Preparation, gradient extraction and surface polarity-dependent antibacterial properties of cockscomb seeds-derived carbon dots

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Cockscomb seeds-derived carbon dots (CSCDs) were fabricated by hydrothermal method with common cockscomb seeds. The prepared CSCDs exhibit selectively antibacterial properties towards Gram-positive bacterias, such as Staphylococcus aureus (*S. aureus*) and Staphylococcus epidermidis (*S. epidermidis*). Then, the CSCDs were separated to three groups with lipophilic, amphipathic and hydrophilic ones by simple gradient extraction. Interestingly, the three groups of CSCDs exhibited different antibacterial activities, and the amphipathic CSCDs(ACSCDs) showed best bacterial inhibition. This phenomenon might stem from the surface properties of the amphipathic CSCDs, which have both hydrophilic region and hydrophilic region on the surface. The structure might help the samples to penetrate the bacterial membrane more easily, and finally cause apoptosis. This work not only supplied a novel antibacterial carbon dots, but also further revealed that surface chemistry might be an important factor which could affect the bactericidal performance of carbon dots.

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Keywords: Carbon dots, Antibacterial, Gram-positive bacteria, Surface polarity

1. Introduction

With the abuse of antibiotics and the aggravation of bacterial drug resistance throughout the world, it is increasingly urgent for the researchers to explore novel antibacterial agents[1]. Antibacterial nanomaterials, such as noble metal nanoparticals (e.g., Cu NPs, Ag NPs), metal oxide nanoparticals(e.g., ZnO, TiO_2), which are highly efficient and difficult to form drug resistance[2], have been widely concerned in recent years. However, these nanomaterials always have some physiological toxicity, which might cause oxidative stress, enzymatic inhibition or protein inactivation and make damage for the living organisms[3-5]. Consequently, it is still of great value to develop novel biosafe nanomaterials with excellent antibacterial property.

Antibacterial carbon dots, which possess good water solubility, excellent biocompatibility and diverse surface functions, have been expected to be promising potential agent for the clinical applications[6-8]. For example, Li et al. fabricated novel carbon dots (CDs) by vitamin C using one-step electrochemical treatment, which has a strong inhibitory effect against both bacteria and fungus[9]. Zhao et al. reported CDs synthesized by protamine sulfate had a strong antibacterial capability against the Staphylococcus aureus (*S. aureus*) and methicillinresistant *S. aureus*[10]. However, these synthetic routes often required complicated process or uncommon raw materials, which might limit their further applications. Besides, the inhibitory mechanisms of CDs on micro-organisms still needs further exploration.

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In this study, Cockscomb seeds-derived carbon dots(CSCDs) were obtained by hydrothermal method using common cockscomb seeds as raw materials, and the prepared CSCDs exhibited excellent biocompatibility and fluorescence properties. Biological test showed that the CSCDs exhibit effective antibacterial properties towards Gram-positive bacterias, such as *S. aureus* and Staphylococcus epidermidis (*S. epidermidis*). Then, the CSCDs were separated to three groups with lipophilic, amphipathic and hydrophilic ones by simple gradient extraction. The three groups of CSCDs exhibited similar physicochemical properties because of the same precursor but show different antibacterial activities, and the amphipathic CSCDs(ACSCDs) possessed best bacterial inhibition. The surface polarity-dependent antibacterial properties of the CSCDs was further expored, which further revealed that surface chemistry might be an important factor which could affect the bactericidal performance of carbon dots.

2. Experimental

2.1. Chemicals and materials

Cockscomb seeds were purchased from Sijiqing Flowers Ltd. (Shijiazhuang, China). The organic solvents were analytical grade and obtained from Jinghai Ltd. (Zhengzhou, China). Experimental strains were got from China Pharmaceutical Culture Collection. Protamine sulfate (PS) was provided from Kang Yuan Biotechnology Co., Ltd (Zhengzhou, China). Deionized water was adopted for all experiments.

