

**Multi-Site Inhibitors for Enteric Coronavirus:  
Antiviral Cationic Carbon Dots Based on Curcumin**

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4 **Multi-Site Inhibitors for Enteric Coronavirus: Antiviral Cationic Carbon Dots**  
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6 **Based on Curcumin**  
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37 ABSTRACT: The research of carbon-based antivirals is still in its infancy, and it  
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39 remains to be explored to develop into safe and effective carbon dots (CDs) with  
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41 antiviral activity at multiple points in life cycle of the virus. Here, we report a  
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43 one-step method to apply curcumin, to prepare of uniform and stable cationic carbon  
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45 dots (CCM-CDs) with antiviral properties. The inhibitory effect of CCM-CDs on viral  
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47 replication was studied by using porcine epidemic diarrhea virus (PEDV) as a  
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49 coronavirus model. Porcine epidemic diarrhea virus (PEDV) is applied as a  
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51 coronavirus model to study the antiviral effect of as-prepared CCM-CDs on its  
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3 replication. The cationic CCM-CDs treatment is found obviously to inhibit the  
4 proliferation of PEDV compared with the common CDs (EDA-CDs). The CCM-CDs  
5 treatment can change the structure of surface protein in viruses, thereby inhibiting  
6 viral entry. And it can also suppresses the synthesis of negative-strand RNA of virus,  
7 the budding of the virus as well as the accumulation of reactive oxygen species (ROS)  
8 by PEDV. Furthermore, CCM-CDs treatment is also found to suppress viral  
9 replication by stimulating the production of interferon-stimulating genes (ISGs) and  
10 pro-inflammatory cytokines. These results offer theoretical support for the  
11 development of CCM-CDs as a hopeful antiviral drug for the treatment of coronavirus  
12 infections, including PEDV.  
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28 *KEYWORDS:* curcumin, carbon dots, antiviral, interferon-stimulating genes,  
29 pro-inflammatory cytokines  
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## 38 **1. INTRODUCTION**

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40 Curcumin (CCM), a polyphenol compound obtained from turmeric roots. Due to its  
41 antioxidant, antiviral, anti-inflammatory, anticancer, antibacterial functions and so on,  
42 the potential application of curcumin in multiple industries is gaining interest.<sup>1-3</sup> Maya  
43 et al. demonstrated that curcumin inhibited the expression and multiplication of HBV  
44 by down-regulating PGC-1 $\alpha$ .<sup>4</sup> Ali et al. found that curcumin inhibited HIV-1 infection  
45 through facilitating the degradation of Tat protein and reducing Tat-mediated  
46 transcription of the TLR promoter.<sup>5</sup> Narayanan et al. evaluated the antiviral activity of  
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4 curcumin against RVFV. And the mechanism confirmed that curcumin inhibited  
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6 RVFV replication by preventing phosphorylation of I $\kappa$ B- $\beta$  NSs protein in the NF- $\kappa$ B  
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8 pathway.<sup>6</sup> However, the pure CCM cannot be widely applied due to its insolubility in  
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10 physiological media and poor bioavailability *in vivo*. To overcome these shortcomings  
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12 of CCM, a widely used strategy is to encapsulate CCM in inorganic-based carriers,<sup>7,8</sup>  
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14 but the process of synthesizing these nanoparticles is relatively tedious and  
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16 time-consuming, furthermore, this treatment does not significantly improve the  
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18 antiviral activity of CCM. Thus, developing simple, safe and effective method to  
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20 improve the bioavailability, solubility, as well as antiviral activity is extremely  
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22 important.  
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29 Due to their different compositions and characteristics, nanoparticles can be  
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31 perfectly practiced as a carrier in efficient delivery of drugs to special sites.<sup>9,10</sup>  
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33 Besides, nanoparticle-based antiviral agents have been reported as potential  
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35 alternatives in the treatment of diseases because of their distinctive biological  
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37 properties derived from morphological (e.g. size, structure) and physicochemical  
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39 features, different from those of traditional small-molecule drugs.<sup>11,12</sup> Antiviral  
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41 nanomaterials, such as silver nanoparticles, functional gold nanoparticles,<sup>13,14</sup>  
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43 peptide<sup>15</sup> and polyvalent nanoarchitectures,<sup>16-18</sup> have aroused widespread research  
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45 interest.<sup>19</sup> Among the nanomaterials, silver nanoparticles (AgNPs) have the highest  
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47 potential for commercialization, and numerous studies have demonstrated that AgNPs  
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49 possess a strong antiviral activity against human immunodeficiency virus (HIV),  
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51 H1N1 influenza virus, herpes simplex virus (HSV) and so on.<sup>20,21</sup> The complicated  
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3 antiviral mechanisms of silver nanoparticles include competing viruses binding to  
4 cells, interactions with DNA, attachment to the cell surface to change membrane  
5 characteristics, inactivating virus particles before entry and enzyme damage.<sup>22</sup>  
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7 However, before the adhibition of metal nanoparticles to therapeutic or preventive  
8 therapy, it is essential to assess the cytotoxicity to most human cells and the  
9 underlying long-term sequelae caused by contacted with these compounds.<sup>23,24</sup>  
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11 Therefore, nanoantibiotics based on non-metallic nanoparticles have appealed  
12 widespread attention owing to their outstanding antiviral characteristic. Recently,  
13 carbon-based nanomaterials have been confirmed to have potent antiviral  
14 properties.<sup>25-27</sup> Sametband et al. found that GO derivatives could suppress viral  
15 infection through competing for virus-cell binding.<sup>28</sup> Barras et al. investigated that  
16 surface functionalized carbon nanodots could function as entry inhibitors through  
17 interaction with the virus at the early stage of viral infection. Meanwhile, our previous  
18 work has demonstrated that CDs might inhibit viral replication via positively  
19 regulating antiviral type I interferon response.<sup>29</sup> In this study, a novel carbon dots  
20 from curcumin as a precursor was prepared, which could improve the bioavailability  
21 of curcumin and achieve synergistic antiviral effect.  
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46 In the present study, it is reported that antiviral cationic carbon dots (CCM-CDs)  
47 are prepared from herbs for the first time and it provides a novel clue to improve the  
48 antiviral activity of herbs. We find that CCM-CDs treatment can significantly inhibit  
49 viral entry, the synthesis of negative-strand RNA in virus, the budding of virus and the  
50 accumulation of reactive oxygen species (ROS) by PEDV. The antiviral activity of  
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3 CCM-CDs may also be ascribed to ISG proteins and pro-inflammatory cytokines  
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6 production.  
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## 10 11 12 **2. EXPERIMENTAL SECTION** 13

