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Recent Progress on the Fabrication of Ultrafine Polyamide-6 Based Nanofibers Via Electrospinning: A Topical Review

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Abstract: Electrospinning is a highly versatile technique to prepare continuous fibers with diameters of the order of nanometers. The remarkable high aspect ratio and high porosity bring electrospun nanofibers highly attractive to various nanotechnological applications such as filtration membranes, protective clothing, drug delivery, tissue-engineering, biosensors, catalysis, fuel cells and so on. In this review, we collectively summarized the recent progress in developments of the electrospun ultrafine polyamide-6 based nanofibers preparation, characterization and their applications. Information of this polyamide-6 and composites together with their processing conditions for electrospinning of ultrafine nanofibers has been summarized in this review. The recent developments made during last few years on these materials are addressed in this review. We are anticipating that this review certainly drive the researchers for developing more intensive investigation for exploring in many technological areas.

Keywords: Polymers; Electrospinning; Ultrafine nanofibers; Experimental parameters

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Introduction

One-dimensional nanostructures that include fibers, wires, rods, belts and tubes have attracted rapidly growing interest due to their fascinating properties and unique applications [1-5]. Electrospinning is currently used as the unique technique that allows the fabrication of continuous fibers with diameters down to a few nanometers. It is the most famous technique for the production of high aspect ratio nanofibers and microfibers. The equipment required for electrospinning is simple, readily available, and inexpensive. Nanofibers are traditionally defined as nanostructures with a diameter below 1000 nm and a length-to-width ratio typically greater than 50. During electrospinning process, a continuous strand of a polymer liquid was drawn through a spinneret by a high electrostatic force to deposit randomly on a grounded collector as a mat. These electrospun fibers possess small inter-fibrous pore size and high surface area to volume ratio than that of the bulk materials [6-8]. The electrospinning process is robust, and results are reproducible, although the process controls to produce samples with high uniformity at a specified diameter are just emerging. The performance and applications of many nanomaterials strongly rely on their morphological and structural properties. Processing nanomaterials in to appropriate structures often improves their performances and can even extend their range of applications [9,10]. In this connection, electrospin nanofibers, the focus of the present review, are recently emerging as important building blocks for variety of technological applications which includes fil-

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tation membranes [11-13], solid phase extraction [14], protective textile [15-17], sensors and biosensors [18-22], catalysis [23, 24], photovoltaic cells [25], wound dressing [26-29], scaffolds in tissue engineering [30-40], drug delivery [41-44], fuel cells [45-48], dye-sensitized solar cells [49,50], super capacitors [51] and some other applications [52-55].

At the present scenario it attained a new dimension for the production of high aspect ratio fibers in sub-nano scale in the range of 5 to 15 nm in diameters. Recently, a variety of materials including polymers, ceramics, semiconductors and metals have been electrospun in to high aspect ratio ultrafine nanofibers structure [56-63]. Because of these interesting morphological features, this cutting-edge technology paves an intensive research in nano-scale. The internal structure of nanofibers can accommodate molecules, chemical reactions, separated phases, and even hold large particles. Experimental factors that affect the final diameter are described [64-67]. Even thinner segments of fibers are occasionally observed, and there is a strong possibility that even single polymer molecules can be held in an extended form by the forces associated with the electrospinning process. Single polymer molecules, extended by the excess electrical charge they carry, may already be present in the electrostatic spray methods used for the injection of molecules into a mass spectrometer.

Recently, growing attempts were focused to control the diameter of electrospun nanofibers to achieve desired functions [68-70]. Ding et al. [71] observed this kind of fishnet-like morphology in electrospun nylon-6 and poly (acrylic acid) (PAA) nanofibers, and they reported that the formation of such kind of morphology was due to applied voltage, relative humidity, kinds of solvents, solution concentration and distance between the capillary tip and the collector in the electrospinning chamber. Nylon-6, polyurethane and poly (vinyl alcohol) (PVA) polymeric nanofiber mats containing spider-net morphology have been synthesized by the addition of metallic salt [8]. Parajuli et al. reported the spider netlike arrangement of fibers with an average diameter of 18 nm by the polymerization of PAA with nylon [72].

More than 100 kinds of polymers have been used to obtain their nanofibers by using electrospinning technology during the past two decades. Electrospinning process of different kinds of polymers and their composites was discussed based on the variety of aspects which include the experimental parameters, reduce the fiber diameter, blending other composites, alignment, orientation and so on [73-75]. Several polymers are combined with other composite materials to obtain composite nanofibers. The resultant composite nanofibers leads to substantial improvements in terms of structural, mechanical, thermal, electrical, and biological properties compared to that of pristine nanofibers. Many unique qualities that include intrinsically high specific surface area, extremely small diameter and high porosity as a result of the electrospun fibers have drawn interests in many research groups around the world [76,77]. Variety of polymer nanofibers has been recently produced in terms of research interest because of their potential applications in diverse fields [78-81]. Recently, Wang et al. reported the comprehensive review of the state-of-the-art research activities related to electrospinning of polymer nanofibers which include their fabrication, novel properties studies and potential applications [82].

The growing interest in the utilization of these nanostructures primarily stems from their unique physical, mechanical and electrical properties associated with their very high surface area. These properties make nanofibers suitable for the creation of numerous technologically advanced products within many fields of application. With development activities related to nanofiber technology intensifying rapidly, one can reasonably project that these nanostructures will achieve widespread commercialization within the near 5 to 10 years. Another important reason for performing this study is to supply a review of nanofiber manufacturing methods, to identify current technical issues, and to illustrate the latest technological developments and how they will useful for further research directions.

The diameters of polymer fibers are around nanometers size, from few tens nanometers to micrometers as example of polymer nanofibers, even though, it is still difficult to electro spin polymer into uniform nanofibers with diameter as small as few hundred nanometers up to now. The high aspect ratio nanofibers are defined as the formation of ultrafine nanostructure in between the main nanofibers. In other words, the ultrafine nanofibers can have the reduced diameter of one order than that of the main nanofibers. Typically, the ultrafine nanofibers consisted of regularly distributed very fine nanofibers with diameters of about 7 to 30 nm whereas the main nanofibers are in the ranges of 100-300 nm in size. Consequently, the peculiar structural morphology of the ultrafine nanofibers made a very deep interest among many research groups since these kind of characteristics can be utilized for many scientific and industrial purposes. Some of the specific applications of these ultrafine nanofibers are of filtrations, sensors, targeted drug delivery systems, and so on.

Electrospun nanofibers are extensively studied and their potential applications are largely demonstrated. Today, electrospinning equipment and technological solutions, and electrospun nanofiber materials are rapidly moving to commercialization. Multifunctional capability, flexible design, rugged, light weight construction and self-powered operation are desired attributes for electronics that directly interface with the human body or with advanced robotic systems. For these applications, piezoelectric materials, in forms that offer the
ability to bend and stretch, are attractive for pressure/force sensors and mechanical energy harvesters. A large area, flexible piezoelectric material that consists of sheets of electrospun nanofibers of the polymer poly(vinylidene fluoride-co-trifluoroethylene) have been reported [83]. Cho et al. [84] have collectively reported about the recent studies in electronic and photonic applications of electrospun organic nanofibers which include organic light-emitting diodes, organic photovoltaics, organics field-effect transistors, lasers and waveguides. Further, there have been number of review articles published which provides an insight into the most prominent aspects of preparation, characterization of electrospun nanofibers and their applications [1,18,85-89]. Persano et al. [90] have briefly reviewed about the industrial up scaling of electrospinning and applications of polymer nanofibers. And in another article, Luo et al. [91] have elaborately reported about the combination interdisciplinary knowledge from the academia and industry to facilitate technological convergence and offers insight for up scaling electrospinning and nanofibers production. They have also discussed about the advances in electrospinning within a framework of large-scale fiber production as well as alternative nanofibers forming methods, providing a comprehensive comparison of conventional and contemporary fiber forming technologies.

In this review, we specifically summarize most recent progress in developments of the ultrafine electrospun nanofibers based on polyamide-6 and their composites. Thus, herein we address the current developments, including preparation, synthesis and characterization of these nanofibers via electrospinning process. At the same time, we provide some potential applications associated with polyamide-6 based electrospun nanofibers.

Synthesis of ultrafine nanofibers

In electrospinning, a strong electrical field is applied to a droplet formed by a polymer solution or polymer melt at the tip of a die acting as one of the electrodes. Figure 1 shows the schematic diagram of electrospinning set up and typical images of nanofibers morphology. The charging of the fluid leads to a conical deformation of the droplet, the well-known Taylor cone [92-94] and eventually to the ejection of a jet from the tip of the cone. Taylor found that the critical field required for such deformation is strongly controlled by the surface tension. The electric dispersion of liquids based on this phenomenon is widely used today, for instance in ink jet printing, for crop spraying, to prepare aerosols from liquids, and to produce propellants for rockets. The tip-to-collector distance was kept as 15 cm. Polymer solution was fed to the 5 ml syringe with plastic micro-tip with a diameter of 0.3 mm and 10 mm length. During the electrospinning process, the drum was rotated at a constant speed by a DC motor to collect the developing nanofibers. When a polymer solution is forced through a spinneret, the process aligns the chain in the direction of the extrusion with a similar alignment of the crystallites if the solid polymer is partially crystalline [95]. The additional orientation that is typically imposed after the spinning causes additional alignment of the crystallites, and the stretching and alignment of the amorphous chains separating the crystallites. In the polymer electrospinning process, several experimental parameters such as solvents, concentration of the polymer solution, applied voltage, surface energy, electrical conductivity, flow rate and humidity play an important role in confining the morphology of the resultant nanofibers [64,96-98].

Choice of system and parameters

Effect of applied voltage

In the electrospinning process, sub-nano diameter polymer fibers can be produced when a high potential difference is applied to a polymer drop suspended at the tip of a capillary. The electrospinning process is affected by a wide range of parameters, because of which controlling the properties of the fibers is difficult.
Typically the polymer solution is taken in a capillary and a high voltage power source is connected to generate an electric field between the tip of the capillary and a grounded collector. The drop is held at the tip of the capillary because of surface tension. As the voltage is increased, the viscoelastic forces are overcome by electric forces because of which the droplet gets distorted.

The formation of ultrafine polyamide-6 nanofibers were investigated by Nirmala et al. [65] by varying the applied voltages. They observed the web-like nanofibers morphology of the electrospun polyamide-6 nanofibers at a certain applied voltage. As the applied voltage was increased to 22 kV, more amount of surface area to volume ratio nanofibers was well formed. The formation of high aspect ratio nanofibers was strongly bound in between the main fibers. High voltage is able to generate more charges to the solution or droplet surface located at the tip of the needle as well as larger electrostatic forces, both of which stretch the jets fully for the favorable formation of the ultrafine nanofibers and completely split nano-nets. At the same time, too high applied voltage, the formation of high aspect ratio nanofibers was reduced and somewhat loosely bound to the main fibers. Therefore, the applied voltage plays an important role in forming the uniform fiber mats. Very clear arrangements of ultrafine fibers strongly bound with the main fibers were observed. The diameter of the polyamide-6 nanofibers were observed to be in the range of 75 to 110 nm, whereas the high aspect ratio structures consisted of regularly distributed very fine nanofibers with diameters of about 9 to 28 nm (Fig. 2).

To further support their claim, the presence of electrically induced double layer in the micro-tip solution was directly evidenced by the TEM observation which further split-up in the formation ultrafine fibers in between the main fibers (Fig. 3). Generally, the polymers bearing reactive functional groups may yield reactions of chemical exchange when they are mixed with solvent, leading to the formation of block, segmented, or random copolymers [99]. Formic acid, a polar protic solvent with high dielectric constant and low polarity is capable of attacking the lactam to produce a series of short chain oligomers, $-\text{CONH}_2^-$ and formate ions ($\text{HCOO}^-$) [96]. The primary step involves the hydrolysis of caprolactam to the amino acid $[\text{H}_2\text{N(CH}_2)_5\text{COOH]}$. The further reaction involves either a direct ring-opening attack of amino acid on caprolactam or a process in which the amino acid zwitterions, $\text{H}_3\text{N}^+(\text{CH}_2)_5\text{COO}^-$, undergoes a ring-opening attack on the cyclic monomer [101]. Therefore, the poly-electrolytic (for example, anionic, cationic and zwitterionic products) polyamide-6 in formic acid was attributed to the partial ionization of the amide groups along the polymer chains. Furthermore, the electro-spinning of polymer from highly polar solvents leads to smaller fiber diameters. Consequently, the applied electrostatic field can facilitate an increased mass throughput of the solution from the spinneret. The results indicated that the polyamide-6 showed an optimal balance of interaction with the solvent and electrical conductivity of the polymer solution, ultimately resulting in high aspect ratio nanofibers.

Further, to better understand the formation of this peculiar morphology in between the main fibers with a probable mechanism (Fig. 4). Too low applied voltage (less than 12 kV) could not sufficiently ionize the
molecules, which was attributed to the generation of bulk and elongated beads within the fiber mat. This is because only a small proportion of the polymer molecules (for example, CO and NH groups’ reaction with free ions) can interact in the low applied voltage. In other words, the solution was not sufficiently ionized. Further increase in applied voltage (19 kV) can just to initiate the splitting up of sub-nanofibers in between the main fibers. At an applied voltage of 22 kV, the solution can be completely ionized. Although the solution retains its overall electrical neutrality, the distributed charges inside the solution were subjected to a spatially dependent electric field that may give rise to electrical stresses [102]. The critical applied voltage causes the formation of an electric double layer next to the solid surface due to the poly-electrolytic behavior of polyamide-6. Furthermore, the induced surface charges can attract the counter ions from the solution. At this stage, the ions in the double layer migrate and drive further ionized solution. Thus, the electric double layer become thinner and then the ionized solution can be aligned as high aspect ratio structures in between the main fibers by relaxing the electrical stress. The ions in the electric double layer interact with the applied voltage, resulting in a nonlinear electro-osmotic flow. This proposed mechanism is directly evidenced by the TEM results in which the formation of double layer and then split-up into ultrafine fibers (Fig. 3). At an applied voltage greater than 22 kV, the solution can be ionized more strongly and became electrically unstable state. At this stage, these highly ionized particles could not retain in between the main fibers to form the high aspect ratio nanofibers. Rather, it detached and simply escaped from the main fibers due to very high ionized state. When we further increased the applied voltage, we observed sparks due to uncontrollable generation of ionized particles from the solutions.

Effect of inorganic salt

Recently, Barakat et al. [8] reported that the use of inorganic salt and polymers to properly study the influence of salt nature, polymer solution and stirring time
on the internal morphology of the electrospun nanofiber mats. They have elaborately studied the effect of inorganic salts (sodium chloride and calcium chloride) addition on nylon 6, poly. (vinyl alcohol) (PVA) and polyurethane. The salts of these metals might be utilized to improve the general characteristics of the electrospun polymer nanofiber mats by modification of the internal structure. It was observed that the addition of the inorganic salts resulted in the formation of multilayers spider-network morphology in nylon 6 and PVA within the electrospun nanofibers mats. The metallic acid (hydrogen hexacholorplatininate solution) led to form trivial spider-nets within both of nylon 6 and PVA nanofibers. On the other hand, for polyurethane, few spider-nets were formed after salt addition due to the low polarity of the utilized solvents (Tetrahydrofuran and N,N-dimethylformamide).

The nature of sol–gel/polymer solution which has been utilized by many researchers to produce metal oxides nanofibers (Fig. 5(A)). The produced polymeric nanofibers embed the ionically balanced sol–gel particles which were formed due to hydrolyzing and polycondensation of the utilized precursor, no spider-net had been observed within the electrospun nanofiber mats of such solutions (Fig. 5(B)). Therefore, calcination of the electrospun nanofiber mat leads to complete elimination of the polymer and decomposition of the condensate precursor to metal oxide in good nanofibrous shape as so many researchers have concluded [103]. However, it is expected that the utilized salt/polymer solution in the present study might have the semblance (Fig. 5(C)). With long mixing time, the ions randomly spread in the solution and might attach with the polymer chains. Therefore, electrospinning of such solutions can be interpreted (Fig. 5(D)). As aforementioned, the instantly formed nanofibers at the tip end would contain solvent, and it is expected that these nanofibers are not ionically balanced. Consequently, one can say that the different charges tinny electric poles which are generated in the nanofibers led to synthesize joints between each two different charge poles (Fig. 5(D)). The electric poles can also present in the recently formed joints which results in creating other connections between these new joints. The low- and high-magnified FE-SEM images of the resultant inorganic salt incorporated nylon-6 nanofibers are shown in Fig. 5(b) and 5(c), respectively. The synthesized joints finally shape the observed spider-network within the electrospun nanofiber mats (Fig. 6). This hypothesis can be supported by TEM results.

![Fig. 5 Schematic diagram showing the mechanism of inorganic salt addition for the spider-net nanofibers (a) and the FE-SEM images of nylon-6 with H$_2$PtCl$_6$ salt ((b),(c)). (Reprinted from [8] with the permission from Elsevier).](image)
Effects of solvents

To investigate the electrospinnability and the formation of high aspect ratio nanofibers in polyamide-6, various solvents have been employed in the form of single and double solvent systems. The formation of ultrafine polyamide-6 nanofibers were extensively analyzed using various solvents including formic acid, dichloromethane, acetic acid, chlorophenol, hexafluoroisopropanol and trifluoroacetic acid via single and mixed solvent system. Generally, the polymers bearing reactive functional groups may yield reactions of chemical exchange when they are mixed with solvent, leading to the formation of block, segmented, or random copolymers [99]. The electrospinning of polymer from highly polar solvents leads to smaller fiber diameters. Formic acid has high dielectric constant which can be reflected in the polarity of the molecule. Consequently, the applied electrostatic field can facilitate an increased mass throughput of the solution from the spinneret. The electrical conductivity was dramatically increased when the polyamide-6 was mixed with formic acid demonstrating that enhanced amounts of free ions in the solution. Well-aligned uniform shaped electrospun polyamide-6 nanofibers were formed with formic acid. This high surface to volume ratio nanofibers was achieved with the use of a new solvent system that involves an acid base reaction to produce weak complexes which serves to increase the conductivity of the polymer solution. Additionally, the weak complex formed dissociates easily and evaporates along with the solvent during the electrospinning process [104].

The electrospun nanofibers of 22 wt% polyamide-6 produced with formic acid and dichloromethane. It was observed that ribbon shaped fibers along with cylindrical fibers. The diameter of ribbon shaped fibers was higher than that of the cylindrical fibers. The reason for the formation of this ribbon shaped fibers is due to the fast evaporation of the solvent during the electrospinning process [105], resulting in the formation of a solid skin that shrinks and collapses upon the evaporation of the remaining solvent.

Electrospinning with two different ratios 3:2 and 4:1 of formic acid and acetic acid, respectively, to check for the high aspect ratio nanofibers (Fig. 7). The resulting electrospun fibers were uniform in dimension and had a smooth surface morphology. The high aspect ratio fibers were not strongly bonded with the main fibers. A possible explanation may be due to the lower dielectric constant and surface tension with those of formic acid. Both solvent systems were successfully able to produce electrospun composites. However, the uniformity of the fiber was improved and the size of the fiber was slightly increased in the solvent combination of formic acid and acetic acid.

Electrospun nanofibers of 22 wt% polyamide-6 produced with formic acid and chlorophenol can also be produced very less amount of high aspect ratio nanofibers. The diameter of the main fibers was slightly increased than that in the previous cases. However, the chlorophenol single solvent system can also be used as good solvent for polyamide-6. However, this solvent system did not produce high aspect ratio nanofibers in polyamide-6 owing to the very low conductivity (0.042 mS/m). It is possible to obtain uniform nanofibers by adjusting experimental parameters such as the solution concentration, applied voltage, etc. Hexafluoroisopropanol is an acidic alcohol and due to its strong hydrogen bonding properties it can be used as a solvent for many different kinds of polymers. Using this solvent, though it was electrospinnable, the resulting polyamide-6 fibers were observed to be thicker. This result is attributed to the high density of this solvent, which tend to a high viscosity and, consequently, the electrospun fibers showed the highest diameter values. The electrospun polyamide-6 nanofibers from hexafluoroisopropanol solvent system had more point-bonded junctions. We could not obtain the high aspect ratio nanofibers for this solvent system. Trifluoroacetic acid is the simplest stable perfluorinated carboxylic acid chemical compound. It is a strong carboxylic acid due to the influence of the electronegative trifluoromethyl group. Trifluoroacetic acid is more acidic than acetic acid. Surprisingly, we did not observe any nanofibers using this solvent. A likely explanation may be due to the strong acidic nature of trifluoroacetic acid that caused a drastic reduction in the molecular weights...
Fig. 7 FE-SEM images of electrospun polyamide-6 nanofibers produced with (a) formic acid + dichloromethane (3:2), (b) formic acid + acetic acid (3:2) and (c) formic acid + acetic acid (4:1); (1) low magnification and (2) high magnification. (Reprinted from [64] with the permission from Springer).

| Materials             | Solvent                        | Key Factor         | Ultrafine Nanofiber Diameter (nm) | Reference |
|-----------------------|--------------------------------|--------------------|-----------------------------------|-----------|
| Polyamide 6           | Formic acid                    | Applied voltage    | 8-40                              | 65        |
| Polyamide 6           | Formic acid + Dichloro Methane (3:2) | Solvent           | 13-34                             | 64        |
| Polyamide 6           | Formic acid + acetic acid (3:2) | Solvent            | 8-38                              | 64        |
| Polyamide 6           | Formic acid + acetic acid (4:1) | Solvent            | 8-32                              | 64        |
| Polyamide 6           | Formic acid + Chlorophenol (1:1) | Solvent            | 8-30                              | 64        |
| Polyamide 6/Chitosan  | Formic acid                    | Solvent            | 20-40                             | 64        |
| Polyamide 6/Lecithin  | Formic acid                    | Solvent            | 10-30                             | 64        |
| PAN/nylon 6           | Formic acid + acetic acid (4:1) | Monomer            | 20                                | 108       |
| PAA/Nylon 6           | Formic acid                    | Monomer            | 19                                | 72        |
| Nylon 6               | Formic acid + acetic acid (4:1) | Inorganic salt     | 10-30                             | 8         |
| PU                    | THF+DMF                        | Inorganic salt     | 10-30                             | 8         |
| PVA                   | Water                          | Inorganic salt     | 10-30                             | 8         |
| PAA                   | Water + ethanol                | Humidity, applied voltage | 10-20                         | 71        |
| Nylon 6               | Formic acid                    | Humidity, applied voltage | 10-20                         | 71        |
| PVA/ZnO               | Water + zinc acetate solution  | Metal oxide        | 25-50                             | 71        |
| Nylon 6/TiO₂          | Formic acid + acetic acid (4:1) | Metal oxide        | 30-50                             | 109       |

Abbreviation in Table 4: PAN (poly acrylonitrile); PAA (polyacrylic acid); PU (polyurethane); PVA (poly vinyl alcohol); THF (tetrahydrofuran); DMF (N,N-dimethylformamide); ZnO (zinc oxide).
owing to the rapid increase of viscosity in the solution [96]. As a result of that the electrosprinnability of polyamide-6 with trifluoroacetic acid was not possible which led to the formation of thin film like structure.

Table 1 summarizes the role of process parameters on the formation of ultrafine polymeric nanofibers. From these results, it is interesting to note that well-aligned uniform electrosprun nanofibers and high aspect ratio nanofibers (diameters in the range from 8 to 35 nm) of polyamide-6 can be produced from formic acid and its mixture solutions. However, the other single solvent system (namely, chlorophenol and hexafluoroisopropanol) except trifluoroacetic acid can also be used as solvents for polyamide-6, in which the expected high aspect ratio nanofibers were not produced. It is believed that the formation of high aspect ratio nanofibers in polyamide-6 can strongly depend on the poly-electrolytic behavior in the solvent. As a result, the electrical conductivity of the polymer solution was increased due to the formation of enormous free ions. By changing the polymer concentration and solvent ratio one can obtain high aspect ratio nanofibers with uniform diameters.

**Effect of humidity**

Bin Ding et al. [71] have recently reported that the formation of the nanowebs of poly(acrylic acid) (PAA) and nylon-6 is considered to be due to the electrically forced fast phase separation of the charged droplets which move at high speed between the capillary tip and the collector. During electrosprinning process, the forces acting on the charged droplet during the high speed flight in the electric field. The forces include electrostatic force, drag force, gravity, Coulombic repulsion force, surface tension and viscoelastic force. The electrostatic force carried the charged droplet from the capillary tip to the collector. The drag force acted on the surrounding air and charged droplet moving at high speed. Also, the deformation of the droplets into films was attributed to the drag force. The Coulombic repulsion force tried to expand the droplet. The surface tension and viscoelastic forces led to the contraction of the charged droplet [106]. The electric field could be increased by increasing the applied voltage at a constant distance. Consequently, the electrostatic and Coulombic repulsion forces of the charged droplet were reinforced with increase in the strength of the electric field. The increased electrostatic force further accelerated the movement of the charged droplet, which led to an increased drag force. The distortion and expansion of charged droplet from a spherical-like to a spindle-like structure in the electric field during electrospraying was reported by Grimm and Beauchamp [107]. The additional expansion could have happened when the Coulombic repulsion and drag forces increased along with the electric field from droplets. Moreover, the increased radial charge repulsion force also had a tendency to expand the charged films. As a result, the deformation of charged droplet was strongly affected by the electric field.

**Effect of monomer**

Parajuli and coworkers [72] have synthesized poly(acrylic acid) monomer in a viscous supporting nylon solution was polymerized and fabricated simultaneously via an electrosprinning process (Scheme 1). The acrylic acid polymerization is achieved via formic acid reduction during the electrosprinning process. Typically, formic acid loses a proton in the acrylic acid solution because of the high acidity. Deprotonated formic acid splits into CO₂ and a hydride ion, which works as a reducing agent [108,109]. If the hydride ion attacks a β carbon of an α, β unsaturated carbonyl monomer (acrylic acid), group-transfer polymerization can be initiated [110]. In the performance of polymerization via electrosprinning, it is difficult to control all parameters, such as the pressure, temperature, and concentration; however, there is sufficient control to allow for the investigation of structural, chemical, and physical modifications of electrospun mats. In another study, Park et al. [111] have reported that the poly(acrylonitrile) (PAN) nanofiber web interpenetrated nylon-6 nanofiber supporters by electrosprinning of an acrylonitrile (AN)/nylon-6 solution.

**Effect of metal oxide**

Mixing of two materials at the nanoscale can form a unique and effective multifunctional nanocomposite material. It was reported that the TiO₂ nanoparticles can form spider-net like electrosprun nylon-6 fiber mats, which can lead to a remarkable increase in the number of reactive sites with a corresponding improvement in hydrophilicity, photocatalytic and antimicrobial activity [112,113]. It was also reported that the preparation of a novel nylon-6/TiO₂ organic–inorganic nanocomposite material in the form of an electrosprun mat, containing two distinct types of fibers (nano and sub nanosized) loaded with TiO₂ nanoparticles, with superior mechanical strength, high hydrophilicity, and good antimicrobial as well as UV blocking ability. This spider-net like nanostructure mat with antimicrobial and hydrophilic properties (antifouling effect) would have great potentiality for water filter applications. Furthermore, the good UV blocking capacity and improved mechanical strength of electrospun mat is highly beneficial for different kinds of protective clothing. Moreover, our research group have recently reported that the electrical characterization of the polyamide-6/TiO₂ composite nanofibers showed a pure resistive behavior of the conductive nanofibers and a good stability of the as-spun nanostructures [114,115]. This significant
Scheme 1  Synthesis of poly(acrylic acid) monomer (Reprinted from [72] with the permission from ACS)

enhancement of electrical properties of these polyamide-6 composite nanofibers can be utilized for quite promising future nanotechnological applications. This observation will continue to drive researchers toward developing future organic based nano-devices.

In another study, it was describe that a novel method to prepare superhydrophobic ZnO surfaces by the combination of electrospinning technique and a wet chemical route, which is simple and straightforward [68]. It shows a very good experimental reproducibility and can produce nanostructured surfaces at a large scale. Here, the fabrication of super-hydrophobic ZnO surfaces is composed of two steps. First, the ZnO nanostructured surface was formed by the calcination of composite nanofibrous films which obtained via electrospinning. Then the as-prepared rough surface was modified by fluoroalkylsilane coating to obtain a super-hydrophobic surface. Additionally, the wettability of pure polymer and composite nanofibrous film surfaces was also investigated.

Application of ultrafine polyamide-6 nanofiber membranes

Nanostructures are attractive materials for nanoscience studies as well as nanotechnological applications. The unique feature of electrospun ultrafine nanofiber, compared to other low dimensional systems, are that have very small diameters, extremely long length, large surface area per unit mass and small pore size. The attractive properties of 1D system arise from their unique chemistry and physics. Nanostructured nanofibers systems are promising for diverse applications, such as the transport and targeted release of drugs and active agents in organisms, tissue engineering, the surface modification of implants, and wound healing.