2.2. Instrumentation and characterization

Morphologies of the CSCD samples were observed by transmission electron microscopy (TEM) images using a Tecnai G2 F20 S-TWIN TEM (FEI, U.S.A.). The zeta potentials of the obtained CSCDs were measured by using a Zetasizer Nano-ZS dynamic light scattering (DLS) instrument (Malvern, England). The X-ray photoelectron spectra (XPS) were measured by a VG Multilab 2000 X-ray photoelectron spectrometer (Thermal Electron, U.S.A.). The Fourier transform infrared (FT-IR) spectra of the CSCDs were obtained with a Bruker FT-IR Spectrometer (Hyperion, U.S.A.). The UV-vis spectra were carried out using a Lambda 752 UV-vis spectrophotometer (PerkinElmer, U.S.A.), while the fluorescence study was obtained on a Fluoro Max-4 Spectrometer (Horiba Jobin Yvon, France). The absorbance of the cells was obtained with a microplate reader (Thermo Labsystems, U.S.A.) at a wavelength of 490 nm.

2.3. Fabriction of CSCDs

CSCDs were fabricatied by hydrothermal method using cockscomb seeds as raw materials. The synthesis procedure was as follows: 0.30 g of dry cockscomb seeds were grinded, then the obtained powder was dispersed uniformly in 30.0 mL of deionized water by ultrasonic treatment. The suspension system was further transferred to a poly(tetrafluoroethylene)-lined autoclave (50 mL) and heated at 200 ℃ for 6 h. After centrifuging for 8 min and separating the precipitate, the obtained brown solution was dialyzed using a dialysis bag (MWCO: 600D) for 36 h. The CSCDs were collected by freeze-drying for further use (yield: ca. 26.5 %).

2.4. Gradient extraction process

Gradient extraction method was used to separate CSCDs based on their surface polarity differences. After dissolving the CSCDs in DI water, two organic solvents: dichloromethane (solvent polarity parameter: 3.1) and ethyl acetate (solvent polarity parameter: 4.4) were used to extract CSCDs in term. The two fractions of extracted CSCDs were denoted as lipophilic (yield: ca. 6.5 %) and amphipathic CSCDs（yield: ca. 29.5 %）, while the fraction retained in water was denoted as hydrophilic CSCDs (yield: ca. 64 %). All the three fractions were freeze-dried and redispersed in DI water before optical characterization.

2.5. Antimicrobial activity tests

For the antimicrobial activity tests, *S. Aureus* and *S. epidermidis* were chosen as the

typical Gram-positive bacterias, while the Escherichia coli (*E. coli*) strains and Pseudomonas aeruginosa (*P. aeruginosa*) strains were adopted as typical Gram-negative bacterias. The experimental strains were cultivated in nutrient broth and then shaked in the incubator at 37 ˚C. For the Kirby-Bauer (K-B) disk diffusion assay, equal amounts of CDs samples (100 ± 5 μg) were firstly loaded onto the paper disks. Then, the microorganism suspension (100 μL, 10^4–10^5 CFU mL^{-1}) was spread uniformly on the nutrient agar surface before placing the disks. After incubating at 37 ℃ for 24 h, the plates were taken out from the incubator, while the inhibition zone diameters of the disks were measured and compared. The antibacterial capacity for the prepared CSCDs was further assessed by minimum inhibitory concentration (MIC) test, while the survival of bacterials was observed visually according to a reported procedure[12-13].

3. Results and discussions

3.1. Results and discussions

CSCDs were prepared using hydrothermal method of cockscomb seeds which was an abundant and renewable biomass resource, while the formation of CSCDs was considered to involve dehydration, crosslinking and carbonization of the components of cockscomb seeds as previous reports[14]. Characterization with TEM revealed the morphology and diameter of the obtained CSCDs (Fig. 1). It could be observed that the CDs are mostly of spherical morphology (diameter: 3-5 nm) and dispersed without apparent aggregation. HRTEM image revealed the interplanar spacing of the CSCDs to be 0.32 nm, which had a good agreement with the <002> lattice spacing value of graphite[15]

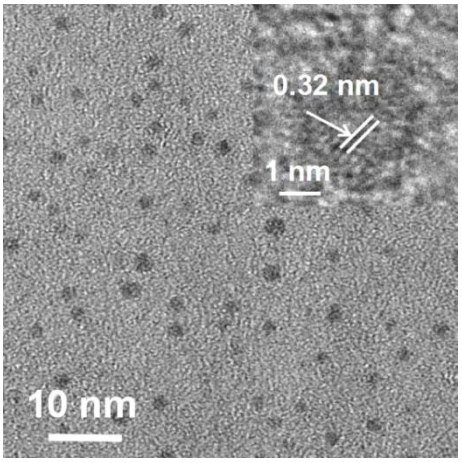

Fig. 1. TEM image of CSCDs, inset: HRTEM images of CSCDs.