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15 **2.1. Preparation of curcumin carbon dots (CCM-CDs).** Curcumin (0.30 g)  
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17 and citric acid (0.60 g) were ground uniformly, sealed and hydrothermally treated in a  
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19 25 mL Teflonlined autoclave at 180 °C for 1 h. 15 mL ultrapure water was added to  
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21 the brown mixture obtained from the reaction, followed by centrifugation to eliminate  
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23 the large particles.<sup>30</sup> Finally, the as-prepared CDs (CCM-CDs) were dialyzed (the  
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25 cut-off molecular weight of the dialysis membrane was 100 D) and then kept in low  
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27 temperature. In the control experiment, 2.0 g citric acid was poured into 8.0 mL  
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29 distilled water, and then 1.0 mL ethylenediamine (EDA) was added to form a  
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31 homogeneous solution. Then, the mixture was reacted at 200 °C for 3 h.<sup>31</sup> The  
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33 obtained CDs (EDA-CDs) were also subjected to dialysis, purification, and kept in  
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35 low temperature.  
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43 **2.2. Cytotoxicity assay.** Briefly, Vero cells were seeded and allowed to grow  
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45 monolayer, and then incubation with CCM-CDs (15.6-250 µg/mL) for 24 and 48 h.  
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47 Then CCK-8 reagent was infected and cultured for 1 h.<sup>32</sup> The relative viability of cells  
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49 was counted based on the expressions: cell survival rate (%) = OD(sample)  
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51 /OD(control) × 100%.  
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56 **2.3. Entry assay.** Briefly, PEDV was exposed with various concentrations of  
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4 CCM-CDs (37 °C/1 h) and then added into 100% confluent Vero cells to allow the  
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6 entry of virions and infection for another 1 h, and then coated with medium. The cells  
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8 continue to be cultured until the control group shows viral lysis plaques. The viral  
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10 titers were measured via plaque assay.<sup>33</sup>  
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14 **2.4. Penetration assay.** Vero cells were precooled (4 °C/30 min), followed by  
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16 infection with PEDV to allow the attachment of virions to cells at 4 °C. After  
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18 incubation for 2 h, medium containing different concentrations of CCM-CDs were  
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20 supplemented and maintaining the cells at 37 °C to start in penetration of virus for 3 h.  
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22 The remaining steps are consistent with the entry assay.<sup>34</sup>  
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27 **2.5. Viral negative-strand RNA replication.** Vero cells were infection with  
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29 PEDV at a MOI of 0.01 (1 h), and then co-cultured with DMEM (supplement 10  
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31 µg/mL trypsin) or CCM-CDs. At 5, 6, 7 and 8 hpi, the cell monolayer was harvested  
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33 using TRIzol reagent and the expression of PEDV negative-strand RNA was detected  
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35 via real-time RT-PCR according to the literature.<sup>35</sup>  
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40 **2.6. Release analysis.** Vero cells were inoculated with PEDV at a MOI of 0.01  
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42 and incubated for the specified time. Then the supernatant was discarded and cells  
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44 were then untreated or treatment with CCM-CDs for 15, 30, 45 and 60 min.  
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46 Subsequently, medium supernatants and cell lysates including progeny PEDV were  
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48 harvested, respectively. Viral titer of samples was tested via plaque assay.  
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53 **2.9. Determination of ROS production.** ROS cumulation suppressed by  
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55 CCM-CDs-exposed Vero cells was measured based on the previously reported.<sup>36</sup>  
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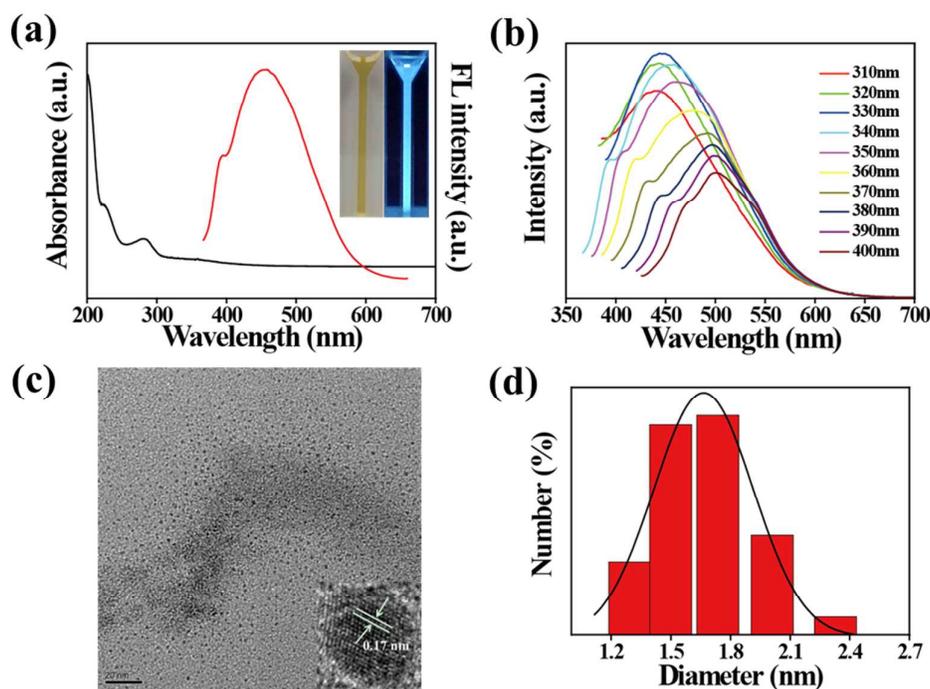
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3 Briefly, PEDV infection of Vero cells was performed by preincubation with 10  $\mu$ M  
4 DCF (30 min/37  $^{\circ}$ C), and then treatment with CCM-CDs for 12 h. The ROS level was  
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6 visualized by a confocal laser scanning microscope.  
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11 **2.10. Statistical analysis.** Independent t test or one-way ANOVA test were used  
12 to analyze the experimental data. The values and error bars represent the mean values  
13 and standard deviations of three independent experiments, respectively. Statistical  
14 significance was expressed as \*\*p < 0.01 and \*p < 0.05.  
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### 22 **3. RESULTS AND DISCUSSION**

