rodrigues et al., 2013; Vecitis et al., 2010) have been exploited to enhance the effectiveness of products used for decontamination. For example, CNT embedded filters substantially reduce the levels of bacteria and viruses in water, and fungi and bacteria in air relative to standard filters (Brady-Estevez et al., 2010; Xu and Yao, 2011). Unfortunately, increasing levels of CNTs in environments such as soil and activated sludge can also cause reductions in populations of ecologically important bacterial species (Chung et al., 2011; Hai et al., 2014; Jin et al., 2013; Parise et al., 2014), upsetting the delicate balance of these ecosystems. Therefore, solutions are required to mitigate the potential negative effects of CNTs on beneficial bacteria in critical ecosystems. One proposed solution focused on the CNT; multiple walled CNTs were coated with a polystyrene-based polymer, which altered the surface property and reduced lung cell toxicity (Tabet et al., 2011). In contrast, the present study proposes a solution that focuses on protecting beneficial bacteria, such as those in wastewater treatment and bioremediation systems, from toxicity of CNTs by cell entrapment in porous polymeric matrices. In fact, cell entrapment is a known alternative process for wastewater treatment and bioremediation.

Entrapped microbial cells are widely used in food (e.g. alcohol fermentation) and pharmaceutical production (e.g. antibiotics) (Kumaravel and Gopal, 2010; Srinivasulu et al., 2003) because of their enhanced resistance to environmental stresses, such as shear forces in mixing vessels, relative to free cells. Cells are typically entrapped in a hydrogel, which may be

natural (alginate, carrageenan, and chitosan) or synthetic (polyacrylamide, polyacrylate, and polyurethane, polyvinyl alcohol) (Aykut et al., 1988; Kumaravel and Gopal, 2010; Manohar et al., 2001; Pramanik et al., 2011). Polyvinyl alcohol (PVA) and alginate are frequently used for cell entrapment because they are economical, nontoxic polymers, physically and chemically stable, and have a low mass transfer resistance (Albert et al., 2011). In addition, alginate is known to be inert and has high water-holding capacity while advantages of PVA over other polymeric materials include durability and mechanical strength.

The goal of this study was to investigate the effect of purified single walled carbon nanotubes (SWCNTs) on the viability bacterial cells entrapped in PVA and alginate. *Escherichia coli* ATCC 8739 was used as a model organism. The strain has been used in laboratory bactericidal studies (Le et al., 2015; Maria-Neto et al., 2012). A beta-D-galactosidase assay, the LIVE/DEAD BacLight assay, and total RNA quantification were used as surrogates of *E. coli* viability during experiments. The advantages of these approaches are that they produce faster results compared to the standard plate count method and can account for viable but non-culturable cells. The galactosidase assay can be applied to entrapped cells *in situ* without a need to remove the bacterial cells from the matrix. A previous study on impact of SWCNTs on *E. coli* ATCC 8739 demonstrated that the three non-culture based methods used in this study provide similar cell viability results as the plate count (Le et al., 2015). Variables examined included SWCNT length and concentration, and initial concentration of entrapped *E. coli* cells. Free cells were included in the study for a comparative purpose. Light and electron microscopies were used to monitor changes in the entrapment bead structure following exposure to SWCNTs.

2. Materials and methods

2.1. Chemicals and cell culture

PVA, boric acid, sodium orthophosphate, sodium alginate, and calcium chloride were purchased from VWR international, Inc. (West Chester, Pennsylvania, USA). The PVA used in this study has a molecular weight of between 77,000 and 79,000 and a degree of hydrolysis of 99.0% to 99.8%. The specific gravity and melting point of the PVA are 1.19-1.31 and 200°C, respectively. The melting, boiling, and flash points of sodium alginate are 300°C, 495.2°C (at 760 mm Hg), and 211.1°C, respectively.

4-methylumbelliferyl- β -D-galactoside, isopropyl- β -D-thiogalactopyranoside, and Xgal were purchased from Sigma Chemical Co. (MO, USA). *E. coli* ATCC 8739 was obtained from the Global Bioresource Center (American Type Culture Collection, VA, USA). The strain was plated onto nutrient agar (NA) and incubated overnight at 37°C. A single colony from the overnight incubation was used to prepare a liquid culture for the experiments. This liquid culture was incubated overnight at 37°C and shaken continuously at 1,500 rpm (Lab-line® orbital shaker 3590, IL, USA). Cells were harvested during stationary growth, pelletized at $4500 \times g$ for 15 min, and washed twice in phosphate buffer saline (PBS) before being entrapped or directly used in experiments (for free cells).