Presently, most patents on electrospinning are focused on applications rather than on new materials or on new processes. Commercialized applications were accomplished by several companies worldwide in specialty filters (air filtration, coalescence filters) with an increasing market in the coming years. Tissue engineering is going to be another successful field of applications for electrospun biodegradable nanofibers (e.g., PLA) because cells including stem cells grow well on these nanofibers. An additional plus is the large potential for loading of electrospun nanofibers by biologically active agents ranging from salts to drugs and proteins. Protective clothing, in particular in combination with special reagents, is expected to enhance the performance of military protective clothing. The main focus here is on trapping of aerosols of chemical and biological warfare agents.
Sensors

The remarkable high surface area-to-volume ratio, small diameter and high porosity bring electrospun nanofibers highly attractive to ultrasensitive sensors and increasing importance in many technological applications. The formation of denser ultrafine nanofibers with addition of chitosan content showed a great improvement in I-V characteristics [70]. For example, the excess chitosan possibly enveloped the ultrafine fiber networks in between polyamide-6/chitosan composite nanofibers with different concentration of chitosan can be enhanced the electrical pathways (Fig. 8). Further, a significant enhancement of the electrical conductance was observed for the samples of composite nanofibers than that of the pristine polyamide-6 nanofibers. This observation can be explained by the charge compensation of the major charge carriers in the composite nanofibers. Consequently, the electrical conductivity of the polyamide-6/chitosan composite nanofibers was prepared with 2 wt% chitosan exhibited the maximum current of 0.4 pA. It is also believed that the enhanced porosity of these composite nanofibers can be utilized for the biosensor applications with improved performance and sensitivity.

Recently, Wang et al. [116] have demonstrated a simple and straightforward strategy of depositing a nanostructured complex, based on a polyethyleneimine functionalized polyamide-6 nanofibers, on a quartz crystal microbalance sensor for humidity detection (Fig. 9). The polyamide-6 nanofiber substrate, comprising common electrospun nanofibers and spider-web-like nanonets fabricated by a versatile electrospinning process, exhibits several fundamental characteristics, such as a remarkable specific surface area, high open porosity and good interconnectivity. The frequency changes by approximately three orders of magnitude with relative humidity varying from 2% to 95%. Finally, they presented the polyethyleneimine functionalized polyamide-6 nanofibers with relatively small hysteresis and long-term stability.
Polyamide-6 is commercially important, and one of the prominent members of the polyamides which has polymorphic, biodegradable, biocompatible and synthetic polymeric material with good mechanical and physical properties. Recently, we have reported one step synthesis of polyamide-6/lecithin homogeneously blended nanofibers via electrospinning for osteoblastic cell culture applications [117]. These as-spun polyamide-6/lecithin nanofibers exhibited a smooth surface and uniform diameters along their lengths. The fine nanofiber structure resulted in a large surface area-to-volume ratio and interconnected porosity (Fig. 10). Human osteoblast cells were utilized for this analysis. The cells spread over the scaffold fibers, linked with fibers by cytoplasmic extensions (Fig. 11). In tissue culture, cell-based tissue engineering is one of the principle research areas. The ability of the cell to regenerate bone was developed by in vitro cell expansion method (Fig. 12). These results suggested the polyamide-6/lecithin composite nanofibers can be utilized for bone regeneration if we increase the mechanical strength of the scaffold by layer-by-layer deposition of polymer fibers. However, these polyamide-6/lecithin composite nanofibers can be used to engineer living tissue in vitro for subsequent transplantation to the defect sites. In an another study, the same authors have reported that the formation of high aspect ratio nanofibers in polyamide-6/chitosan composites and the in vitro cytotoxic analysis based on the peculiar spider-net like structures [118].

**Photocatalytic membranes**

The efficiency of free TiO$_2$ nanoparticles in a polymer matrix is almost same, which confirms that the surface area of the TiO$_2$ nanoparticles does not significantly decrease upon the incorporation of the nanoparticles into the polymer fiber [112]. For this purpose, a used fiber mat was kept in water for 7 days and reused after washing five times with distilled water. Then the photo degradation of methylene blue dye was carried out under the same conditions as before. The efficiency of the reused mat is nearly similar to that of the initially used mat. A slight decrease in the photocatalytic efficiency of the reused mat was observed, which may be due to the deposition of byproduct particles on the surfaces of the NPs. Initially, the efficiency of the reused nanofibers was less than the initially used one, but after
some time it was nearly equal, which indicates the presence of some foreign particles in the used mat. Moreover, the change in shape of the Ag nanoparticles due to the intense solar radiation may be the cause of decreased photocatalytic efficiency.

**Multi-component textile material**

The mixing of these two materials at the nanoscale can form a unique and effective multifunctional nanocomposite textile material. It is expected that the TiO$_2$ nanoparticles can form spider-net like electrospun nylon-6 fiber mats, which can lead to a remarkable increase in the number of reactive sites with a corresponding improvement in hydrophilicity, photocatalytic and antimicrobial activity. This spider-net like nanostructure mat with antimicrobial and hydrophilic properties (antifouling effect) would have great potentiality for water filter applications [113]. Furthermore, the good UV blocking capacity and improved mechanical strength of electrospun mat is highly beneficial for different kinds of protective clothing.

**Concluding remarks**

Polymer nanofibers with high aspect ratio structure
were successfully produced by electrospinning technique. Electrospinning has potential advantages in cost, simplicity and innovation in preparing polymer nanofibers with controllable nanostructures for the industrial and biological applications. The large surface area to volume ratio polyamide-6 and their composite nanofibers with diameters less than 50 nm can be achieved. This review collectively summarizes the process feasibility for the formation of ultrafine polymeric nanofibers with variety of parameters such as with and without additives. Further, we explored the prominent application point of view with these ultrafine polymeric nanofibers. We believe that this topical review will definitely open new directions as per the nanotechnological applications are concerned. We anticipate that the continuous research activities on these ultrafine polymeric nanofibers can significantly progress towards the rapid improvements based on the next generation nano-device applications.

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Self-assembly Polyrotaxanes Nanoparticles as Carriers for Anticancer Drug Methotrexate Delivery

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Abstract: α-Cyclodextrin/poly(ethylene glycol) (α-CD/PEG) polyrotaxane nanoparticles were prepared via a self-assembly method. Anticancer drug methotrexate (MTX) was loaded in the nanoparticles. The interaction between MTX and polyrotaxane was investigated. The formation, morphology, drug release and in vitro anticancer activity of the MTX loaded polyrotaxane nanoparticles were studied. The results show that the MTX could be efficiently absorbed on the nanoparticles, and hydrogen bonds were formed between MTX and α-CDs. The typical channel-type stacking assembly style of polyrotaxane nanoparticles was changed after MTX was loaded. The mean diameter of drug loaded polyrotaxane nanoparticles were around 200 nm and the drug loading content was as high as about 20%. Drug release profiles show that most of the loaded MTX was released within 8 hours and the cumulated release rate was as high as 98%. The blank polyrotaxane nanoparticles were nontoxicity to cells. The in vitro anticancer activity of the MTX loaded polyrotaxane nanoparticles was higher than that of free MTX.

Keywords: Polyrotaxane nanoparticles; Drug delivery; Methotrexate; Anticancer activity

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Introduction

Polyrotaxanes have supramolecular architectures with cyclodextrins (CDs) threaded in poly(ethylene glycol) (PEG) chains, they are attractive materials to biomaterials scientists and chemists [1-5]. As CDs and PEG have been approved by Food & Drug Administration (FDA) in pharmaceutical formulations, the polyrotaxanes exhibit great potential applications in drug delivery. The excellent biocompatibility, multiple functionality and specific self-assembly capabilities are the advantages of polyrotaxanes as drug carriers.

Drug-carriers conjugate is an important form for polyrotaxanes to deliver drugs. With the modification of hydroxyl groups on the CDs, anticancer drugs could be immobilized on polyrotaxanes via cleavable bonds to form drug-polyrotaxane conjugates [6,7]. The drug release was triggered by the broken of the cleavable bonds. High drug loading content was expected to achieve in the conjugates due to the large amount of hydroxyl groups. Targeting moieties such as ligands were also conjugated to polyrotaxanes for multivalent recognition. As CDs could slide along and rotate around the PEG chains, this flexible motion avoided the mismatch of ligand-receptor interaction. This active recognition mode was reported as a new strategy to enhance targeting efficiency [8,9].

Polyrotaxanes were also fabricated non-viral gene vectors. Cationic modified CDs could condense DNA plasmids efficiently [10,11]. The sliding and rotation of CDs along the PEG chains provided a “live” style to condense DNA. The N/P ratio of the
DNA/polyrotaxane complex was much lower than that of the DNA/cationic polymer complex under the same DNA condensation condition. Stimuli-sensitive bonds were introduced in the end-caps of polyrotaxane vectors to control the dissociation of the DNA/polyrotaxane complex. The released free DNA was favorable to penetrate into nuclei for efficient transfection. The polyrotaxanes as non-viral gene vectors avoided the over tight condensation of DNA comparing with other non-viral vectors such as cationic polymers.

Polyrotaxane nanoparticles were fabricated via supramolecular self-assembly to deliver anticancer drugs. Early papers have reported polyrotaxane nanoparticles as drug carriers, however, the self-assembly of polyrotaxane nanoparticles were driven by the crystallization of polyrotaxanes [12-14], whose compact architectures hindered the loading of anticancer drugs. In our previous work, we reported a new strategy, small π–π conjugated molecules such as coumarin derivative and cinnamic acid were immobilized on the terminal groups of PEG chains to prepare amphiphiles [15,16], α-cyclodextrins (α-CDs) were threaded in the amphiphiles and the polyrotaxanes self-assembled into nanoparticles. Anticancer doxorubicin was loaded in the nanoparticles via hydrophobic group as well as π–π stacking interactions between drugs and the end-cap moieties.

Methotrexate (MTX) is an antimetabolite and antifolate drug. It inhibits the metabolism of folic acid for cancer treatment. Herein, we tried to explore the loading of MTX in polyrotaxane nanoparticles. The interaction between MTX and polyrotaxane was investigated. The formation, morphology, drug release and in vitro anticancer activity of the MTX loaded polyrotaxane nanoparticles were studied.

Materials and methods

Materials

PEG (Mn=2000), cinnamic acid and α-CD were purchased from Aldrich and vacuum dried at room temperature for 24 h before used. N,N′-dicyclohexylcarbodiimide (DCC), MTX and DMSO-d6 were purchased from Aldrich and used as received. Dulbecco’s modified Eagle’s medium (DMEM), 100× mycillin, and fetal bovine serum (FBS) were used to culture cells for cytotoxicity test. Other chemicals were purchased from Kelong Chemical Co. (Chengdu, China) and used without further purification.

Measurements

Proton nuclear magnetic resonance (1H NMR) spectra were performed on Brukerav-400 spectrometer with DMSO-d6 as solvents. Tetramethylsilane (TMS) was used as the internal reference. Fourier transform infrared (FT-IR) spectra were recorded on a Thermo Scientific Nicolet iS10 spectrophotometer. Ultrasound was performed on Ultrasonic purifier KQ-300DE, the frequency was at 70%. X-ray diffractionmetry (XRD) patterns were obtained at room temperature on an X’Pert pro MPD X-ray diffractometer, the powder samples were mounted on a sample holder and scanned from 5° to 50°. Ultraviolet visible (UV-vis) spectra were recorded on Lambda 650 in a range from 200 nm to 500 nm. Dynamic light scattering (DLS) experiments were performed on a Malvern ZetasizerNano ZS at an angle of 90° at 25°C. Transmission electron microscopy (TEM) was performed on Hitachi H600-4. Atomic force microscopy (AFM) imaging was performed using MFP3D (Asylum Research) with tapping mode.

Synthesis of cinnamic acid modified PEG

20 g of PEG (Mn=2000) and 4.44 g of cinnamic acid were dissolved in 200 mL of dichloromethane. 7.98 g of DCC dissolved in 100 mL of dichloromethane was slowly dripped into the mixture in an ice bath. The mixture was stirred in dark at room temperature for 24 h after the solution of DCC was dripped over. The insoluble N,N-dicyclohexylurea (DCU) was filtered. The filtrate was concentrated in a rotary evaporator. The modified PEG was precipitated in excessive diethyl ether. The product was filtered and dried in vacuum.

Preparation of polyrotaxanes

1 g of cinnamic acid modified PEG was dissolved in 20 mL of distilled water, the solution was added to 10 mL of saturated aqueous solution of α-CDs. The mixture was ultrasonically agitated for 10 min and stand at 4°C overnight to precipitate white paste. The product was collected by centrifugation, washed with distilled water three times to remove the unthreaded α-CDs. The white precipitate was vacuum dried at room temperature to get polyrotaxanes.

Biocompatibility test

HepG2 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) at 37°C in 5% CO2 atmosphere. HepG2 cells harvested in a logarithmic growth phase were seeded on 96-well at a cellular density of 1 × 104 cells/well and incubated for 1 day. Polyrotaxanes were dissolved and diluted in phosphate buffer solution (PBS) buffer with the concentration ranged from 1 × 10^{-4} to 1 mg/mL. The polyrotaxanes and HepG2 cells were incubated for 72 h. The CCK-8 assay at 490 nm was performed and the percentage of cell viability was then determined.
Preparation of MTX loaded polyrotaxane nanoparticles

Polyrotaxane (10 mg) and MTX (5 mg) were dissolved in 1 mL of DMSO. The solution was added into 10 mL of distilled water dropwise with vigorous stirring in dark. The solution was transferred to a dialysis tubing (MWCO=3500) and dialyzed at 4°C against distilled water for 48 h. The solution in tubing was freeze-dried to obtain MTX loaded polyrotaxane nanoparticles.

The MTX loaded polyrotaxane nanoparticles were dissolved in 3 mL of DMSO to determine the drug loading content. The MTX concentration was tested using the UV-vis spectrophotometer at 388 nm. The drug loading content was calculated based on the standard curve obtained from MTX in DMSO. The drug loading content (DLC) and encapsulation efficiency (EE) were calculated according to the following formula:

\[
\text{DLC (wt \%) = \left(\frac{\text{weight of loaded drug}}{\text{weight of drug loaded nanoparticles}}\right) \times 100\%}
\]

\[
\text{EE (\%) = \left(\frac{\text{weight of loaded drug}}{\text{weight of drug in feeding}}\right) \times 100\%}
\]

In vitro drug release

The drug loaded polyrotaxane nanoparticles were dispersed and diluted in PBS solution to get final concentration of 2 mg/mL. The diluted solution (0.5 mL) was transferred to dialysis tubing (MWCO=3500). The tubings were then immersed in a flask containing 25mL of PBS solution (pH=7.4), shaking at a speed of 100 rev/min at 37°C. At specific time intervals, 1mL of solution was withdrawn from the release medium and replaced with fresh PBS solution.

Results and discussion

The schematic illustration of the self-assembly of the drug loaded polyrotaxane nanoparticles was presented in Scheme 1. The cinnamic acid modified PEG threaded in the cavity of α-CDs to receive polyrotaxanes. The MTX was loaded in the polyrotaxane nanoparticles. Two drug loaded polyrotaxane nanoparticles with different α-CD concentrations in PEG chains were prepared.

\(^{1}\)H NMR spectrum was used to characterize the formation of polyrotaxane. The protons of CH\(_2\)CH\(_2\)O in PEG chain appeared at δ=3.5 ppm. The assignment of the protons in α-CDs in the polyrotaxanes was shown in Fig. 1. The proton signals of the hydroxyl groups in b-OH and c-OH appeared at δ=5.52 and 5.44 ppm, respectively. The signal in δ=4.8 ppm was attributed to the proton in carbon (a) in the α-CD backbone. The peak at δ=4.48 ppm was assigned to the proton in f-OH. As the chemical environment of the protons in the α-CD backbone was similar, the chemical shifts of the

![Scheme 1](image-url)
protons in the α-CD backbone (c, e, b and d) were located from δ=3.8 to 3.3 ppm, and the signals were split into multiple peaks. The protons in both PEG and α-CDs were detected in the spectrum, which revealed that the cinnamic acid modified PEG was threaded in the cavities of α-CDs [15]. The mole ratio of EG/CD was calculated from the integrities of OCH$_2$CH$_2$ in PEG and CH(Ca) in α-CDs. It was reported that the maximum ratio of EG/CD in polyrotaxane was 2.0-2.1 [17,18], The calculated EG/CD ratios of the two polyrotaxanes were 2.9 and 4.3 when the feeding concentrations of α-CDs were 1.45 and 0.73 g/mL (Table 1), which implied that though the cinnamic acid modified PEG could thread into the cavity of α-CDs, the threading capability was weakened by the cinnamate moiety modification.

Fig. 1 The $^1$H NMR spectrum of α-CDs/PEG polyrotaxane.

Table 1 The parameters of MTX loaded polyrotaxane nanoparticles

| Entry   | α-CD (g/mL)$^a$ | EG/CD$^b$ | DLC (%) | EE (%) | Size (nm)$^c$ |
|---------|----------------|-----------|---------|--------|--------------|
| TX/PN-L | 0.73           | 4.3       | 18.7    | 43     | 150          |
| MTX/PN-H| 1.45           | 2.9       | 20.8    | 57     | 140          |

$^a$ PN: polyrotaxane nanoparticle.

$^b$ The concentration in feeding.

$^c$ Calculated from $^1$H NMR spectra.

In order to explore the interactions between MTX and polyrotaxane nanoparticles, XRD, UV-vis and FT-IR spectra of the drug loaded polyrotaxane nanoparticles were measured. XRD is a powerful tool to study the crystal structure of materials. The XRD spectra of blank polyrotaxane nanoparticles, MTX and MTX loaded polyrotaxane nanoparticles were presented in Fig. 2. In the XRD spectrum of blank polyrotaxane nanoparticles, a very strong peak at 2θ=19.7° was presented in the diffraction patterns. It was the characteristic peak associated with a channel-type crystalline structure by virtue of the polymeric nature of the guest molecules [19]. The XRD spectrum demonstrated that the cinnamate moiety modified PEG was threaded in α-CDs to form necklace-like polyrotaxanes, which was consistent with the result of $^1$H NMR spectrum. Free MTX showed strong crystallization capability in the XRD spectrum. However, in the spectrum of MTX loaded polyrotaxane nanoparticles, no strong crystal peaks were observed, it was likely amorphous material, the weak peaks located in the sites of neither polyrotaxane nor MTX. It clearly displayed that the loaded MTX was in amorphous state and it destroyed the crystal structure of polyrotaxane nanoparticles.

The MTX loaded polyrotaxane nanoparticles were dispersed in distilled water to test their UV absorbance (Fig. 3(a)). The spectra of MTX and blank nanoparticles were used as controls. As the strong absorbance of water was below 250 nm, the peaks above 250 nm were the absorbance of materials. There were two peaks in the UV spectrum of MTX, a strong peak at 302 nm and a weak shoulder at 350 nm. The UV absorbance peak of blank polyrotaxane nanoparticles appeared at around 280 nm. Comparing with the spectra of MTX loaded polyrotaxane nanoparticles and MTX, it could be found that all the peaks of MTX had red shifts. The peak at 302 nm was shifted to 306 nm and the absorbance at 350 nm was shifted to 378 nm. This red shift revealed that interactions between MTX and polyrotaxane nanoparticles were formed. The UV absorbance of MTX loaded polyrotaxane nanoparticles...
with different concentrations were tested, difference absorbance strength was observed and no red shift was found (Fig. 3(b)). It implied that the red shift was due to the interaction between MTX and polyrotaxane nanoparticles [20,21].

![Graph showing UV spectra](image)

**Fig. 3** The UV spectra of polyrotaxane nanoparticles, MTX and MTX loaded polyrotaxane nanoparticles (a); and MTX loaded polyrotaxane nanoparticles with different concentrations (b).

The FT-IR spectra of MTX, polyrotaxane nanoparticles and MTX loaded polyrotaxane nanoparticles were presented in Fig. 4. Strong vibrations were observed around 3200 to 3600 cm\(^{-1}\), which were attributed to the stretching vibration of hydroxyl and amino groups. The vibration peaks of polyrotaxane nanoparticles, MTX and MTX loaded polyrotaxane nanoparticles were at 3410, 3439 and 3423 cm\(^{-1}\), respectively. After MTX was loaded in polyrotaxane nanoparticles, the vibration of MTX was shifted to low wavenumber region, and the vibration of hydroxyl in polyrotaxanes was shifted to higher wavenumber region, which demonstrated the formation of hydrogen bonding [22].

![Graph showing FT-IR spectra](image)

**Fig. 4** The FT-IR of blank polyrotaxane nanoparticles, MTX and MTX loaded polyrotaxane nanoparticles.

Two drug loaded polyrotaxane nanoparticles of MTX/PN-L and MTX/PN-H were used to investigate the release profiles (Fig. 6). They were similar, most of the loaded MTX was released within the first four hours, and the cumulated release rates of MTX/PN-L and MTX/PN-H nanoparticles were as high as 98% and 96%, respectively. The release of MTX/PN-H nanoparticles was a little slower than that of MTX/PN-L nanoparticles. Comparing to other anticancer drug loaded polymeric nanoparticles reported previously [23-27], the release of MTX loaded polyrotaxanes nanoparticles was very fast. There were two reasons to explain this phenomenon, one was that the hydrophilicity of MTX was better than other anticancer drugs such as doxorubicin and 9-nitro-20(S)-camptothecin, which resulted faster diffusion from the nanoparticles to the medium. The other reason was that the hydrophobic moieties of the polyrotaxane nanoparticles could not provide enough hydrophobic interaction to encapsulate the MTX inside the nanoparticles, most of the drug was absorbed on the surface of polyrotaxane nanoparticles via Van der Waals’ force and hydrogen bonding interaction. Thus, the MTX was released very fast from the polyrotaxane nanoparticles. In cancer therapy, sustaining low dose of anticancer drug was not preferable for cancer treatment, which could not kill cancer cells efficiently and induced multidrug resistance in cancer cells. The polyrotaxane nanoparticles could trigger fast release of MTX, which was potentially favorable for the cancer treatment.
Fig. 5 The morphologies of blank polyrotaxane nanoparticles (a) and MTX loaded polyrotaxane nanoparticles (b), the samples were PN-H and MTX/PN-H.

The two blank PN-L and PN-H nanoparticles were incubated with HepG2 cells to evaluate their cytotoxicity. The concentrations of the two nanoparticles were ranged from $1 \times 10^{-4}$ to 1 mg/mL. The cell viabilities of HepG2 cells were presented in Fig. 7. All the cell viability of the samples were higher than 90% even when the concentration of nanoparticles was as high as 1 mg/mL, which was much higher than the concentration for anticancer activity study. The cell viability results revealed that the blank polyrotaxane nanoparticles were non-toxic to HepG2 cells.

The in vitro anticancer activity of MTX loaded PN-L and PN-H nanoparticles was tested by CCK-8 assay. The drug loaded polyrotaxane nanoparticles were incubated with HepG2 cells for 72 h to evaluate their in vitro anticancer activity. The result showed that MTX loaded PN-L and PN-H nanoparticles killed HepG2 cells in a dose-dependent manner. The value of half maximal (50%) inhibitory concentration (IC50) of
MTX loaded PN-L and PN-H nanoparticles calculated from the inhibition cures in Fig. 8 were 6.1 and 5.5 ng/mL, and that of free MTX was 7.0 ng/mL, the lower IC50 represented better anticancer activity. These results indicated that the HepG2 cells were more sensitive to MTX loaded PN-H nanoparticles. The in vitro anticancer activities of the two nanoparticles were higher than that of free MTX, which implied that the in vitro anticancer activity of MTX was enhanced after loaded in polyrotaxane nanoparticles.

Conclusions

α-CD/PEGpolyrotaxane nanoparticles were fabricated to load anticancer drug MTX. The size of drug loaded nanoparticles was around 150 nm and the drug loading content was around 20%. The MTX was absorbed on the polyrotaxane nanoparticles and hydrogen bonding between MTX and polyrotaxane nanoparticles was formed. The loaded MTX destroyed the crystal of polyrotaxanes and the UV absorbance peaks of MTX loaded polyrotaxane nanoparticles exhibited red shift. The MTX loaded polyrotaxane nanoparticles were in spindle shape, which were different from regular spherical shape to blank polyrotaxane nanoparticles. The loaded MTX was rapidly released from polyrotaxane nanoparticles and the cumulated release rate was as high as 98% within the first four hours. The polyrotaxane nanoparticles were non-toxic to HepG2 cells and the anticancer activity of MTX loaded polyrotaxane nanoparticles was better than that of free MTX.

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Singular Sheet Etching of Graphene with Oxygen Plasma

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Abstract: This paper reports a simple and controllable post-synthesis method for engineering the number of graphene layers based on oxygen plasma etching. Singular sheet etching (SSE) of graphene was achieved with the optimum process duration of 38 seconds. As a demonstration of this SSE process, monolayer graphene films were produced from bilayer graphenes. Experimental investigations verified that the oxygen plasma etching removes a single layer graphene sheet in an anisotropic fashion rather than anisotropic mode. In addition, etching via the oxygen plasma at the ground electrodes introduced fewer defects to the bottom graphene layer compared with the conventional oxygen reactive ion etching using the powered electrodes. Such defects can further be reduced with an effective annealing treatment in an argon environment at 900-1000°C. These results demonstrate that our developed SSE method has enabled a microelectronics manufacturing compatible way for single sheet precision subtraction of graphene layers and a potential technique for producing large size graphenes with high yield from multilayer graphite materials.

Keywords: Graphene; Plasma; Singular sheet etching

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Introduction

The outstanding electronic, optical and physical properties [1-5] of graphene, a typical two-dimensional nano material have drawn tremendous attention from the scientific community for structuring electronic and photonic devices with higher performance in such applications as high-speed transistors, DNA sequencing, and biochemical sensors [6-10]. To date, significant progress has been made in producing graphene with both high yield and large size. Methods such as epitaxial synthesis [11], chemical vapor deposition [12,13], and chemical deoxidization [14] have been demonstrated for producing graphene of high quality. However, it is still a challenge for researchers to generate graphene with the desired number of graphene layers using the existing methods. Reproducible control of the number of graphene layers is highly desired for the reproducibility of graphene-based devices in practical applications as well as for the fundamental study of graphene characteristics. In particular, layer engineering of graphene will contribute greatly to the in-depth understanding of inter-layer transport properties in a multilayer structured graphene sensor previously developed in our group [15]. The influence of the number of graphene layers on graphene’s electronic and optical properties has also been reported by other researchers [16]. To address this challenge, several efforts have been made towards realizing the layer engineering of graphene [17-20]. For example, Tour et al. [21] reported the layer-by-layer removal of graphene sheets using a wet etching method by coating graphene surfaces with zinc and dissolving the zinc with dilute acid, providing a promising method for engineering the number of graphene lay-

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ers. However, the requirement of acid treatments makes this method incompatible with microelectronics manufacturing. Very recently, the atomic layer etching of graphene was also reported by oxidation of carbon sp\(^2\) bonds to sp\(^3\) bonds with oxygen radicals and the consequent bombardment with argon atoms [22]. Although this technique allowed for a single sheet of graphene to be removed, a large number of defects were introduced to the graphene due to the combination of radical oxidation and argon atom bombarding.

In this study, we developed a simpler and less invasive method for engineering the number of graphene layers using pure oxygen plasma etching. By carefully tuning the variables of the oxygen plasma etching (e.g., power, oxygen flow rate, operating pressure and process duration), we were able to achieve single sheet etching (SSE) of graphene thin films, which removed one single graphene sheet at a time, with both the oxygen reactive ion etching and oxygen plasma etching. Because plasma strength at the ground electrode is lower than that at the powered electrode, the SSE by oxygen plasma can potentially introduce a relatively smaller number of defects to graphene. As a technical demonstration, monolayer graphene has been produced from bilayer graphene by the SSE method. It should be noted that defect formation by oxygen plasma is inevitable even with indirectly coupled gentle oxygen plasma [23], which can significantly affect graphene properties, such as electrical, optical, and thermopower properties [23-27]. To address this issue, we also demonstrated post-etch annealing treatment that can effectively restore disordered graphene. Our process is completely compatible with microelectronics manufacturing and shows the prospect of being a promising post-synthesis method for engineering the number of graphene layers and producing thin graphene films with both large size and high quality.

