Biopolymer-directed synthesis of high-surface-area magnetite colloidal nanocrystal clusters for dual drug delivery in prostate cancer†

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Typical biopolymers, including soybean, casein, γ-poly(glutamic acid) (PGA), agarose and chitosan, were investigated as stabilizers in the synthesis of nanoporous magnetite colloidal nanocrystal clusters (MCNCs) by the hydrolysis and reduction of iron(III) chloride hydrate in ethylene glycol at 200 °C. The cluster sizes, morphologies, porous structures and magnetization of the resulting MCNCs were significantly affected by the different biopolymers. Of those members, soybean protein enabled the formation of spongy MCNCs with a surprisingly high specific surface area of 207 m² g⁻¹ and a characteristic mesopore diameter of 6.3 nm. Use of the other biopolymers (e.g. PGA and casein) led to the formation of MCNCs with lower specific surface areas (>100 m² g⁻¹) but considerably enhanced saturation magnetizations (~60 emu g⁻¹). Our results further shed light on the role played by the biopolymers in the structural evolution of the porous nanocrystal clusters. Analysis by characterizations of TEM and TGA showed that the decomposition of the biopolymer chains may have occurred during the transition from solid to porous clusters. As such, it is most likely that the biopolymers including soybean, casein and PGA serve as sacrificial templates to direct the formation of high-surface-area MCNCs. Taking into account the comprehensive properties of the different MCNCs, the PGA-stabilized MCNCs were selected as a drug delivery vehicle to simultaneously encapsulate therapeutic docetaxel (DOC) and ceramide (CER) via the hydrogen bonding interaction, for the treatment of prostate cancer. The inhibitory and apoptotic effects of the loaded DOC and CER co-delivered within MCNCs were evaluated in the prostate cancer cell line (PC-3) in vitro.

Introduction

Colloidal nanocrystal clusters (CNCs) are an extensively studied class of nanomaterial that is attracting increasing scientific attention across a wide range of disciplines.¹,² To date, various CNCs have been prepared in which inorganic nanocrystals have been endowed with a colloidal cluster structure and thus enhanced applicability.³ In particular, magnetite colloidal nanocrystal clusters (MCNCs) consisting of multiple single-domain magnetite nanocrystals formed in a three-dimensional, oriented attachment are very attractive due to the fact that assembly of 10–20 nm magnetic nanocrystals into a sub-micrometer-scale colloidal particle affords a high magnetic susceptibility as well as a low coercive force in the superparamagnetic regime.⁴ As such, MCNCs display a rapid response to an external magnetic field and are recyclable, which are highly desirable attributes for in vitro biological experiments such as sensing⁵ and DNA or protein separation.⁶,⁷ Also, the particle sizes of MCNCs could be controllable, giving rise to the appropriate size (less than 400 nm) for in vivo biomedical applications including magnetic resonance imaging⁸ and targeted drug delivery.⁹ Among the various synthetic methodologies, Li et al. pioneered the one-pot solvothermal synthesis that is one of the most intriguing methods for preparing cluster-structure magnetic nanoparticles.¹⁰ Uniformly sized MCNCs with enhanced magnetic performance have been attained by high-temperature hydrolysis and reduction of iron chloride hydrate in ethylene glycol. To achieve applicability in biomedical fields, Yin et al. synthesized highly water-dispersed MCNCs using poly(acrylic acid) as a stabilizer,¹¹ and Zhao et al. fabricated sodium citrate-stabilized MCNCs to improve biocompatibility with the aim of targeted drug delivery.¹² Both groups employed a similar solvothermal synthetic approach. Although these systems were rationally designed, synthesis of MCNCs of large size gives rise
to clusters with a limited surface area (approximately 10 m² g⁻¹) and low-capacity storage so as to restrict their application. Therefore, the synthesis of high-surface-area MCNCs needs to be undertaken. Recently, internally hollow MCNCs have been solvothermally prepared by controlling the ripening evolution of MCNCs with the aid of structure-directing agents. Compared with solid MCNCs, these are unique in that the mesoscopic void space greatly improves the amount of drug that can be loaded. However, their inferior colloidal stability in water limits their practical use and furthermore, the internally hollow microstructure is responsible for a sharply decreased magnetization. A surfactant-aided self-assembly method enables the generation of MCNCs with high surface areas (approximately 140 m² g⁻¹), which probably arise from their inherent loose structures. However, the presence of non-covalent interactions among nanoparticles means that the structural stability of these MCNCs cannot be ensured upon external intervention. In this context, it is a substantial challenge to find an alternative approach to the preparation of high-surface-area MCNCs with exceptional structural stability that are thereby applicable to targeted drug delivery in complicated physiological environments.