The FTIR spectrum (Fig. 2a) showed C-O-C stretching vibration at 1090 cm^{-1}, C=O vibrations at 1670 cm^{-1}, C-H vibrations at 2960 cm^{-1} and a broad and strong band representing O-H/N-H at 1380-1290 cm^{-1}. XPS spectrum in the full range (Fig. 2b) indicated that the CDCD samples were mainly composed of four elements: carbon (78.4%), nitrogen (8.14 %), oxygen (12.37 %) and S (1.09%). XPS precise analysis (Fig. 4) further conformed that the C1s band could be deconvoluted into three types: graphitic or aliphatic, oxygenated, and nitrous carbon atoms [17]. The XPS results indicated that the prepared CDs were functioned by plenty of polar groups, which was in agreement with the FTIR analysis results. The zeta potential of these CSCDs was measured to be +16.2 mV, while the positive charge of these CDs might be caused by the nitrogenous functional groups of CDs.[18]

Attributed to the π-π* transitions of C=C/C=N bonds[19], the UV-vis absorption analysis of the prepared CSCDs (Fig. 3a) exhibited an obvious absorption peakcentered at 273 nm. As shown in Fig. 3b, the as-obtained CSCDs exhibited excitation-dependent PL behavior, while the

optimum EX/EM wavelengh was measured to be 340/442 nm. The fluorescence quantum yield and average lifetime in in aqueous solution was determined to be 12.8 % (λex = 360 nm, using quinine sulfate as reference) and 8.70 ns, respectively.

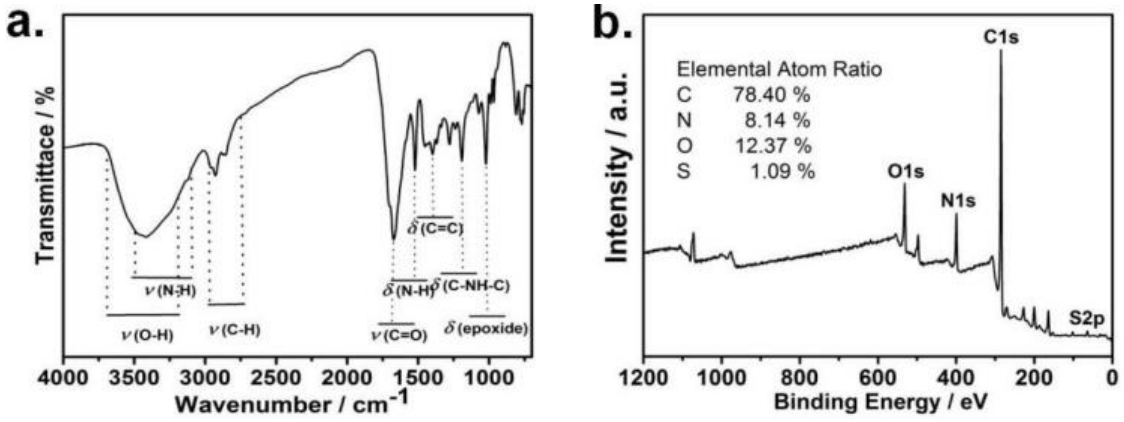

Fig. 2. FTIR analysis (a) and XPS survey scan spectrum (b) of CSCD.