2.2. Single-walled carbon nanotubes

SWCNTs with a diameter of 1-2 nm, and a length of 0.5-2 μm (short SWCNTs) and 5-30 μm (long SWCNTs) were obtained from Cheap Tube Inc. (Brattleboro, VT, USA). They were purified as described in Le et al. (2015) to remove silica, cobalt, and amorphous carbon before being used in experiments. Characteristics of the purified CNTs including Raman spectra and purity are described in Le et al. (2015).

2.3. Cell entrapment

E. coli ATCC 8739 cells were entrapped in PVA according to Pramanik et al. (2011). For alginate entrapment, the procedure described by Konsoula and Liakopoulou-Kyriakides (2006) was modified. The modification involved hardening the alginate beads overnight.

2.4. *Experimental setup, procedure, and design*

Experiments were performed in 50 ml flat-bottomed glass bottles using either 6.5 log₁₀ CFU or 9.0 log₁₀ CFU *E. coli* ATCC 8739 per test for both entrapped cells and free cells. The concentration of bacteria in the environment can vary greatly. For example, it is 10⁶ - 10⁷ CFU/ml for surface waters polluted by sewage or exceeds 10⁹ CFU/ml for activated sludge. The cell concentrations chosen represented the low and high values based on these numbers. To obtain the chosen cell concentrations, the quantity of cells in the liquid culture prepared as described in subsection 2.1 was determined by the plate count method on NA at 37°C overnight. Based on the plate count result, the volumes of the liquid culture that would give the above two CFU values were determined and used for harvesting as described in subsection 2.1. Harvested cells were entrapped to obtain 45 spherical beads (the number of beads used in each test, 3-4 mm diameter for each bead). The 45 beads with entrapped cells were suspended in 50 mL PBS and used in the test. For the test with free cells, harvested cells were suspended in 50 mL PBS and used in the test. The two different starting *E. coli* concentrations were tested because Le et al. (2015) reported that the resistance of bacteria to SWCNTs is affected by bacterial concentration.

Entrapped and free bacterial cells were treated with 0, 5, 10, 20, 50, 100, 200, 500, or 1,000 µg of SWCNTs/ml. There are many entry points for nanomaterials including SWCNTs into the environment and it is difficult to determine their relevant concentrations. Therefore, the wide range of SWCNT concentrations was used. To achieve the chosen concentrations, SWCNTs were added to the tests from a purified 10 mg/ml stock. The mixture was stirred using a magnetic

stirrer at 800 rpm for 90 min at room temperature. Following the tests, the viability of free and entrapped cells was determined using a beta galactosidase enzyme assay, the LIVE/DEAD *BacLight* commercial viability assay, and by quantification of total RNA.

2.4.1. *Beta galactosidase assay*

The galactosidase assay was modified from Fiksdal (1994). β -D galactosidase cleaves lactose into galactose and glucose. This assay measures the rate at which β -D galactosidase catalyzes the release of the fluorophore 4-methylumbelliferone from the substrate 4-methylumbelliferyl- β -D galactoside. The fluorophore was quantified spectrophotometrically at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The modified procedure was carried out as described in Le et al. (2015), with the exception that the filtration step was not required.

2.4.2. *LIVE/DEAD BacLight assay*

The L7012 LIVE/DEAD *BacLight* bacterial viability assay (Invitrogen, Grand Island, NY, USA) was used with a microplate reader to determine the viability of *E. coli* ATCC 8739. The assay uses two fluorescent dyes, SYTO 9 and propidium iodide (PI). SYTO 9, a green fluorescent dye, can penetrate both live and dead cells, while the red-fluorescent nucleic acid stain PI can only diffuse through damaged membranes. Viable cells with an intact membrane appear green, while non-viable cells show red fluorescence. The percent viability of cells was determined based on the ratio of red and green fluorescence in samples.