There have been many reports on the use of biopolymers to adjust the characteristics of advanced materials; for example, a biomimetic or biotemplating approach has been used to control the size, shape, crystal structure, orientation and organization of nanoscale matter. This bottom-up strategy is chemically diverse because biopolymers are believed to be a renewable and highly diverse source of nanometer-scale ordered complexes. Hitherto, they have been applied as templates in two ways for the construction of well-structured inorganic nanomaterials. The first is in directing the self-assembly of pre-synthesized nanoparticles based on the morphological characteristics and the functionality of the biopolymers. For example, linear single-stranded DNA or two-dimensional protein crystals have been utilized to guide the periodic assembly of nanoparticles for generation of nanowires or nanostructured arrays. The second method involves the use of biological species to prepare inorganic nanostructures in situ by virtue of specific interactions or molecular recognition for guiding the evolution of one-dimensional structures. For instance, protein fibrils with a spatially elongated conformation have been applied as templates to enable the formation of magnetic nanowires based on the multiple ion-binding sites of proteins. Despite the above-mentioned potential advantages, biotemplating techniques for the synthesis of MCNCs still remain largely unexplored. MCNCs are commonly obtained from solvothermal synthesis because of the larger surface-to-volume ratio of the nanocrystals that provides a strong thermodynamic driving force for their attachment. Since they depend on aggregation-based growth, it would seem most promising to explore specific-structured MCNCs by using biopolymers as templates to tune the aggregating characteristics of magnetite nanoparticles. This approach would afford clusters with high surface areas through the formation of rough surfaces or discrete inner cavities.

Thus, we employed a variety of biopolymers, such as soybean, casein, γ-poly(glutamic acid) (PGA), agarose and chitosan, as structure-directing agents in the solvothermal synthesis of MCNCs based on the high-temperature hydrolysis and reduction of iron chloride hydrate in ethylene glycol with NH₄OAc as an alkaline source. The mode of attachment, crystalite size and crystallinity of the primary magnetite nanoparticles were largely affected by the used biopolymers. Although variations in cluster microstructures, specific surface areas, and magnetic properties were exhibited, they were controllable so that the as-synthesized MCNCs simultaneously had permanent mesopores, high specific surface areas and rapid magnetic responses, all of which were comparable or superior to those of primary Fe₃O₄ nanocrystals. In addition, the effects of the reaction conditions on the evolution of the porous structures were deliberately investigated. This afforded the insight that biopolymeric chains that undergo thermal decomposition will serve as a self-sacrificing template responsible for the formation of a hyperbranching or spongy structure as the reaction progresses. Due to the advanced characteristics of the biopolymer-synthesized MCNCs, we embarked on a thorough study of MCNCs-assisted chemotherapy for prostate cancer by using PGA-stabilized MCNCs to encapsulate two drugs together (i.e. docetaxel (DOC) and ceramide (CER)). Aided by the loaded CER, the resistance of PC-3 cells to DOC was notably reduced. The inhibitory and apoptotic effects of the DOC/CER-entrapped MCNCs were also evaluated in vitro.

Results and discussion

Synthesis and characterization of nanoporous magnetite colloidal nanocrystal clusters (MCNCs) stabilized with various biopolymers

Various biopolymeric stabilizers were used to manage the nucleation and clustering-based growth of MCNCs. Specifically, agarose and chitosan are polysaccharides that are both able to anchor Fe₃O₄ nanoparticles with multiple functional groups (–OH, –NH₂, and –N–C=O) and to endow the resulting MCNCs for diverse surface functions. Also, it is reported that chitosan can act as a reductant to controllably synthesize the Ag or Au nanocrystals. PGA subunits are linked by peptide bonds and each has one carboxylic acid that is easily deprotonated to provide the strong electron-donating power for the chelation with metal ions. It is therefore likely that the ionized PGA will bind to Fe₃O₄ nanocrystals to form the stable conjugates, albeit without the reducing function. Soybean and casein are well-known thermally stable proteins, and both can strongly adsorb on the Fe₃O₄ nanoparticle surface through multiple bonds between iron and functional groups such as –COOH, –NH₂, –S and –OH units. Furthermore, soybean protein contains phytochemicals, e.g. sucrose and stachyose and thus will provide synergistic chemical reduction power with the aid of the production of metal nanoparticles; PGA and casein don’t have a similar reducibility. All of the biopolymeric stabilizers are low-cost and commercially available and produce MCNCs that can be dispersed in aqueous solution and have strong affinities for biological systems.