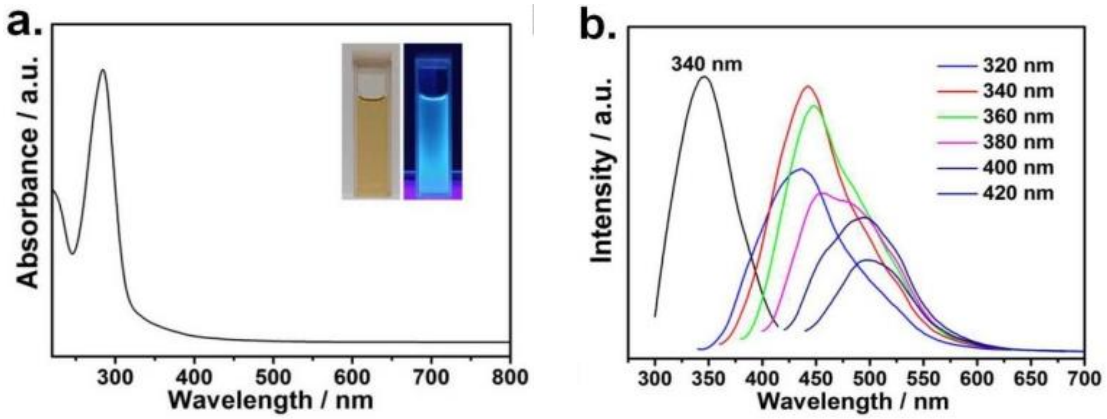

Fig. 3. Optical analysis of the CSCDs in aqueous solution. (a) UV-vis absorption spectra. (b) PL emission spectrum at different EX wavelength (320-420 nm), the EX spectrum (black line) monitored at the maximum emission peak.

The antibacterial properties of CSCDs were confirmed using MIC test and K-B method [20]. As presented in Fig. 5a-d, the disks loaded with CSCDs were surrounded by clear inhibition zones for Gram-positive strains (Fig. 5 a, b). Furthermore, the MICs of the CSCDs towards *S. aureus* and *S. epidermidis* were determined to be 50 and 45 μg/mL(Tab. 1). However, it has no obvious inhibitory effect on Gram-negative ones (Fig. 5 c, d; Tab 1). Thus, the CSCDs showed much better bactericidal effect tawards Gram-positive bacteria than that of Gram-negative ones at the same concentration. For the former case, the selective inactivation of the CSCDs could be attributed to the specific cell membrane structures. Gram-positive strains had a loose and negatively charged cell wall, which might be combined and attacked readily by the surface positive CSCD samples. Consequently, the CSCDs could pass through cell membranes and bind to DNA, disturb the genetic process and finally kill the Gram-positive bacterials more easily compared with the Gram-negetive ones[21].

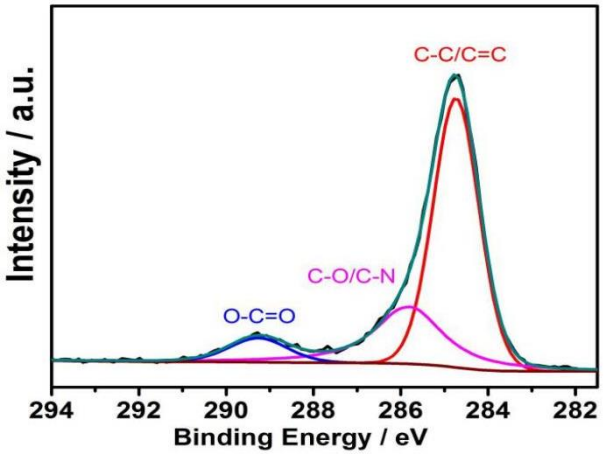

Fig. 4. High-resolution C 1s spectra analysis of the CSCDs.