**Methods**

Graphenes were first produced with mechanical exfoliation from highly oriented pyrolytic graphite (HOPG) flakes and then transferred onto a Si substrate with a 300 nm thick SiO\(_2\) layer [28]. Optical microscope and micro-Raman spectrograph (Holo Probe Micro-Raman Spectrograph) were used to locate and identify both monolayer and bilayer graphenes. The micro-Raman spectrograph used in this study has an excitation wavelength of 532 nm, a minimal laser spot size of ~5 µm for a 100× magnification, and a power of 0.53 mW. Prior to oxygen plasma etching, all samples were cleaned with acetone rinse for 5 minutes, followed by thermal cleaning in an argon and hydrogen environment. Acetone is used to remove tape residues and other organic contaminations. No chemical effects on graphene at room temperature have been reported in previous literatures. Following the acetone cleaning, the graphene samples were rinsed with isopropanol alcohol and then DI water to remove the acetone residue. We believe that the chemical effect on graphene doping can be negligible. The heat treatment has been shown to effectively remove unavoidable contaminations (e.g., tape residues, resist residues, and adsorbed water) from the as-produced graphene surfaces [29,30]. In our experiments, the treatment was carried out in Ar/H\(_2\) (3% H\(_2\) in volume percentage) atmosphere, with a continuous...
gas flow of 2000 sccm at 380°C for 40 minutes. As a demonstration of the cleaning efficacy, the left panel in Fig. 1(a) shows the AFM (atomic force microscope, DI 3100) image of the as-produced monolayer graphene that was identified with Raman spectroscopy. Fine granular like particles and contaminations were observed on both the graphene and the SiO$_2$/Si substrate surfaces. These particles can be effectively removed by heating the as-produced sample in Ar/H$_2$ atmosphere, as indicated in the right panel of Fig. 1(a).

The SSE of graphene with oxygen plasma was conducted in a plasma etching system (March, PX-250) under the following conditions: RF (radio frequency) of 13.56 MHz, RF power of 70 W, oxygen flow rate of 10 sccm (standard cubic centimeter per minute), base pressure of 70 mTorr, and processing pressure of 313-326 mTorr. These parameters were specifically chosen in order to ignite stable oxygen plasma while reducing plasma strength to minimize plasma induced structural damages and disorders of graphene. During the experiments, the graphene samples were placed on the ground electrode for etching, as schematically displayed in Fig. 1(b). Specifically, the powered electrode refers to the electrode plate that is connected to an RF bias, while the ground electrode plate is connected to the inner wall of the vacuum chamber and also the ground of the etching system. Figure 1(c) shows the experimental setup. The ground and powered electrodes are made of aluminum and have dimensions of 7 in. × 8 in. × 0.093 in. The distance between a ground electrode and the powered one is 2 in. It is clear that the intensity of plasma is higher in the powered electrode than the ground ones based on the brightness. The gentle plasma generated on the ground electrode explains why it required longer time to etch a single layer and generated fewer defects as confirmed later.

The process durations for single sheet removal of graphene were carefully tuned after a large number of experiments and varied according to the location of graphene samples (at powered electrode or ground electrode). AFM and SEM (scanning electron microscopy, Hitachi-4700) were used to inspect the surface topologies of graphene samples before and after being treated with oxygen plasma. Micro-Raman spectroscopy was used to identify the number of graphene layers as well as to study quantitatively the defects of the plasma treated graphene samples.

**Results and discussion**

**SSE of graphene with ground electrode oxygen plasma**

In order to characterize the process parameters for SSE of graphene, both monolayer and bilayer graphene films were subjected to oxygen plasma etching for different etching durations. The etching time was increased by a step of 2 seconds. AFM, SEM and Raman spectroscopy were used to investigate graphene topologies after each treatment. Figure 2 shows the evolution of a graphene sample treated by the SSE process with ground oxygen plasma for an optimum process duration of 38 seconds. In this case, the graphene sample had a step with a monolayer region and a bilayer region, which were identified with micro-Raman spectroscopy (Fig. 2(a)). After one SSE step, the monolayer region was completely removed, while the bilayer region was thinned down to a monolayer graphene, as shown in Fig. 2(b). By carefully comparing Figs. 2(a) and 2(b), it can be seen that the border (the upper edge) of the bilayer region did not change obviously, which implies that the edges are not a preferred direction during the etching process. After the second etching, the bilayer region was completely etched away (Fig. 2(c)), leaving the SiO$_2$/Si substrate. All SEM images were taken at the same location of the sample after each oxygen plasma treatment. Graphene residues were found at the boundary between the monolayer and bilayer regions. This was mainly caused by graphene fragments and folded graphene edges, resulting in thicker graphene areas (> 2 layers).

To further demonstrate the repeatability and controllability of the SSE method with the ground electrode plasma, we applied the optimum recipe to multiple bilayer graphene samples and successfully obtained monolayer graphene films from the bilayer ones, verified by...
Fig. 3 Demonstration of producing a monolayer graphene from a bilayer graphene with the oxygen plasma etching. Illustrations (a), (b), and (c) show the SEM image, the AFM image (upper left panel), the 2D band of its Raman spectrum (upper right panel) and its height profile from the AFM image (bottom panel) before being etched with oxygen plasma (a), after the first (b) and the second (c) SSE treatments, respectively. Specific regions from which the AFM images and Raman spectra were taken are highlighted by the red squares.

AFM and Raman spectroscopy. Figure 3 shows the evolution of a representative bilayer sample when being subjected to the SSE process. After the first etching (Fig. 3(b)), while the SEM and AFM images had no distinguishable difference between the untreated graphene and the treated one, their Raman spectra and AFM profiles clearly showed the changes resulting from plasma etching. Particularly, the thickness of the bilayer graphene decreased from \( \sim 1.259 \) nm to \( \sim 0.810 \) nm, resulting an apparent difference of 0.449 nm. Graphene thickness measurement using an AFM is typically not atomically precise due to sample contamination and/or an unclear or worn AFM cantilever. The step size 0.449 nm is slightly larger than 0.335 nm (one layer) but obviously much smaller than 0.670 nm (two layers), indicating one atomic carbon layer was removed. The 2D band of its Raman spectrum comprised only one Lorenzian peak, which is another indicator of a monolayer graphene [31,32]. After the second treatment (Fig. 3(c)), the SEM and AFM images taken from the same regions indicate that the bilayer graphene was completely removed with only folded thicker portions of the graphene left on the substrate. This was further confirmed with the Raman spectrum taken from the same location, which contained no specific peak of graphene.

Etching mechanism of oxygen plasma

We have demonstrated that the SSE of graphene can be realized with the oxygen plasma, but it still remains unclear how the oxygen plasma interacts with graphenes. An answer to that question is essential for further understanding the effects of oxygen plasma on the remaining graphene sheets after the etching process. Two possible etching mechanisms include isotropic etching and anisotropic etching. If the single sheet removal of graphenes by oxygen plasma is isotropic, the etching rate should be uniform everywhere on a graphene sheet regardless of graphene defects and edges. Initiatively, when a single atomic layer is removed from the top, a single or a couple of chains of atoms at the edge will be removed. This is because removing an atom from the top will need sufficient energy to break three bonds, while at the edges, only one or two bonds need to be broken due to the existence of dangling bonds. That means the etching cannot be an isotropic process. For graphene sheets, there are two possible preferred directions for anisotropic etching: vertical (top surface) and horizontal (edges). Experimentally, it is possible to detect a removed single layer using Raman or optical microscopy, but the loss of a couple of chains of atoms at the edge is beyond the resolution of these instruments. Consequently, the sizes of the graphene sheet and the defective regions will not change during the etching in the case of anisotropically vertical etching, but will be projected to the bottom graphene sheet after the etching process, as illustrated in Fig. 4(a). While in the anisotropically horizontal etching mode, the etching rates at the graphene edges and defective regions are anticipated to be higher than that at other intact regions since the edges/defects of graphene tend to react faster with chemicals (e.g.
Fig. 4  Schematic cartoons show two possible etching mechanisms of ground electrode oxygen plasma: (a) Anisotropic vertical etching; (b) Anisotropical horizontal etching. In the anisotropic vertical etching mode, the defect patterns on the top graphene sheet will be copied to the bottom graphene sheet with similar sizes. However, in the anisotropical horizontal etching mode, the defect patterns will expand during etching and be copied to the bottom graphene sheet in an obviously. (c)-(e) SEM images of monolayer graphene samples before and after being subjected to oxygen plasma etching for 32 s, 30 s and 28 s, respectively. The monolayer regions of the graphene samples were marked with arrows. In each sub-figure, the top and bottom SEM images show the graphene sample before and after being etched with the corresponding process duration, respectively.

oxygen plasma) than pristine graphene [33]. As schematically displayed in Fig. 4(b), the defective regions of the top graphene sheet will expand, resulting in larger defective areas on the bottom sheet after the etching process. Additionally, the size of the graphene sheet will shrink due to the anisotropic etching of oxygen plasma.

Experiments were conducted in order to investigate which one of these two possible mechanisms will better explain the single sheet removal of graphene. In this study, the etching rate of graphene was relatively fast and the time interval was too short, making it difficult to control the etching time accurately on the same graphene sheet. Therefore, different monolayer graphene samples were etched for various plasma durations and inspected with SEM to evaluate the time evolution of oxygen plasma etching. Etching conditions, such as RF power, oxygen flow rate and working pressure, except the process duration, were kept the same for each sample. Figures 4(c)-(e) show the SEM images of three monolayer graphene samples before and after being etched by the oxygen plasma at the ground electrode with the plasma durations ranging from 28 to 32 seconds. It can be seen that, first, no noticeable area expansions of defective regions in the monolayer samples were observed during the etching. Second, no remarkable shrinkage of the monolayer graphene dimensions due to the possible faster etching rates at the graphene edges was observed during the etching processes. These results suggest that the single sheet removal of graphenes by oxygen plasma is most likely anisotropically vertical etching.
Comparison of the oxygen plasma etching and the oxygen reactive ion etching

With a similar means of investigation, singular layer etching of graphene can also be achieved with oxygen reactive ion etching at the powered electrode for a shorter etching duration of 17 seconds. Figure 5 provides the demonstration of a monolayer graphene produced from a bilayer graphene using the oxygen reactive ion etching. A ~0.4 nm thickness decrease of the graphene was observed after one SSE plasma treatment. The evolution of its Raman 2D band also demonstrated that a monolayer graphene was produced from the bilayer graphene.

It is known that the strength of oxygen plasma varies at the powered electrode and the ground electrode (as shown in Fig. 1(c)), which is anticipated to result in different levels of physical damage and defects on the post-etch graphene sheets. Therefore, we investigated the plasma induced defects on a variety of plasma-treated graphene samples by measuring the intensity ratios of the D band and G band \(I_D/I_G\) in their Raman spectra. A higher \(I_D/I_G\) ratio indicates a larger number of defects. Figure 6(a) and 6(b) show the Raman spectra taken from two bilayer graphene samples before and after SSE by the oxygen reactive ion etching and the oxygen plasma etching, respectively. The Raman spectra show that the sample etched with the oxygen reactive ion etching at the powered electrode had an \(I_D/I_G\) ratio of ~1.18 (the bottom panel of Fig. 6(a)), while the \(I_D/I_G\) ratio of the graphene etched with the oxygen plasma at the ground electrode was ~0.94 (the bottom panel of Fig. 6(b)). The statistical analysis of the Raman \(I_D/I_G\) ratio of graphene samples after etching with the powered electrode oxygen plasma was ~1.05, while the average \(I_D/I_G\) ratio after etching with the oxygen plasma was ~0.86. These results indicate that the oxygen plasma introduced fewer defects compared to the powered electrode oxygen plasma, which is mainly due to the relatively low strength of oxygen plasma at the ground electrode.

Furthermore, it is found that the amount of defects can be reduced significantly with a post-etch annealing treatment. The annealing process was carried out in an Ar environment at 900-1000\(^\circ\)C for 1 h. Figure 6(d) shows the Raman spectrum of a monolayer graphene, which was produced with the SSE method, after the annealing process. Compared with its Raman spectrum before the annealing process, which was shown in the bottom panel of Fig. 6(a), it can be seen that the intensity of the D band peak has been greatly suppressed by the annealing process. The recovery of disordered graphene by high temperature Ar annealing may be

![AFM images, height profiles and the 2D bands of its Raman spectrum of a bilayer graphene](image)

Fig. 5 AFM images, height profiles and the 2D bands of its Raman spectrum of a bilayer graphene (a) Before and (b) After the SSE with powered electrode oxygen plasma, demonstrating the production of a monolayer graphene from a bilayer graphene.
related to the thermally induced reconstruction of graphene lattice and dangling bonds [34] as well as graphene dehydrogenation [35]. It should be mentioned that electron beam irradiation during the SEM step is known to have effects on the transformation of the crystalline order and electronic properties of mono/bilayer graphene films [36]. During our quantitative studies of defects, the as-etched graphene samples were examined only by the micro-Raman spectroscopy in order to eliminate additional defect formation due to the electron beam irradiation. Therefore, the formation and partial recovery of defects on our graphene substrates are mainly attributed to the oxygen plasma etching and their successive annealing treatment.

Fabrication of suspended graphene

Our method can also be applied in controlling the number of layers in the case of suspended graphene. In this approach, tranches were made on the SiO$_2$ (300 nm)/Si substrates through photolithography and induced coupled plasma reactive ion etching (ICP-RIE), respectively. First, SiO$_2$ was completely removed and Si substrate was etched down by around 10 µm. Next, graphene layer was prepared using micro mechanical exfoliation and then deposited over the tranches and the holes. After that, graphenes were located by optical microscope and Raman spectroscope. Then graphene layer was etched layer-by-layer using oxygen plasma, showing that the SSE method provides a possible way to produce graphenes directly from graphite flakes. Figure 7 shows a suspended graphene sheet with a thickness of ~2 nm produced through layer by layer etching of a graphite flake of an original thickness of ~15 nm.

Fig. 7 SEM image of a suspended graphene sample of 2 nm thickness produced from graphite flake of ~15 nm using layer by layer oxygen plasma etching. Inset is the Raman spectra.

Conclusions

In this paper, we developed a method for engineering
the number of graphene layers with oxygen plasma that is simple and compatible with microelectronics manufacturing. SSE processes, which remove only one single graphene sheet at a time, were achieved with both the powered electrode oxygen plasma and the ground electrode oxygen plasma. Production of monolayer graphene films from bilayer graphene samples was demonstrated with our SSE processes. The results show that the oxygen plasma etching of graphene was an anisotropically vertical etching process. It was also found that etching with the oxygen plasma etching introduced fewer defects onto the bottom graphene layer compared with oxygen reactive ion etching. That is because the oxygen plasma strength was lower at the ground electrode than at the powered electrode. The plasma induced defects can be obviously suppressed by an annealing process after oxygen plasma etching. Our results provide a post-synthesis approach to the single sheet precision subtraction of graphene layers and the possibility of producing large size graphene thin films directly from multilayer graphene or even graphite flakes. These films can be used for constructing novel graphene based devices by selectively etching the graphene at specific regions.

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RhB Adsorption Performance of Magnetic Adsorbent Fe₃O₄/RGO Composite and Its Regeneration through A Fenton-like Reaction

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Abstract: Adsorption is one of the most effective technologies in the treatment of colored matter containing wastewater. Graphene related composites display potential to be an effective adsorbent. However, the adsorption mechanism and their regeneration approach are still demanding more efforts. An effective magnetically separable absorbent, Fe₃O₄ and reduced graphene oxide (RGO) composite has been prepared by an in situ coprecipitation and reduction method. According to the characterizations of TEM, XRD, XPS, Raman spectra and BET analyses, Fe₃O₄ nanoparticles in sizes of 10-20 nm are well dispersed over the RGO nanosheets, resulting in a highest specific area of 296.2 m²/g. The rhodamine B adsorption mechanism on the composites was investigated by the adsorption kinetics and isotherms. The isotherms are fitting better by Langmuir model, and the adsorption kinetic rates depend much on the chemical components of RGO. Compared to active carbon, the composite shows 3.7 times higher adsorption capacity and thirty times faster adsorption rates. Furthermore, with Fe₃O₄ nanoparticles as the in situ catalysts, the adsorption performance of composites can be restored by carrying out a Fenton-like reaction, which could be a promising regeneration way for the adsorbents in the organic pollutant removal of wastewater.

Keywords: Magnetic adsorbent; Fe₃O₄ nanoparticles; Reduced graphene oxide; Fenton-like reaction; Regeneration

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Abbreviation

\[ Q_e \] — Equilibrium adsorption capacity of adsorbent, mg/g;  
\[ C_i \] — The initial aqueous phase concentration of absorbates, mg/L;  
\[ C_e \] — The equilibrium concentration of absorbates, mg/L;  
\[ V \] — The volume of solution, mL;  
\[ M_s \] — The mass of adsorbent, g;  
\[ Q_{max} \] — The maximum adsorption capacity of adsorbent, mg/g;  
\[ K_L \] — The Langmuir constant, L/mg;  
\[ K_f \] — Freundlich constant, L/g;  
\[ n \] — Freundlich constant.

Introduction

Colored matter containing wastewater discharged from dyeing, textile, leather, cosmetics or food industries has brought serious environmental problems in our daily life. Even at a low concentration, dyes in water would bring aesthetically displeasing, and most of them are toxic to both aquatic life and humans [1]. The complex conjugated molecular structures make them stable and recalcitrant to oxidant and bacteria, also resistant
to be bleached by sunlight. Accordingly, traditional biological or chemical oxidation methods are difficult to remove them from wastewater. Simultaneously, dyes are always highly soluble in water, rendering them hard to be dealt with by precipitation or flocculation. Therefore, cost-effective method is still urgently demanded to remove colored substance from wastewater. Adsorption is an effective and classic depth processing in the field of wastewater treatment, by which a substance at the interfacial layer of the adsorbent would accumulate owing to the operation of surface forces [2]. Compared to other advanced water-treatment technology, adsorption has such merits as a low invest-cost, simplicity of design and operation, and no discharge of harmful substances. Although the history of adsorption in wastewater treatment is more than one hundred years, there are still two challenges in the practical applications. One is to realize selective adsorption, which is the prerequisite for recovering valuable substances from wastewater without being disturbed by coexisting matters [3,4]. Another is to obtain adsorbents possessing excellent adsorption performance and the ability to be facilely separated and regenerated, which is significant to lower the operating cost [5,6].

In previous years, carbonaceous materials, including active carbon and carbon nanotubes, have been widely investigated and applied in the removal of diverse pollutants from water due to their high adsorption capacity [1,7]. Compared with them, graphene and graphene oxide (GO) nanosheets, a type of two-dimensional nanomaterial with a single layer framework of six-member carbon rings, also show strong adsorption ability for both organic or inorganic pollutants because of their large theoretical specific surface area of 2630 m$^2$/g and special π-π conjugate planar geometric structure [8-12]. Moreover, the oxygen-containing groups and surface amphiphilicity can be tuned by synthesizing modified GO or reduced GO (RGO) for specific adsorption objectives [13,14]. On the other hand, multifunctional graphene-related nanocomposites would bring additional properties to graphene and become a focused field of graphene materials [11,15,16]. Among them, incorporation of magnetic nanoparticles with graphene has attracted tremendous attention. The inherent superparamagnetic behavior of magnetic nanoparticles enables them to be easily separated and collected by an external weak magnetic field [17,18]. Moreover, the presence of Fe$_3$O$_4$ nanoparticles can prevent the aggregation and restacking of graphene sheets, and consequently retain a high surface area [19]. In addition, Fe$_3$O$_4$ nanoparticles can serve as both a powerful adsorbent [17,20] and a catalyst for heterogeneous Fenton-like reaction [21,22]. The superparamagnetic Fe$_3$O$_4$ nanoparticles possess excellent adsorption capacity for arsenic removal [17], and the performance was much improved when they are dispersed over RGO to form a hybrid adsorbent [23]. In addition, considering the accumulated organic molecules at the interface layer by adsorption is similar to a concentrated process, combining Fenton-like oxidation after adsorption would significantly enhance the reaction kinetics and possibly develop into a new regeneration approach [5].

In general, the Fe$_3$O$_4$/GO (or RGO) nanocomposites can be synthesized by solvothermal, self-assembly with covalent bond, and in-situ chemical coprecipitation methods, and display efficient adsorption performance in the removals of organic dyes and inorganic metal ions in wastewater [19,23-27]. The abundant oxygen-containing groups on GO enable a hydrogen bond and electrostatic interactions with organic adsorbates, while the hydrophobic polyaromatic islands of unoxidized benzene rings of RGO offer hydrophobic interaction and π-π stacks toward the organic molecules, especially conjugation molecules. However, much more efforts are required to understand the optimized structural factors for adsorption. Furthermore, it was found that under a photo-Fenton reaction GO would transform into graphene quantum dots [28]. Although GO is unstable in the Fenton conditions, RGO is recalcitrant in the heterogeneous Fenton-like reaction, because the less oxygen containing groups in RGO makes the initial decomposition unfeasible [28], and the oxidation species in the Fenton-like system are localized over the surface of catalysts and less accessible to the graphene partial. Due to the large specific surface area, tunable structures and components of RGO, its application in Fenton(-like) reactions has attracted increasing attentions in the latest years [29-31].

Herein, a Fe$_3$O$_4$/RGO composite with efficient adsorption performance for rhodamine B (RhB) removal has been synthesized by optimizing the iron source, and the structural controllable adsorption mechanism was discussed by the analyses of isotherms and kinetics. Moreover, the regeneration of the adsorbent has been preliminarily studied via a way of heterogeneous Fenton-like reaction.

**Experimental**

**Materials**

Ferrite chloride (FeCl$_3$·6H$_2$O), graphite (Spec. pure) and active carbon (AC) powder were procured from Sinopharm Chemical Reagent Co., Ltd. RhB was obtained from Shanghai Hyperheal Biotech Co., Ltd. Ferrous chloride (FeCl$_2$·4H$_2$O) was purchased from Aladdin Chemistry Co. Ltd. All chemicals used in this study without special states were of analytical grade and used as received. All solutions were prepared with deionized water.
Synthesis of Fe₃O₄/RGO adsorbents

Graphene oxide was prepared by a modified Hummers’ method [32, 33]. Briefly, graphite oxide was obtained by oxidizing graphite powder with the presence of H₂SO₄, NaNO₃ and KMnO₄. The graphite oxide was washed with diluted HCl solution and exfoliated by ultrasonic treatment at 400 W for 30 min. The Fe₃O₄/RGO adsorbents were synthesized through an in situ method. In a typical synthesis, FeCl₂·4H₂O and FeCl₃·6H₂O (weight ratio 3:1) were firstly dissolved in 60 mL GO aqueous suspension (1 mg/mL). The mixture was mechanical agitated in a nitrogen atmosphere at 90°C for 20 min, to ensure that ferric and ferrous salts were completely dissolved and anchored at the surface of GO nanosheets by chemical interactions. Then 20 mL aqueous ammonia was dropwise added, following with 4 h reaction at 90°C to complete the precipitation and formation of Fe₃O₄ and the reduction of GO. Finally the precipitation was magnetically separated, washed with copious water, and vacuum dried overnight at 60°C. The obtained dark powder is a composite of Fe₃O₄ and RGO. By changing the amount of iron source, various composites with different theoretic weight ratios of Fe₃O₄ (according to the amount of total iron dosages) to RGO (according to the GO dosages) were synthesized, that is 1:1, 2:1, 3:1 and 4:1 and assigned as M-1 to M-4, respectively. The control sample of Fe₃O₄ nanoparticles were also prepared according to the same procedure but without the addition of GO in the reaction solution.

Adsorption kinetics and isotherms

The kinetics of the adsorption process was determined in batch experiments with 50 mg/L RhB solutions. A 100 mL volume of the test solution and 0.2 g weight of adsorbent were shaken in a flask at a speed of 150 r/min at 30°C in a thermostatic oscillatory water bath. At several points in time, 5 mL sample was taken out from the water bath, and the supernatant solution was collected by preliminarily magnetic separation and subsequently centrifugation to ensure completely removal of solid adsorbents. The residual concentration was analyzed by a UNICO UV-1202 spectrometer. The control (no sorbent) test displays there is negligible change of the dye concentration in the whole process.

RhB adsorption isotherms were determined by batch adsorption experiments at 30°C in the dark. Briefly, various amounts of solid adsorbent were introduced into 50 mL flasks, receiving 20 mL RhB aqueous solution with an initial 50 mg/L concentration. The samples were shaken for 2 h at the same conditions in the kinetic experiment. The time was sufficient to reach apparent adsorption equilibrium based on the results of adsorption kinetics. Then the equilibrium concentra-

tion in the aqueous phase was measured. The adsorbed capacity was calculated according to equation (1):

\[
Q_e = \frac{(C_i - C_e)V}{M_s}
\]

where \(Q_e\) (mg/g) is the equilibrium adsorption capacity of adsorbent, \(C_i\) (mg/L) is the initial aqueous phase concentration, \(C_e\) (mg/L) is the equilibrium concentration, \(V\) (mL) is the volume of solution, \(M_s\) (g) is the mass of adsorbent.

A Fenton-like reaction was carried out to regenerate the saturated adsorbents. Briefly, 0.04 g adsorbents saturated with RhB were transferred into a 30 mL H₂O₂ (30 mmol/L, pH=3.0) aqueous solution and shaken for 30 min at 50°C to completely remove the adsorbed RhB molecules. The regenerated adsorbents were magnetically separated from the solution and dried for recycled adsorption tests.

Characterization

The nanometric structure and the morphology of the composites were observed by a transmission electron microscope (TEM, JEM-2100F, JEOL, Japan). The crystal structure of Fe₃O₄ and Fe₃O₄/RGO composites were measured using a powder X-ray diffraction (XRD, D/max-2200/PC, Rigaku Corporation, Japan) with Cu Kα radiation at 40 kV and 30 mA. Raman spectra were obtained on a Senterra R200-L dispersive Raman microscope (Bruker Optik Gmbh, Germany) with a 532 nm laser source. The chemical states of elements in the nanocomposites were determined by X-ray photoelectron spectroscopy (XPS, AXIS ULTRA DLD, Kratos Ltd., Japan). The Brunauer-Emmett-Teller (BET) specific surface areas of the samples were analyzed by a Micromeritics ASAP 2010 M+C nitrogen adsorption apparatus (USA). All of the samples were degassed at 180°C before nitrogen adsorption measurements were taken. Fourier transform infrared spectroscopy (FTIR) was performed at a scanning range of 4000–400 cm⁻¹ on a Nicolet 6700 Infrared-Raman Spectroscope (Thermo Fisher Co., USA) with DTGS detector.

Results and discussion

Characterizations of adsorbents

The morphology and microstructure of GO and Fe₃O₄/RGO composite (M3) were characterized by TEM. As shown in Fig. 1(a), GO displays a typical flake-like and crumpled shape of graphene morphology. Corrugation and scrolling are the intrinsic nature of graphene, because its ultra-thin and large two-dimensional structure would become thermodynamically stable via bending [34-36]. The GO nanosheets are transparent and very stable under the electron beam.
The ordered graphitic lattices are visible in the HRTEM image of the insert in Fig. 1(a). The folding and scrolling edges of nanosheets allow for a cross-section view of several GO layers. The TEM in Fig. 1(b) and 1(c) show that the spherical structure Fe$_3$O$_4$ nanoparticles grow over the nanosheets of RGO regularly with diameters ranging from 10 to 20 nm. The dispersion of Fe$_3$O$_4$ nanoparticles is well, and there is little observable aggregation of those nanospheres, which is attributed to the in-situ growth of Fe$_3$O$_4$ nanoparticles followed with the chemical interaction between ferric or ferrous ions and the carboxylate or hydroxyl groups.

XRD patterns were measured to study the crystal phases and components of Fe$_3$O$_4$ and the Fe$_3$O$_4$/RGO composites, as shown in Fig. 2. The presence of four characteristic peaks at about 30.1°, 35.5°, 43.1° and 57.1° are observed for all of the diffraction patterns, which are identical to the indices $d_{(220)}, d_{(311)}, d_{(400)}$ and $d_{(511)}$ of inverse spinel Fe$_3$O$_4$ (PDF No. 75-1609), respectively. No other peaks of the hematite or other crystal phase iron oxides were detected in the XRD patterns, indicating that the Fe$_3$O$_4$ nanoparticles in the as prepared composites were pure Fe$_3$O$_4$ with inverse spinel structure [37]. It can be observed that with the increases of iron oxide, the intensity of characteristic peaks in the composites is enhanced.