In a typical experiment, the MCNCs were synthesized by the hydrolysis and reduction of iron(m) chloride hydrate in ethylene glycol with a biopolymeric stabilizer and NH₄OAc as an alkaline to promote interaction with Fe³⁺ ions. The photographs in Fig. S1 (ESI™) record the reaction phenomena without or with the addition of stabilizers at different time intervals in 1 h. The solution without the biopolymers was eventually brick red. When
PGA was added, the mixture, which was originally yellow, turned black as the reaction progressed. The results indicate that the high-molecular-weight PGA is gradually dissolved and complexes with iron ions leading to the formation of metal–biopolymer complexes, which frequently show specific colours as a result of the variation of electronic transitions. Analogously, it can be seen that the pre-treated solutions with the addition of the other biopolymers were all darker than that of the solution without biopolymers after 1 h.

The TEM images in Fig. 1 display the controllable structures of MCNCs as synthesized with different biopolymeric stabilizers. As a control, Fig. 1a shows uniform MCNCs formed without the addition of a biopolymeric stabilizer, which have a solid micro-structure with a smooth periphery and dense texture. Fig. 1b displays the relatively regular colloidal cluster synthesized by chitosan. Compared with the bare MCNCs, the chitosan seems to cover the nanoparticle cluster that does not show the clear appearance of the primary magnetite nanoparticles. In addition, the grain size of the resulting MCNCs is predominantly approximately 250 nm, far bigger than that of the bare MCNCs (ca. 200 nm). Fig. 1c and d exhibit the cluster structures synthesized using agarose and PGA, respectively. The clusters are both flower-like with a rough surface, which are more branched than those shown in Fig. 1a and b. In the case of PGA addition, the resulting MCNCs (Fig. 1d) are composed of smaller primary nanoparticles that are randomly arranged but attached with respect to each other in an oriented fashion. As such, the branching structure is more evident than that offered by the agarose-synthesized MCNCs. Grain sizes are estimated to be approximately 150 nm. Fig. 1e and f display similarly structured MCNCs that were synthesized by utilizing casein and soybean, respectively. A close inspection shows that the primary nanoparticles are the smallest compared with the others and that they cluster into sponge-like spheres of irregular shape. Much clearer and enlarged TEM images of the five biopolymer-stabilized MCNCs are supplied in Fig. S2 (ESI†) to further elucidate their various structural characteristics. Additionally, we used the DLS to characterize the hydrodynamic sizes of the different MCNCs synthesized with or without biopolymers. The result in Table S1 (ESI†) reveals that the chitosan- and agarose-stabilized MCNCs have relatively large particle sizes, while the PGA-, casein- and soybean-stabilized MCNCs are smaller. Meanwhile, the polydispersity index (PDI) was obtained for determining the size and mass distribution in water. All of the biopolymer-synthesized MCNCs have a PDI of less than 0.2, indicative of a narrow size distribution. This is better than the PDI of the MCNCs synthesized without biopolymers (PDI = 0.228). Additionally, the PDI of the PGA-stabilized MCNCs is the smallest. It is likely that the fixed PGA with abundant uncoordinated –COOH groups affords a negatively charged MCNC surface and thus improves the dispersibility of MCNCs in water. The dispersion of PGA-stabilized MCNCs can remain stable in water for a couple of hours (Fig. S3 in ESI†), which is beneficial in bio-related applications.