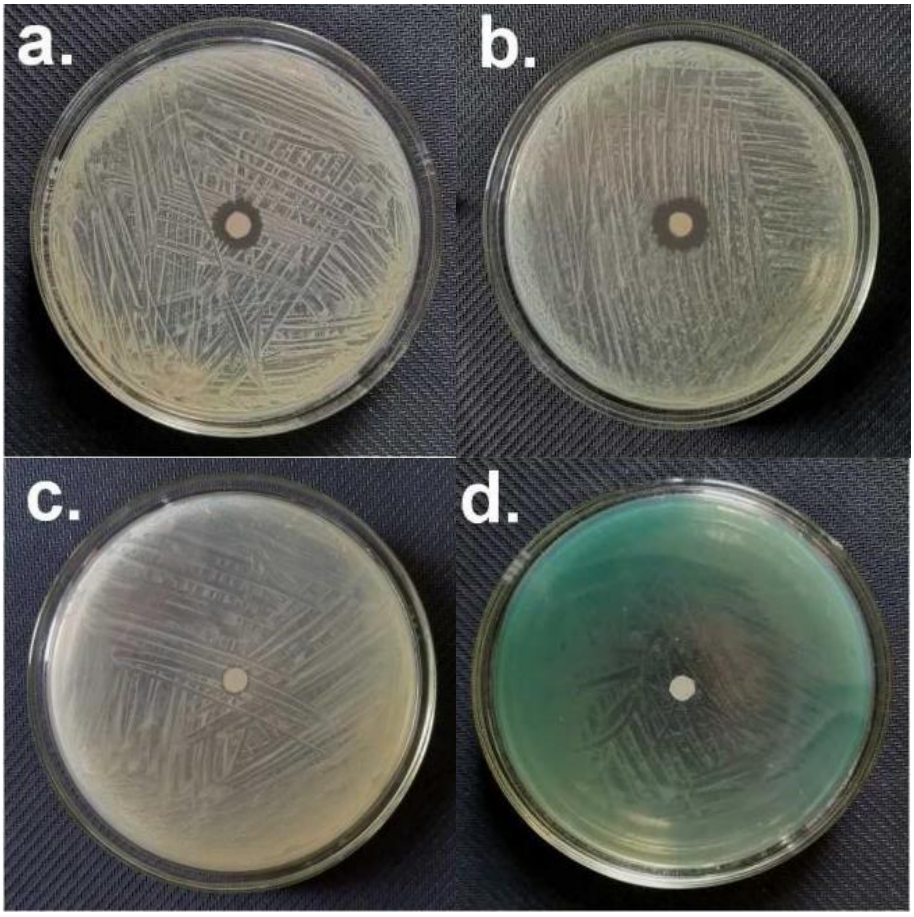

Fig. 5. Representative images of K-B test disks for (a) S. aureus, (b) S. epidermidis, (c) E. coli. and (d) P. aeruginosa.

3.2. Surface polarity-dependent antibacterial properties of CSCDs

Cockscomb seeds are common biomaterial resource consisted of various phytocompounds, such as protein, cellulose, quercetin. So, there is some reason to deduce that the prepared CSCDs is a mixture with different surface properties. Herein, gradient extraction was used to separate the raw CSCDs based on the surface polarity. Two organic solvents: dichloromethane and ethyl acetate were applied to extract the CSCDs gradiently. The two fractions of extracted CSCDs were

denoted as E-CSCDa and E-CSCDb, while the CSCDs remained in the aqueous system was denoted as E-CSCDc, respectively.

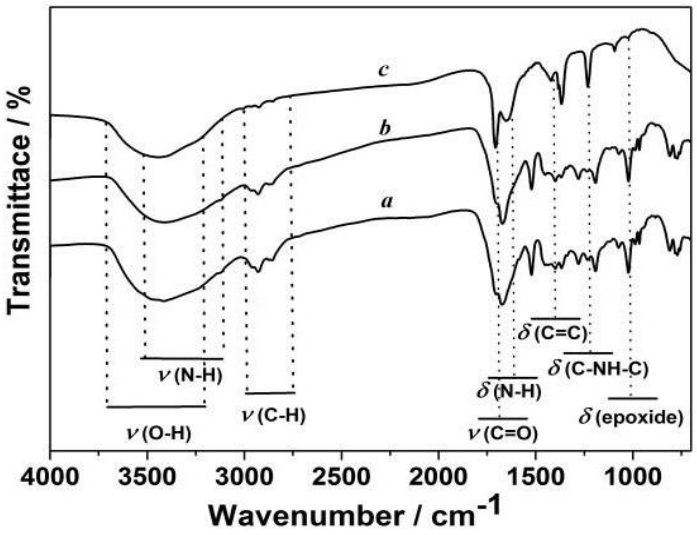

Fig. 6. The FTIR contrastive analysis of the three CSCDs: E-CSCDa (a);CSCDb (b) and E-CSCDc (c).