Figure 3 shows the Raman spectra of GO and the composite M3. In the range below 800 cm$^{-1}$, some bands including 215.9, 273.3, 390.7, 482.3 and 642.1 cm$^{-1}$ appear in the spectrum of M3. These bands are also observed in the composite of Fe$_3$O$_4$/MWCNTs and attributed to the vibration modes of Fe-O and Fe-C bonds [5]. There is no observable Raman mode at around 300 or 410 cm$^{-1}$, indicating the absence of hematite phase in the composite [38,39]. On the other hand, Raman spectroscopy can provide information on the disorder and defect structures of carbonaceous materials. Both spectra of GO and M3 show the fundamental $D$ and $G$ bands of graphene at around 1340 and 1580 cm$^{-1}$, respectively. The former one is corresponding to A1g symmetry mode of the disordered $sp^3$ carbon with the structural defects, amorphous carbon, or edges that break the symmetry and selection rule, while the latter one is attributed to the in-plane vibration of $sp^2$-bonded carbon domains [33,40]. Moreover, the intensity ratio of the two bands ($I_D/I_G$) is used to measure the degree of ordering in the carbon materials.
The ratio $I_D/I_G$ of GO is calculated as 2.70, indicating the presence of a large amount of $sp^3$ carbons in the GO nanosheets. $D$ and $G$ bands of GO are centered at 1332 and 1581 cm$^{-1}$, respectively. In comparison to GO, M3 has a much lower $I_D/I_G$ value (1.86), and the center of the two bands red shift to 1341 and 1589 cm$^{-1}$, respectively. It represents that GO has been partially reduced, and there are chemical interactions, but not only physical adsorption between Fe$_3$O$_4$ nanoparticles and RGO carbon [41].

The chemical states of elements in M3 were further determined by XPS analyses, and the results are shown in Fig. 4. The binding energies of peaks at about 284, 530 and 711 eV are corresponding to the C 1$s$, O 1$s$ and Fe 2$p$, respectively. The carbon spectrum could be deconvoluted into two bands at 284.6 and 287.3 eV, corresponding to C=C/C–C, and C=O respectively. The absence of C–O group in the nanocomposite indicates the reduction of GO. In Fig. 4(c), the two peaks at 710.9 and 724.8 eV of Fe 2$p$ are corresponding to Fe 2$p^{1/2}$ and Fe 2$p^{3/2}$ of Fe$_3$O$_4$, respectively [42]. This can also be supported by the deconvoluted peak at 530.2 eV in the O 1$s$ spectrum (Fig. 4(d)), which is the binding energy of crystal oxygen in Fe$_3$O$_4$. Another peak located at 531.8 eV can be attributed to the residual C=O group over the RGO.

$N_2$ adsorption-desorption isotherms for Fe$_3$O$_4$ and the composites are shown in Fig. 5. According to the IUPAC classification, Fe$_3$O$_4$ displays a type II isotherm, which is the normal form for non-porous materials. It is obviously that the composites exhibit higher $N_2$ adsorption quantity than pure Fe$_3$O$_4$, because the highly dispersion of Fe$_3$O$_4$ nanoparticles over the RGO support results in enhanced surface area and improved porosity and adsorb ability. The isotherm curves of the composites are quite different with Fe$_3$O$_4$ and ascribed to type IV. The sharp steps at the relative pressure around 0.4 $P/P_0$ is associated with capillary condensation of $N_2$ molecules inside the pores. However, the Type H$_2$ characteristic of their hysteresis loops above 0.4 $P/P_0$ presents that the distribution of pore size and shape is not well-defined, which appears in many typical porous adsorbents (e.g. inorganic oxide gels and porous glasses), and is attributed to the complicated mechanism related with pores with narrow necks and wide bodies and effects of the whole network [43]. M2 has the highest $N_2$ adsorption, corresponding to its largest specific surface area, which is found to be 296.2 m$^2$/g by using a BET equation, higher than 264.6 and 208.7 m$^2$/g for M1 and M3, respectively. Comparing to the 13.1 m$^2$/g surface area of Fe$_3$O$_4$ nanoparticles, the composites has significantly enhanced surface area, which
is the result of the presence of two dimensional RGO nanosheets and the highly dispersive Fe$_3$O$_4$ nanoparticles over the surface of RGO. According to above analyses, the adsorbent of Fe$_3$O$_4$/RGO with tunable surface properties can be obtained by changing the dosage of iron sources.

**Adsorption performance of the Fe$_3$O$_4$/RGO composites**

In order to evaluate the adsorption performance of the Fe$_3$O$_4$/RGO composites toward organic dyes in wastewater, the basic dye RhB was employed as the model pollutant. Figure 6 is the adsorption kinetics of RhB on various adsorbents. Fe$_3$O$_4$ nanoparticles display low adsorption capacity due to its small surface area and lack of chemical interaction between the nanoparticles and RhB molecules. The active carbon (AC) shows a high dye adsorption capacity, which can be attributed to its large surface area (>500 m$^2$/g), hydrophobia surface and porous structure. RhB adsorption reaches equilibrium at 300 min, achieving more than 98% RhB decolorization. As our expectation, all composites show high adsorption capacity when reaching equilibrium. However, according to Fig. 6, it is interesting to observe that the composite M1, which has the highest amount of RGO and even a larger surface area than M3, shows relatively poorer adsorption performance, both in the capacity and kinetic rate. The RhB adsorption on M1 approaches balance after more than 10 h, while the time on M2 and M3 is only 20 and 10 min, respectively. It is known that RGO has the ability to adsorb RhB efficiently at very low concentration, indicating specific interactions toward RhB molecules [24,44-46]. However, the adsorption kinetic depends much on the chemical components of RGO. Composites M2 and M3 display even better adsorption capacities and faster kinetic rates than AC, although AC has more than double surface area than the composites. It also suggests that the RhB adsorption mechanisms on AC and composites are different. Figure 6(b) shows the RhB adsorption kinetics on M3 at different pH conditions. In both acidic and basic solution, the adsorption rate and capacity show negligible decrease. The binding mechanisms of RhB on other molecular or materials have been discussed and most of them are pH dependent [46,47], including hydrogen bond, electrostatic interaction and hydrophobic interaction. Here the RhB adsorption on the composite is efficient in a wide pH range from 3.6 to 9.6. The reason is that the influences of pH on these mechanisms are different. The function of hydrogen bond is strong at acidic solution, whereas the role of electrostatic interaction would be important at basic conditions due to the more negative charged materials. In additional, in a higher pH solution, the inhibited aggregation of RhB molecules also favors a better adsorption [48]. Moreover, the planar structure of RGO and the aromatic conjugate RhB molecules renders a strong π-π stack between them, which enables the efficient adsorption work at a wide pH range.

The isotherms have been measured to explore the adsorption process. Figure 7 shows the adsorption isotherms of RhB on various adsorbents. The most frequently-used isotherm models are Langmuir and Freundlich models. The former one is represented for the...
monolayer adsorption by the equation (2), whereas the latter one is described by equation (3) for the multilayer adsorption.

\[
\frac{C_e}{Q_e} = \frac{1}{Q_{\text{max}}} K_L + \frac{C_e}{Q_{\text{max}}} \quad (2)
\]

\[
\ln Q_e = \ln K_f + \frac{1}{n} \ln C_e \quad (3)
\]

where \(Q_{\text{max}}\) (mg/g) is the maximum adsorption capacity of adsorbent; \(K_L\) (L/mg) is the Langmuir constant related to energy of adsorption; \(K_f\) (L/g) and \(n\) are Freundlich constants corresponding to the factors affecting the adsorption capacity and intensity of adsorption, respectively. The linear fitting results and parameters by both models are listed in Table 1. According to the linear correlation coefficient in the two models, RhB adsorption on \(\text{Fe}_3\text{O}_4/\text{RGO}\) composites can be well described by Langmuir model, but poorly by Freundlich model. The fitting results suggest that RhB adsorption on composites is ascribed to monolayer adsorption, an interaction between the groups on RGO and RhB molecules playing principal role for the effective adsorption. However, the better linear fitting for active carbon and GO is Freundlich model, indicating RhB adsorptions on them are more complicated than monolayer format, which could be ascribed to the porous structure of AC and the different mechanism on RhB reduction by GO. The adsorption capacity of GO was measured by centrifuging the suspension after adsorption equilibrium. \(Q_{\text{max}}\) for GO is only 14.2 mg/g, which is comparable to the reference result [49], but much lower than those of composites. This indicates in the composites, the micro components of those carbon rings have been much altered. In the composites, M2 possesses the largest \(Q_{\text{max}}\) and smallest \(K_L\), corresponding to its highest adsorption capacity and lowest adsorption energy. Considering the presence of \(\text{Fe}_3\text{O}_4\) nanoparticles, we normalize the maximum adsorption capacity of adsorbent according to the carbon amount in the composites. Then \(Q_{\text{max}}\) of M2 is 432.91 mg/g, more than 3.7 times higher than AC, and at the same time the adsorption rate is about thirty times faster. However, compared to M2, M3 shows even higher adsorption rate but a little lower adsorption capacity, indicating the influential factors to adsorption rate and adsorption capacity are different. The excellent adsorption performance of composites can be attributed to the combination effects of electrostatic interactions, hydrophobic interactions and \(\pi-\pi\) stacks toward the organic conjugation molecules. However, there is only van der Waal’s hydrophobic interaction for the adsorption on AC, which is relatively weak and results in a much slower adsorption rate and a smaller adsorption capacity [46,48]. It was reported that GO can act as a surfactant because of its hydrophilic edges and hydrophobic basal plane [13]. The dosage of iron source contributes to the extent of GO reduction. With the reduction, the increase of hydrophobic characteristic and yet partially retained hydrophilic groups must be one reason for the improved adsorption. A strong hydrophobic interaction toward RhB molecules induces the fast adsorption. Moreover, the enhanced \(\pi\) electrons in the conjugated structure of

![Fig. 7 Adsorption of RhB onto various adsorbents (a) and Langmuir isotherms of \(\text{Fe}_3\text{O}_4/\text{RGO}\) composites (b); inset in (b) is the Freundlich isotherms of AC and GO.](image-url)

| Adsorbent | Freundlich Model | Langmuir Model |
|-----------|------------------|----------------|
| \(n\)     | \(K_f\) (L/g)    | \(Q_{\text{max}}\) (mg/g) | \(Q_{\text{max}}\) (mg/gC) | \(K_L\) (L/mg) | \(R^2\) |
| M1        | 6.67            | 41.68 0.905    | 66.67 133.34   | 3.75 0.999 |
| M2        | 8.33            | 78.26 0.969    | 142.86 432.91  | 0.88 0.984 |
| M3        | 9.09            | 61.91 0.966    | 92.08 368.32   | 1.28 0.995 |
| M4        | 10              | 49.26 0.979    | 71.02 355.10   | 0.91 0.995 |
| AC        | 7.69            | 54.67 0.989    | 91.66 116.66   | 1.74 0.974 |
| GO        | 1.21            | 2.23 0.978     | 14.20 14.20    | 0.23 0.976 |

Table 1 Fitted parameters for RhB adsorption on different adsorbents
RGO lead to a facile $\pi-\pi$ stack with the molecules with aromatic groups. Considering the fastest adsorption kinetic and relatively higher adsorption capacity of M3, this composite was chosen for the following studies.

**Recycle and regeneration**

The recyclable property of the composites was investigated by the magnetically separable performance and the regeneration after heterogeneous Fenton-like reaction or methanol washing. Magnetic hysteresis of M3 was measured as shown in Fig. 8. The composite has a typical ferromagnetic hysteresis, with a saturated magnetization of 51.76 emu/g. It was known that Fe$_3$O$_4$ nanoparticles can be separated at a very low magnetic field due to its superparamagnetic behavior [17]. Incorporation of Fe$_3$O$_4$ nanoparticles with RGO would makes the composites be easily separated from aqueous solution.

If the adsorbent can be regenerated, the adsorbent could be repeatedly used for dyestuff wastewater purification with a low running cost. Traditional method for adsorbent regeneration is to eliminate the adsorbates by washing with a certain solution or calcining at a high temperature. However, these methods would be requiring a high cost, causing secondary pollution or time-consuming. A control test shows that the adsorbed RhB on M3 could not release into the water at a wide range of pH solution. However, the adsorbent can be regenerated by rinsing with methanol. The regenerated M3 by rinsing with methanol was used for RhB adsorption, and the adsorption performance is almost restored, as shown in Fig. 9. As we known, Fe$_3$O$_4$ in nano sizes can act as a heterogeneous Fenton-like catalyst [21,22]. According to the Haber-Weiss mechanism, the primary processes can be described by equation 4 to 6, in which the catalytic H$_2$O$_2$ decomposition includes the adsorption of H$_2$O$_2$, the reduction of Fe$^{III}$ and the formation of radicals by Fe$^{II}$ and Fe$^{III}$ species [50].

\[
\begin{align*}
\equiv \text{Fe}^{III} + \text{H}_2\text{O}_2 & \xrightarrow{k_{ads}} \equiv \text{Fe}^{III}(\text{H}_2\text{O}_2)_{ads} \quad (4) \\
\equiv \text{Fe}^{III}(\text{H}_2\text{O}_2)_{ads} & \xrightarrow{k_1} \text{Fe}^{II} + \text{HO}_2 + \text{H}^+ \quad (5) \\
\equiv \text{Fe}^{II} + \text{H}_2\text{O}_2 & \xrightarrow{k_2} \equiv \text{Fe}^{III} + \text{OH} + \text{OH}^- \quad (6)
\end{align*}
\]

Fe$_3$O$_4$/RGO composites could be a more suitable Fenton-like catalyst due to the enhanced dispersion of Fe$_3$O$_4$ nanoparticles over GO nanosheets and the concentrated RhB over the surface of adsorbents. To regenerate the adsorbent by Fenton-like reaction, the RhB saturated adsorbents were treated in H$_2$O$_2$ solution to remove adsorbed dye molecules. The final solution is colorless, indicating that adsorbed RhB was degraded and escaped from the adsorbents, which was further confirmed by the FTIR spectra, as shown in Fig. 10. Compared to the as-prepared M3, there are several obvious new bands in the spectrum of RhB saturated adsorbents, such as the bands at around 821, 1331 and 1460 cm$^{-1}$, corresponding to the rock vibration of -CH$_2$-, the stretching vibration of C-N, and the bending vibration of -CH$_2$-, respectively [46,51]. After Fenton-like reaction, these bands originated from RhB molecules have disappeared in the sample of M3$_F$, indicating that RhB molecules on the adsorbent have been degraded and removed. Furthermore, the regenerated M3$_F$ was employed for RhB adsorption to evaluate the adsorption capacity of the treated composites, as shown in Fig. 9. After twice treatments, although the RhB adsorption on M3$_F$ becomes slower, the removal efficiency of RhB is still more than 90% after 60 min adsorption by M3$_F$, indicating that the adsorption capacity of M3 can be retained after regenerated by the Fenton-like treatment method. Further optimizing the conditions in the Fenton-like reaction could obtain a promising regeneration technology for Fe$_3$O$_4$/RGO adsorbents.
Conclusions

Effective magnetic absorbents, Fe₃O₄/RGO composites have been synthesized by an in situ coprecipitation and reduction method. Fe₃O₄ nanoparticles in sizes of 10-20 nm are well dispersed over the RGO nanosheets. According to the adsorption kinetics and isotherms toward RhB, the composite with a Fe₃O₄ to RGO ratio of 2:1 shows the highest adsorption capacity and relatively faster adsorption rate, more than 3.7 times higher and thirty times faster than commercial active carbon.

Considering the monolayer RhB adsorption behavior by the Langmuir model isotherms, strong interactions between RhB molecules and RGO nanosheets, which depend much on the chemical components of RGO, contribute mainly to the surprising fast adsorption rates. Furthermore, with Fe₃O₄ nanoparticles as the heterogeneous Fenton-like catalysts, the adsorbed RhB can be degraded and removed from the adsorbents after a Fenton-like reaction, which could be developed into a promising regeneration way of adsorbents in the removal of organic molecules.

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A Bionic Fish Cilia Median-Low Frequency Three-Dimensional Piezoresistive MEMS Vector Hydrophone

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Abstract: A bionic fish cilia median-low frequency three-dimensional MEMS vector hydrophone is reported in this paper. The piezoresistive reasonable position was obtained through finite element analysis by ANSYS and the structure was formed by MEMS processes including lithography, ion implantation, PECVD and etching, etc. The standing wave barrel results show that the lowest sensitivity of the hydrophone is $-200$ dB and reach up to $-160$ dB (in which the voltage amplification factor is 300). It has a good frequency response characteristics in $25 \text{ Hz } \sim 1500 \text{ Hz}$ band. Directivity tests displayed that the hydrophone has a good “8”-shaped directivity, in which the resolution is not less than 30 dB, and asymmetry of the maximum axial sensitivity value is less than 1.2 dB.

Keywords: MEMS vector hydrophone; Bionic; ANSYS; Median-low frequency

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Introduction

With the development of underwater acoustical warfare, high frequency noise radiated by underwater motion platform has greatly reduced. Especially after anechoic tile using on majority of submarines, the working frequency of sensor has dropped to below 3 kHz, which makes the underwater acoustic detection to the median-low frequency [1,2]. To obtain the spatial gain with small scale sensor array at low frequency and precisely azimuth information as the underwater target, vector hydrophone is a best choice [3,4]. Mostly, traditional co-vibrating vector hydrophones adopt move coil or piezoelectric principle [5].

In recent years, the detection for underwater acoustic signals with a variety of new sensing mechanism was reported. For example, a PVDF film hydrophone made by Britain and France has been used in their submarines. Bionics is the application of biological methods and systems found in nature to the study and design of engineering systems and modern technology. The two-dimensional bionic vector hydrophone developed by North University in China is a new type of vector hydrophone, which has the advantages of small size, vector character, low-cost, easy installation, etc [6]. However, as a two-dimensional vector hydrophone, it is not useful in spatial localization. Therefore, there is an urgent need to develop a three-dimensional MEMS vector hydrophone.

In this paper, we developed a three-dimensional MEMS vector hydrophone based on fish cilia and piezoresistive principle. The detailed design, fabrication, and performance test are discussed in the following sections.

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Sensor design and analysis

The lateral line, as a sensory organ, is peculiar to fish and amphibians. Figure 1 shows the fish lateral line which consists of cilium shape mechanoreceptors or neuromasts. It is covered with a jelly-like cupola located on the skin or in the canals along the body. When the pressure of water is changed by acoustic waves, the fluid motion gets into pore through lateral line and delivers to mucus. This will cause the flowing of mucus which makes the displacement of hair cell. Sensory cells can be stimulated and the stimulation is transmitted to medulla oblongata by nerve fiber and the fish will react according to the signal.

According to the above fish lateral line structure, a three-dimensional MEMS vector hydrophone was designed as shown in Fig. 2. The sensor hair was imitated by rigid cylinder (X and Y direction) and long cantilever beam (Z direction). The sensory cells and efferent nerve were imitated by the piezoresistors and metal lead, respectively. Moreover, the piezoresistors of X and Y direction were linked by full wheat stone bridge. Whereas, the piezoresistors on each of two cantilever beam of Z direction were linked by half wheat stone bridge to form two half bridge, and the signal of two half bridges was added to make up for the deficiencies of low sensitivity of the half bridge.

According to the acoustics theory, when $k\alpha \ll 1$ ($k$ is wavenumber and $\alpha$ is the hydrophone radial size), the scattering of incident acoustic wave caused by vector hydrophone can be ignored. In the condition of far field, the vector hydrophone is equivalent to water particles, as shown in Fig. 3.

\[
\begin{align*}
    m_1 \vec{v}_1 &= m_1 \vec{v}_1' + m_2 \vec{v}_2
\end{align*}
\]

where, $m_1$ and $m_2$ is the mass of water particles and nerve fiber respectively; $\vec{v}_1$ is the vibration velocity of water particles before the collision; $\vec{v}_1'$ and $\vec{v}_2$ is respectively the vibration velocity of water particles and nerve fiber after the collision.

As shown in Fig. 4, the orthogonal decomposition of

\[
Z
\]

\[
X
\]

\[
Y
\]
\( \vec{v}_2 \) is \( \vec{v}_{2x}, \vec{v}_{2y}, \vec{v}_{2z} \),
\[
\begin{align*}
\vec{v}_{2x} &= v_2 \cos \theta \cos \alpha \\
\vec{v}_{2y} &= v_2 \sin \theta \cos \alpha \\
\vec{v}_{2z} &= v_2 \sin \alpha
\end{align*}
\]
so,
\[
\begin{align*}
\theta &= \arctan \left( \frac{\vec{v}_{2y}}{\vec{v}_{2x}} \right) \\
\alpha &= \arctan \left( \frac{\vec{v}_{2z}}{\sqrt{\vec{v}_{2x}^2 + \vec{v}_{2y}^2}} \right)
\end{align*}
\]

From all above we can come to the conclusion that the acoustic propagation characteristics can be detected by testing movement of the nerve fiber.

**Simulation and fabrication**

It is well known, the piezoresistive effect represents the change in electrical resistivity of a semiconductor when mechanical stress is applied. The piezoresistance expression of silicon cantilever is
\[
\Delta R/R = \sigma_l \pi_l + \sigma_t \pi_t
\]
For the piezoresistors with \( P \)-type (110) crystal orientation, \( \pi_l = 71.8 \times 10^{-11} \text{ Pa}^{-1} \), and \( \pi_t = -66.3 \times 10^{-11} \text{ Pa}^{-1} \), respectively. Generally, when external force acts on the cantilever, its shear stress \( \sigma_t \) can be neglected for far less than normal stress \( \sigma_l \). So the mechanical sensitivity of the vector hydrophone would be expressed as:
\[
S = 71.8 \times 10^{-11} \frac{\sigma_l V_{in}}{P}
\]  
(7)
In order to obtain a maximum sensitivity, the piezoresistor cannot be placed in the maximum stress area and the nonlinear area.

The stress of the MEMS hydrophone was analyzed using ANSYS where 1 Pa loads were added along the Y direction of the rigid cylinder and Z direction to the cantilever beam. The stress-contour of the structures are shown in Fig. 5(a) and Fig. 6(a), whereas, the stress curves of one beam obtained by path definition in ANSYS are shown in Fig. 5(b) and Fig. 6(b).

According to the piezoresistor distribution principle, the simulation piezoresistor distribution graph was marked in Fig. 5(b) and Fig. 6(b), where there were about 100 \( \mu \text{m} \) from the end of cross-beam and 200 \( \mu \text{m} \) from the roots of silicon cantilever. Moreover, the transverse stress of cross-beam X direction was approximated to zero in symmetrical distribution as shown in Fig. 5 and Fig. 6.

The MEMS hydrophone microstructure was fabricated by standard MEMS process using 4-inch SOI wafers with the electrical resistivity of 2~4 \( \Omega \cdot \text{cm} \), the active layer thickness of 20 \( \mu \text{m} \), the buried oxide layer thickness of 2 \( \mu \text{m} \) and handle wafer thickness of 400 \( \mu \text{m} \). The MEMS fabrication technological processes are
shown in Fig. 7, including oxidation, ICP etching, ion implantation, films growth and vapor plating processes.

**Bionic package and test**

Figure 8 shows the images of the hydrophone microstructure. To perform an underwater test of the MEMS vector hydrophone, the microstructure must be packaged to avoid damage. The package structure not only has a protective effect, but ensures the external sound signal to the maximum transfer to biomimetic cilia. According to the neuromast model shown in Fig. 1(c), bionic package structure was made. The bionic package structure is composed of two parts: transferal acoustic cap manufactured by the polyurethane [8,9] with better acoustic properties and silicone oil which is used to fill into the acoustic cap. The detailed package structure is shown in Fig. 9.

To verify the rationality and feasibility of the hydrophone structure, the receiver sensitivity and directivity pattern of the MEMS bionic vector hydrophone was investigated in the standing wave tube calibration device. Figure 10 shows the principle of calibration device. The receiver sensitivity test adopted the antitheses calibration method compared the voltage output of tested MEMS hydrophone with the reference hydrophone. The test frequency is in the range of 25 Hz-3.5 kHz. The vector hydrophone was fixed parallel to the acoustic wave propagation direction of the standing wave tube calibration device along direction X, Y and Z, respectively. Put the experiment data, which were recorded at the frequency of 1/3 octave band, into the formula [10],

$$M_x = M_0 e x \sin k d / e_0 \cos k d$$  \hspace{1cm} (6)

where $M_0$ is the receiving sensitivity of reference hydrophone, $e_x$ and $e_o$ is respectively the open-circuit
A mechanical rotary rod was used to rotate the vector hydrophone along horizontal axis in order to change the receiving direction of sound source. We recorded the vector hydrophone sensitive in each direction. When putting the test data into the following formula [11],

\[ L = 20 \log \left( \frac{e_\theta}{e_{\text{max}}} \right) \quad (7) \]

where \( L \) is the normalized data, \( e_\theta \) and \( e_{\text{max}} \) respectively the voltage of arbitrary direction and the maximum direction of the vector hydrophone, the directivity pattern can be obtained. Figure 12 shows the directivity pattern at the frequency 500 Hz. It can be seen there is a smooth “8” shape graph for the vector hydrophone. The directional resolution, which is the ratio of hydrophone axial maximum sensitivity with transverse minimum sensitivity at one certain frequency, is no less than 30 dB.

**Conclusions**

A median-low frequency three-dimensional MEMS vector hydrophone is presented in this paper. The hydrophone has the advantages of small size, simple
manufacturing and high sensitivity. According to the test results, the hydrophone has a good frequency response in the range of 25 Hz\textasciitilde1500 Hz, and its sensitivity can reach up to \(-180\) dB. Directivity tests displayed that the hydrophone has a good “8”-shaped directivity, whose resolution was not less than 30 B. The improvement of the hydrophone sensitivity and expanding its available band are under ongoing.

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Electrochemical Aptasensor Based on Prussian Blue-Chitosan-Glutaraldehyde for the Sensitive Determination of Tetracycline

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Abstract: In this paper, a novel and sensitive electrochemical aptasensor for detecting tetracycline (TET) with prussian blue (PB) as the label-free signal was fabricated. A PB-chitosan-glutaraldehyde (PB-CS-GA) system acting as the signal indicator was developed to improve the sensitivity of the electrochemical aptasensor. Firstly, the PB-CS-GA was fixed onto the glass carbon electrode surface. Then, colloidal gold nanoparticles (AuNPs) were dropped onto the electrode to immobilize the anti-TET aptamer for preparation of the aptasensor. The stepwise assembly process of the aptasensor was characterized by cyclic voltammetry (C-V) and scanning electron microscope (SEM). The target TET captured onto the electrode induced the current response of the electrode due to the non-conducting biomolecules. Under the optimum operating conditions, the response of differential pulse voltammetry (DPV) was used for detecting the concentration of TET. The proposed aptasensor showed a high sensitivity and a wide linear range of $10^{-9} \sim 10^{-5}$ M and $10^{-5} \sim 10^{-2}$ M with the correlation coefficients of 0.994 and 0.992, respectively. The detection limit was $3.2 \times 10^{-10}$ M (RSD 4.12%). Due to its rapidity, sensitivity and low cost, the proposed aptasensor could be used as a pre-scanning method in TET determination for the analysis of livestock products.

Keywords: Aptasensor; Tetracycline; Colloidal gold nanoparticle; Chitosan

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Introduction

Tetracyclines (TCs) have been extensively used as a bacteriostatic and antibiotic drug in livestock production to control bacterial infections and to increase the growth rate of animals owing to its broad-spectrum antibacterial activity [1]. Tetracycline (TET), oxytetracycline hydrochloride (OTC), chlortetracycline (CTC) and doxycycline hydrochloride (DXC) are members of the polyketide family, which includes a number of important pharmaceutical compounds. They are widely used as bactericidal agents in both human and veterinary medicine during recent decades [2]. However, the heavy use of TET and other TCs in aquaculture and farming industry may lead to the antibiotics residues in diary food products [3-6]. It has been concerned that the use of antibiotics in food-producing animals, particularly long-term use for growth promotion, contributes to the emergence of antibiotic-resistant bacteria in animals. These resistant bacteria may spread from animals to human beings via the food chain. They may also transfer their antibiotic-resistant genes into human pathogenic bacteria, leading to failure risk of antibiotic treatment for possibly life-threatening human conditions [7]. To safeguard human health, many organizations in the world have officially established a maximum antibiotics residues limit of 100 µg/kg in the food-producing species [8-10]. So it is of grave importance
to develop a quick and convenient method to test and identify antibiotics residues in livestock products.

Over the past decade, many studies had been established for the determination of TET residues. Kurittu et al. [11] used microbiological inhibition test technique to measure TET residues, but the test lacked specificity and sensitivity. Therefore, a lot of traditional analytical methods have been developed for the replacement of microbiological assays, such as high performance liquid chromatography (HPLC) [12-14], capillary electrophoresis (CE) [15,16], fluorescence (FL) [17,18] and mass spectrometric detection (MS) [19,20]. These traditional analytical methods are highly sensitive and specific, but they are not suitable for widespread and rapid detection. Because they need expensive equipments and complicated procedures for sample pretreatment. In view of these disadvantages, a simple, sensitive and selective method for the determination of TET residue is required.