Fig. 1  Representative TEM images of the MCNCs without (a) and with the stabilizers chitosan (b), agarose (c), PGA (d), casein (e) and soybean (f). Carbon mapping images of the individual MCNC synthesized by using chitosan (g), PGA (h) and soybean (i). All scale bars are 100 nm. The green dots denote carbon in the elemental mappings.
Based on the aforementioned results, we attribute the variation in grain sizes of the MCNCs to the vital role of biopolymeric stabilizers in the clustering of the nanocrystals. Since the isoelectric points of PGA, casein and soybean are all in the acidic pH region, they are positively charged under alkaline conditions. It is thus likely that the PGA-, casein- and soybean-stabilized Fe₃O₄ nanocrystals may have improved stability and their clustering will be limited due to the electrostatic repulsion, thereby leading to smaller cluster sizes. Chitosan and agarose are uncharged in the alkaline medium. It seems impossible that the self-assembly of chitosan- or agarose-coated nanocrystals is suppressed very much. Meanwhile, the carbonization of chitosan and agarose may take place under solvothermal conditions. This can form a carbon-containing residue covering the MCNC surface and is responsible for the larger grain sizes than those of the other biopolymer-synthesized MCNCs.

Apart from the polysaccharide-synthesized MCNCs, unique morphologies, which are acquired with the assistance of PGA, casein and soybean, are observed. To gain insight into the formation origin, we performed elemental mappings to ascertain the distribution of carbon throughout the representative individual MCNCs synthesized by using chitosan, PGA and soybean, respectively. As displayed in Fig. 1g-1, the distribution of carbon not only elucidates the presence of the biopolymer components in all three samples, but also identifies differences among them based on the abundance of carbon in each. It is found that the soybean-stabilized MCNC shows the greatest concentration of carbon in the particle area and the chitosan-stabilized MCNC has far lower carbon content than the others. TG analysis confirmed again that the weight loss of the adsorbed biopolymers increased from 9.7% to 10.6%, 11.6%, 12.3%, and 14.3%, corresponding to the samples solvothermally synthesized for 24 h with chitosan, agarose, PGA, casein and soybean, respectively (Fig. S4 in ESI†). The difference should stem from the variation in the process of the biopolymer-assisted agglomeration of primary Fe₃O₄ nanocrystals. To the best of our knowledge, heating will cause the gelation of soybean protein due to an increasing number of disulfide bonds. This will also happen to the casein as the protein chains can be interacted with calcium phosphate or gold nanoparticles to form a network. Thus, it is most likely that the network-like assemblies are generated by interlocking Fe₃O₄ and proteins, and as the reaction progresses these assemblies agglomerate to colloidal microspheres with a loose texture. As such, the carbon as displayed in the elemental mapping is abundant in the protein case.

The powder X-ray diffraction (PXRD) patterns for all of the MCNCs are compiled in Fig. 2A. They reveal that the characteristic peaks are well indexed to the cubic structure of Fe₃O₄ crystals, according to JCPDS 75-1610. Among them, the primary magnetite nanocrystals from the bare MCNCs give rise to the strongest and sharpest X-ray diffraction peaks, indicative of the formation of large-size, highly crystalline Fe₃O₄. When the biopolymeric stabilizers were utilized in the synthesis of the MCNCs, the PXRD patterns are markedly weakened and give lower S/N ratios. In the case of soybean, in particular, the main diffraction peaks are broadened. This implies that smaller sized crystallites predominate within the clusters and that an imperfectly oriented attachment exists in the crystal. To further elucidate the effects of biopolymeric stabilizers on their magnetic properties, all of the MCNCs were subjected to measurement on a vibrating sample magnetometer at 300 K. As shown in Fig. 2B, all behaved as a single superparamagnetized domain that normally would present a relatively low coercive force when the applied magnetic field is close to zero. There is a decreasing tendency to exhibit saturation magnetization when the biopolymeric stabilizers were used. The bare MCNCs displayed an extremely high saturation magnetization of up to 80.0 emu g⁻¹. When chitosan, agarose, PGA, casein and soybean were used, the values of the saturation magnetization were reduced to 74.0 emu g⁻¹, 66.2 emu g⁻¹, 57.1 emu g⁻¹, 47.7 emu g⁻¹ and 26.9 emu g⁻¹, respectively. The reason may be that the biopolymers largely restrict particle growth and thus the primary magnetite nanocrystals are so tiny that they elicit a considerably decreased magnetization in the resulting MCNCs. To investigate whether there is a direct relationship between these factors, we compiled the data in Table 1. The half-peak widths derived from the PXRD peaks at 35.5° were used to estimate the crystallite sizes of the primary magnetite nanocrystals via the Sherrer equation. When the particle sizes and saturation magnetization values corresponding to the different biopolymers are compared, the same trends in variation are observed. This implies that the magnetic performance is dependent on the nanocrystal size. However, we are aware that PGA- and casein-synthesized MCNCs have similar grain sizes but very different magnetizations. The results of a thermal gravimetric (TG) analysis in Fig. S4b (ESI†) demonstrate that 12.3% of casein is retained within the MCNCs, more than the PGA content (11.6%). Also, casein contains two inorganic elements, i.e. calcium and phosphorus, which will survive in thermal decomposition. Thus, a lower magnetization is obtainable.