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25 **3.1. Preparation and characterization of CCM-CDs.** The optical properties of  
26 CCM-CDs was firstly characterized through UV-vis spectroscopy. In Figure 1a, the  
27 characteristic absorption peaks at about 282 nm and 225 nm should be attributed to  
28 n- $\pi^*$  and  $\pi$ - $\pi^*$  transition, respectively. The FL spectra showed that the maximum  
29 fluorescence emission wavelength was 460 nm, suggesting a blue light emission.  
30 Figure 1b showed the red shift of the emission wavelength of CCM-CDs from 460 to  
31 501 nm in the excitation wavelength range from 310 to 400 nm. This phenomenon  
32 was called the excitation-dependent FL behavior, which was thought to be connected  
33 with the distribution of different sizes and surface states of CCM-CDs.<sup>37</sup> Besides, the  
34 fluorescence spectra of CCM-CDs were detected after changing the time of pyrolysis.  
35 As displayed in Figure S1, the maximum fluorescence emission wavelength of the  
36 CCM-CDs had an obvious red shift with the increase of pyrolysis time, which was  
37 consistent with the conclusion of quantum confinement effects.<sup>38</sup> In Figure 1c, the  
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4 resulting CCM-CDs were uniform in size and spherical in shape. The lower right  
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6 corner of Figure 1c displayed the average size of CCM-CDs was  $1.5 \pm 0.3$  nm by  
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8 high-resolution TEM (HRTEM) calculation. The Gaussian fitting curve revealed that  
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10 the average diameters of CCM-CDs were 1.7 nm (Figure 1d) and the zeta potential of  
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12 CCM-CDs was  $15.6 \pm 2.05$  mV. Since the surface of the CCM-CDs was rich in  
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14 hydroxyl groups and the solution is slightly acidic (pH = 6.8). The hydroxyl groups  
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16 were hydration with the positively charged  $H_3O^+$  in the solution,<sup>39</sup> resulting in a  
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18 certain degree of positive charge on the surface of the CCM-CDs.  
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**Figure 1.** (a) UV-vis absorption spectra and FL emission spectra of CCM-CDs in aqueous solution. (b) Excitation-dependent FL for CCM-CDs. (c) TEM and HRTEM images. Scale bar: 20 nm. (d) Size distribution of CCM-CDs.

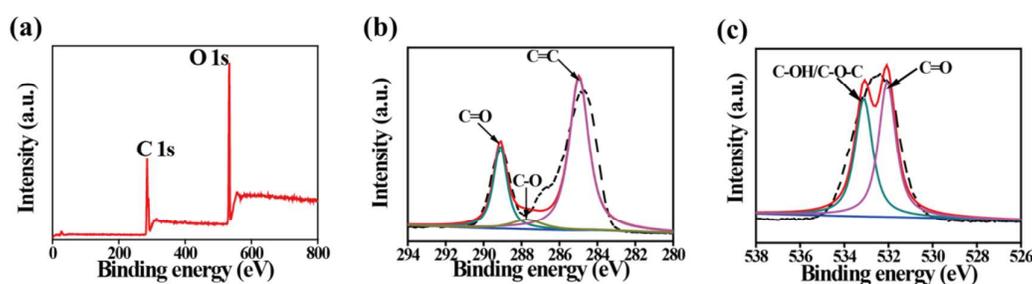
The fluorescence lifetime of the CCM-CDs was evaluated through