As shown from the FTIR spectra in Fig. 6, It could be concluded that the bond intensity of the polar functional groups such as O-H/N-H and C=O gradually increased from E-CSCDa to E-CSCDc. In addition, the intensity of less polar groups such as C-H and C-O-C decreased [22].

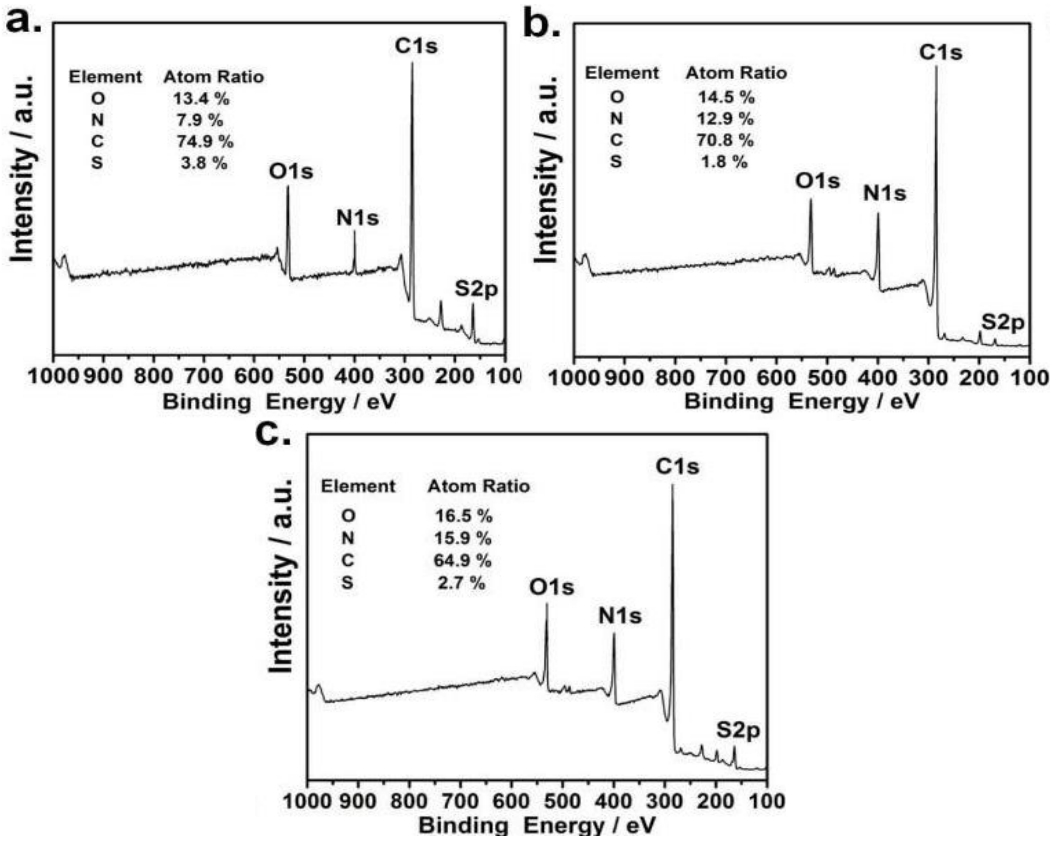

Fig. 7. XPS spectrum of the four CSCD fractions: E-CSCDa (a), E-CSCDb (b), E- CSCDc (c).

It could be shown from the XPS survey spectra (Fig. 7) that the content of surface heteroatoms increased successively from E-CSCDa to E-CSCDc, which refleted the increasing of surface polar functional groups. The E-CSCDa was best classified as lipophilic fraction because of the abundant surface hydrophobic groups, the E-CSCDb could be classified as amphiphilic fraction due to the simultaneous presence of hydrophobic moieties and hydrophilic functional groups, and the final E-CSCDc was best to be denoted as hydrophilic ones. it is shown from the TEM images that the three samples have similar morphologies and size distributions (Fig. 8). Furthermore, the zeta potential of three CSCDs was measured to be +14.7, +16.3 and +16.5 mV. Then, it seemed that the gradient extraction had no obvious influence on the morphology and surface charge of CSCDs.