Nitrogen adsorption–desorption measurements at 77 K were used to analyse the porous nature of MCNCs. The N₂ sorption isotherms are displayed in Fig. S5 (ESI†). It is evident that all of the profiles can be identified as type-IV isotherms, implying the characteristic of mesoporosity. The Brunauer–Emmett–Teller (BET) model was used to estimate the surface areas and pore volumes for the various biopolymer-stabilized MCNCs, as shown in Table 2. The bare MCNCs, as expected, give the lowest surface area due to dense packing of the primary nanoparticles. Apart from chitosan, the other biopolymeric stabilizers

![Image](http://pubs.rsc.org/supplemental/images/2012/07/45777B.png)
apparently enhance the surface areas of the resulting MCNCs, which are many times that of the control. In particular, soybean-synthesized MCNCs present superior nanoporosity with a specific surface area of \(207 \text{ m}^2\text{ g}^{-1}\) and a pore volume of \(0.34 \text{ cm}^3\text{ g}^{-1}\). The Barrett–Joyner–Halenda (BJH) model was used to obtain a narrow distribution profile of the pore sizes that was predominantly positioned around 6.3 nm. Casein, PGA and agarose also improve the porosity of their synthesized MCNCs.

To our knowledge, Fe\(_3\)O\(_4\) nanoparticles with a diameter of less than 20 nm have a high specific surface area up to \(140 \text{ m}^2\text{ g}^{-1}\). Thus, the biopolymer-synthesized MCNCs have surface areas that are sufficiently comparable to those of isolated magnetic nanoparticles. Taking into account the analysis of their microstructures, we reason that their nanoporous nature is probably derived from the following two aspects: (1) primary magnetite nanoparticles of smaller sizes are more susceptible to producing inner voids as they are attached to each other along a common crystallographic orientation; (2) a cluster structure with an open branching or sponge-like pattern can provide discrete inner slits or cavities.

**Evolution of the porous structure of MCNCs as the reaction proceeds**

To our knowledge, high-molecular-weight biopolymers normally have a limited solubility in solvents, but it was found that the solvothermal conditions lead to a considerable improvement in solubility. Due to the elevated temperature and strong polarity of ethylene glycol, conformational transition and thermolysis of the biopolymeric chains are more likely to occur. Therefore, we embarked on an investigation of the effects of the reaction conditions on the evolution of the porous structure and adopted PGA-stabilized MCNCs as a representative for a better understanding of this process.

Morphology variations at different time intervals were monitored. The TEM images in Fig. 3 exhibit the tendency for shape alteration to occur with a prolonged reaction time. Fig. 3a shows the spherical nanoparticle clusters after 6 h of reaction. The gray biopolymer component is clearly observed to coat the MCNCs. When the reaction time was extended to 10 h, 16 h and 24 h, the moiety at the periphery gradually disappeared, as displayed in Fig. 3b–d, respectively. Simultaneously, branching tips were exposed, making the branching structure more explicit. During this structural evolution, the size of the primary magnetite nanoparticles was nearly invariant, whereas the agglomerated sizes constantly decreased from ca. 300 nm to 150 nm. The TG analysis in Fig. S4a (ESI†) shows that the weight loss of the organic moiety decreases from 19.1% to 12.7% to 11.6%, corresponding to MCNCs synthesized at 6 h, 10 h and 24 h, respectively. This implies that the PGA retained on the MCNCs is lost gradually as the solvothermal reaction is prolonged. As is well known, PGA or PGA complexes decompose at around 200 °C, which is derived from the cyclodepolymerization of the main chain. We reason that after the initial PGA-tethered-Fe\(_3\)O\(_4\) clusters are formed, the