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4 time-correlated single photon counting (Figure S2). The average lifetime  $\tau$  can be  
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6 calculated by the equation:  $\tau = (B_1\tau_1^2 + B_2\tau_2^2)/(B_1\tau + B_2\tau_2)$ .<sup>40</sup> Table S1 presented the  
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8 fitting parameters of  $\tau_1$ ,  $\tau_2$ ,  $B_1$ ,  $B_2$  and  $\tau$ , and the lifetime of the CCM-CDs was  $8.81 \pm$   
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10 1.25 ns. Taking the quinine sulfate (QY = 54%) as the standard, the quantum yield  
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12 (QY) of CCM-CDs was calculated to be 3.6% based on the previously reported  
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14 procedure.<sup>41,42</sup>  
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19 In Figure S3a, both curcumin and CCM-CDs showed different functional groups  
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21 except for the phenolic hydroxyl. The absorption band at  $3200-3400 \text{ cm}^{-1}$  was due to  
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23 the stretching vibration of phenolic hydroxyl groups, which appeared either in  
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25 curcumin or CCM-CDs. Phenolic hydroxyl groups in curcumin may play vital roles in  
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27 its anti-oxidant, anti-inflammatory and radical scavenging activities, implying the  
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29 potential vital roles of the phenolic hydroxyl groups in the antiviral activity of  
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31 CCM-CDs. Besides, the band observed at  $1622 \text{ cm}^{-1}$  in curcumin are due to C=O  
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33 vibration bands, which shifted to  $1650 \text{ cm}^{-1}$  in CCM-CDs. The peaks at 1217 and  
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35 1019  $\text{cm}^{-1}$  for curcumin explicitly demonstrated the exist of C-O-C and C-O, which  
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37 disappeared in CCM-CDs. In Figure S3b, the X-ray diffraction pattern demonstrated  
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39 the crystalline nature of the sample. Due to their nanoscale size, the diffraction peaks  
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41 of the CCM-CDs distinctly broadened, suggesting the existence of CDs. The  
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43 characteristic bands in the Raman spectra of CCM-CDs were located at around 1345  
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45 and 1595  $\text{cm}^{-1}$ , corresponding to the D band and G band, respectively (Figure  
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47 S3c).<sup>43,44</sup>  
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56 The overall XPS analysis of the CCM-CDs showed that there were two bands at  
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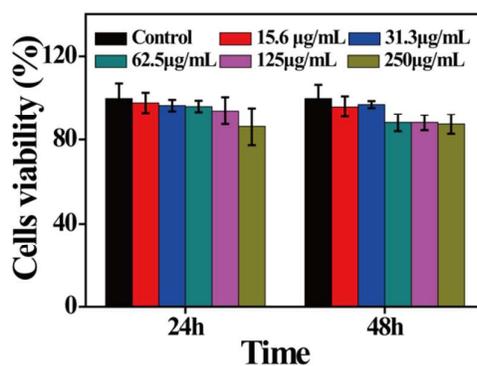
284.1 and 533.1 eV, corresponding to C 1s and O 1s (Figure 2a). The C 1s peaks at 284.6 and 288.8 eV (Figure 2b) attributed to the C-C and C=O functions, indicating that the synthetic CCM-CDs surface was rich in hydrophilic groups.<sup>7</sup> A contribution at 531.8 and 533.0 eV of O 1s showed due to the C=O and C-OH/C-O-C functions (Figure 2c).<sup>45</sup> The surface composition of the CCM-CDs as determined by XPS was accordance with the corresponding FT-IR conclusion. These results indicated that the remaining curcumin and citrate are not carbonized and remained on the surface of carbon dots as protective groups.



**Figure 2.** XPS-survey spectra of CCM-CDs derived from curcumin. Overall spectrum (a), C 1s (b) and O 1s (c) high-resolution survey spectra of the CCM-CDs.

**3.2. Toxicity of CCM-CDs on cells.** *In vitro* cytotoxicity of CCM-CDs against Vero cells and PK-15 cells was evaluated via CCK-8 assay (Figure 3 and Figure S4). As presented in Figure 3, the survival rate of Vero cells exposed with various concentrations of CCM-CDs for a different incubation time (24 and 48 h). As expected, Vero cells were co-incubated with CCM-CDs concentration ranges from 15.6 to 125  $\mu\text{g/mL}$  *in vitro* and cell viability was observed to exceed 90% after 24 and 48 h, confirming the low cytotoxicity of the as-prepared CCM-CDs. To test whether

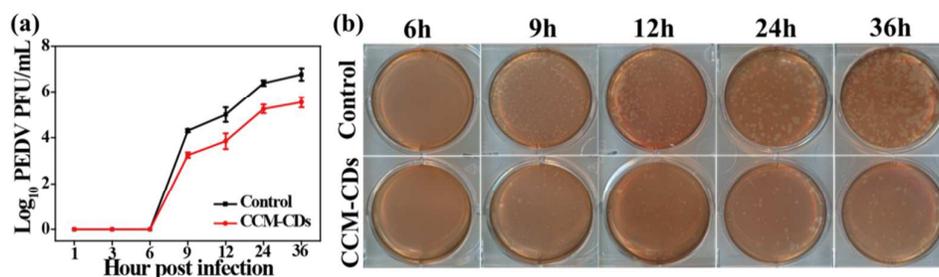
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4 DMSO had an effect on the stability of CCM-CDs at low concentrations, the particle  
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6 size of CCM-CDs in DMSO was measured. The average hydrodynamic size was 1.6  
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8 nm (Figure S5). This was close to the size measured when the solvent was water,  
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10 indicating that the effect of DMSO on CCM-CDs could be ignored. Thus, 125  $\mu\text{g}/\text{mL}$   
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12 CCM-CDs were used for the following antiviral experiments.  
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31 **Figure 3.** The effects of different concentrations of CCM-CDs on Vero cells viability  
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33 were detected by CCK-8 assay.  
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37 **3.3. Antiviral activity of CCM-CDs against PEDV infection.** The inhibitory  
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39 effect of the CCM-CDs was investigated through one-step growth curve assay to  
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41 evaluate the infectivity of PEDV. Specifically, the replication of PEDV in Vero cells  
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43 was determined with treatment and untreated of 125  $\mu\text{g}/\text{mL}$  CCM-CDs, followed  
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45 by quantifying of viral titers. As displayed in Figure 4a, compared with the control  
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47 groups, the virus titer of the experimental groups were obviously reduced,  
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49 demonstrating the efficient inhibitory effect of the CCM-CDs on PEDV infection.  
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51 Meanwhile, it can be visually observed that the plaque numbers were distinctly  
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53 reduced when exposure with CCM-CDs (Figure 4b). The reduction of plaque numbers  
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and decrease of virus titers indicated that CCM-CDs can effectively inhibit virus replication.



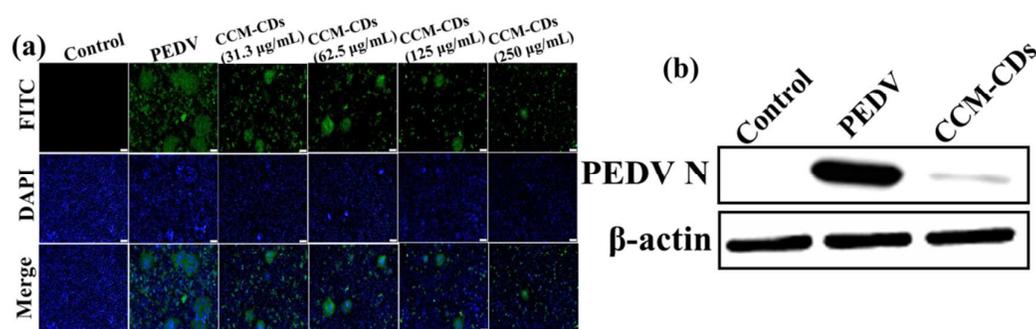
**Figure 4.** One-step growth curves of virus in the absence and presence of CCM-CDs

(a). Virus titers were detected in the presence and absence of CCM-CDs and photos were taken after 2-3 days post infection (b).