The LIVE/DEAD *BacLight* assay followed a protocol recommended by the kit manufacturer. Entrapped cells were released before the LIVE/DEAD assay was performed. Alginate beads were immersed in a 0.3 M sodium citrate solution at pH 5.0 with shaking until complete dissolution was achieved (Roukas, 1996). PVA beads were dissolved at 65-70°C in DI water with continuous shaking for 10 min (Pramanik and Khan, 2009). The obtained solutions, which

contained the cells, were centrifuged at $14,000 \times g$ for 10 min and pellets were used in the LIVE/DEAD assay.

2.4.3. Total RNA quantification

Entrapped cells in alginate beads were released in the same manner as described above for the LIVE/DEAD assay. PVA beads were cut and immersed in phosphate buffer (pH 6.8). The mixture was centrifuged at $2,383 \times g$ for 10 min to release the cells into the supernatant (Pramanik et al., 2011). After their release from sodium alginate and PVA, the cells were pelletized at $14,000 \times g$ for 10 min. Total RNA was extracted and purified using the GenElute™ Bacterial Total RNA Miniprep Kit (Qiagen, MD, USA). The total RNA concentration in ng/ml was determined from a 260 nm to 280 nm absorbance (A_{260}/A_{280}) value using a Nanodrop spectrophotometer (model 1000 uv/vis). The RNA quantification for free cells followed the same procedure excluding the cell release step.

2.4.4. Microscopy

Following their exposure to 500 $\mu\text{g/ml}$ of short and long SWCNTs, PVA and alginate beads were examined microscopically under a bright-field mode in order to evaluate the attachment of SWCNTs on the bead surface (BX61™, Olympus, PA, USA). PVA and alginate beads exposed to 500 $\mu\text{g/ml}$ of long SWCNTs were examined by scanning electron microscopy analysis. PVA beads were examined according to a procedure described by Chen et al. (1998). Briefly, the beads were dehydrated in a graded series of ethanol and critical point dried using a Tousimis Autosamdri-810 critical point drier using liquid carbon dioxide as a transitional fluid. Alginate beads were rinsed in a CaCl_2 solution (0.1 M) for 15 min twice, and placed in a mixture of 2.5% glutaraldehyde and 0.1 M CaCl_2 for 1 hour. They were rinsed in 0.1 M CaCl_2 twice, and dehydrated by successive transfers through 30% ethanol in 0.07M CaCl_2 (30 min), 50% ethanol

in 0.05M CaCl₂ (30 min), 70% ethanol in 0.03 M CaCl₂ (30 min), 90% ethanol in de-ionized water (30 min), and 100% ethanol (60 min). PVA and alginate beads were cut in half, attached to aluminum mounts by silver paint, and coated with gold using a Balzers SCD 030 sputter coater. Images of the bead specimens were taken using a JEOL JSM-6490LV scanning electron microscope.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) and *t*-test were performed using Excel 2011 statistical tools. A *p*-value < 0.05 was used as a criterion for significance level. ANOVA was used to determine whether cell viability results (represented by galactosidase activity, LIVE/DEAD assay result, and RNA quantity) from different conditions (entrapment matrix including no entrapment, SWCNT concentration, cell concentration, and type of SWCNTs) are statistically different. When ANOVA showed that different conditions provided significantly different viability results, *t*-test was performed.

3. Results and discussion

Effects of SWCNTs on the viability of entrapped and free bacterial cells, determined by three different viability assays, galactosidase enzyme assay, LIVE/DEAD *BacLight* assay, RNA quantification are shown in Figures 1 to 3, respectively. The viability of bacteria following exposure to SWCNTs for 90 min is expressed as a percentage of that in bacteria in time matched controls without SWCNTs. The viability of entrapped and free bacteria in controls, determined by three different viability assays, did not differ significantly between 0 and 90 min. Images of the beads are presented in Figures 4 to 6. Images in Figure 4 were taken by a digital camera. A fluorescence microscope under a bright-field mode and a scanning electron microscope were used to capture images in Figures 5 and 6, respectively.

3.1. Effect of SWCNTs on the viability of cells

The viability of alginate and PVA entrapped cells decreased with increasing concentration of SWCNTs (Figures 1 to 3). All three viability indicators provided results with a similar trend; viability reductions were significantly greater for free cells than entrapped cells at all SWCNT concentrations, with the exception of 5 and 10 $\mu\text{g/ml}$ for some experimental conditions (length of SWCNTs and initial cell concentration). For example, total RNA was greater in alginate and PVA entrapped cells than in free cells, with the exception of 5 $\mu\text{g/ml}$ of short SWCNTs and initial cell concentration of 6.5 $\log_{10}$ CFU per test (Figure 3). Another common trend is the viability difference between free cells and entrapped cells is more obvious for the cases with long SWCNTs and initial cell concentration of 6.5 $\log_{10}$ CFU per test.