In recent years, new rapid electrochemical methods for TET detection appeared frequently, such as immunosensors [21], enzyme sensors [22] and aptasensor [23]. Aptamers are nucleic acid ligands selected from random sequence pools in vitro, which can be naturally folded into different three-dimensional structures and have the capability of binding specifically to biosurfaces [24-27]. Aptamers against a specific target can be obtained by an iterative approach called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [25]. Compared to antibodies, aptamers have more advantages, such as low cost, stability, simplicity, easy modification, target versatility and can be synthetically manufactured. To the best of our knowledge, electrochemical aptasensors for TET detection have been applied to detect TET in the analysis of the food safety [23,28]. Kim et al. [28] developed a electrochemical aptasensor based on the screen printed gold electrode using square-wave voltammetry (SWV) technique for the detection of TET, but the proposed aptasensor suffered the drawbacks of low sensitivity, and the stability and reproducibility of the method were not discussed.

Nanometer materials, as the new modified sensing interface, can facilitate the immobilization of biomolecules and improve electrochemical properties of the transducer, such as owning low-background current, high signal to noise ratio and fast electron transfer [29]. Advanced inorganic nanoparticles are currently the most popular material with a wide range of application including optics, electricity and sensors [30]. Among various nanomaterials, colloidal gold nanoparticles (AuNPs) have gained considerable attention because of their large specific surface area, good biocompatibility and high surface free energy [31,32]. Chitosan (CS) possesses excellent membrane-forming ability, high permeability towards water, good adhesion, outstanding biocompatibility, nontoxicity, high mechanical strength and susceptibility to chemical modification due to the protonation and insolubility in solution with pH above pKa (6.3) for the deprotonation [33]. It possesses abundant primary amino groups, so it has a high affinity for Au.

In this work, we proposed a novel label-free electrochemical aptasensor based on prussian blue (PB) as the signal for the determination of TET. Herein, PB-chitosan-glutaraldehyde (PB-CS-GA) was synthesized for the first layer modificatory material of the electrode, which could adsorb AuNPs and act as “molecular wire” to facilitate the electron transfer. AuNPs serving as the second layer modificatory material were used for two purposes: (a) to further accelerate the electron transfer (b) to bond and immobilize the anti-TET aptamer onto the electrode via Au-N bond. TET was captured by the anti-TET aptamer immobilized AuNPs/PB-CS-GA modified electrode, which was folded into specific three-dimensional structures. Each step modification of the electrode was investigated by cyclic voltammetry (C-V), and the concentration of TET was analyzed by differential pulse voltammetry (DPV). Due to the inhibition ability of the charge transfer of non-conducting biomolecules, the decrease level of peak currents before and after the TET captured by anti-TET aptamer indicated the concentration of TET. The constructed aptasensor preparation process and determination ability toward its target were studied in detail and it was applied to determine TET in milk samples with satisfactory results.

Experimental

Materials

The anti-TET oligonucleotides purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) with the following sequences were designed by Niazi [34] and Zhou [23]: 5'-NH2-(CH2)5-GTG ACG GAA TTC GCT AGC CCC CCG GCA GGC CAC GGC TTG GGT TGG TCC CAC TGC GCG TGG ATC CGA GTA CGA CCA GTG G -3’ and probe aptamer 5’-GCA TGC CTT AAG CGA TCG GTG GGG GGC CGT CCG CAC CCA ACC AGG GTG ACG CGC ACC TAG GCT CGA GGT GCA C-6 FAM (FITC)-3’. Chloroauric acid (HAuCl4) and ethanol were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Chitosan (CS) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Phosphate buffer solution (PBS, pH = 7.4) was prepared from 1.45 g Na2HPO4·12H2O, 0.1 g KH2PO4, 0.1 g KCl and 4 g NaCl in 500 mL ultrapure water with its pH adjusted by HCl and NaOH solutions. The other chemicals were analytical reagent grade and used without further purification. Ultrapure water (18.2 MΩ-cm) was produced.
by Millipore (PALL, Germany).

Apparatus and measurements

The C-V and differential pulse voltammetry (DPV) were carried out on a CHI660D electrochemical workstation (Chenhua Co., Shanghai, China). A three-electrode system was selected to measure electrophenomenal response. Modified glass carbon electrode (GCE) (3 mm in diameter) was used as a working electrode. Platinum wire acted as auxiliary electrode, and saturated calomel electrode was used as a reference electrode. Scanning electron microscope (SEM) was carried out on JSM-6360LV SEM (Japan). Fourier transformation infrared (FT-IR) spectroscopy was recorded with a Nicolet 380 FTIR spectrometer (Thermo Fisher Scientific Co., USA).

Preparation of colloidal gold nanoparticles

The colloidal AuNPs were prepared according to the reference with slight modifications [35]. First of all, all glassware used in these preparations needed to be thoroughly cleaned in aqua regia (HCl:HNO₃ = 3:1). Then the glassware was rinsed with ultrapure water, and oven-dried prior to be used. Then 2.5 mL of 1% sodium citrate solution was quickly added to 100 mL 0.01% HAuCl₄ solution, and the mixture solution was brought to boiling with vigorous stirring. Then the color changed from pale-yellow to claret-red within 2 min. Keeping the solution boiling for 10 min, the mixture was continued to be stirred for another 10 min until the solution returning to room temperature. Finally, the colloidal AuNPs were stored in a refrigerator with a dark-colored glass bottle.

Preparation of CS-GA composites

1% (1 g/100 mL) chitosan solution was prepared by dissolving 1.0 g chitosan flakes into 100 mL 1.0% acetic acid and stirring for 3 h. Then 5% GA solution was added into the 1% CS solution by 10 min sonication to get CS-GA solution. The solution was stored in refrigerator.

Fabrication of the aptasensor

The GCE was sonicated in “piranha solution” (H₂SO₄:30% H₂O₂ = 1:1), and rinsed with ultrapure water before dried naturally. Next, the GCE was polished to a mirror-like appearance with 0.05 µm alumina slurry, and then sonicated in nitric acid (V/V, 1:1), ethanol and ultrapure water for 5 min, respectively. Firstly, the bare GCE was immerged in the CS-GA solution containing 2.5 mM FeCl₃, 2.5 mM K₃[Fe(CN)]₆, 0.1 M KCl, 0.01 M HCl with a constant potential of 0.4 V for electrodeposition for 300 s. PB was deposited onto the surface of the electrode when FeCl₃ (Fe³⁺) and K₃[Fe(CN)]₆ (Fe(CN)₆³⁻) were coexistant in the electrodeposition solution, and the reactions were as follows:

\[
\text{Fe}^{3+} + \text{e}^− \rightarrow \text{Fe}^{2+} \quad \text{Fe}^{2+} + \text{Fe(CN)}_{6}^{4−} \rightarrow \text{KF} \text{e}[\text{Fe(CN)}_{6}]
\]

After that, the electrode was cyclic scanned in the potential range from −0.2 to +0.6 V at the scan rate of 50 mV/s until the C-V curve stable, and the PB-CS-GA/GCE was obtained. Then 5 µL AuNPs were added onto the surface of PB-CS-GA/GCE and dried in the air at room temperature (noted as AuNPs/PB-CS-GA/GCE). Then the AuNPs/PB-CS-GA/GCE removed physically adsorbed colloidal AuNPs with ultrapure water and dried by nitrogen. 5 µL anti-TET aptamer of 5 mM was dropped onto the AuNPs/PB-CS-GA/GCE. Then a rubber cap was coated on the electrode to prevent evaporation of the aptamer solution. 4 h later, unbound anti-TET aptamer was removed by ultrapure water and then anti-TET/AuNPs/PB-CS-GA/GCE was passivated by 1 M ethanolamine (EA) for 1 h to obtain EA/anti-TET/AuNPs/PB-CS-GA/GCE (ethanolamine was used to remove non-specific adsorption on the anti-TET/AuNPs/PB-CS-GA/GCE surface). Finally, the EA/anti-TET/AuNPs/PB-CS-GA/GCE was rinsed with ultrapure water and stored at 4°C in refrigerator. The obtained electrode was used as the aptasensor in this work, and the stepwise assembly of the proposed aptasensor was shown in Scheme 1.

Hybridization of anti-TET aptamer with probe aptamer

5 µL anti-TET aptamer with 5 mM concentration was dropped onto the AuNPs/PB-CS-GA modified glass slide. 4 h latter, 2 µL probe aptamer (2 mM) was added and the hybridization reaction was carried out at room temperature in the dark environment for 2 h. The obtained sample was rinsed with 0.1 M Tris-HCl buffer to remove unbound probe aptamer, and then dried in the air atmosphere.

Electrochemical measurements

The electrochemical characteristics of the modified electrode were characterized by C-V and DPV in PBS (pH = 7.4). Electrochemical measurements were established in a conventional electrochemical cell. The formation of anti-TET/TET complexes was performed by dropped 10 µL of different concentration of the target TET on the EA/anti-TET/AuNPs/PB-CS-GA/GCE at 37°C for 1 h. After being carefully washed with ultrapure water to remove unbound TET, the electrode was characterized by DPV for the detection of TET. The DPV measurements were performed in the potential range from −0.1 V to +0.4 V with pulse amplitude of 50 mV.
Results and discussion

Sensing mechanisms of the aptasensor

In this study, the aptasensor was constructed by covalently attaching an amino-modified anti-TET aptamer on the AuNPs/PB-CS-GA coated GCE surface. It contained 76-base sequence and specifically binded to TET with high affinity. The AuNPs were employed as the carriers of the anti-TET aptamer to amplify the change of peak currents. The PB was used as a mediator to generate the electron flow between bulk solution and work electrode, which was shown in Scheme 1. The anti-TET aptamer modified electrodes presented a stable and free configuration on the GCE surface to give a significantly strong faradaic current. The formation of anti-TET/TET complex hindered the electron-transfer for the PB and Prussian white (PW) transition, thus producing a detectable decreasing signal. The electrochemical data analysis was carried out by the change of the DPV peak currents before and after the TET treatment in PBS (pH = 7.4). The reaction between PB and PW was as follows:

\[ \text{KFe}^{III}\text{[Fe}^{II}(\text{CN})_6] + e^- + K^+ \rightarrow \text{K}_2\text{Fe}^{II}\text{[Fe}^{II}(\text{CN})_6] \]

PB
PW

Morphology characterization of the aptasensor

The surface morphology of the PB-CS-GA/GCE and AuNPs/PB-CS-GA/GCE were characterized by SEM. The SEM image of the PB-CS-GA modified GCE was shown in Fig. 1(a). It exhibits the morphology with a rough, porous, and homogeneous layer with packed uniform particles. When the AuNPs were covered over the PB-CS-GA modified GCE, the surface exhibited a film with many globular features (Fig. 1(b)). Furthermore, to monitor anti-TET aptamer immobilizing process, fluorescent images before and after the probe aptamer assembly were indicated. In Fig. 1(c) and Fig. 1(d), the results showed that the FITC labeled anti-TET aptamer immobilized on AuNPs/PB-CS-GA modified glass released visible green fluorescence under the fluorescent microscopy, while the anti-TET aptamer covered AuNPs/PB-CS-GA modified glass exhibited no fluorescence.

Characterization of the aptasensor

The FT-IR was applied to character the formation and properties of the films. FT-IR spectra of CS, PB and PB-CS-GA were measured and shown in Fig. 2. It could be seen in FT-IR spectra of CS (Fig. 2(a)), the band around the wave number of 3400 cm\(^{-1}\) showed an obvious absorption peak which could be attributed to the stretching vibration of —OH or —NH groups. The absorption peaks around the wave number of 1560 cm\(^{-1}\) and 1400 cm\(^{-1}\) corresponded to the bending vibration of N—H and C—H, respectively. The FT-IR spectra in Fig. 2(b) showed stretching vibration of —CN group which was contained in molecule of PB from 2080 to 2040 cm\(^{-1}\). Figure 2(c) contained all the characteristic peaks appeared in CS and PB, and another typical absorbance band attributed to stretching vibration of —C==O group (around 1640 cm\(^{-1}\)) existing in GA, which indicated that the PB-CS-GA was successfully deposited onto the surface of the electrode.
Fig. 1 SEM images of (a) PB-CS-GA/GCE; (b) AuNPs/PB-CS-GA/GCE; Fluorescence microscope images of the anti-TET modified glass (c) and the probe aptamer hybridized with the anti-TET (d).

Fig. 2 The FT-IR spectra of (a) CS; (b) PB and (c) PB-CS-GA.

C-V is an effective and convenient technique for probing the feature of the modified electrode surface. Herein C-V was employed to investigate the electrochemical properties of each immobilization step. Figure 3 showed the C-Vs of different electrodes in the PBS buffer (pH = 7.4). No redox peak can be observed for the bare GCE (Fig. 3(a)). Figure 3(b) showed reversible oxidation-reduction peaks of PB in PBS. Compared to the bare GCE, the electrochemical response of the electrode indicated the conversion of PW and PB on the electrode. The current clearly increased when AuNPs were deposited on the electrode, indicating that the use of AuNPs significantly enhanced the conductivity of the electrode as a conductive wire or an electron-conducting tunnel (Fig. 3(c)). While the anti-TET aptamer was immobilized successfully on the modified electrode, the peak currents decreased (Fig. 3(d)). This was because the immobilized anti-TET aptamer hindered electron transmission. After EA was used to block nonspecific sites and the TET was captured by the anti-TET aptamer, the diffusion of the conductive ion participated in the reaction between PB and PW was hindered, so the peak current further decreased in turn (Fig. 3(e) and 3(f)).

Fig. 3 C-Vs of the bare GCE (a); PB-CS-GA/GCE (b); AuNPs/PB-CS-GA/GCE (c); anti-TET/AuNPs/PB-CS-GA/GCE (d); EA/anti-TET/AuNPs/PB-CS-GA/GCE (e) and TET/EA/anti-TET/AuNPs/PB-CS-GA/GCE (f) in the pH 7.4 PBS in the potential range of potential range from −0.2 to +0.6 V at scan rate of 50 mV/s.
Optimization of experimental conditions for aptasensor

Optimal concentration of the anti-TET for the aptasensor

The concentration of the anti-TET was optimized to obtain a high sensitivity of the fabricated electrochemical aptasensor. The effect of the concentration of anti-TET on ∆I (the decrease of DPV peak currents before and after the TET treatment) was investigated from 2 µM to 6 µM. The results were shown in Fig. 4(a). It could be seen that the ∆I of aptasensor increased rapidly with the increase of anti-TET concentration in the range of 2 ∼ 5 µM. After the concentration was higher than 5 µM, the ∆I almost kept constant, which indicated 5 µM anti-TET was sufficient to cover the AuNPs/PB-CS-GA modified GCE surface. Therefore, 5 µM anti-TET was selected as the optimum loading amount in this study.

Optimal incubation time for the aptasensor

The incubation time of the anti-TET with the TET was an important parameter for the aptasensor. It could be seen that a visible difference in the increase of ∆I with different incubation times. As shown in Fig. 4(b), the ∆I increased gradually with the augment of incubation time. However, when the incubation time reached 60 min, the signal response sustained a stable value. The result indicated that the interaction of aptamer with TET reached to equilibrium. Thus, the incubation time was controlled to be 60 min.

Performance of the aptasensor

Detection of TET

DPV technique could provide a better peak resolution and current sensitivity, which was very suitable for detecting the concentration of TET. The performed aptasensors were evaluated by the relationship between the ∆I and TET concentrations. Under the optimal experimental conditions, DPVs for TET detection were obtained. As shown in Fig. 5(a), the decrease of peak current was proportional to the logarithm of the TET

Fig. 4  Optimization of the experimental parameters: effects of (a) the concentration of the anti-TET and (b) the TET incubation time.

Fig. 5  (a) DPVs of the aptasensor incubated with different concentrations of TET (a-i: the concentrations of TET are 0 M, 1 × 10⁻⁹ M, 1 × 10⁻⁸ M, 1 × 10⁻⁷ M, 1 × 10⁻⁶ M, 1 × 10⁻⁵ M, 1 × 10⁻⁴ M, 1 × 10⁻³ M, 1 × 10⁻² M) were performed in the pH 7.4 PBS in the potential range from -0.1 V to +0.4 V with the parameters: pulse amplitude 50 mV, pulse width 0.2 s, pulse period 0.5 s; (b) The linear relationship between the peak current changes (ΔI) in the DPV and the logarithmic values of the TET concentrations. Error bars showed the standard deviations of measurements taken from three independent experiments.
concentration. In Fig. 5(b), curve a indicates the current variables of DPV showing a good linear relationship with the logarithmic values of the TET concentrations in two ranges of $1 \times 10^{-9} \text{M} \sim 1 \times 10^{-5} \text{M}$ and $1 \times 10^{-5} \text{M} \sim 1 \times 10^{-2} \text{M}$. The linear equation was $\Delta I (\mu\text{A}) = 5.738 + 0.386 \log C (\text{M})$ and $\Delta I (\mu\text{A}) = 14.464 + 2.094 \log C (\text{M})$, with a correlation coefficient of 0.994 and 0.992, respectively. The limit of detection (LOD) of TET was calculated as three times higher than the standard deviation for the average measurements of blank samples (LOD = 3 RSD/slope) [36]. Therefore, the LOD was determined to be as low as $3.2 \times 10^{-10} \text{M}$ with relative standard deviation (RSD) of 4.12%, lower than that of TET detection in other ways showed in Table 1. As comparison, the current responses of the aptasensor (without PB-CS-GA composite, EA/anti-TET/AuNPs/GCE, curve b in Fig. 5(b)) were recorded in 0.1 mol/L PBS (pH = 7.4) containing 5.0 mM K$_3$[Fe(CN)$_6$], and the linear range was $1 \times 10^{-8} \text{M} \sim 1 \times 10^{-2} \text{M}$ with the detection limit of $7.4 \times 10^{-9} \text{M}$. It was observed that the EA/anti-TET/AuNPs/PB-CS-GA modified electrode led a wider dynamic measurement range and a higher sensitivity compared with EA/anti-TET/AuNPs modified electrode. Based on the above work, we could conclude that PB mixed into CS-GA as the signal indicator could improve the sensitivity of the electrochemical aptasensor.

**The specificity of the aptasensor**

The specificity performance of the aptasensor for TET was further studied in this work. The selectivity test was carried out by measuring and comparing the response of TET (5 µM) to some possible interferences including a structurally similar tetracycline derivative OTC (5 µM) and two structurally distinct molecules of Kanamycin Monosulfate (5 µM) and Gentamycin Sulfate (5 µM). It could be observed in Fig. 6, the TET sample existed a significant peak current change, while the same concentrations of the other three chemicals had slight emissions. These tests indicated that the developed strategy could be used to identify TET with high specificity.

**Stability and reproducibility of the aptasensor**

The stability of the aptasensor was studied by comparing the $\Delta I$ after a storage period of 7, 14 and 21 days. The electrodes were stored in refrigerator at 4°C and $\Delta I$ was periodically measured. The response current retained above 96%, 93% and 89% of its initial response, respectively, which indicated the storage stability of the aptasensor was quite good.

The reproducibility of the aptasensor was evaluated by detecting the $\Delta I$ response with five electrodes prepared in the same way. The five aptasensors were incubated with $10^{-5} \text{M}$ TET, and an acceptable reproducibility with relative standard deviation (RSD) of 5.8% was obtained. The experimental result indicated the proposed aptasensor had a good reproducibility.

**Determination of TET in milk samples**

In order to evaluate the feasibility of the aptasensor system for possible applications, the standard addition method was used. The milk samples were prepared as follows: the milk was firstly diluted at the ratio of 1:10 and ultracentrifugated at 30 000 rpm for 90 min. In this way, the intermediate layer milk serum was obtained without fat and casein. Then we added TET to the collected TET-free milk serum to the final concent-

### Table 1  Comparison with other methods for the determination of TET

| Method of detection | Limit of detection (M) | Linear range (M) | References |
|---------------------|------------------------|-----------------|------------|
| Fluorescence optical fiber sensor | $1.06 \times 10^{-7}$ | $6.98 \times 10^{-7}$-$8.73 \times 10^{-5}$ | [37] |
| Molecularly Imprinted Technique | $5.58 \times 10^{-8}$ | $2.25 \times 10^{-7}$-$2.25 \times 10^{-5}$ | [38] |
| high-throughput suspension array technology | $3.4 \times 10^{-9}$ | $1.5 \times 10^{-8}$-$6.7 \times 10^{-6}$ | [39] |
| high pressure liquid chromatographic | $2.1 \times 10^{-8}$ | $-$ | [40] |
| aptasensor | $3.2 \times 10^{-10}$ | $1 \times 10^{-9}$-$1 \times 10^{-2}$ | this work |

Fig. 6 The specificity of the aptasensor in the presence of 5 µM TET, 5 µM OTC, 5 µM Kanamycin Monosulfate, 5 µM Gentamycin Sulfate.

Determination of TET in milk samples

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tration of $5 \times 10^{-8}$ M, $5 \times 10^{-6}$ M and $5 \times 10^{-4}$ M. As shown in Table 2, the recovery tests were performed using 3 replicates with the aptasensor that prepared in the same way. The recovery of 92~106% indicated that the aptasensor was available for the analysis of TET in milk samples.

| Sample | Added (M) | Total found (M) | RSD (%), n=3 | Recovery (%) |
|--------|----------|----------------|-------------|-------------|
| 1      | $5 \times 10^{-8}$ | $4.8 \times 10^{-8}$ | 5.3 | 96 |
| 2      | $5 \times 10^{-6}$ | $5.3 \times 10^{-6}$ | 6.2 | 106 |
| 3      | $5 \times 10^{-4}$ | $4.6 \times 10^{-4}$ | 5.4 | 92 |

Conclusions

In this work, a novel label-free aptasensor based on AuNPs/PB-CS-GA for sensitive determination of tetracycline was developed. The PB-CS-GA has performed higher sensitivity of the electrochemical aptasensor due to the PB as the signal indicator. In addition, the AuNPs/PB-CS-GA system showed good conductibility and biocompatibility and exhibited higher sensitivity and stability. Its electrochemical signal had a good linear relationship with the TET concentration in the range of $1 \times 10^{-9}$ M~$1 \times 10^{-5}$ M and $1 \times 10^{-5}$ M~$1 \times 10^{-2}$ M. This strategy contributed to improve the sensitivity and stability of aptasensor, thus, it provided a novel promising platform of aptasensor for TET detection.

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Temperature-dependent of Nonlinear Optical Conductance of Graphene-based Systems in High-intensity Terahertz Field

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Abstract: For multi-photon processed with the linear dispersion in the high-intensity terahertz (THz) field, we have systematically investigated the temperature-dependent nonlinear optical response of graphene-based systems, including single layer graphene, graphene superlattice and gapped graphene. In the intrinsic single layer graphene system, it demonstrates that, at low temperature, nonlinear optical conductivities of the third- and fifth-order are respectively five and ten orders of magnitude larger than the universal conductivity with high-intensity and low frequency THz wave. In the graphene superlattice and gapped graphene systems, the optical responses enhanced because of the anisotropic massless and massive Dirac fermions.

Keywords: Nonlinear; Graphene; Terahertz; Conductance; Transmittance

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Introduction

Graphene is a single atomic layer of carbon atoms tightly packed into a highly symmetric two-dimensional honeycomb lattice [1]. The spectrum of graphene quasiparticles, electrons and holes are described by an effective Dirac equation with a vanishing effective mass [2]. The massless energy dispersion of graphene quasiparticles leads to many unique physical properties, such as the electron-hole symmetry and a half-integer quantum Hall effect [3-5], a finite conductivity of $4 e^2/h$ even when the carrier concentration tends to zero [3], the strong suppression of weak localization [6-8] and ultrahigh carrier mobility [1]. Therefore, graphene is considered as a promising material for electronic applications.

The optical properties of graphene have been studied both experimentally [9-17] and theoretically [18-30]. In the terahertz to the far-infrared (FIR) spectral regime, optical conductance of graphene-based systems attracts many researchers’ interests due to the ongoing search on viable terahertz detectors and emitters. Graphene is traditionally a poor conductor in this part of the spectrum, with the universal conductivity $\sigma_0 = e^2/4h$ leading to absorb only 2.3% at normal incidence per graphene layer [11]. As discussed by Miknailov et al. [21,22], they developed a quasi-classical kinetic theory to predict the stronger nonlinear electromagnetic response of graphene under electromagnetic radiation at different frequencies ranging from microwaves to infrared. These studies show that irradiation of graphene by an electromagnetic wave of frequency $\omega$ should lead to excitation of odd high-harmonics (i.e. $3\omega, 5\omega, 7\omega, \cdots$). This may make graphene as a simple and natural frequency multiplier and open up exciting opportunities for using graphene in terahertz electronics. Subsequently, a series of theoretical works were carried out to seek for the strong nonlinear terahertz

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responses of graphene-based systems, such as single layer graphene (SLG) [23-25], bilayer graphene (BLG) [26], graphene p-n junction [27], graphene nanoribbons (GNRs) [28], semihydrogenated graphene (SHG) [29] and Kronig-Penney type (KP) graphene superlattice [30]. As studied by Hendry et al. in 2010 [15], they first measured the coherent nonlinear optical response of single- and few-layer graphene using four-wave mixing. Their results demonstrate that graphene exhibits a very strong nonlinear optical response in the near-infrared spectral region. The large optical nonlinearity originates from the interband electron transitions and is eight orders of magnitude larger than the nonlinearities observed for dielectric materials without such transitions. Recent experimental progress presented the results on studying the carrier dynamics in epitaxially grown graphene at higher photon energies from 10 to 250 meV [16], as well as the nonlinear THz response of graphene excited at electric field strengths in the nonperturbative regime [17].

The theoretical progresses have been shown that the linear energy dispersion of quasiparticles, electrons and holes in graphene leads to the strongly nonlinear optical response of this system in the terahertz to the FIR regime [21]. As a consequence, graphene is a rather strong nonlinear material. Wright and Xu et al. [23,24] adopted a quantum-mechanical approach to study the linear, third-order and fifth-order nonlinear optical response of intrinsic SLG under an electric field of around $10^5$ V/cm, which indicates these relative nonlinear terms make graphene a potential applications for photonic and optoelectronic device. Zhou et al. [25] also treated the same topic with more rigorous perturbation theory. Yee Sin Ang et al. [28, 29] investigated the optical conductivity of SHG and KP graphene superlattice. It has been shown that SHG possesses a unique potential as a two-color nonlinear material and the total optical response of the graphene superlattice was enhanced due to the formation of anisotropic Dirac fermions in the terahertz frequency region. It was also revealed that the band structure isotropy is not a prerequisite for the strong optical nonlinearity in graphene.

Previously letters only studied the third- and fifth-order nonlinear optical response to each graphene system with frequency respectively, and did not mention the temperature factors when calculating the relationship between the transmittance and the frequency in the terahertz regime. The main purpose of this paper is to theoretically investigate the temperature-dependent nonlinear optical conductance of different graphene-based systems in high-intensity terahertz field based on the simplified model for multiphoton process with linear dispersion. Firstly, we investigated the strong nonlinear optical response of intrinsic single layer graphene systematically, and obtained relationship between transmittance versus temperature and intensity. Then, we studied the nonlinear optical response of KP graphene superlattice, considering the anisotropy massless Dirac fermions. Moreover, the strong optical nonlinearity existed in gapped graphene was revealed.

**Theory and discussion**

**Massless Dirac fermions**

We adopted the approach that couples the massless Dirac fermions to the time-dependent electric field quantum mechanically to calculate the nonlinear terms, both in high-order electrical field and in multiple frequencies. When a time-dependent external field $E(t) = E_0 e^{i\omega t}$ is along the $x$-axis, the low energy tight binding Hamiltonian of graphene-based systems can be written as,

$$ H = v_F \begin{pmatrix} 0 & p_- + eA_x \\ p_+ + eA_y & 0 \end{pmatrix} $$

where $p_\pm = p_x \pm i\lambda p_y$, $A_\pm = A = (E_0/\omega)e^{i\omega t}$ and $v_F \approx c/300 = 10^6$ m/s is the Fermi velocity. $\lambda$ is an anisotropy parameter, which modifies the $y$-direction group velocity by $v_y = \lambda v_F$, and can be continuously tuned in the range of $0 < \lambda \leq 1$. The massless Dirac fermions is perfectly isotropic in all directions in the SLG which is the isotropic case $\lambda = 1$, but travels anisotropically with $v_F$ in the direction along the periodicity of the KP potential ($x$-direction) and reduced velocity of $\lambda v_F$ in the direction perpendicular periodicity ($y$-direction) in the KP graphene superlattice. The time-dependent two-component wave function can be expanded in the basis set,

$$ \psi(p,t) = \sum_{n=0}^{\infty} \begin{pmatrix} \alpha_n(p) \\ \beta_n(p) \end{pmatrix} e^{i(n\omega\tau - \varepsilon/\hbar)t} $$

where $\varepsilon = v_F|p|$, by substituting Eq. (1) and (2) into the Schrödinger equation $ih(\partial\psi/\partial t) = H\psi$. Due to the orthonormal relation of $e^{in\omega t}$, we can write the coupled recursion relations for the spinor components,

$$ \alpha_n = \frac{eE_0v_F}{i\omega\hbar} \times \frac{v_F p_- \alpha_{n-1} + (v_F p - n\omega)\beta_{n-1}}{n\omega(n\omega - 2v_Fp)} $$

$$ \beta_n = \frac{eE_0v_F}{i\omega\hbar} \times \frac{v_F p_+ \beta_{n-1} + (v_F p - n\omega)\alpha_{n-1}}{n\omega(n\omega - 2v_Fp)} $$

The recursion relation couples the $n$ photon processes to the $n - 1$ photon processes. From the solutions to Eq. (3) we can calculate the $n$th order total current,

$$ J_n = \frac{1}{2\pi^2} \int N(\varepsilon) p d\varepsilon d\theta Re \left( \sum_{n=0}^{\infty} \alpha_i^* \beta_{n-i} + \beta_i^* \alpha_{n-i} \right) $$

where $N(\varepsilon) = n_F(-\varepsilon) - n_F(\varepsilon) = \tanh(\varepsilon/2k_B T)$ is the temperature factor.
The linear \((n = 1)\) optical conductivity of graphene-based systems, which is a single photon process where an electron absorbs a photon making a transition from the valence band to the conduction band is described as,

\[
\sigma_1 = \sigma_1(\omega) = \frac{\sigma_0}{\lambda} N \left( \frac{\hbar \omega}{2} \right)
\]  

(5)

Because of time-reversal symmetry, the second and fourth order solutions make no contributions to the total current.