The protective effects of CCM-CDs were detected by fluorescent inverted microscopy. In Figure S6a, no cytopathic effect (CPE) was noticed in the negative control group. Cells treated with PEDV showed lysis and detachment from the cell monolayer at 12 hours post infection (hpi) (Figure S6b). However, the cytopathic effect was gradually diminished when treated with different concentrations of CCM-CDs (Figure S6c and S6d). These results suggested that CCM-CDs interfered with the infectivity of PEDV.

To study the inhibitory effect of CCM-CDs, the expression of PEDV N proteins in Vero cells was detected. In Figure 5a, the green fluorescence signals of the experimental groups were dramatically reduced, which is directly reflected in the decrease of the expression level of PEDV N protein. These results were confirmed by western blot analysis (Figure 5b). To test whether the quenching effect of carbon dots

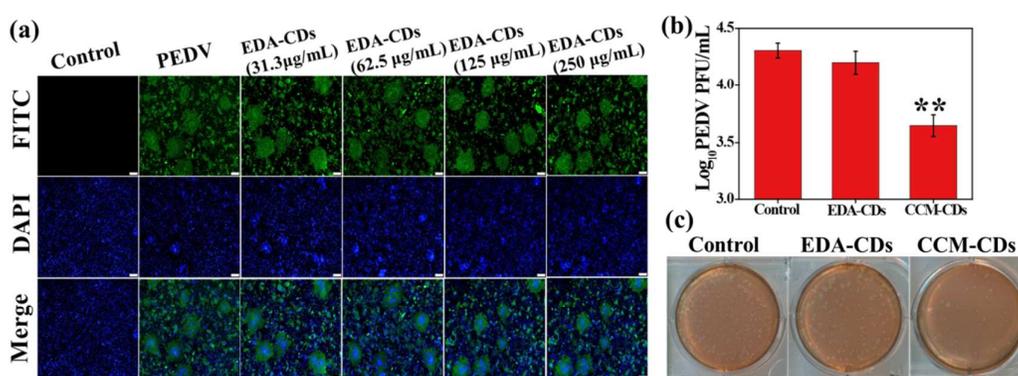
will influence the fluorescence, the fluorescence image was observed by using a confocal laser-scanning microscope. A weak blue fluorescence can be observed, indicating that CCM-CDs can be taken by Vero cell (Figure S7). However, the fluorescence of CCM-CDs can be neglected in comparison with that of FITC and DAPI. These results excluded the probability that the aforementioned reduction of viral titers was resulted from cellular toxicity of the CCM-CDs.



**Figure 5.** The effect of CCM-CDs on PEDV. (a) The effect of different concentrations of CCM-CDs on PEDV-infected Vero cells by indirect immunofluorescence assay. Scale bar: 100 µm. (b) The expression level of PEDV N protein in the presence of 125 µg/mL CCM-CDs at 12 hpi by western blot analysis.

The antiviral activity of CCM-CDs was also compared to that of EDA-CDs as ethylenediamine (EDA) is a common surface passivation reagent for the preparation of high yield CDs.<sup>31</sup> The optical properties and surface functional groups of the EDA-CDs were displayed in Figure S8. In Figure 6a, EDA-CDs were found almost impossible to suppress the virus infection at all tested concentrations. In Figure 6b and 6c, the virus titer values and plaque numbers revealed that CCM-CDs had a superior antiviral effect to EDA-CDs. The CCM-CDs showed a higher zeta potential (+15.6

mV) than EDA-CDs (-5.1 mV). Moreover, the effect of a positively charged CDs (33 mV) on PEDV N protein was also analyzed by western blot. From the Figure S9, it can be seen that the positively charged CDs showed a decrease in PEDV N protein expression than that of the control groups. We speculate that the surface charge of the CCM-CDs plays a small part in their antiviral efficacy. The positively charged CCM-CD on the surface undergoes strong electrostatic interactions with PEDV or cell membranes, thereby competing to bind the virus to cells.



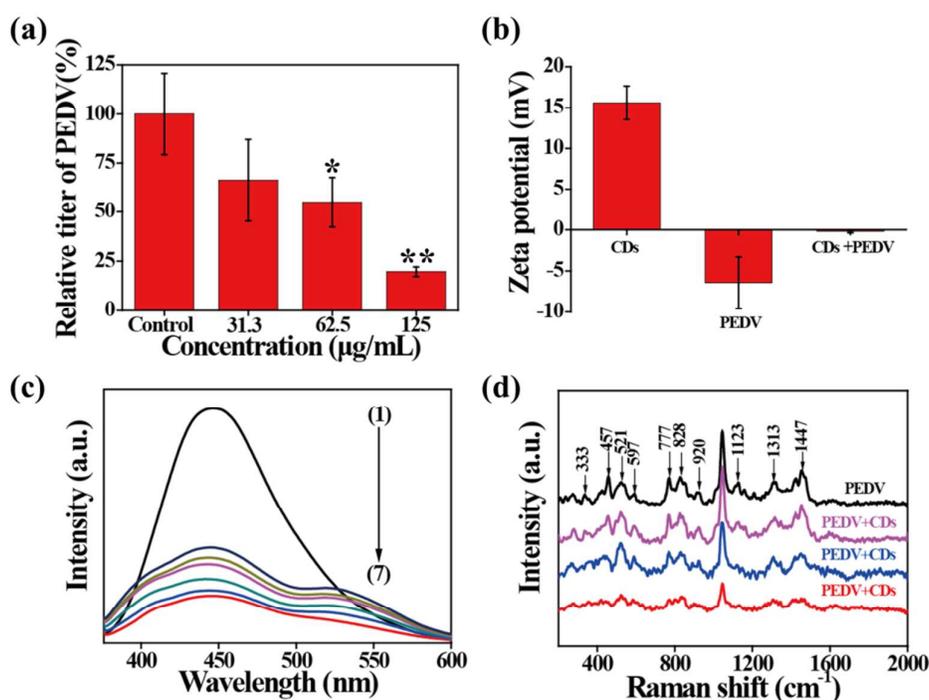
**Figure 6.** The effect of EDA-CDs on PEDV. (a) The effect of different concentrations of EDA-CDs on PEDV-infected Vero cells by indirect immunofluorescence assay. Scale bar: 100 µm. (b) The titer of PEDV when exposed or unexposed to 125 µg/mL EDA-CDs or CCM-CDs. All error bars were determined according to the three replicate experiments. (c) Virus titers were calculated in the presence and absence of EDA-CDs or CCM-CDs. Pictures were taken at 12 hpi.