A previous study has shown that SWCNTs exhibit toxicity primarily through physical contact with bacteria (Le et al., 2015), suggesting that entrapment matrices may protect the bacteria by preventing physical contact between the cells and SWCNTs. It should be noted that entrapment also can protect bacteria from diffusible stressors such as high concentrations of alcohol and antibiotics, which can readily enter the entrapment matrix (Chen et al., 1998; Nishio et al., 1998). The matrix type had no effect on viability reductions at all SWCNT concentrations for both short and long SWCNTs (ANOVA and t -test, $p > 0.05$). In this study, physical separation provided by PVA and alginate reduced direct contact between the entrapped cells and SWCNTs. Since the cells were uniformly distributed over the beads, most of the cells entrapped inside were protected. For almost all cases, there was no significant difference in cell viability based on all three indicators between the PVA and alginate entrapped cells at all concentrations of both short and long SWCNTs (ANOVA and t -test, $p > 0.05$).

3.2. Effect of the bacterial concentration on the viability of entrapped cells exposed to SWCNTs

The resistance of entrapped *E. coli* to SWCNTs was dependent on the initial cell concentration, with greater resistance observed at the higher concentration (Figures 1A and B, 2A and B, and 3A and B compared with Figure 1C and D, 2C and D, and 3C and D, respectively). This observation is also applied to free cells. High cell concentrations are considered one of the stressful conditions due to competition for substrate and other resources such as dissolved oxygen, and led to adaptations for survival. As a result, cells in high concentrations are more tolerant to unfavorable environment. Liu et al. (2000) suggested that alterations of genetic and proteomic regulations and physiology of *E. coli* induced by increasing cell concentration led to more resistance to adverse conditions. Chaithawiwat et al. (2016) observed *E. coli* cells from stationary phase being less susceptible to nanoscale zero-valent iron compared to those from log growth phase.

3.3. Effect of the SWCNT length on the viability of entrapped cells

In general, long SWCNTs were more toxic for entrapped *E. coli* than short SWCNTs, which is similar to previous findings using free cells (Le et al., 2015). Longer CNTs are more likely to wrap around free cells than short CNTs (Chen et al., 2013), and, possibly as a consequence, are more likely to aggregate with free cells than to self-aggregate (Yang et al., 2010). This greater propensity to aggregate with bacteria is proposed to explain the greater toxicity of long CNTs. However, the aggregation cannot be used to explain the greater toxicity of long SWCNTs on entrapped bacteria, suggesting an alternative toxicity mechanism. It is possible that long SWCNTs penetrate deeper into the matrix than short SWCNTs, resulting in greater contact. This advantage may be negated at higher SWCNT concentrations (500 and 1000 µg/ml), as the pores on the surface of beads become blocked, preventing entry of any SWCNTs into the beads. This

would explain the lack of a substantial difference in the bactericidal effect between short and long SWCNTs at 500 and 1000 µg/ml.

3.4. Differences among the three methods for viability assessment

Overall, after exposure to SWCNTs, the viability of the entrapped cells was higher than that of the free cells based on all three viability methods used including galactosidase enzyme assay (Figure 1), LIVE/DEAD *BacLight* assay (Figure 2), and RNA quantification (Figure 3). Estimates of viability reductions based on total RNA were greater than the other two methods, particularly at high SWCNT concentrations. A previous study suggested LIVE/DEAD *BacLight* and beta galactosidase assays as more conservative bacterial viability indicators compared to the plate count method due to the presence of viable but non-culturable cells (Le et al., 2015). Among the three viability methods used in this study, total RNA should be the least reliable method as Condon (2007) reported the activation of RNase in *E. coli* cells that are under stress.