**Table 1** Effect of the various biopolymeric stabilizers on the crystallite sizes of primary Fe\(_3\)O\(_4\) nanocrystals and the saturation magnetizations of the resulting MCNCs

<table>
<thead>
<tr>
<th>Stabilizer</th>
<th>Half-peak width (degree)(^a)</th>
<th>Crystallite size (nm)(^b)</th>
<th>Saturation magnetization (emu g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>0.22</td>
<td>35.7</td>
<td>80.0</td>
</tr>
<tr>
<td>Chitosan</td>
<td>0.59</td>
<td>13.3</td>
<td>74.0</td>
</tr>
<tr>
<td>Agarose</td>
<td>0.69</td>
<td>11.4</td>
<td>66.2</td>
</tr>
<tr>
<td>PGA</td>
<td>0.78</td>
<td>10.1</td>
<td>57.1</td>
</tr>
<tr>
<td>Casein</td>
<td>0.79</td>
<td>9.9</td>
<td>47.7</td>
</tr>
<tr>
<td>Soybean</td>
<td>0.89</td>
<td>8.8</td>
<td>26.9</td>
</tr>
</tbody>
</table>

\(^a\) Half-peak width was obtained at the designated peak on 35.5°, and the unit was converted to rad as needed. \(^b\) The crystallite size was calculated from Sherrer’s Equation.

**Table 2** Porous characteristics of the MCNCs synthesized with various biopolymer stabilizers

<table>
<thead>
<tr>
<th>Stabilizer</th>
<th>Surface area (m(^2) g(^{-1}))(^a)</th>
<th>Pore volume (cm(^3) g(^{-1}))</th>
<th>Pore size (nm)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>19</td>
<td>0.04</td>
<td>—</td>
</tr>
<tr>
<td>Chitosan</td>
<td>27</td>
<td>0.10</td>
<td>—</td>
</tr>
<tr>
<td>Agarose</td>
<td>60</td>
<td>0.10</td>
<td>6.9</td>
</tr>
<tr>
<td>PGA</td>
<td>102</td>
<td>0.28</td>
<td>10.0</td>
</tr>
<tr>
<td>Casein</td>
<td>126</td>
<td>0.34</td>
<td>13.6</td>
</tr>
<tr>
<td>Soybean</td>
<td>207</td>
<td>0.34</td>
<td>6.3</td>
</tr>
</tbody>
</table>

\(^a\) Calculated by the BET method. \(^b\) Calculated from the N\(_2\) desorption branch by the BJH model.

Fig. 3 Representative TEM images of the PGA-stabilized MCNCs synthesized at (a) 6 h, (b) 10 h, (c) 16 h and (d) 24 h, respectively. All scale bars are 100 nm.
branching morphology of MCNCs are gradually produced due to the thermolysis of embedded PGA in ethylene glycol. Therefore, PGA possibly serves as a self-sacrificial template in the synthesis of the branched MCNCs.

For the other biopolymeric stabilizers, soybean and casein are well-known heat-stable proteins but do not remain chemically stable at 200 °C. Therefore, similar trends in weight loss were found (Fig. S4b and S4c in ESI†) and this probably leads to the porous structure as a result of formation of the biopolymer-embedded MCNCs. Since agarose and chitosan are susceptible to pyrolysis and dehydration, they function less well in biopolymer-directed clustering of Fe3O4 nanocrystals, which is responsible for the carbon residue apparently covering the dense MCNCs.