**3.4. Mechanism of viral inhibition.** The proposed antiviral mechanisms of CCM-CDs cover direct action on viral entry, penetration, replication and budding.<sup>46</sup> And thus a set of experiments were carried out to identify which stage(s) of the viral

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4 life cycle were suppressed by CCM-CDs.  
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7 The influence of CCM-CDs on virus entry was evaluated by using plaque  
8 reduction analysis. PEDV samples were first treated with different concentrations of  
9 CCM-CDs, then inoculated into the cells (37 °C/1 h). In Figure 7a, CCM-CDs  
10 showed a strong concentration-dependent inhibitory effect on PEDV, suggesting that  
11 they blocked PEDV infection at the early stages of viral entry. The inhibition  
12 efficiency of CCM-CDs (125 µg/mL) on virus entry was over 50%. Furthermore, the  
13 zeta potentials of CCM-CDs, PEDV and CCM-CDs pretreated with PEDV were  
14 measured, and they were +15.6, -6.42 and -0.18 mV, respectively (Figure 7b),  
15 implying that the positively charged CCM-CDs may cause virus aggregation through  
16 electrostatic interaction, resulting in reduced viral infectivity. These results were  
17 verified through fluorescence and Raman spectral analysis. Figure 7c showed that the  
18 fluorescence intensity and the maximum emission of CCM-CDs decreased gradually  
19 and exhibited an apparent red shift along with the increase of PEDV concentration,  
20 indicating the occurrence of interaction between CCM-CDs and PEDV.<sup>47</sup> To  
21 demonstrate the interaction between CCM-CDs and PEDV, Raman displacement tests  
22 were carried out for PEDV when it was exposed or unexposed to different  
23 concentrations of CCM-CDs. From Figure 7d, it can be seen that, with the amount of  
24 CCM-CDs increasing, the Raman spectra exhibited most obvious differences in the  
25 shifts of 333, 457, 521, 597, 777, 828, 920, 1123, 1313 and 1447 cm<sup>-1</sup>. The peaks at  
26 521 and 777 cm<sup>-1</sup> were attributed to S-S disulfide stretch in proteins and  
27 cytosine/uracil ring breathing, respectively.<sup>48</sup> The peak at 828 cm<sup>-1</sup> was assigned to  
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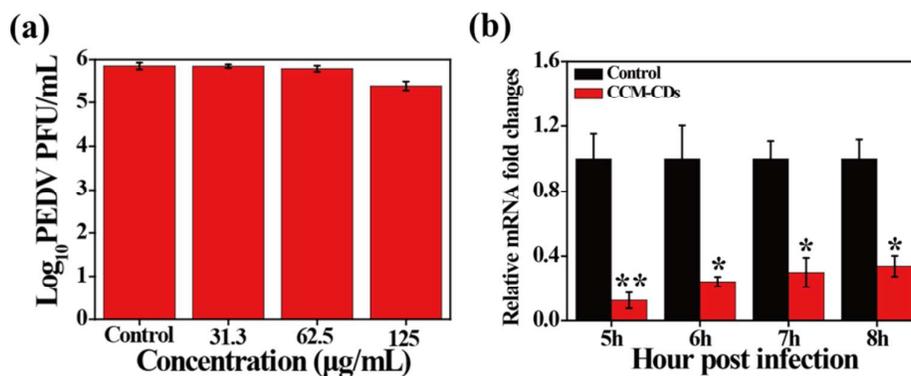
the out-of-plane ring breathing tyrosine. The bands at 920, 1123, 1313 and 1447  $\text{cm}^{-1}$  corresponded to C-C stretch of proline ring, C-C stretching mode of lipids/protein,  $\text{CH}_3\text{CH}_2$  twisting mode of collagen/lipids and  $\text{CH}_2$  bending mode of proteins and lipids.<sup>49,50</sup> These observation suggested that the addition of CCM-CDs had changed the structure of the protein.



**Figure 7.** (a) The dose relationship between the viral entry inhibitory efficiency and the amount of CCM-CDs added. (b) The zeta potentials of the CCM-CDs, PEDV and CCM-CDs pretreated with PEDV, respectively. (c) Fluorescence spectra of CCM-CDs. CCM-CDs (125  $\mu\text{g/mL}$ ) were exposed to PEDV (1-7: 0,  $1 \times 10^5$ ,  $2 \times 10^5$ ,  $3 \times 10^5$ ,  $4 \times 10^5$ ,  $5 \times 10^5$ ,  $6 \times 10^5$  PFU/mL). (d) Raman spectral analysis of PEDV and CCM-CDs-treated PEDV. Black line is PEDV ( $10^5$  PFU/mL), pink line represents PEDV ( $10^5$  PFU/mL) pretreated with 31.3  $\mu\text{g/mL}$  CCM-CDs, blue line represents PEDV ( $10^5$  PFU/mL) pretreated with 62.5  $\mu\text{g/mL}$  CCM-CDs and red line indicates

PEDV ( $10^5$  PFU/mL) pretreated with 125  $\mu\text{g/mL}$  CCM-CDs, respectively.