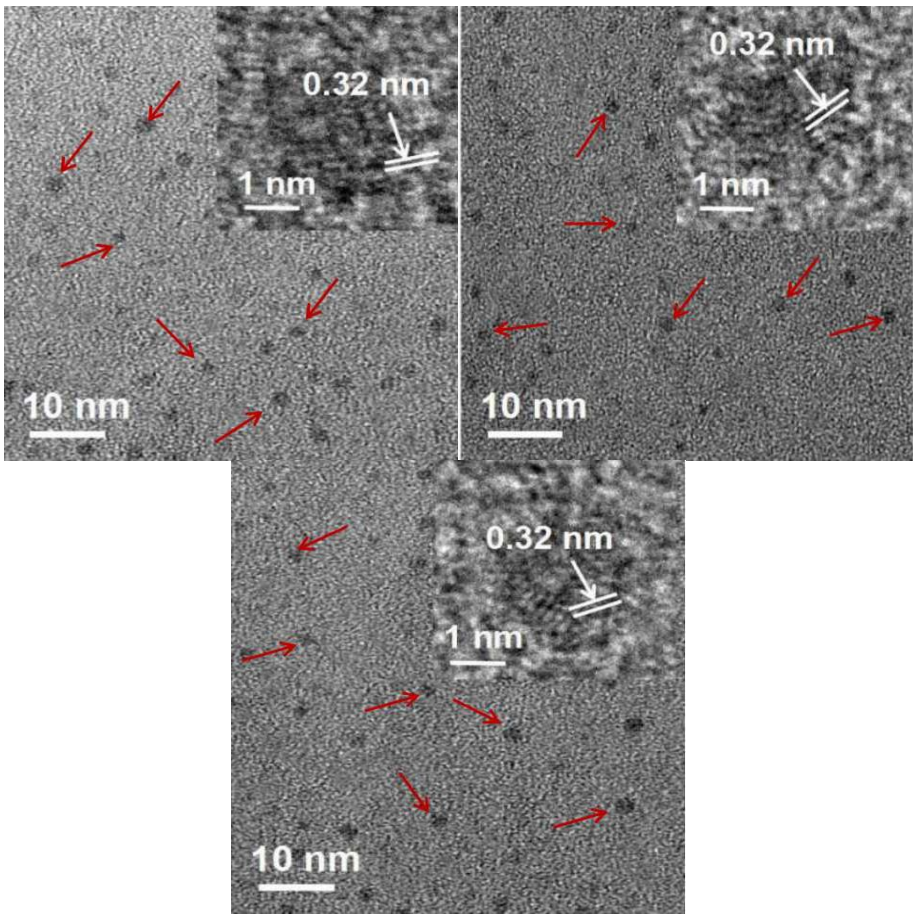

Fig. 8. TEM images of the three separated samples (inset: HRTEM images). (a) E-CSCDa; (b) E-CSCDb; (c)E-CSCDc.

To compare the antibacterial properties of the three surface-state CSCDs, the K-B method and MIC test were further adoped[23]. As shown in Fig. 9a-d, the three CSCDs were observed to have no obvious inhibitory effect against Gram-negative strains (*E. coli*, *P. aeruginosa*). However, the seperated CSCDs could selectivly inhibit the growth of Gram-positive ones (*S. aureus*, *S. epidermidis*). Furthermore, the disk with E-CSCDb were surrounded by the largest inhibition zone for both *S. aureus* and *S. epidermidis* strains, the disk with E-CSCDc was smaller, and E-CSCDa was the smallest. The MIC test showed the MIC increased as the order of: E-CSCDb <E-CSCDc< E-CSCDa (Table 2). In a word, both the diffusion disk method and MIC test implied that E-CSCDb held the strongest bactericidal ability.

For the bactericidal substance, the antibacterial capability is considered to be influenced by surface charge, partical size, and so on[24]. However, the surface polarity was proved to be

another influence factor for the antibacterial effect in this research. For the E-CSCDa, the water-soluble capacity was considered to be poor caused by the lack of surface polar functional groups, which might restrict them to diffuse freely in physiological system and resulted in weak sterilization property. For the E-CSCDc, abundant polar functional groups are beneficial for the samples to diffuse in aqueous system. However, the hydrophilic structure might be not positive for the E-CSCDc to penetrate the lipid bilayer membrane structure (important part of the bacterial membrane) of which the center was hydrophobic [25]. For the E-CSCDb, the samples are amphiphilic and similar to the phospholipid molecule, which might help the samples to exchange with the phospholipid molecules and penetrate the bacterial membrane, bind with DNA, and finally cause apoptosis [26-27].