3.5. Microscopy

PVA beads were much darker following exposure to SWCNTs compared to control beads. In contrast, the appearance of alginate beads did not change (Figure 4). The PVA matrix has a porous structure throughout the bead (Figure 5A), which is less dense than the alginate matrix pores (Figure 5B). As shown in Figure 5C, SWCNTs adsorbed onto the outer surface and penetrated the PVA beads to a depth of 10-200 µm. Although SWCNTs also were observed on the surface of alginate beads, the quantities were far less than those seen in PVA beads (Figure 5D). SEM images confirmed that SWCNTs penetrated into the PVA beads (Figure 6) while no SWCNTs were observed inside alginate beads (data not shown). The extent to which SWCNTs penetrated into PVA beads increased with SWCNT concentration. The very dense or less porous microstructure of alginate could limit the diffusion of SWCNTs inside the matrix. Although the

transport of SWCNTs into the PVA beads might be facilitated by the highly macroporous structure of the matrix, PVA may form a crystalline coating around CNTs (Mansur et al., 2012) which might reduce their antibacterial effect. Another possible reason for the similar effect of alginate and PVA entrapments is that the majority of the entrapped cells was in the inner part of the beads.

4. Conclusions

Alginate and PVA entrapment similarly protect bacteria from the harmful effects of SWCNTs. The three viability indicators used in this study provided results that have similar trends. The bactericidal effect on entrapped cells was higher with increasing SWCNT concentration, lower initial cell concentration, and longer SWCNTs. Entrapped cells may be applied in treatment and remediation of water and wastewater contaminated with SWCNTs. This is the first report on nanomaterial toxicity mitigation by using cell entrapment.

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Figure captions

Fig. 1. Effect of SWCNTs on viability of free *E. coli* and *E. coli* entrapped in alginate and PVA based on the galactosidase enzyme assay. (A) Initial *E. coli* cell concentration $\sim 6.5 \log_{10}$ CFU test⁻¹ and short SWCNTs, (B) Initial *E. coli* cell concentration $\sim 6.5 \log_{10}$ CFU test⁻¹ and long SWCNTs, (C) Initial *E. coli* cell concentration $\sim 9.0 \log_{10}$ CFU test⁻¹ and short SWCNTs, and (D) Initial *E. coli* cell concentration $\sim 9.0 \log_{10}$ CFU test⁻¹ and long SWCNTs.

Fig. 2. Effect of SWCNTs on viability of free *E. coli* and *E. coli* entrapped in alginate and PVA based on the LIVE/DEAD BacLight assay. (A) Initial *E. coli* cell concentration $\sim 6.5 \log_{10}$ CFU test⁻¹ and short SWCNTs, (B) Initial *E. coli* cell concentration $\sim 6.5 \log_{10}$ CFU test⁻¹ and long SWCNTs, (C) Initial *E. coli* cell concentration $\sim 9.0 \log_{10}$ CFU test⁻¹ and short SWCNTs, and (D) Initial *E. coli* cell concentration $\sim 9.0 \log_{10}$ CFU test⁻¹ and long SWCNTs.

Fig. 3. Effect of SWCNTs on viability of free *E. coli* and *E. coli* entrapped in alginate and PVA based on RNA quantity. (A) Initial *E. coli* cell concentration $\sim 6.5 \log_{10}$ CFU test⁻¹ and short SWCNTs, (B) Initial *E. coli* cell concentration $\sim 6.5 \log_{10}$ CFU test⁻¹ and long SWCNTs, (C) Initial *E. coli* cell concentration $\sim 9.0 \log_{10}$ CFU test⁻¹ and short SWCNTs, and (D) Initial *E. coli* cell concentration $\sim 9.0 \log_{10}$ CFU test⁻¹ and long SWCNTs.

Fig. 4. Entrapment beads before and after exposure to 500 $\mu\text{g/ml}$ of long SWCNTs. (A) PVA beads, and (B) alginate beads.

Fig. 5. Microscopic images of the cross section of (A) PVA beads before exposure to long SWCNTs, (B) PVA beads after exposure to 500 $\mu\text{g/ml}$ of long SWCNTs, (C) Alginate beads before exposure to long SWCNTs, and (D) Alginate beads after exposure to 500 $\mu\text{g/ml}$ of long SWCNTs.

Fig. 6. SEM images of outer layer of PVA beads after exposure to 500 $\mu\text{g/ml}$ of long SWCNTs: (A) Bundle/Web of SWCNTs and (B) A close up image of the box in (A) showing SWCNT bundle/web (red arrow).

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.