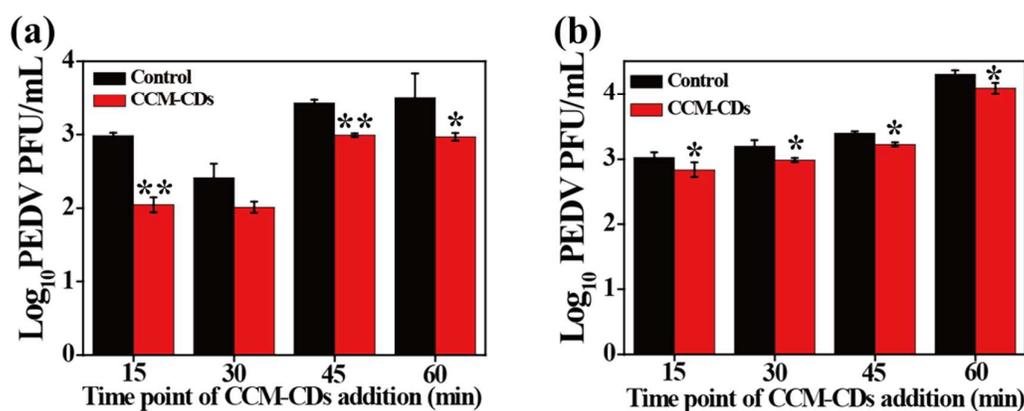
In order to test whether CCM-CDs can inhibit viral penetration, different concentrations of CCM-CDs were injected after attaching PEDV to the cell surface ( $4\text{ }^\circ\text{C}/2\text{ h}$ ), then penetration was started through transferring temperature to  $37\text{ }^\circ\text{C}$ . In Figure 8a, no noticeable difference was observed from viral titers after treatment or untreated with CCM-CDs, indicating that the CCM-CDs did not inhibit viral penetration. It is well documented that the synthesis of PEDV negative-strand RNA begins soon after penetrating into the infected cells. The inhibitory activity of CCM-CDs on negative-strand RNA synthesis was evaluated. The negative-strand RNA level of PEDV showed a remarkable down-regulation in CCM-CDs-treated cells compared to the untreated control at different hours post infection, suggesting that CCM-CDs can effectively inhibit PEDV at the replication step (Figure 8b).



**Figure 8.** (a) The dose relationship between the viral entry inhibitory efficiency and the amount of CCM-CDs used. (b) The relative synthesis of PEDV negative-strand RNA was measured after infection for various times.

To probe the influence of CCM-CDs on virion budding, viral titers in the

intracellular and supernatant after various times of CCM-CDs treatment were quantified. In Figure 9a, significant differences were detected in PEDV intracellular titers between CCM-CDs-treatment groups and the control groups. Whereas, the virus titer in the supernatant treated with CCM-CDs was only slightly less those in the control group, indicating that CCM-CDs could inhibit viral budding (Figure 9b).



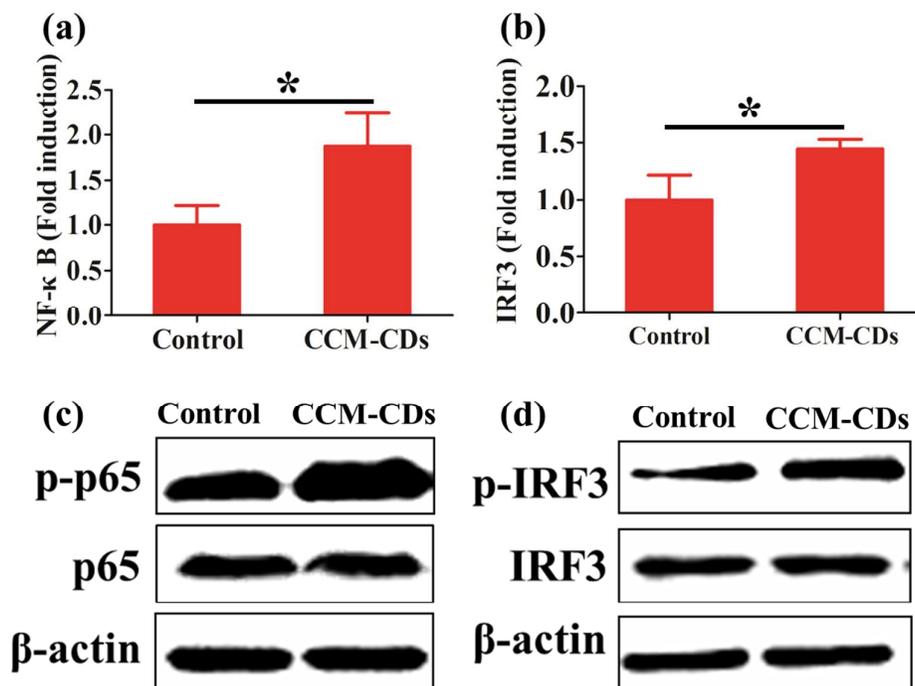
**Figure 9.** The titer of PEDV was quantified after treatment with CCM-CDs at different time points. Viral titer in intracellular (a) and in supernatant (b).