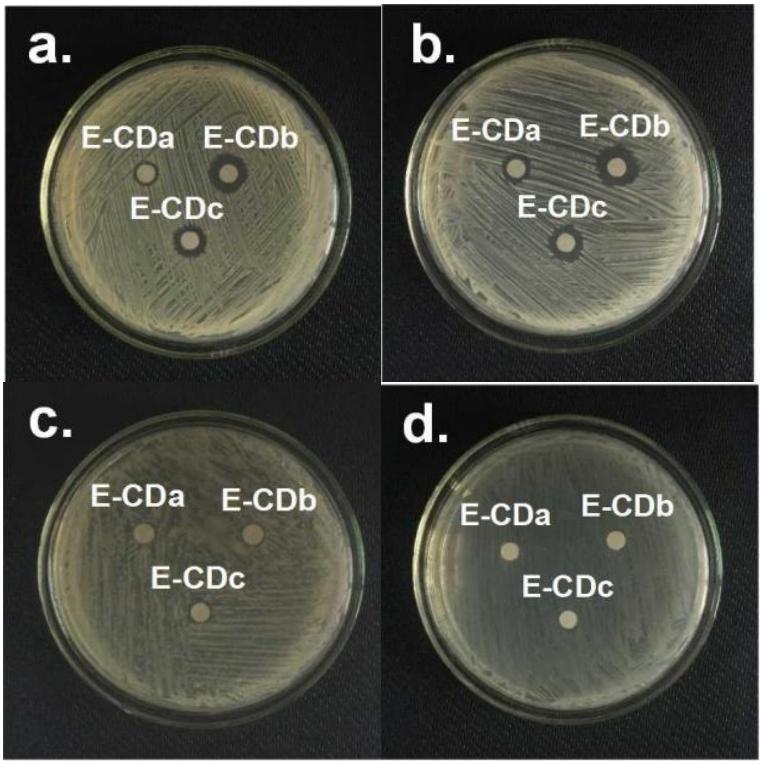

Fig. 9. Representative photograghs of agar plates containing disks impregnated with CSCD fractions for (a) S. aureus, (b) S. epidermidis, (c) E. coli. and (d) P. aeruginosa.

Table 1. MIC (μg mL^{-1}) of CSCDs for the four microorganisms.

	Strain no.	MIC
S. Aureus	ATCC 25923	55
S. epidermidis	ATCC 12228	45
E. coli	ATCC 11303	>1500
P. aeruginosa	ATCC 27853	>1500

Table 2. MIC of the separated CSCD fractions for the four microorganisms.

	Strain no.	MIC_a	MIC_b	MIC_c
S. Aureus	ATCC 25923	140	50	80
S. epidermidis	ATCC 12228	120	40	70
E. coli	ATCC 11303	>1500	>1500	>1500
P. aeruginosa	ATCC 27853	>1500	>1500	>1500

Notes: *MICa, the MIC (μg mL^{-1}) of E-CSCDa; MICb, the MIC (μg mL^{-1}) of E-CSCDb; MICc, the MIC (μg mL^{-1}) of E-CSCDc.*

4. Conclusions

In summary, novel CSCDs were obtained by hydrothermal method using common cockscomb seeds as raw materials. These CSCDs exhibited excellent biocompatibility, good fluorescence property and effective antibacterial properties towards Gram-positive bacterias, such as *S. aureus* and *S. epidermidis*. The CSCDs were further separated to three groups with lipophilic, amphipathic and hydrophilic ones by simple gradient extraction, and the amphipathic CSCDs was testified to possess best bacterial inhibition. Then, surface polarity-dependent antibacterial properties for the CSCDs were discussed. This work may supply a new perspective to explore the relationship between surface science and biological carbon nanomaterials.

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