The third-order nonlinear \((n = 3)\) optical conductivity is made up of two components: (i) \(\sigma_3(\omega)\), single-frequency term which corresponds to the absorption of two photons and the simultaneous emission of one photon; and (ii) \(\sigma_3(3\omega)\), triple-frequency term which corresponds to the simultaneous absorption of three photons, is described as,

\[
\sigma_3 = \sigma_3(\omega) + \sigma_3(3\omega) = \frac{\sigma_0}{\lambda} \frac{2e^2 v_F^3 I}{\varepsilon_0 c \hbar^2 \omega^3} \left[ N_{51}(\omega)e^{i\omega t} + N_{53}(\omega)e^{3i\omega t} \right]
\]  

(6)

where \(I(W/cm^2)\) is the intensity and \(\varepsilon_0\) is vacuum permittivity. The temperature factor are given by \(N_{53}(\omega) = 13/48 N(\hbar \omega/2) - 2/3N(\hbar \omega)+15/16N(3\hbar \omega/2)\) and \(N_{51}(\omega) = 2N(\hbar \omega)\).

The fifth-order nonlinear \((n = 5)\) optical conductivity is made up of three components: (i) \(\sigma_5(\omega)\), single-frequency term which corresponds to the absorption of three photons and the simultaneous emission of two photons; (ii) \(\sigma_5(3\omega)\), triple-frequency term which corresponds to the absorption of four photons and the simultaneous emission of one photon; (iii) \(\sigma_5(5\omega)\), five-frequency term which corresponds to simultaneous absorption of five photons, is described as,

\[
\sigma_5 = \sigma_5(\omega) + \sigma_5(3\omega) + \sigma_5(5\omega) = \frac{\sigma_0}{\lambda} \frac{4e^4 v_F^4 I^2}{\varepsilon_0^2 c^2 \hbar^4 \omega^5} \left[ N_{51}(\omega)e^{i\omega t} + N_{53}(\omega)e^{3i\omega t} + N_{55}(\omega)e^{5i\omega t} \right]
\]  

(7)

where

\[
\begin{align*}
N_{51}(\omega) &= \frac{159}{256} N \left( \frac{3\hbar \omega}{2} \right); \\
N_{53}(\omega) &= \frac{2}{15} N(\hbar \omega) - \frac{867}{15360} N \left( \frac{3\hbar \omega}{2} \right) + \frac{16}{135} N(2\hbar \omega); \\
N_{55}(\omega) &= \frac{413}{23040} N \left( \frac{\hbar \omega}{2} \right) - \frac{2048}{23040} N(\hbar \omega) + \frac{6102}{23040} N \left( \frac{3\hbar \omega}{2} \right) - \frac{8192}{23040} N(2\hbar \omega) + \frac{4925}{23040} N \left( \frac{5\hbar \omega}{2} \right).
\end{align*}
\]  

(8)

Considering both the linear and nonlinear (the third and fifth-order) terms, the total interband optical conductivity can be described by the formula,

\[
\sigma_{\text{inter}}(\omega) = \sigma_1 + \sigma_3 + \sigma_5
\]

\[
= \sigma_0 \left\{ N \left( \frac{\hbar \omega}{2} \right) + \frac{2e^2 v_F^3 I}{\varepsilon_0 c \hbar^2 \omega^3} \left[ N_{51}(\omega)e^{i\omega t} + N_{53}(\omega)e^{3i\omega t} + N_{55}(\omega)e^{5i\omega t} \right] \right\}.
\]  

(9)

For intrinsic single layer graphene \((\lambda = 1)\), the temperature dependence of linear \(\sigma_1\) and third-order nonlinear optical conductivities \(\sigma_3(\omega)\) and \(\sigma_3(3\omega)\) in unit of \(\sigma_0\) is plotted in Fig. 1(a) at a frequency of 1 THz under the intensity of 53 W/cm\(^2\). We can find that the two nonlinear terms of the third-order conductivity decrease with temperature. At low temperature, the nonlinear term \(\sigma_3(3\omega)\) less than the linear conductance \(\sigma_1\), and then stays as the same as the \(\sigma_1\) even at room temperature. And the \(\sigma_3(\omega)\) is greater than the linear conductance in the whole temperature regime. But when the frequencies below 0.7 THz, all the two third-order nonlinear terms are larger than the linear conductance in the whole temperature regime, and decrease rapidly with temperature than at 1 THz.

Figure 1(b) shows the intensity dependence of the third-order nonlinear optical conductance. It is demonstrated that when the intensity below 100 W/cm\(^2\), the nonlinear term is so tiny that can be neglected. The ratio of third-order nonlinear optical conductivity and \(\sigma_0\) increases with the intensity and the growth rate gradually slowed down. In the same intensity, the ratio decreases with increasing frequency. For high intensity and low frequencies, the nonlinear terms dominate; on the contrary, the linear terms dominate. The nonlinear conductance can be around an order of magnitude larger than the linear conductance when the intensity is 4000 W/cm\(^2\).

Figure 2(a) shows the temperature dependence of linear \(\sigma_1\) and fifth-order nonlinear optical conductivities \(\sigma_5(\omega)\), \(\sigma_5(3\omega)\) and \(\sigma_5(5\omega)\) in unit of \(\sigma_0\) at a frequency of 1 THz under the intensity of 120 W/cm\(^2\). We can find that the three nonlinear terms decrease with increased temperature, and the \(\sigma_5(\omega)\) decreases rapidly than the other two terms. At the low temperatures, the nonlinear term \(\sigma_5(3\omega)\) is less than the linear conductance, and then stays as the same as the linear conductance even at room temperature. The \(\sigma_5(\omega)\) is larger than the linear conductance in the whole temperature regime. But the \(\sigma_5(5\omega)\) is less than the linear conductance and about one order magnitude lower than the \(\sigma_5(\omega)\) in the low temperature regime. However, when the frequencies below 0.7 THz, all fifth-order nonlinear terms are larger than the linear conductance in the whole
Fig. 1 For the SLG, (a) The linear and third-order nonlinear conductivity vs temperature for frequency of 1 THz and 0.7 THz; the intensity is 53 W/cm²; (b) The third-order nonlinear conductivity vs intensity for frequency of 0.6, 0.8, 1 THz at room temperature.

Fig. 2 For the SLG, (a) The linear and fifth-order nonlinear conductivity vs temperature for frequency of 1 THz and 0.7 THz; the intensity is 120 W/cm²; (b) The fifth-order nonlinear conductivity vs intensity for frequency of 0.6, 0.8, 1 THz at room temperature.
temperature regime, and decrease rapidly with increased temperature than those behave at 1 THz. Figure 2(b) illustrates the intensity dependence of the fifth-order nonlinear optical conductance. It is shown that when the intensity is weak, the nonlinear term is so tiny that can be neglected. The ratio of fifth-order nonlinear optical conductivity and $\sigma_0$ increases rapidly with the intensity which is faster than the third-order terms. Contrasting with Fig. 1(b) we can discover that the fifth-order nonlinear conductance can be about three orders magnitude larger than the third-order nonlinear conductance when the intensity is 4000 W/cm$^2$.

In general, we find that the nth order conductivity term is proportional to the parameter $Z = (ev_F l^{0.5}/\hbar)^n$. This means that at sufficiently high intensity and low frequency, nonlinear terms are relevant. To the present THz experiments, the value of intensity 1000 W/cm$^2$ is not so large for THz pulses, but it is very large for continuous wave (CW) sources. For example, if CW THz laser generated 1 THz with a spot diameter 300 micron, it would need around 1 W power to achieve this intensity. For lower frequency, it would require more power because the spot size should inevitably increase. However, with the development of THz technologies, this intensity value will eventually reach. Such a high-intensity THz wave yields non-negligible numbers of photo-generated electrons and holes. In the absence of fast recombination processes, the photo-generated electrons and holes should increase the distribution functions and, thus, decrease the nonlinear conductivities. As a result, the actual nonlinear conductance is not so large above the intensity 1000 W/cm$^2$.

In order to contrast the third- and fifth-order nonlinear optical response more effectively, we plot the frequency dependent on the third- and fifth-order nonlinear optical conductance of SLG in unit of $\sigma_0$ for two different temperatures in Fig. 3. The intensity is 1000 W/cm$^2$. All nonlinear terms decrease rapidly with increased frequency. When frequencies below 1 THz, the nonlinear term dominates and the fifth-order nonlinear terms exceed the third-order nonlinear terms, which is about two orders of magnitude larger than that behaves in 0.5 THz. The huge nonlinearities in the intensity below 1 THz are associated with the strong interaction of carriers with low-energy photons, which is characterized by the perturbation Hamiltonian inversely proportional to the photon energy. Within the frequency range 1.5–3 THz, the third-order nonlinear response is stronger than the fifth-order nonlinear response; however, they are so tiny that can be neglected completely. At the intensity around 1000 W/cm$^2$ and $f = 1$ THz, the value of $Z = 10$ for third-order conductance and $Z = 120$ for fifth-order conductance. However, for $f = 3$ THz, the resulting at about $Z = 10^{-1}$ for third-order and $Z = 10^{-2}$ for fifth-order nonlinear conductance, which is so small in the higher frequency that can be totally neglected.

![Fig. 3](image_url)

In Fig. 4(a) we plotted the total interband optical conductivity of the frequency with $I = 1000$ W/cm$^2$ at $T = 0$ K and 300 K, respectively. For frequencies below 0.5 THz, because the third- and fifth-order nonlinear terms play an important role compared with the linear term; the total interband optical conductivity is strongly decrease with frequency, about $10^5 \Omega^{-1}$ at 0.1 THz, which is more than five orders of magnitude larger than the universal conductivity. In the high-frequency part of the terahertz regime, the nonlinear terms play so small roles in the total interband optical conductivity that can be neglected completely. In the frequency range 3–10 THz, the total interband optical conductivity is almost unchanged with the frequency nearly equal to the universal conductivity $\sigma_0 \approx 6.08 \times 10^{-5} \Omega^{-1}$.

We plotted the temperature dependent the total interband optical conductance with $I = 1000$ W/cm$^2$ for three different frequencies in Fig. 4(b). In the same frequency, the total interband optical conductance decreases with increasing temperature. The total interband optical conductance at 0 K is around two orders of magnitude higher than the total interband optical conductance at 300 K for a frequency of 0.1 THz, and is about an order magnitude larger than that behaves at 1 THz. The large optical nonlinearity originates from the interband electron transitions and the temperature-dependence of the nonlinear conductivities is due to the decrease in the electron and hole distribution functions at integer multiples of half the photon energy. In the high-frequency part of the terahertz regime, the total interband optical conductivity is nearly equal to $\sigma_0$ at zero temperature and decreases slightly at room temperature.
Optical transitions in graphene include interband and intraband transitions. Based on linearization of the tight binding Hamiltonian of graphene near the Dirac points of the first Brillouin zone, its total optical conductivity can be expressed as the sum of the two contributions,

$$\sigma(\omega) = \sigma^{\text{inter}}(\omega) + \sigma^{\text{intra}}(\omega)$$  \hspace{1cm} (10)

The Drude model can estimate the intraband optical conductivity, which can be approximated as follows,

$$\sigma^{\text{intra}}(\omega) = \frac{ie^2E_F}{\pi\hbar^2(\omega + i\tau^{-1})}$$  \hspace{1cm} (11)

where $\tau$ is the relaxation time, the Fermi level $E_F$ is determined by the carrier concentration $n_0 = (E_F/hv_F)^2/\pi$. The linear intraband conductivity associated with intraband transitions can be described as follows,

$$\text{Re}(\sigma_1^{\text{intra}}) = \frac{e^2}{2\pi\hbar} \left( \frac{E_F}{\hbar} \right) \frac{\tau}{1 + (\omega\tau)^2}$$  \hspace{1cm} (12)

At the intensity of 1000 W/cm$^2$, the generation rate of electrons and holes is about $10^{13}$ cm$^{-2}$ in 1 ps. For the relaxation time of 1 ps, the part of the linear intraband optical conductivity of SLG is around 0.13 $\Omega^{-1}$ at 0.1 THz and 0.0044 $\Omega^{-1}$ at 1 THz. Thus, the intraband conductivity is much weak than the interband conductivity that can be neglected completely.

According to the relation between the real part of optical conductivity and the transmittance [31], the transmittance of intrinsic SLG as follows,

$$T_r = \frac{4\varepsilon_0^2}{(2\varepsilon_0 + \sigma(\omega)/e)^2}$$  \hspace{1cm} (13)

In Fig. 5, considering the third- and fifth-order nonlinear responses and the temperature factor, we plot the transmittance versus the temperature for a frequency of 0.1, 0.2, 0.3 and 1 THz respectively with $I = 1$ W/cm$^2$. At low frequencies (0-0.2 THz), the transmittance is strongly decreased in the entire temperature regime by the nonlinear terms effect, in which the fifth-order terms play more important roles than the third-order terms. The transmittance decreases with decreasing temperature, and almost all absorbed at zero temperature for 0.1 THz. While the frequency is greater than 0.3 THz, both the third- and fifth-order nonlinear optical responses play so small roles in the total conductivity, consequently the transmittance is nearly equal to a constant slightly larger than 97.7% at room temperature.

Figure 6 illustrates the transmittance versus the intensity for a frequency of 0.1, 0.5, 1 and 10 THz respectively at room temperature. Considering the fifth-order nonlinear responses, the transmittance decreases quickly with increased intensity. When the intensity is around $10^3$ W/cm$^2$ and the frequency below 0.5 THz, the single layer intrinsic graphene is almost entirely absorption. However, in the high-frequency part of the terahertz regime, the transmittance is nearly equal to a constant slightly larger than 97.7%, which is the same as the situation in the visible regime, and almost not changed by the increase of the intensity.

In the KP graphene superlattice ($0 < \lambda < 1$), the group velocity of quasiparticle no longer travels with uniform $v_F$ in all directions. From the Eq. (6) and (7), the third- and fifth-order nonlinear optical conductances of graphene superlattices are all universally enhanced by a factor of $1/\lambda$. In Fig. 7(a), the third-order nonlinear optical conductivities $\sigma_3(\omega)$ and $\sigma_3(3\omega)$ at
Fig. 5  The transmittance of intrinsic SLG vs temperature for frequency of 0.1, 0.2, 0.3 and 1 THz; the intensity is 1 W/cm².

Fig. 6  The transmittance of intrinsic SLG vs electric field for frequency of 0.1, 0.5, 1 and 10 THz at room temperature.
$T = 300$ K and $I = 1000$ W/cm$^2$ are plotted for different $\lambda$. In comparison to the isotropic case (SLG), the nonlinear responses of graphene superlattice are universally enhanced by the anisotropy factor when the external field aligns with the superlattice periodicity. The temperature dependence of the third-order nonlinear optical conductivities is plotted in Fig. 7(b) at 1 THz and the intensity of 1000 W/cm$^2$. The two nonlinear terms decrease with increasing temperature, which is the same as the isotropic case but enhanced by a factor of $1/\lambda$. Figure 7(c) shows the intensity dependence of the third-order nonlinear optical conductance for different $\lambda$. For the KP graphene superlattice, the $y$-direction group velocity is reduced to $v_y = \lambda v_F$, the $y$-component momentum $p_y$ in an equi-energy slice becomes larger, so the overall larger momentum of the charge carrier is the reason for the enhancement of the optical conductance. In a more general case, when $E$ is in any angle $\phi$ as measured from the $x$-axis, the combine effect of the $x$-directional $\sigma(x)$ and $y$-directional $\sigma(y)$, the total angular dependence conductivity, measured along the $\phi$-direction, as follows, $\sigma(\phi)^2 = \sigma(x)^2 + \sigma(y)^2$, the ratio as follows, $\sigma_n(\phi,j\omega)/\sigma_n(j\omega) = (\cos^2(\phi)/\lambda^2 + \lambda^2 \sin^2(\phi))^{1/2}$. The $\phi$-dependence of the total third-order nonlinear conductivity is demonstrated in Fig. 7(d). The maximum conductance occurs at $\phi = 0^\circ, 180^\circ$ when the $E$ is along the $x$-axis main enhanced by the factor of $1/\lambda$. When the $\phi$ in the vicinity of $90^\circ$, $\sigma(y)$ becomes dominant and the influence of the factor $\lambda$ over the factor of $1/\lambda$.

**Massive Dirac fermions**

The graphene with a finite band gap that could be created via breaking the symmetry of A and B sublattices in graphene-based system, the low-energy quasiparticles into massive Dirac fermions which are described by the finite-mass Dirac equation $H_\Delta = \sigma_x p_x + \lambda \sigma_y p_y + (\Delta/2) \sigma_z$. For the typical band gap value of $\Delta = 0.03$ eV, the linear and third-order nonlinear optical conductivities at low temperature are plotted in Fig. 8. It is shown that the gapped graphene also exists a strong nonlinear optical response by the three-photon...
process $\sigma_3(3\omega)$ even when the energies lower than the band gap (sub-gap frequency regime) where the linear response is forbidden. And by changing anisotropy parameter, the nonlinear optical response is similarly tuned-up $1/\lambda$. So this kind of gapped graphene as two-color nonlinear material can be applied to nonlinear optics since the linear process is fully suppressed in the terahertz regime.

Fig. 8 The linear and third-order nonlinear optical conductivities of gapped graphene at low temperature 77 K. The intensity is 1000 W/cm$^2$.

**Conclusion**

Depends on a theoretical investigation of the linear and nonlinear (the third- and fifth-order) optical conductivity, we have shown that graphene-based systems exhibit strong nonlinear optical response in the terahertz regime. Our results demonstrate that at low temperature, the third- and fifth-order nonlinear optical conductivities in the intrinsic single layer graphene system are more than five and ten orders of magnitude respectively, larger than the universal conductivity at the temperature, third- and fifth-order nonlinear optical responses make the transmittance of 1 THz. In addition, the strong temperature-dependent intensity around 1000 W/cm$^2$, larger than the universal conductivity at the terahertz regime. Our results demonstrate that at low temperature, the third- and fifth-order nonlinear optical responses make the transmittance of 1 THz. In addition, the strong temperature-dependent intensity around 1000 W/cm$^2$, larger than the universal conductivity at the terahertz regime.

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A Cationic [60] Fullerene Derivative Reduces Invasion and Migration of HT-29 CRC Cells in Vitro at Dose Free of Significant Effects on Cell Survival

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Abstract: Nanomaterials with unique characteristics exhibit favorable therapeutic and diagnostic properties, implying their enormous potential as biomedical candidates. C₆₀ has been used in gene- and drug-delivery, as imaging agents, and as photosensitizers in cancer therapy. In this study, the influences of a cationic functionalized fullerene on cellular behavior of human colorectal cancer cell line (HT-29) were investigated. Results indicated that HT-29 treated with the studied compound showed a lower sensitivity but a significant impairment in migration and invasion by interfering with the activities of matrix metalloproteinases (MMP-2 and 9). The presence of fullerene also altered the capacity of adhesion-related proteins to perform their activity, thereby inducing dramatically adverse effects on the cell physiological functions such as cell adhesion. Thus, our study suggests that this compound is a new potential anti-metastatic effector and a therapeutic component for malignant colorectal cancer.

Keywords: Fullerene; Cancer; Anti-metastatic drug; Nanomedicine

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Introduction

Current status of anticancer chemotherapy of solid malignant tumors indicates the necessity for agents active against tumor metastases. Metastasis, the spread of a primary tumor from its initial location to distant localities, is the main cause of death in cancer patients. Colorectal cancer (CRC) can lead to metastasization and represents one of the leading causes of cancer-related mortality [1]. Its progression to a highly advanced, metastatic stage (mCRC) still decreases the overall 5-year survival to less than 8-10% [2].

The rapid development of nanotechnology and its applications has allowed for a wide variety of nanoparticles to provide a broad range of opportunities in multidisciplinary fields and particularly in medicine, for clinical therapy and diagnosis [3]. Recent reports show that carbon nanomaterials, in particular fullerenes, inhibit various angiogenic signalling pathways and, therefore, can be potentially used in anti-angiogenic therapy [4-7]. Based on these properties, we might expect that fullerenes can have significant effects on tumour metastases either preventing their formation or inhibiting their growth. In this context it might be interesting to note that the endohedral metallofullerenol Gd@C₈₂(OH)₂₂ has been demon-
strated to interfere with the neoplastic growth, as well as with tumour metastasis, in a mouse cancer model, with almost no toxicity to normal cells in vivo and in vitro [8]. Also the water-soluble pristine (unmodified) C₆₀ inhibits the transplantable malignant Lewis lung carcinoma growth and metastasis in C57Bl/6J male mice [7]. Limited knowledge exists on the capacity of the fullerenes to interfere with tumor invasion and the mechanism involved in the fullerene anti-metastatic effect remains to be elucidated. To explore this further, the effect of fullerene on key steps of tumor metastasis, including cell adhesion, migration and invasion, was investigated.

Our previous studies demonstrated that a cationic fullerene derivative (C₆₀+), a fulleropyrrolidinium salt, was able to effectively inhibit tumor cell proliferation in vitro [9] and, recently published data from our laboratory, obtained by RNA-sequencing on MCF7 cells [10], indicated that the expression profile of several proteins involved in cell-cell adhesion junctions was altered when treated by C₆₀+. Therefore, the question if compound C₆₀+ plays a role in the regulation of migration and adhesion of HT-29 colorectal cell line naturally arose.

The present study examined the hypothesis that derivative C₆₀+ participates in colorectal cancer cell invasion. Cells treated with it exhibited significant impairment in a series of preliminary migration and adhesion assays in vitro. Thus, compound C₆₀+ may be a new potential anti-tumor effector and therapeutic component for malignant colorectal cancer.

## Experimental

The synthesis and the characterization of the fullerene derivative C₆₀+ was performed as previously described [9,11].

The human colorectal carcinoma cell line were purchased from the ECACC N° 86012803 (HT-29). HT-29 was maintained in RPMI-1640. The culture medium was supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine 2 mM; cells were grown at 37°C in a 95% air and 5% CO₂ humidified incubator.

HT-29 were harvested by trypsinization and plated into 96-well culture plates at approximately 1.5 x 10⁴ per well. After incubated for 24 h, different concentrations of C₆₀+ (1, 5, 10, 25 and 40 µM) dissolved in culture medium were added to each well. Then the samples were incubated 48 h at 37°C in the humidified atmosphere (5% CO₂). The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to assess the metabolic activity of cells treated as described above. 20 µl stock MTT (5 mg/mL) were added to each well, and cells were then incubated for 4 h at 37°C. Cells were lysed with isopropanol HCl 0.04 N. Absorbance was measured at 540 and 630 nm using a microplate reader (Automated Microplate Reader EL311, BIOTEK® Instruments, Vermont, USA). All measurements were done in six replicates, and at least three independent experiments were carried out.

A 96-well plate was pre-coated with fibronectin, collagen I, collagen IV, Poly-L-lysine, laminin (Sigma-Aldrich, St. Louis, USA) and Matrigel™ (20 µg/mL) for 4 h at 37°C or 4°C overnight and subsequently blocked with PBS-BSA 0.1% (w/v) for 15 min at 37°C. Sub confluent tumor cells were treated with C₆₀+ (25 µM) for 48 h and then grown in serum-free medium for additional 24 h. Cells were trypsinized with 1 mM EDTA, resuspended in serum free medium with 0.1% BSA for 30 min at room temperature to ensure re-expression of integrins on the cell surface and seeded in the plate (5 x 10⁴ cells/well). HT-29 were allowed to attach to each substrate for 1 h at 37°C and subsequently fixed with trichloroacetic acid 10% (v/v) for 1 h at 4°C and stained with sulphorhodamine B 0.4% (w/v). The absorbance was read at 570 nm and related to the adhesion rate.

Effects of the C₆₀+ to inhibit cancer cells motility were tested performing a conventional Boyden chamber assay.

HT-29 cells, sown 72 h before, were left in serum-free medium containing 0.1% bovine serum albumin (BSA) for 24 h after being treated with C₆₀+ (25 µM) for 48 h, at 37°C. At the end of the treatment, 5 x 10⁴ cells were sown in 200 µL of serum-free medium containing 0.1% BSA, in the upper side of a polystyrene-free polycarbonate filter (6.5-mm diameter and 8-µm pore size) set in a Transwell® cell culture chamber (Corning Costar Italia, Milan, Italy) in triplicate. The lower compartment was filled with the appropriate culture medium, supplemented with 10% FBS. Plates were left in the incubator for 72 h, at 37°C, 5% CO₂, 100% relative humidity. At the end of the incubation, cells that had not invaded were mechanically removed from the upper surface of the filter by wiping them with a cotton bud. Cells that had migrated to the lower surface were fixed with 1.1% glutaraldehyde for 15 min, washed with deionised water and air-dried. Transwells® were then stained with 0.1% crystal violet in 200 mM borate buffer, pH 9.0 for 20 min at room temperature. After washings with deionised water and complete drying, the dye was dissolved in 10% acetic acid and the absorbance was read at 590 nm by a SpectraCount spectrophotometer (Packard Bell, Meriden, CT, USA).

Invasive capability was measured in a Transwell® cell culture chamber (Corning Costar Italia, Milan, Italy) according to a method modified from Albini et al. [12]. In brief, the surface of a polystyrene-free polycarbonate filter (6.5-mm diameter and 8-µm pore size)
size) was coated with 30 µg per 50 µL of Matrigel® (Beckton Dickinson, Bedford, MA, USA) diluted in DMEM and air-dried overnight at room temperature. The filters were reconstituted with DMEM immediately before use. HT-29 cells, sown 72 h before, were left in serum-free medium containing 0.1% bovine serum albumin (BSA) for 24 h after to being treated with C60+ (25 µM) for 48 h, at 37°C. At the end of the treatment, cells were harvested with trypsin–EDTA, and 5 x 10⁴ cells were sown in 200 µL of serum-free medium containing 0.1% BSA, in the upper compartment chamber, in triplicate. The lower compartment was filled with the appropriate culture medium, supplemented with 10% FBS. Plates were left in the incubator for 96 h, at 37°C, 5% CO2, 100% relative humidity. At the end of the incubation, the plate was processed as described in the migration assay.

To perform gelatin zymography, supernatant was collected from sub confluent cell culture treated for 48 h with C60+ and left 24 h in serum-free medium. Proteins present in the supernatant were concentrated by centrifugation and quantified using Nano Drop spectrophotometer. 180 µg of total mixture of proteins were separated on 10% polyacrylamide gel containing 0.06% gelatin (Sigma-Aldrich, St. Louis, USA). Then the gel was washed for 30 minutes using 2.5% Triton X-100 solution to remove SDS and then incubated in an activating buffer (50 mm pH 7.5 TRIS, 5 mM CaCl₂, 0.2 M NaCl) for 20 h at 37°C. Gelatinase activity was demonstrated by gel staining with 0.5% Commassie blue and destaining with 30% methanol solution and 10% acetic acid. To assess activity, the stained gel was photographed and analyzed using ImageJ software.

Data were subjected to computer-assisted analysis by Graph Pad Instat 3 and statistical significance or regression analysis, were reported in the text.