**3.5. CCM-CDs activation of antiviral innate immunity.** Innate immune response offers the first defense line against the invasion of pathogens and limits their spread.<sup>51,52</sup> Incoming viruses can activate nuclear factor- $\kappa$ B (NF- $\kappa$ B) and/or interferon-regulatory factors (IRFs) to induce the expression of hundreds of antiviral cytokines, including interferons (IFNs) and proinflammatory cytokines. Antiviral IFNs signal via the JAK/STAT pathway to drive the synthesis of a great number of IFN-stimulated genes (ISGs) or through direct stimulation in an IFN-independent pathway to collaboratively stop different stages of viral replication during viral replication cycle.<sup>53</sup> To investigate whether CCM-CDs can trigger host innate immune

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4 response, we examined the influence of CCM-CDs on the expressions of ISGs and  
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6 proinflammatory cytokines in vitro by measuring their mRNA levels using RT-PCR  
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8 (Figure S10 and S11). After CCM-CDs treatment, an obviously up-regulated  
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10 expression could be observed in interferon inducible protein 10 (IP-10) (Figure S10a),  
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12 interferon stimulated gene 54 (ISG-54) (Figure S10b), MxA (Figure S10c) and  
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14 interferon stimulated gene 20 (ISG-20) (Figure S10f). The mRNA expression levels of  
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16 interleukin 8 (IL-8) (Figure S10d) and interleukin 6 (IL-6) (Figure S10e) were also  
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18 5.8- and 3.0-fold more in cells treated with CCM-CDs than in the untreated controls.  
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23 It was found that TRIM32 can be used as an antiviral factor mainly by activating the  
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25 interferon immune response and promoting the expression of IL-8 and IL-6.<sup>54</sup> This  
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27 was consistent with our conclusion.  
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32 The cooperativity of the transcription factors IRF3 and NF- $\kappa$ B are necessary for  
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34 the induction of ISGs and pro-inflammatory cytokines. To examine whether  
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36 CCM-CDs induced expression of IRF-3 and NF- $\kappa$ B, Vero cells were first exposed or  
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38 unexposed with CCM-CDs, then the IRF3-Luc, NF- $\kappa$ B-Luc luciferase reporter  
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40 plasmids and the internal control plasmid pRL-TK were co-transfected. As  
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42 demonstrated in Figure 10a and 10b, the CCM-CDs could upregulate IRF3 and  
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44 NF- $\kappa$ B promoter activity. Moreover, the production of phosphorylated IRF3 (p-IRF3)  
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46 and p65 (p-p65) proteins in CCM-CDs treatment group was dramatically higher than  
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48 that in control group (Figure 10c and 10d). The phosphorylation of IRF3 and NF- $\kappa$ B  
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50 subunit p65 was considered to be the sign of IRF3 and NF- $\kappa$ B. Collectively, all these  
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52 data revealed that innate immune response might be triggered in vitro by the  
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CCM-CDs treatment.



**Figure 10.** CCM-CDs upregulated the expression of IRF3 and NF-κB promoter. Cells were cotransfected with the NF-κB-Luc (a) and IRF3-Luc (b) together with the pRL-TK plasmid. When exposure with CCM-CDs for 12 h, the luciferase test was carried out. CCM-CDs treatment stimulated phosphorylation of IRF3 and p65 (c, d). Cells were treated with CCM-CDs for 12 h, and then cells were harvested and detected by western blot assays with specific antibodies.

**3.6. Inhibition on ROS generation by CCM-CDs.** The infection of certain viruses occurs simultaneously with the overexpression of reactive oxygen species (ROS), leading to DNA damage by regulating apoptotic signaling pathways. In order to probe whether CCM-CDs can suppress ROS generation, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) test was performed. The

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3 stronger fluorescent intensity of DCF was observed in PEDV-infected cells compared  
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6 to the control group (Figure S12a and S12b). However, after treatment with  
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8 CCM-CDs, the fluorescence intensity turned weak (Figure S12c). Since most  
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10 curcumin was carbonized at high temperatures, and the prepared CCM-CDs was  
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12 centrifuged and dialyzed to remove unreacted small molecules. And the presence of  
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14 free curcumin was excluded, which indicated that CCM-CDs could inhibit ROS  
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16 generation induced by PEDV infection..  
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#### 21 **4. CONCLUSION**

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24 In conclusion, the fluorescent CCM-CDs were obtained by a simple one-step method  
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26 of pyrolysis of curcumin and its detailed characterization was performed. The  
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28 characterization results showed that the CCM-CDs had an ultrasmall size (diameter *ca.*  
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30 1.5 nm ), rich hydrophilic groups and a positive potential (+ 15.6 mV).  
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35 This is the first report showing that CCM-CDs obtained by pyrolysis of herbs  
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37 have prominent antiviral activity against PEDV infection. Compared with the  
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39 common CDs synthesized by pyrolysis of ethylenediamine, the cationic CCM-CDs  
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41 possess better antiviral properties. The underlying mechanism analysis indicates that  
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43 CCM-CDs exposure can inhibit the viral entry through changing the structure of viral  
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45 surface protein, and prevent the synthesis and budding of negative-strand RNA in  
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47 virus. It has also been demonstrated that CCM-CDs could obviously suppress the  
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49 accumulation of ROS caused by the PEDV. Furthermore, CCM-CDs treatment can  
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51 suppress virus reproduction by activating the production of interferon-stimulating  
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3 genes (ISGs) and pro-inflammatory cytokines of Vero cells.  
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7 Our results provide a novel clue to improve the antiviral activity of herbs. The  
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9 integrated data indicate that CCM-CDs can be potentially applied against not only  
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11 PEDV but also other viruses. However, further evaluation needs to be performed on  
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13 the prophylactic and therapeutic effects of CCM-CDs using an appropriate animal  
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15 model.  
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## 19 20 **ASSOCIATED CONTENT**

### 21 22 **Supporting Information**

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26 Supplementary data including: synthesis routes of CCM-CDs (Scheme S1),  
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28 fluorescent spectra (Figure S1), fluorescence decay curves (Figure S2 and Table S1),  
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30 primers sequence (Table S2), FTIR spectra, Raman spectra and XRD patterns of  
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32 CCM-CDs (Figure S3), cytopathic of Vero cells (Figure S4), size distribution (Figure  
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34 S5), cytopathic effects of Vero cells (Figure S6), fluorescence microscopy images  
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36 (Figure S7), characterization of EDA-CDs (Figure S8), western blot (Figure S9),  
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38 real-time RT-PCR assay (Figure S10 and S11) and indirect immunofluorescence assay  
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40 (Figure S12) .  
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3 \*These authors jointly supervised this work.  
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7 **Notes**  
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10 The authors declare no competing financial interest.  
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Abstract Graphic