Inhibition and apoptosis of PC-3 cells by dual-drug-loaded MCNCs

As reported, prostate cancer usually responds to androgen deprivation therapy but is likely to transform to incurable hormone-refractory prostate cancer after 12–18 months.29 Utilization of chemotherapeutic agents such as docetaxel (DOC) is an important clinical option but the main challenge is how to tackle the problem of resistance to DOC. Ceramide (CER), the central molecule involved in sphingolipid metabolism, has been recognized as a cellular second-messenger that is able to trigger apoptotic events in many normal and cancer cells.30 In addition, it has been well demonstrated that CER can alter resistance to chemotherapeutic agents.31 As such, it is anticipated that if chemotherapeutic agents coupled with CER are used to treat prostate tumors, it will significantly enhance cytotoxicity.32 However, CER is prone to enzymatic degradation in systemic circulation and also does not penetrate well through the cell membranes.33 Thus, there is a need for improved systemic delivery that can encapsulate DOC and CER, facilitate cellular internalization, and protect the drugs against enzymatic degradation for maximum therapeutic effect. In this context, we investigated co-encapsulation of DOC and CER within porous MCNCs. Although the soybean- or casein-coated MCNCs have high surface areas and large pore volumes, their magnetic response is relatively weaker, which will limit the effectiveness of magnetic targeted drug delivery. Therefore, the PGA-coated MCNCs were used with consideration of their appropriate porosity and rapid magnetic response as well as the presence of multiple –COOH groups that can interact with both drugs for improvement of their loading. Experimental evidence of DOC and CER loading was directly demonstrated by using TG analysis in Fig. S7 (ESI†). Compared with the blank sample, a high drug loading capacity of 23 wt% for DOC and CER together was achieved, reflecting that the high-surface-area MCNCs can deposit a desirable amount of drugs as well as maintaining a high saturation magnetization after drug loading (44.7 emu g\(^{-1}\)). Note that the required amount of CER needed to treat prostate cancer is quite small relative to DOC. Consequently, the weight loss of CER is not clearly visualized in the TG curve because of the very small feeding amount of CER.

The in vitro drug releasing experiments were conducted at pH of 7.4 and 5.0, respectively, and only DOC release from the DOC+CER-loaded MCNCs was monitored by UV-vis spectrometry since the CER spectrum was overlapped by the DOC absorption curve. When the pH was 7.4, the cumulative release of DOC from the DOC+CER-loaded MCNCs was plotted against incubation time in Fig. S7a (ESI†). A burst release was observed at the early stage and the subsequent release was very slow. After 48 h, the released amount was as low as 16.8%. This result implies that most of the incorporated drugs remained in the MCNC pores at pH 7.4 and a small amount of the drugs adsorbed on the surface of the MCNCs was quickly released. In the acidic buffer solution, the releasing behaviour of the DOC+CER-loaded MCNCs was different. We found that the MCNC carrier was degraded quickly at a pH of 5.0 and the loaded drugs were released simultaneously. Fig. S7b (ESI†) is the UV-vis spectra for detecting the absorption changes of the dispersion of the DOC+CER-loaded MCNCs during the drug release. A shoulder peak appears at 462 nm, which is ascribed to the absorption of iron ions due to the acidic degradation of the MCNC carriers.34 The inset of Fig. S8b† shows that the DOC has a maximum absorption wavelength at 228 nm, which can be overlapped completely by the absorption curve of the iron ions. Thus, it is difficult to monitor the release of DOC at a pH of 5.0. However, it can be seen that the MCNC carrier is totally decomposed within 12 h. This means that the loaded drugs will be released in the same process. This is undoubtedly an excellent property for a drug delivery system since the release of the drug is pH-dependent and the carriers could be “degradable” and less toxic against normal cells and tissues.

The cell viability of PC-3 cells was measured using the MTT assay. As shown in Fig. 4, there is no statistical difference between the cell viabilities upon addition of MCNCs at 24 h, 48 h and 72 h. In other words, the MCNCs are of negligible toxicity towards the PC-3 cells, confirming again their biocompatibility. All other experimental groups involving DOC, CER, DOC+CER, or DOC- or CER- or DOC+CER-loaded MCNCs
evidently inhibited cell viability ($p < 0.05$), exhibiting a time-dependence. The most significant variation was found at 72 h ($p < 0.01$) when the DOC+CER-loaded MCNCs gave the lowest cell viability ($p < 0.05$). It is reported that CER is able to enhance the anti-proliferative and apoptotic effects of paclitaxel.\textsuperscript{31} As both DOC and paclitaxel are taxanes, we were concerned about the issue of whether or not CER could promote the chemotherapeutic effect of DOC. Undoubtedly, the results show the enhanced efficacy of a combination of free DOC and CER. Furthermore, the DOC+CER-loaded MCNCs exhibit a more efficient inhibitory effect on PC-3 cells than the two free drugs used together.