Results and discussion

Fullerene derivative C₆₀⁺ inhibited HT-29 proliferation

The effects of C₆₀⁺ (Fig. 1(a)) on the viability of HT-29 cells after 48 h challenge with concentrations ranging from 1 to 40 µM are reported in Fig. 1(b). C₆₀⁺ caused a statistically significant reduction of cell viability (about 40% inhibition), as determined by the MTT test only at the highest dose tested, being the other dosages only marginally capable to influence HT-29 cell growth during the experiment. This result is consistent with unpublished data showing different degrees of cytotoxicity of this fullerene derivative in vitro on a number of tumor cell lines: MCF7 cells were particularly sensitive to the cytotoxic effects [9], whereas other cell lines, e.g. MDA-MB231, were much less sensitive, as reported here for HT-29 cells. The scenario depicted by our RNA-seq analysis suggested that C₆₀⁺ induced an alteration of the gene expression targeting mTOR signaling at different levels in MCF7 cell line [10]. Among the many factors potentially determining an inhibition of mTOR signaling, we proposed a decrease of available cellular energy as the most plausible. We here hypothesize that the mechanism of action of C₆₀⁺ in the HT-29 cell line could be the same, given that HT-29 represents a cell line with high in vitro invasive and in vivo metastatic behavior.

Cell adhesion analysis

Cell adhesion to the extracellular matrix (ECM) is an important step that regulates different cellular functions including cell migration and proliferation, differentiation, and tissue organization [13]. This event is mediated by cell surface receptors, the integrins, α and β chain heterodimers with short cytoplasmic tails, the enzymatic activity of which activates a variety of intracellular signalling pathways [14]. Integrins were shown to be involved in the processes of cancer cell invasion and metastasis [15], and, most notably, the integrin-ECM interactions were also demonstrated to have a role for cell survival and resistance to chemotherapy in many types of solid cancers, including colon cancer [16].

In order to mimic the detachment of metastatic cells from the primary mass and their adhesion in a secondary site, we performed a study where HT-29 cells
were exposed to C$_{60}^+$ at 25 µM for 48 h, then harvested from the plate and allowed to adhere to different substrates components of the ECM such as laminin, fibronectin and collagens. Data reported in Table 1 show that C$_{60}^+$ causes a similar inhibition of the adherence ability of HT-29 cells independently whether the substrate used was a component of the ECM, an unspecific substrate (poly-L-lysine) or no substrate at all. However, in all the cases, the HT-29 cells, after such treatment, showed a marked reduction (60-70% inhibition) of their ability to adhere as compared to untreated cells.

Table 1 Inhibitory potential of C$_{60}^+$ to the interaction between HT-29-ECM substrates. After treatment with C$_{60}^+$, cells were seeded on 96-well plate functionalized with fibronectin, collagen I and IV, laminin, poly-L-lysine and Matrigel (20 µg/mL) and left to adhere 1 h at 37°C with 5% CO$_2$. Unbound cells were removed while adhered cells were fixed, stained with sulphorodamine B and absorbance read at 570 nm. Results obtained for each substrate are expressed as percentage of adhesion inhibition of treated HT-29, considering the untreated HT-29 adhesion value as 100%

| Substrate         | % Inhibition of Adhesion by C$_{60}^+$ |
|-------------------|---------------------------------------|
| No substrate      | 78                                    |
| Poli-L-lys        | 69                                    |
| Laminin           | 63                                    |
| Fibronectin       | 66                                    |
| Collagen I        | 68                                    |
| Collagen IV       | 62                                    |

This effect might be related to a number of hypotheses. Integrins might be activated by extracellular events, aside from the intracellular signaling events [17], such as ligand binding, divalent cation concentration, mechanical stress, all of them potentially perturbed by the fullerene treatment. However it must be taken into account also the possible effect of this compound on cell viability that, although not leading to cell death, might keep the treated cells to a lower stage of “biological activity”.

Cell migration, invasion and zymography assays

Migration and invasion are two essential steps of the pathological events leading to cancer metastasis. We studied cell migration using a conventional Boyden chamber assay (Fig. 2(a)).

HT-29 cells, treated with C$_{60}^+$ for 48 h, were left to migrate for 72 h from the upper chamber through the insert, following the chemotactic signal (serum) coming from the lower side of the chamber. Data reported in Fig. 2(a) shows a pronounced (80%) and statistically significant reduction of the migration ability of the treated cells as compared to control constituted by untreated HT-29 cells.

Similar effects were obtained when we studied the invasion process. In this case, cells were seeded on a layer of matrigel previously deposited on the Boyden chamber grid and evaluated after 96 h. Compound C$_{60}^+$ inhibited more than 75% the capacity of the treated HT-29 cells to cross the matrigel barrier and to accumulate in the lower compartment of the chamber (Fig. 2(b)).

The compared analysis of the effects of C$_{60}^+$ on migration and invasion suggests that this compound does not reduce the capacity of HT-29 cells to degrade the ECM. In fact, it seems that the effects of the considered fullerene derivative on these cells might consist of a more general reduction of the cells ability to move or a...
reduced capacity to respond to the chemotactic signal, operated by the serum present in the lower wall. On the contrary, the treated cells have a reduced gelatinolytic ability, as shown by a study of MMP2 and MMP9 activity, performed on the supernatant obtained by the HT-29 cells exposed to C_{60}+ at 25 µM for 48 h (38-42% inhibition, Fig. 3), suggesting that the treated cells have a lower capacity to degrade the ECM.

![Image](http://dx.doi.org/10.5101/nml.v6i2.p163-168)

Fig. 3 Gelatinolytic activity of MMP-2 and MMP-9 was evaluated in HT-29 cells treated with C_{60}+ at 48 h.

The activation of the zymogen form of MMP2 (pro-MMP2) is a cell-surface event that is mediated by members of the membrane-type (MT) subfamily of MMPs as MT1-MMP and by the tissue inhibitor of metalloproteinase (TIMP-2), a member of the family of MMP inhibitors [18,19]. It has been demonstrated that this inhibitor directly interacts with MMP2 or through MT1-MMP/TIMP-2 complex formation reducing the enzymatic activity of MMP2 [20-22].

Recently we found that C_{60}+ increases 4-folds the expression level of TIMP-2 after 24 and 48 h of treatment [10] in the human breast cancer cell line MCF7. This result could suggest a similar effect of this compound also on HT-29 colon cancer cell line and a consequent reduced gelatinolytic activity of MMP2 detected after treatment of cells with fullerene C_{60}+.

Moreover the complex pro-MMP2 and TIMP-2 is able to inhibit other MMPs such as MMP9 through the formation of a ternary complex pro-MMP2-TIMP-2-MMP9 [23-25]. This data suggest that the reduced MMP9 activity detected in treated HT-29 cells, could be due to the increased level of TIMP-2 after C_{60}+ treatment and its modulation on MMP9 activity via MMP2 involvement.

Conclusions

The in vitro study of fullerene derivative C_{60}+ on the ability of HT-29 cells to migrate and invade, events that mimic important steps of the process of metastasis formation, showed promising effects. The compound, in fact, inhibited the capacity of HT-29 cells to re-adhere, to migrate and to invade and these effects were, at least in part, associated with the decrease of the gelatinolytic activity of the treated cells. The treated HT-29 cells did not present significant differences in the adherence process in presence of the various ECM substrates and this seems to rule out an effect of C_{60}+ on cell integrins. Rather, it seems that C_{60}+ might act on a more generalized pathway responsible for multiple effects of the metastatic capacity of the cells.

Anyhow, these data, although preliminarily and considering the role attributed to phenomena such as adhesion and migration for the process of metastasis formation suggest the ability of this fullerene derivative to interfere with metastases of solid tumors, in agreement with other data already reported in the literatures [7,8].

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A Novel Photothermal Nanocrystals of Cu$_7$S$_4$ Hollow Structure for Efficient Ablation of Cancer Cells

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Abstract: Cu$_{2-x}$S nanocrystals (NCs), characterized by low cost, low toxicity, high stability and high photothermal conversion efficiency, provide promising platforms as photothermal agents. Herein, a novel two-step synthesis has been developed for Cu$_7$S$_4$ nanocrystals with hollow structure using the as-prepared copper nanoparticles as starting solid precursor followed by hot-injection of sulfide source. The Cu$_7$S$_4$ NCs exhibit intense absorption band at Near-infrared (NIR) wavelengths due to localized surface plasmon resonance (LSPR) mode, which can effectively convert 980 nm-laser energy into heat. Moreover, the localized high temperature created by Cu$_7$S$_4$ NCs under NIR irradiation could result in efficient photothermal ablation (PTA) of cancer cells in vivo, demonstrating a novel and promising photothermal nanomaterials.

Keywords: Semiconductor nanocrystals; Near-infrared absorption; Photothermal

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Introduction

Near-infrared (NIR)-laser-driven photothermal ablation (PTA) therapy has attracted great attention in recent years as a minimally invasive and potentially more effective treatment alternative to conventional approaches [1-16]. In order to promote the photothermal conversion efficiency and gain access to efficient PTA therapy, it is the developing novel photothermal conversion agents that we are focusing on. Currently, four types of photothermal agents have been extensively developed in PTA therapy. The first type are organic compounds (e.g., indocyanine green (ICG) dye [1] and poly aniline nanoparticles [2]), which may suffer from limitations such as photobleaching or unsatisfactory photothermal conversion efficiency. The second type are carbon nanomaterials (including carbon nanotubes [3-5] and graphene [6-9]) whose absorption coefficient in the NIR region is usually not as high as expected. The third type are noble metal nanostructures, such as nanorods [10-12], nanoshells [13,14], nanocages [15,16], nanostars [17] and Pd- nanosheets [18]. These noble metal nanostructures exhibit intense NIR photoabsorption, and also are the most widely explored kind of photothermal agents. However, their practical applications are limited by the high cost. Alternatively, copper-containing semiconductor nanocrystals, characterized by low production cost, high stability, low toxicity, and high photothermal conver-
experimental photothermal agents [19-23]. CuS nanoparticles [24], CuS superstructures [21], Cu₇S₄ nanocrystals [19], and Fe₃O₄@Cu₂₋ₓS [22], Cu₀S@SiO₂ [20] nanocomposites have been developed as 980 nm laser-driven photothermal agents for efficient PTA of cancer cells in vitro and in vivo. To meet the severe requirements of future PTA therapy, it is still necessary to develop novel kind of copper chalcogenides based photothermal agents. Herein, we have developed a novel two-step method for preparation of Cu₇S₄ nanocrystals with hollow structure and investigated them as a potential of 980 nm-laser-driven photothermal agents.

Experimental

Characterization: Sizes, morphologies, and microstructures of the samples were determined by field emission transmission electron microscope (TEM, JEOL, JEM-2100F). Powder X-ray diffraction (XRD) was conducted by a D/max-2550 PCX-ray diffractometer (Rigaku, Japan). UV-Vis-NIR absorption spectra were measured on a UV-2550 Spectrophotometer (Shimadzu, Japan).

Synthesis of Cu₇S₄ NCs: All of the chemicals were bought from Aladdin and are analytically pure, which were used as received without further purification. The preparation of copper NCs followed a modified procedure [25]. Briefly, 0.5 mmol of copper acetylacetonate was dissolved in 15 mL of oleylamine in a 100 mL flask, which was purged with dry nitrogen gas to remove residual water and oxygen. The reaction mixture was heated to 230°C at a rate of 5°C/min by a heating mantle while being magnetically stirred and then kept at 230°C for 10 min to form copper NCs. Subsequently, a certain amount of sulfur (e.g. 0 mmol, 0.1 mmol, 0.2 mmol, 0.3 mmol, 0.5 mmol and 0.6 mmol) dissolved in 5 mL of oleylamine was quickly injected into the solution. The resulting mixture was maintained at 230°C for 30 min. After the solution was cooled to room temperature, the Cu₇S₄ NCs were purified by washing with n-hexane. Subsequently, the Cu₇S₄ NCs dispersed into 15 mL of chloroform for later use.

Synthesis of polymer-modified Cu₇S₄ NCs: The as-prepared Cu₇S₄ NCs were coated with an amphiphilic hydrolyzed poly (maleic anhydride) pre-modified with dodecylamine according to a literature report [23,28]. In a 50 mL round bottom flask, 530 µL of the amphiphilic polymer stock solution (0.8 M monomer units in CHCl₃), 5 mL of the above Cu₇S₄ NCs chloroform solution and 25 mL of anhydrous CHCl₃ were combined and vortexed with magnetically stirring for 45 minutes at room temperature. Subsequent rotary evaporation of the solvent resulted in a dark-brown film of polymer coated Cu₇S₄ NCs attached to the inner wall of the flask. 10 mL of aqueous sodium borate buffer (SBB, pH12) was then added to the flask and subject to ultrasonication for 15 min. After phase transfer from chloroform to aqueous solution, the hydrophilic Cu₇S₄ NCs were collected by centrifugation. The aqueous solution of the Cu₇S₄ NCs was stored at 4°C for later use.

Measurement of photothermal performance: A 980 nm laser was delivered through a quartz cuvette containing aqueous dispersion (0.3 mL) of the aqueous solution containing Cu₇S₄ NCs with different concentrations (i.e., 6.25, 12.5, 25, 50, 80, 100 and 150 µg/mL). The light source was an external adjustable power (0-0.8 W) 980 nm semiconductor laser device laser module (Xi’an Tours Radium Hirsh Laser Technology Co., Ltd. China). The output power density was independently calibrated to be 0.72 W/cm² using a handy optical power meter (Newport model 1918-C, CA, USA). A thermocouple with an accuracy of 0.1°C was inserted into the aqueous dispersion of Cu₇S₄ NCs, which was oriented perpendicular to the path of the laser beam. The temperature was recorded one time per 5 s.

In vitro cytotoxicity of Cu₇S₄ NCs

Hepatocarcinoma cell lines Hep3B cells were seeded in a 96-well plate at a density of 1 × 10⁴ cells/well for 24 h at 37°C in 5% CO₂ to allow the cells to attach. The culture medium was changed and cells were incubated with complete medium containing the Cu₇S₄ NCs at a series of concentrations (i.e., 50, 100, 150, 200 and 250 µg/mL) at 37°C with 5% CO₂ for further 24 h. After that, 0.1 mL of 3-(4,5)-dimethylthiahiazo-2-yl)-2,5-diphenyldiazotetrazolium bromide (MTT) solution (5 mg/mL, Sigma, Cat.M2003) was added to each well of the microtiter plate and then was incubated in the CO₂ incubator for 4 h. The cells then were lysed by the addition of 100 µL of dimethylsulfoxide (DMSO). The spectrophotometric absorbance of formazan was measured using a plate reader at 570 nm. Four replicates were done for each treatment group.

In vivo photothermal ablation of cancer cells

Severe combined immunodeficiency (SCID) mice were inoculated subcutaneously with 4 × 10⁶ hepatocarcinoma cell lines Hep3B cells on the left side of the rear leg 4-5 weeks before the experiments. When tumors grew to 8-12 mm in diameter, the SCID mice were randomly labeled as control and treatment samples. Two nude mice were first anaesthetized by trichloroacetaldehyde hydrate (10%) at a dosage of 40 mg/kg body weight, and then the treatment and control were injected with 0.15 mL of PBS solution containing the Cu₇S₄ NCs (150 µg/mL) and saline, respectively, at
the central region of the tumors with a depth of ∼4 mm. After 2 h, the tumors of the control and treatment group were irradiated with 980 nm laser (an output power density of 0.72 W/cm²) for 10 min. After that, the two SCID mice were scarified and tumors were removed, embedded in paraffin, and cryosectioned into 4 µm slices. These slides were stained with hematoxylin/eosin, the examined under a Zeiss Axiovert 40 CFL inverted fluorescence microscope, and images were captured with a Zeiss AxioCam MRc5 digital camera.

**Results and discussion**

Cu$_7$S$_4$ nanocrystals (NCs) were synthesized by a two-step method, as shown in Fig. 1. As a first step, copper nanoparticles were synthesized using oleylamine as reducing agents, according to reported method [25]. Then the as-prepared copper nanoparticles were used as solid precursor, which could react with sulfide source introduced by hot-injection. As a result, the hollow-structure Cu$_7$S$_4$ NCs could be successfully fabricated.

The copper nanoparticles were prepared without injection of sulfide source (e.g. 0 g). Figure 2(a) showed the typical transmission microscopy (TEM) image of the copper nanoparticles. The particles are spherical and have an average diameter of 15 nm. The high magnification of TEM images (Fig. S1) showed that the copper nanoparticle was crystalline with a certain amount of surface oxidation. As the copper nanoparticle show a high activity to react with oxygen [25], the oxidation likely occurred before XRD measurement when the specimen was exposed to air. Therefore, the crystalline structures of the copper nanoparticles were indexed to CuO (JCPDS card No. 44-0706), as shown in Fig. S3. The resulting UV-vis spectrum of the copper nanoparticles (Fig. S2) exhibited a well-defined surface plasmon absorption peak occurred at around 580 nm, which was consistent with previous reports, confirming the successful synthesis of copper nanoparticles [25].

The resultant copper nanoparticles with a uniform diameter could be used as a sacrificial solid precursor for the synthesis of Cu$_7$S$_4$ NCs with hollow structure by injection of an appropriate sulfide source (e.g. 0.6 mmol). Compared to copper seeds synthesized from the first step (Fig. 2(a)), the diameter of Cu$_7$S$_4$ NCs, after 30 min aging of the second injection of sulfide source, were within an average size of 15-30 nm, as shown in Fig. 2(b). Moreover, the solid nanoparticles were converted into some hollow structures, as shown in high magnification of TEM (Fig. 2(c) and 2(d)). This hollowing process could be explained by the Kirk end all diffusion [26]. The diffraction peaks of the hollow nanoparticles prepared with the S source of 0.6 mmol (Fig. 3) could be indexed with those of Cu$_7$S$_4$ (JCPDS 50 nm 100 nm 50 nm 5 nm S 200°C 50 nm 5 nm
Encouragingly, the Cu$_7$S$_4$ NCs dissolved in chloroform showed an intense absorption band in NIR region owing to the localized surface plasmon resonance (LSPR) mode, as shown in Fig. 4. Specifically, the Cu$_7$S$_4$ NCs exhibit a minimum of absorption around $\sim$670 nm and an increased absorption with the increase of wavelength with the maximum absorption peak of $\sim$1100 nm, in analogy to the reported Cu$_{2-x}$S [19,24]. This stronger absorption in NIR region of Cu$_7$S$_4$ NCs prepared by injecting sulfide source makes them a potential 980 nm-laser-driven photothermal agents for ablation of cancer cells.

We also investigated the effect of sulfide amount on crystal structure and morphology of copper sulfide nanoparticles. The resultant copper nanoparticles, as sacrificial templates, were used to react with sulfide source with different doses (e.g. 0.1 mmol, 0.2 mmol, 0.3 mmol and 0.5 mmol). The corresponding X-ray diffraction (XRD) patterns were displayed in Fig. 3. The diffraction peaks of all as-prepared Cu$_7$S$_4$ NCs could be indexed with those of Cu$_7$S$_4$ (JCPDS card No. 33-0489), confirming the successful preparation of Cu$_7$S$_4$.

![Fig. 3 XRD of the Cu$_7$S$_4$ NCs prepared with different sulfide source.](image)

![Fig. 4 UV-Vis-NIR absorbance spectrum of chloroform solution containing the Cu$_7$S$_4$ NCs prepared with the sulfide source of 0.6 mmol.](image)

![Fig. 5 TEM images and high magnification of TEM of Cu$_7$S$_4$ NCs prepared with the sulfide source of ((a), (b)) 0.1 mmol, ((c), (d)) 0.2 mmol, respectively.](image)
nanocrystals (orthorhombic, space group P62/nma JCPDS No.33-0489) from Cu nanoparticles by injecting of different doses of sulfide source. It seems that the amount of sulfide source has no obvious effect on the crystal structure of final product. It should also be noted that the difference in morphology and size can be largely dependent on the amount of sulfide source. When adding 0.1 mmol of sulfide source, the obtained nanocrystals with spherical shape and relatively uniform diameter of 25 nm, as shown in Fig. 5(a) and 5(b). With the increase of the sulfide source from 0.1 mmol to 0.2 mmol, the uniformity of size decreased. Specifically, two obvious different sizes (including average diameter 15 and 35 nm) of nanocrystals coexisted in the final product, as shown in Fig. 5(c). Moreover, the nanoparticles exhibit obvious hollow structure, as shown in Fig. 5(d). Further increase of sulfide source to 0.3 mmol resulted in more triangular morphology existing in the product as shown in Fig. 6(a) and 6(b). Besides, 0.5 mmol of sulfide source could lead to less obviously hollow core structure in final products as shown in Fig. 6(c) and 6(d).

Due to their strong absorption in NIR region, the Cu$_7$S$_4$ NCs prepared with 0.6 mmol of sulfide source were chosen as an example to investigate the photothermal conversion performance and the potential as 980 nm-laser-driven photothermal agents. The as-synthesized Cu$_7$S$_4$ NCs were passivated with oleylamine and could not be dispersed into aqueous or physiological solution, which limited these nanocrystals applying directly to biological system. To obtain hydrophilic Cu$_7$S$_4$ NCs, an amphiphilic polymer was coated on the surface of Cu$_7$S$_4$ NCs by hydrophobic self-assembly [23, 28]. The polymer-modified Cu$_7$S$_4$ NCs could be easily dispersed in water or phosphate buffered saline (PBS) at pH 7.4.

The photothermal conversion performance of the Cu$_7$S$_4$ NCs was examined under the irradiation of 980 nm laser with power density of 0.72 W/cm$^2$ by detecting the extent of temperature increase, as shown in Fig. 7. Pure water was used as a negative control. The temperatures of all the Cu$_7$S$_4$ NCs increased with the irradiation time, and the temperature increased more rapidly with increasing the concentration of Cu$_7$S$_4$ NCs. The temperature of Cu$_7$S$_4$ NCs aqueous dispersion (e.g. 150 µg/mL) could be elevated by 15.7°C with the irradiation of 980 nm laser for 400 s, compared with pure water (i.e., 0 µg/mL) that was only increased by less than 4°C. These confirmed the Cu$_7$S$_4$ NCs can rapidly and efficiently convert the 980 nm-laser energy into thermal energy. Moreover, with the increase of the concentration (i.e., from 6.25 to 12.5, 25, 50, 80 and 100 µg/mL), the temperature of the Cu$_7$S$_4$ NCs aqueous dispersion could be increased by 6.5, 8, 8.7, 10.1, 12.2 and 14.1°C, respectively (Fig. 7(b)). As is well-known, hyperthermic therapy takes advantage of heat between 40 and 45°C to damage cancer cells [29]. Assuming that the temperature of in vivo human body is 37°C, tumor region injected with an aqueous dispersion of Cu$_7$S$_4$ NCs can easily be heated to over 45°C within 400 s by irradiation with a 980 nm laser (0.72 W/cm$^2$), which probably efficiently induces cancer cells death [29].

The influence of the Cu$_7$S$_4$ NCs on the viabilities of cancer cells were used to evaluate the biocompatibility
The Cu$_7$S$_4$ NCs with various concentrations (i.e., 50, 100, 150, 200 and 250 µg/mL) were incubated with Hepatocarcinoma cell lines Hep3B cells for 24 h and then the cell viabilities were tested by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. There was no obvious adverse effect on cell viabilities, even the concentration reaching 250 µg/mL, as shown in Fig. 8, which meant a low cytotoxicity induced by Cu$_7$S$_4$ NCs.

To evaluate photothermal ablation effect of cancer cells, two nude mice bearing evident tumors were randomly divided into control and treatment group. The control mouse was injected with 200 µL of saline in the central region of the tumor, while the treatment mouse was injected with 200 µL of PBS containing Cu$_7$S$_4$ NCs (e.g., 150 µg/mL). After 2 h, the tumors of the two mice were irradiated with 980 nm of laser for 10 min (see detail in experiment section and the experimental setup shown in Fig. S4). Subsequently, PTA effects of tumor tissues in mice were confirmed by histological examination (hematoxylin/eosin (H&E) staining), as shown in Fig. 9. In the case of control group, there were little change regarding the cells’ sizes and shapes, nuclear modifications as shown in Fig. 9(a) and 9(c). In the case of treatment group, under the same irradiation conditions, the common signs of thermal cell necrosis are presented on considerable regions of the examined tumor slide (Fig. 9(b)). Furthermore, more destruction of the tumor cells, such as shrinkage of the malignant cells, loss of contact, cosinophilic cytoplasm, and nuclear damage could be observed from Fig. 9(d). These facts suggested that in vivo cancer cells could be efficiently destroyed by the high temperature arising from the excellent photothermal effect of Cu$_7$S$_4$ NCs. Therefore, it is safe to conclude that Cu$_7$S$_4$ NCs have great potential to be used as an excellent photothermal agent for PTA therapy.

### Conclusion

Using the copper nanoparticles as a starting precursor, we have successfully synthesized Cu$_7$S$_4$ NCs with hollow structure, which exhibited intense absorption band at NIR wavelengths due to LSPR. The hydrophilic Cu$_7$S$_4$ NCs coated with amphiphilic polymer could effectively convert 980 nm-laser energy into heat and further destroy cancels cells in vivo. Therefore, these Cu$_7$S$_4$ hollow NCs demonstrated great superiority as an excellently potential photothermal agent, as a result of their small size and high photothermal conversion efficiency as well as their low cost.

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Fig. 9  (a)-(d) Representative H&E stained histological images of in vivo tumor sections treated by the irradiation of 980 nm laser (0.72 W/cm²) over a period of 10 min injected with ((a), (c)) saline and ((b), (d)) PBS solution of the Cu7S4 NCs (150 µg/mL), respectively.

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Supporting information

A Novel Photothermal Nanocrystals of Cu$_7$S$_4$ Hollow Structure for Efficient Ablation of Cancer Cells

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Supplementary figures

Scheme S1 The high magnification of TEM images of copper nanoparticles.

Scheme S2 UV-vis-NIR absorbance spectra of the Cu nanoparticles prepared with S source (e.g. 0 mmol).

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Scheme S3  XRD of the CuO prepared with S source (e.g. 0 mmol).

Scheme S4  Photograph of the experimental setup for \textit{in vivo} photothermal ablation of cancer cells.
Fabrication, Characterization and Thermo-physical Property Evaluation of SiC Nanofluids for Heat Transfer Applications

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Abstract: Nanofluids (NFs) are nanotechnology-based colloidal suspensions fabricated by suspending nanoparticles (NPs) in a base liquid. These fluids have shown potential to improve the heat transfer properties of conventional heat transfer fluids. In this study we report in detail on fabrication, characterization and thermo-physical property evaluation of SiC NFs, prepared using SiC NPs with different crystal structures, for heat transfer applications. For this purpose, a series of SiC NFs containing SiC NPs with different crystal structure (α-SiC and β-SiC) were fabricated in a water (W)/ethylene glycol (EG) mixture (50/50 wt% ratio). Physicochemical properties of NPs/NFs were characterized by using various techniques, such as powder X-ray diffraction (XRD), scanning electron microscopy (SEM), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS) and Zeta potential analysis. Thermo-physical properties including thermal conductivity (TC) and viscosity for NFs containing SiC particles (α- and β-phase) were measured. The results show among all suspensions NFs fabricated with α-SiC particles have more favorable thermo-physical properties compared to the NFs fabricated with β-SiC. The observed difference is attributed to combination of several factors, including crystal structure (β- vs. α-), sample purity, and residual chemicals exhibited on SiCNFs. A TC enhancement of ~20% while 14% increased viscosity were obtained for NFs containing 9 wt% of particular type of α-SiC NPs indicating promising capability of this kind of NFs for further heat transfer characteristics investigation.

Keywords: SiC nanoparticles; Nanofluids; Thermal conductivity; Viscosity; Thermo-physical property

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Introduction

Heat transfer fluids are involved in many industrial processes to remove excess heat. Conventional heat transfer fluids such as water (W), ethylene glycol (EG) and their mixture (W/EG) have shown poor thermal conductivity (TC) characteristics. On the other hand, developing effective cooling methods for high-tech applications such as transportation, microelectronics, cutting fluid, energy supply, manufacturing and biomedical applications have been prioritized to accelerate further development of these kinds of technologies [1-6]. Hence, conventional working fluids for cooling are being replaced with newer fluids with enhanced TC than the pure and conventional liquids. Pioneer investigations of Choi [7], Lee and Choi [8] and Masuda et al. [9]
reported that the effective TC of suspensions containing suspended nanoparticles (NPs) can be higher than those of normally used industrial heat transfer liquids. Such kind of new fluids, called as nanofluids (NFs), is a new class of dispersions containing nano-sized solid particles (NPs) dispersed in conventional heat transfer fluids as base liquid [7]; and are considered to be new enhanced heat transfer fluids, since they might offer appealing possibilities due to their improved heat transfer performance [10-14]. Consequently, there is a demand for effective and novel heat transfer fluids to solve these challenges. During last decade, there has been a rapid development in NFs preparation methods. There are two major techniques, which are usually utilized for making NFs: one-step method and two-step method. In the one-step method [10,15], NPs are directly prepared in the base liquid, thus forming the NFs in a combined process. Usually, physical vapor deposition (PVD) technique [10], solution chemical method [15] or microwave-assisted route [16] is used for one-step preparation of NFs. There are some advantages in this preparation method, including minimum agglomeration and better stability. In the two-step fabrication method [17-18], NPs are first synthesized/acquired, followed by dispersing them into the base liquid (such as W or mixture of W and EG) by techniques including high shear [19-20] and ultrasound [21]. Currently, availability of NPs from commercial sources and different suppliers has attracted researchers to this method, since they can have several choices for a certain nanoparticle, which allow effective and comparative research.

To date, several kinds of solid particles/NPs and base liquids have been utilized in order to fabricate NFs for efficient heat transfer applications: NPs of metals such as Ag [22], Cu [23] and Au [24], oxides such as Fe$_3$O$_4$, mesoporous SiO$_2$, CuO and Al$_2$O$_3$ [25-28] and carbon based materials such as CNTs [29] are some examples in this regard. Among various materials used for preparing NFs, SiC is one of the promising materials to fabricate efficient NFs for cooling applications. It has one

### Table 1 Summary of available literature on thermo-physical properties of SiC NFs

| Material | Base Liquid | NP Loading (wt%) | pH | Size (nm) | T (°C) | % TC Enhancement | % Increase in Viscosity | Ref. |
|----------|-------------|------------------|----|-----------|--------|-----------------|--------------------------|------|
| α-SiC    | W/EG        | 13               | 9.4| 16*       | 22.5   | 1.5             | 52                       | [35] |
| α-SiC    | Water       | 13               | 9.4| 29*       |        | 15              | 35                       |      |
| α-SiC    | Water       | 13               | 9.4| 66*       | 15.5   | 17              | 17                       |      |
| α-SiC    | Water       | 13               | 9.4| 90*       | 17     | 17              | 17                       |      |
| SiC      | Water       | 3                | 6.3| 22.5      | 3.7    | 102             |                          | [32] |
| SiC      | Water       | 9.5              | 11 | 22.5      | 7.2    |                 |                          |      |
| α-SiC    | Water       | 24               |    | 22.5      |        | 28              | 260                      | [33] |
| α-SiC    | Water       | 3                | 9-10| 170*     | 4      | 4               | 38                       | [31] |
| α-SiC    | Water       | 6                | 22.5| 9        | 4      | 16              | 185                      |      |
| α-SiC    | Water       | 12               |    | 22        |        |                 |                          |      |
| α-SiC    | Water       | 13.2             | 10 | 26* (spherical) | 4 | 9 | 16 | Not Reported | [31] |
| α-SiC    | Water       | 13.2             | 10 | 600* (Cylinder) | 4 | 16 | Not Reported | [31] |
| α-SiC    | EG          | 13.2             |    | 26* (spherical) | 4 | 10 | Not Reported | [31] |
| α-SiC    | EG          | 13.2             |    | 600* (Cylinder) | 4 | 16 | Not Reported | [31] |

*Data obtained by DLS Measurements

*Data estimated from BET measurements
of the highest TC values (360-490 W/m·K; depending on crystal structure of SiC) [30] and is commercially available. Table 1 shows the summary of the literature on SiC NFs for heat transfer applications. For the first time, Xie et al. [31] investigated TC of SiC NFs at 4°C and reported some TC enhancement methods, yet they did not report on the viscosity of NFs. Won Lee et al. [32] developed water based NFs with β-SiC NPs and investigated TC and viscosity of suspensions at 9.5 wt% particle loading. Singh et al. [33] worked on water based commercial suspension containing α-SiC NPs and reported about TC and mechanical effects of SiC NPs in water media. Timofeeva et al. [34] studied the effect of different particle size and interface on thermo-physical properties and heat transfer characteristics of water based NFs with α-SiC NPs. In another study, Timofeeva et al. [35] reported on a series of NFs with α-SiC NPs indifferent base liquids (water and W/EG mixture) and NPs sizes. They have investigated the effect of temperature and base liquid on the heat transfer characteristics of water and W/EG based α-SiC NFs.

Although few studies have been performed on the use of SiC NF for heat transfer applications, there is no comparative study on the effect of SiC crystal structure on the thermo-physical properties of NFs containing α-SiC and β-SiC NPs. Therefore, the objective of this work is to fabricate SiC NFs with different SiC-crystalline phases in order to investigate comparatively physicochemical and thermo-physical properties including TC, and viscosity of SiC NFs in W/EG as the base liquid.

Experimental

Materials and methods

Silicon Carbide (SiC) particles with different crystal structures of alpha type (α-SiC) and beta type (β-SiC) were purchased from various suppliers/institutes including Superior Graphite (USA), PlasmaChem GmbH (Germany) and ENEA (Italy). Ethylene glycol (EG) and sodium hydroxide (NaOH) were acquired from Sigma Aldrich and Merck KGaA (Germany). All the reagents were used as received without further purification. The materials are abbreviated for ease as shown/listed in Table 2.

Fabrication of α-SiC NF and β-SiC NF via two-step method

A series of NFs were fabricated by dispersing a known weight of α-SiC and β-SiC NPs in W/EG (50:50) base liquid, via two-step method. The suspension was sonicated for 15 min and the pH value was adjusted to ~9.5 using NaOH in order to obtain stable NFs containing 9 wt% α-SiC and β-SiC NPs. All NFs (Table 3) were stable without any visual precipitation within a week. In order to study the real effect of α-SiC and β-SiC NPs on the thermo-physical properties of NFs, NPs were used as received and the use of surfactant/surface modifiers was avoided. Fabricated NFs were evaluated for TC and viscosity properties. Formulating NFs for obtaining optimum thermo-physical properties such as optimal TC enhancement, minimum impact on viscosity and longer stability is always desired. In this case, several factors such as selection of optimum particle loading play essential role. In this study, the optimum particle loading (9 wt%) was selected after an extensive test campaign of NFs with various NP concentration, where optimizing basis was maximizing the TC enhancement of NFs with minimal impact of NPs addition on viscosity. Moreover, by doing the literature survey about the relevant research (Table 1) concentration of 9 wt% was selected in order to achieve NFs with optimal properties. Therefore, all NFs were made with NPs loading of 9 wt%. As Table 1 shows, at higher

| Table 2 | SiC NPs tested for TC and viscosity |
|---------|------------------------------------|
| Material | Supplier/Institute | NP ID | Surface Area*(m²/g) |
| SiC-Alpha type | Superior Graphite (USA) | (α1-SiC) | 18 |
| SiC-Alpha type | PlasmaChem GmbH (Germany) | (α2-SiC) | 18 |
| SiC-Beta type | PlasmaChem GmbH (Germany) | (β1-SiC) | 80 |
| SiC-Beta type | ENEA (Italy) | (β2-SiC) | 90 |

*Data provided by the supplier.

| Table 3 | SiC NFs tested for TC and viscosity properties |
|---------|---------------------------------------------|
| Sample | NP ID | Crystal Structure | Base Liquid (50:50) by wt% | NPs loading (wt %) | pH |
| α1-SiC NF-W/EG | (α1-SiC) | Hexagonal | W/EG | 9 | ~9.5 |
| α2-SiC NF-W/EG | (α2-SiC) | Hexagonal | W/EG | 9 | ~9.5 |
| β1-SiC NF-W/EG | (β1-SiC) | Cubic | W/EG | 9 | ~9.5 |
| β2-SiC NF-W/EG | (β2-SiC) | Cubic | W/EG | 9 | ~9.5 |
concentrations, although NFs with higher TC was achieved, very high viscosity values were observed for NFs, which is unfavorable for their use in practical heat transfer applications.

Characterization techniques

Microstructure and morphology of α-SiC and β-SiC particles were evaluated by using scanning electron microscopy (SEM; FEG-HR Zeiss-Ultra 55). Transmission electron microscopy (TEM) analysis of the particles was performed using JEOL 2100 at 200 kV acceleration voltage. Nicolet Avatar IR 360 spectrophotometer, in the range of 500-4000 cm⁻¹ was used for Fourier transform infrared spectroscopy (FTIR) analysis of solid particles and solid/liquid samples. Powder X-ray diffraction (XRD) was performed on a Philips X’pert pro super diffractometer with Cu Kα source (λ=1.5418 Å). Zeta potential analysis of α-SiC and β-SiC particles was performed for evaluating NFs stability region. Average solvodynamic particle size distribution of β-SiC particles was evaluated by Beckmann-Coulter DelsaNano C system. TC of NFs was measured by using TPS 2500 instrument (HotDisk model 2500), which works based on the Transient Plane Source (TPS) method. The validity of the TPS instrument was checked by comparing with a standard source for thermodynamic properties of water (IAPWS reference) [36]. Compared to the reference the accuracy of measurement for distilled water was within 2% [36]. Finally, viscosity of NFs was evaluated using a DV-II+ Pro-Brookfield viscometer.

Results and discussion

XRD analysis

There are about 250 crystalline forms for Silicon carbide material [37]. Figure 1(a) displays β-SiC, which is formed at temperatures below 1700°C, has a cubic crystal structure [38], while as Fig. 1(b) shows the schematics of α-SiC, which is formed at temperatures higher than 1700°C, has a hexagonal crystal structure. In order to identify the crystal structure of SiC NPs, XRD analysis was performed. Figure 1(c) shows the XRD pattern of β1-SiC and β2-SiC NPs. The diffraction peaks at 35.8°, 41.5°, 60.0°, 72.0° and 75.7° can be indexed as the (111), (200), (220), (311) and (222) reflections of β-SiC phase, respectively (JCPDS#01-074-2307). The diffraction peak at 34.5° is attributed to a small amount of α-SiC phase. β2-SiC sample exhibits excess Si phase and all the extra peaks were indexed with Si reference pattern (JCPDS#00-027-1402). Figure 1(d) presents the XRD patterns of α1-SiC and α2-SiC particles. Both types of α-SiC show hexagonal crystal structure and all observed peaks were indexed with the JCPDS # 01-073-1663 for hexagonal SiC phase.

Morphology analysis

SEM micrographs of α-SiC and β-SiC NPs are presented in Fig. 2(a)-(d). Figure 2(a) and 2(c) represent the spherical morphology of β-SiC with estimated primary particle size of (60±10) nm and (30±10) nm for β1-SiC and β2-SiC, respectively. while Fig. 2(b) and 2(d) present the SEM micrographs of α1-SiC and α2-SiC, respectively. Since there is a very wide size dispersion for both α1 and α2-SiC NPs, it is rather difficult to perform size estimation from the micrographs; however, a rough estimate (by counting > 200 particles) showed the primary particle size of (115±35) nm and (85±20) nm for α1-SiC and α2-SiC, respectively. SiC NPs with α-type structure shows larger particle size than β-type SiC NPs, which may influence the thermophysical properties of the resultant NFs.

![Fig. 1](image-url)  
Fig. 1  The unit cell (crystal structure) and XRD pattern of (a, c) β-SiC and (b, d) α-SiC particles, respectively.
Figure 3(a)-(d) show the TEM micrographs of β-SiC and α-SiC NPs, respectively. Near spherical morphology for β-SiC NPs was observed (Fig. 3(a) and 3(c)) while hexagonal morphology for α-SiC particles were clearly visible (Fig. 3(b) and 3(d)). The morphology of α-SiC particles may be dominated by the anisotropic crystal structure, allowing the crystal to grow in certain directions more than the other directions (a.k.a. Crystal habit). Compared to the SEM micrographs presented in Fig. 2(a) and 2(c), β-SiC NPs in TEM micrographs highly agglomerated but much smaller particles were observed. Selected area electron diffraction (SAED) pattern, shown in inset images in Fig. 3(c) and 3(d), were indexed for cubic structure β-SiC (ICDD No: 01-074-2307) and hexagonal crystal structure α-SiC (ICDD No: 01-073-1663), respectively. These electron diffraction results reveal purity and good crystallinity of β-SiC NPs and α-SiC particles. Moreover, there is good match between SAED pattern and XRD analysis.
Dynamic light scattering (DLS) analysis

DLS analysis was performed to estimate the dispersed size of SiC NPs in liquid media, in order to understand the influence of effective size of dispersed NPs in the liquid media. DLS analysis results are shown in Fig. 4(a) and 4(b) for both α and β-SiC in pure water and W/EG media. Figure 4(a) displays DLS results for α1-SiC and α2-SiC in pure water and W/EG mixture as base liquids. We included pure water in our DLS analysis, since it is also one of the most commonly used base liquids. We included pure water in our DLS analysis, since it is also one of the most commonly used base liquids. A wide range of particle size distribution (150-4500 nm) with an average peak value of ∼1290 nm was obtained for α1-SiC particles in water media. For α2-SiC in water media, a much wider range of size between 30-10000 nm, with an average peak value of ∼1800 nm has been obtained, indicating strong aggregation of particles in water base liquid. When it comes to W/EG media, both in case of α1- and α2-SiC, smaller average sizes with narrower size distribution was observed when compared to the water media, indicating W/EG can affect significantly the dispersion property of SiC particles. EG may act as dispersant and covalently bond onto the particles surface, which allow stabilization of smaller aggregates/agglomerates as compared to pure water media. In comparison, α2-SiC NPs showed larger dispersed particle size than that of α1-SiC. The average dispersed size of β-SiC NPs in both liquids are presented in Fig. 4(b). When β-SiC NPs were dispersed in pure water it is seen that, the size of β1-SiC NPs is in the range of ∼100-800 nm with an average diameter of 340 nm while the size estimation for β2-SiC in water media showed the range of 130-450 nm with an average hydrodynamic diameter of ∼260 nm. A narrow NPs size distribution with smaller average hydrodynamic size was obtained for β2-SiC NPs. In W/EG media, the size of β1-SiC NPs is in the range of 100-450 nm with a peak average of 245 nm while those range and average number are 100-200 nm and 145 nm for β2-SiC NPs. A narrower NPs size distribution with a smaller average dispersed size is estimated for β2-SiC NPs. In the same way, as observed for α-SiC NPs, the presence of EG reduces the aggregate/agglomerate size in the suspensions. Looking at the all results presented in Fig. 4(a) and 4(b), β-SiC NPs have smaller dispersed size than α-SiC NPs, both the NPs exhibit smaller average solvodynamic size in W/EG base liquid compared to the pure water media, indicating a better dispersability in W/EG media. A comparison between SEM, TEM and DLS results for β-SiC NPs display that for all the α and β-SiC NPs the primary particle size obtained from SEM and TEM micrographs is less than the predicted sizes from DLS method. This difference may be due to the aggregation/agglomeration of α-SiC and β-SiC NPs in the base liquid media, in addition to the adsorbed liquid layer on particles’ surface.

FTIR analysis

In order to study the surface characteristics of particles, FTIR analysis was carried out. FTIR spectra for all “as-received” SiC particles are presented in Fig. 5. The absorption band between 860 cm⁻¹ and 760 cm⁻¹ is attributed to the Si—C bond. For all the SiC particles, the absorption between 1100 cm⁻¹ and 1000 cm⁻¹ are assigned to Si—O—Si or Si—O—C vibrations, respectively. For α1-SiC particles the band at 1200 cm⁻¹ is attributed to the Si—C, α2-SiC particles show absorption bands at 1180 cm⁻¹ and 1380 cm⁻¹, which can be attributed to the Si—C and amorphous carbon, respectively. Moreover, in both α1-SiC and α2-SiC particles, there are two bands between 1525 and 1620 cm⁻¹, attributed to the C≡O groups, which may be due to the un-reacted precursor or residual chemicals due to the method used for their fabrication. By looking at β1-SiC, in addition to Si—C bond Si—O—Si at 780 and 1050 cm⁻¹, respectively. There are two more bands at 1200 and 1315 cm⁻¹. The first band is assigned to Si—C and the second one to amorphous carbon, respectively. As Fig. 5 shows, compared to other samples, β1-SiC and α2-SiC (both are from the same supplier) present shoulder around 800 cm⁻¹, which may be attributed to the presence of O at the interface due to the synthesis procedure of high-temperature reduction of SiO₂, based on information provided by the supplier.
IEP values in the pH range of 2-3, while negatively charged particles in the pH region of 9.5 to 10. Therefore, the pH of NFs was adjusted at 9.5 to obtain stable suspensions. Figure 6 indicates that α-SiC may be due to higher silica content (more intensive Si—O—Si bands in FTIR) of α2-SiC, which shifts the IEP to more acidic pH region (IEP of SiO2 is about 2) [47]. The β2-SiC NPs exhibit slightly lower IEP than β1-SiC NPs. Although β2-SiC showed higher Si—O—Si bands in FTIR, it exhibited lower IEP due to probably the high content of Si (assessed from XRD).

**Zeta potential analysis**

Characterizing NPs dispersions and understanding the role of various parameters, which may affect colloidal properties, are important for any NFs. In the literature, very limited investigations can be found on NFs that report on the effect of pH, particle surface chemistry, primary particle size and crystal phase on properties such as Isoelectric Point [40-46]. The pH of a suspension has important role not only on the rheological property of suspension but also in terms of fabrication of stable suspension, which is related to the electrostatic charge on particles. It is well known that for fabricating stable NFs, the pH value of the NF must be far from the Isoelectric Point (IEP), where the overall charge on the particles becomes zero. If the pH gets close to the IEP the particles tend to agglomerate and finally precipitate, since there are not enough repulsive forces between them. When the pH is set far from the IEP the absolute electrical charge on particles is increased, which cause repulsive interaction between particles due the collision. In order to identify the optimum pH values for stable NFs formulation, Zeta potential analyses were performed for both the α-SiC and β-SiC particles in the pH region from 2 to 10. The results are presented in Fig. 6 where the IEP obtained for the α-SiC and β-SiC particles show great variations, one very important common point of exhibiting highly negatively charged particles in the pH region of ~9.5 and 10. Therefore, the pH of NFs was adjusted at 9.5 to obtain stable suspensions. Figure 6 indicates that β-SiC NPs has higher IEP values compared to the α-SiC. The α1-SiC and α2-SiC particles have very close IEP values in the pH range of 2-3, while β-SiC NPs display similar IEP values in the pH range of 5-6. The possible reasons for this observation may be due to different synthesis methods of SiC particles, and impurities present, resulting in different surface chemistry of SiC particle with α- and β- type. As FT-IR analysis revealed (Fig. 5), different SiC NPs exhibited different surface chemistry. By doing a comparison between FT-IR test results and Zeta potential analysis (Fig. 6), one can see that having lower IEP for α2-SiC than α1-SiC may be due to higher silica content (more intensive Si—O—Si bands in FTIR) of α2-SiC, which shifts the IEP to more acidic pH region (IEP of SiO2 is about 2) [47]. The β2-SiC NPs exhibit slightly lower IEP than β1-SiC NPs. Although β2-SiC showed higher Si—O—Si bands in FTIR, it exhibited lower IEP due to probably the high content of Si (assessed from XRD).
α type when the primary NP size increased the IEP also increased (primary size has not been calculated for α-SiC as the particles are non-spherical, though the magnitude of BET surface area is used for size comparison; large BET surface area revealing smaller primary particles – c/o Table 1). The same point is valid for β-SiC, for which an increase of IEP was observed with increasing primary NP size. A two-three orders of magnitude difference between the IEP values of α-SiC and β-SiC NPs may also be attributed to be sensitive to the SiC NPs crystal structure/phase. Our results on IEP of SiC NFs showed that IEP may be affected by the primary size of dispersed particles, impurities involved, and different surface chemistry (due to the fabrication method of particles) of NPs.

**TC and viscosity measurements of NFs**

Thermo-physical properties of NFs including TC and viscosity were performed for the all fabricated NFs at 20°C. In order to evaluate the TC of NFs, 9 wt% NFs with α-SiC and β-SiC and W/EG as base liquid were prepared and TPS method was used to test the TC of NFs. The TC evaluation results, measured at 20°C, are listed in Table 4, showing higher TC values ($K_{nf}$) for all SiC NFs than the base liquid W/EG. Moreover, Table 4 shows that TC of NF with α type SiC are higher than that of β types. The reason is not clear, however, several factors may play the role for facing this difference. It may be due to the dissimilar crystal structure of SiC NPs which also have different magnitude of TC (360 W/m-K for β and 490 W/m-K for α-type) [30]. This point indicates the possibility of influence of SiC NPs crystal phase on TC of NFs. The size of NPs may also have a role. The TC results clearly displayed that for all type of SiC NPs the TC values increase with increasing NP size, which is compatible with the literature [52].

Even sample impurity and different surface chemistry of SiC NPs, as observed from XRD and FTIR analysis, may also result in different TC for NFs. Table 4 also shows that the α1-SiC NF exhibited higher TC values than α2-SiC NF. As a result, NF with α1-SiC showed the highest TC values, while NF containing β2-SiC revealed the minimum TC value which may be due to the all above mentioned reasons such as having largest and smallest particle size for α1-SiC NPs and β2-SiC NPs, respectively. Table 4 lists the relative TC, defined as the ratio of TC of SiC NFs ($K_{nf}$) over the TC of base liquid ($K_{bl}$). The maximum TC enhancement of 20% was obtained for α1-SiC NF at 20°C. Timofeeva et al. [35] presented several results for α-SiC NFs with W/EG base liquids (Table 1). In the best case they reported 17% TC enhancement for the particles with average size of 90 nm. A comparison between the TC enhancements of α1- SiC NF- W/EG (present study) at 20°C and that reported by Timofeeva et al. [35], where they used commercial NFs, shows that NFs for the present work has higher TC even at ~4 wt% lower particle loading, indicating higher effective thermal performance of NF presented in this work.

It is important to clarify that having greater relative TC value only is not enough for utilizing a NF as effective coolant. In order to choose the efficient NF with optimum characteristics for heat transfer applications, not only TC but also viscosity must be evaluated. The internal resistance of a fluid to flow is described by viscosity [53], which plays an important role in all thermal applications involving flowing fluids [54]. This property is expected to be higher when compared to their base liquids, but this increase makes a negative impact on the pumping power and heat transfer coefficient. These parameters are very essential in practical heat transfer applications. For instance, in laminar flow, the pressure drop is directly proportional to the viscosity. Moreover, Prandtl and Reynolds numbers are influenced by viscosity of fluids and heat transfer coefficient is a function of these numbers. Therefore, viscosity is as important as TC in engineering systems involving fluid flow [55]. This is an important criterion for the use of this type of NFs in convective heat transfer applications. The viscosity tests were carried out at 20°C for all NFs with W/EG as the base liquid, where all the samples exhibited Newtonian behavior. The results are listed in Table 4, which show that all NF with α-type SiC particles have lower viscosity values than NF containing β-type SiC particles. Three possible reasons can be enlisted for the observed difference as the effect of the crystal structure or difference in primary particle size (BET surface area) for α-SiC NF and β-SiC NPs and even to the level of impurities as observed from XRD and FTIR analysis. The smaller surface area of α-SiC particles (Table 2) results in a smaller contact (solid-liquid interface) area between α-SiC particles and W/EG base liquid and therefore exhibit smaller viscosity value than α-SiC particles. Timofeeva et al. [35] reported 17% TC enhancement for the particles with average size of 90 nm. A comparison between the TC enhancements of α1- SiC NF- W/EG (present study) at 20°C and that reported by Timofeeva et al. [35], where they used commercial NFs, shows that NFs for the present work has higher TC even at ~4 wt% lower particle loading, indicating higher effective thermal performance of NF presented in this work.

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**Table 4** TC and viscosity evaluation results of SiC NFs tested at 20°C

| Sample                  | NPs loading (wt%) | TC (W/mk) | $K_{nf}/K_{bl}$ | Viscosity (cP) | $\mu_{nf}/\mu_{bl}$ |
|------------------------|-------------------|-----------|-----------------|----------------|---------------------|
| Base Liquid (W/EG)     | –                 | 0.392     | –               | 3.897          | –                   |
| α1-SiC NF W/EG         | 9                 | 0.4704    | 1.20            | 4.4245         | 1.14                |
| α2-SiC NF W/EG         | 9                 | 0.4626    | 1.18            | 4.3646         | 1.12                |
| β1-SiC NF W/EG         | 9                 | 0.4469    | 1.14            | 5.299          | 1.36                |
| β2-SiC NF W/EG         | 9                 | 0.4195    | 1.07            | 6.2352         | 1.60                |
β-SiC at the same particle loading. The increased viscosity values for α1-SiC and α2-SiC particles are very close as the surface area for both particles are almost the same. Secondly, for the same reason α2-SiC NF exhibited the lowest and β2-SiC NFs the highest viscosity values at 20°C. The relative viscosity, which is defined viscosity of NF (μnf) to the viscosity of base liquid (μbl), are listed in Table 4. The minimum increase of ~12% in viscosity was achieved for SiC NF with 9 wt% α2-SiC particle loading, which has small surface area (larger average particle size), while the maximum increase of ~60% in viscosity was obtained for SiC NF with β2-SiC at the same particle loading. NFs containing β2-SiC exhibit higher increase in viscosity compared to the β1-SiC NF at the same NPs loading may be due to its smaller size, and impurity content, which provides a larger surface area per unit volume. A direct comparison between increase of viscosity in this work and the reported values in the literature (Table 1) is not possible because of having different factors such as particle loadings, particle size and dissimilar temperature. Selecting NFs with similar particle loadings, Won Lee et al. [32] reported 7.2% TC enhancement while increase in viscosity was 102% for a water based β-SiC NF with ~9.5 wt% particle loading indicating that although it exhibit nearly the same relative TC compared to the worst case in this study (β2-SiC NF with 9 wt% NPs loading), shows 42% more increase in viscosity. Our findings show favorable thermo-physical properties (higher TC enhancement with lower increase in viscosity) compared to all series of water based α-SiC NFs reported by Timofeeva et al. [34] (Table 3). They have utilized commercial water based NFs, prepared by dispersing α-SiC NFs, and reported 7-12.5% TC enhancement with 17.5-60% increase in viscosity of the NFs containing 13 wt% of SiC NPs with different sizes. Timofeeva et al. [35] also reported on α-SiC NFs with W/EG showing 17% increase in viscosity at 13 wt% SiC NPs loading (Table 1). The observed differences between this work and Timofeeva et al [34,35] may be due to the different surface chemistry of α-SiC particles (IEP= 4 [34]), type of the base liquid, or additives, besides different NPs loading. The increased viscosity will result in use of a greater pumping power, which might counterweigh the benefits of higher TC enhancement values. The tradeoff between thermo-physical properties including increases in viscosity values and relative TC is very essential in order to utilize the NFs for heat transfer applications.

Figure 7 summarizes the comparison between TC enhancement and viscosity increase, for all fabricated NFs, where both α-SiC NFs show that the TC increase is higher than the viscosity increase at 20°C, while reverse behavior is observed for β-SiC NFs. This may imply that α-SiC NFs formulations can be proper candidates as efficient NFs for convective heat transfer applications. Moreover, a comparison between α1-SiC NF and α2-SiC NF shows that although α1-SiC NF has 2% higher viscosity increase, it exhibits 2% greater TC enhancement value indicating a promising capability of this NF as efficient coolant in heat transfer applications. Among all the fabricated NFs in this work, NFs fabricated by α1-SiC particle is the optimum choice, which is therefore selected for further thermal characteristics investigations, and results are to be reported elsewhere.

![Fig. 7 Comparison of the TC enhancement with increase in viscosity (at 20°C) for NFs, with W/EG as base liquid, containing 9 wt% α- and β- type SiC particles.](image)

**Conclusions**

We presented on the fabrication and evaluation of highly stable SiCNFs for heat transfer applications. The NFs were prepared by dispersing SiC particles with different crystal structure in W/EG base liquid, using colloidal stabilization strategies. A detailed physico-chemical evaluation showed different characteristics of SiC NPs, including crystal structure, primary/dispersed particle size, surface functionality, surface charge, and purity levels of SiCNP s used. Interpretation of the results from different analytical techniques showed that IEP of the SiC NPs, and the viscosity of NFs, may be affected by the primary size, surface chemistry of NPs (due to the different NPs fabrication routes), as they may also be dependent on SiC crystal structure (α- vs. β-type). Thermo-physical properties, TC and viscosity, of SiC NFs were performed at 9 wt% NPs loading at 20°C. TC enhancement of the NF, over the base liquid, due to the presence of SiC particles are observed for NFs containing both α- and β-type SiC NPs. W/EG based NFs with α-SiC exhibited higher TC than that with β-SiC, which may be attributed to the effect of the crystal structure (α-type has higher TC value), or the phase purity of the as-received materials. Among all fabricated W/EG based NFs containing SiC with different crystal structure (α- vs. β-type), α1-SiC NF displayed the highest TC enhancement of 20%, while only 14% increase in viscosity, revealing its...
promising characteristics for heat transfer applications.

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