It appears that MCNCs alone could not inhibit the growth of PC-3 cells whereas they do improve the inhibitory effect on PC-3 cells when carrying the two drugs together. We attempted to undertake a meticulous study to better understand the role of MCNCs in promoting the death of PC-3 cells. Apoptosis is a form of programmed cell death that plays an essential part in the normal development and homeostasis of organisms and gives us another chance to investigate through a specific death program. As shown in Fig. 5, when PC-3 cells were treated with DOC-, CER- and DOC+CER-loaded MCNCs for 24 h, their apoptosis rates rose to 19.6%, 14.3% and 25.6%, respectively, which correspond to increases of 4.1%, 1.1% and 4.9% compared with the apoptosis rates of DOC, CER and DOC+CER. This indicates that the MCNCs indeed assist in inducing cell death when they are used together with the drugs.

The Bcl-2 family of proteins includes proapoptotic and antiapoptotic members, which are key regulators of programmed cell death. Several studies have reported that the mechanisms of apoptosis induced by both DOC and CER correlated with two simultaneous processes, namely, inhibition of the expression of Bcl-2 (an antiapoptotic member) and enhancement of the level of Bax (a proapoptotic member).\textsuperscript{35} In addition, the caspase family is a group of cysteinyl aspartate-specific proteases that are highly conserved in multicellular organisms. Caspase-3, a member of this family, has been identified as a key mediator of apoptosis.\textsuperscript{36} As a result, based on these three specific proteins, a mechanism of apoptosis was proposed through comparison of the behaviour of dual-drug-loaded MCNCs with their control groups.

As shown in Fig. 6, it appears that there is no statistical difference in the expression of Bcl-2 and Bax in PC-3 cells between the DOC+CER-loaded MCNCs and the control groups. In a set of Bcl-2 tests, we found that the expression of Bcl-2 was significantly lower in the six drug-containing groups than in the MCNC group and the negative control. Of those drug-containing groups, the free DOC+CER and the DOC+CER-loaded MCNCs both gave the lowest expression of Bcl-2. Also, they could attain an enhanced expression of Bax compared with the others. As shown in Fig. 7, the MCNCs had no obvious effect on caspase-3 activity in PC-3 cells. The three drug-containing MCNCs presented a higher caspase-3 activity than those of MCNCs, DOC, CER and DOC+CER. In addition, the caspase-3 activity of the DOC- and CER-loaded MCNCs were both lower than that of the DOC+CER-loaded MCNCs. As a result, it could be surmised that MCNCs have no obvious

\textbf{Fig. 5} Apoptosis rates of PC-3 cells after treatment of various agents at 24 h.

\textbf{Fig. 6} Effects of the negative control (A), DOC (B), CER (C), DOC+CER (D), MCNCs (E), DOC-loaded MCNCs (F), CER-loaded MCNCs (G) and DOC+CER-loaded MCNCs (H) on the protein levels of Bcl-2 and Bax in PC-3 cells.

\textbf{Fig. 7} Effects of the agents on the caspase-3 activity of PC-3 cells.
effect on apoptosis and, upon loading of dual drugs, the most significant apoptosis-related behaviours are the enhancement of caspase-3 activity, the inhibition of Bcl-2 expression and the increase in Bax expression. However, we could not distinguish between the various expressions of Bcl-2 and Bax promoted by the DOC+CER- versus DOC-loaded MCNCs. In spite of this, our results verify that a combination of DOC and CER largely improves the efficacy of apoptosis through the inhibition of resistance to drugs; MCNCs are also biocompatible without changing the mechanisms of apoptosis. A further study is under way to specifically investigate the variations in apoptosis induced by dual-drug-loaded MCNCs.

Conclusions
To summarize, structurally tunable MCNCs with enhanced magnetic properties, high specific surface area, permanent nanoporosity, diverse surface functions and prominent biocompatibility were synthesized by a solvothermal route. Various biopolymeric stabilizers, such as soybean, casein, PGA, agarose, and chitosan, were applied to control the formation of nanoporous Fe3O4 clusters based on the hydrolysis and reduction of iron(III) chloride hydrate in ethylene glycol at 200 °C. The results show that the colloidal morphologies, grain sizes, nanoporosity, and magnetic properties of the MCNCs are strongly dependent on the nature of the different biopolymeric stabilizers. Soybean promoted the synthesis of sponge-like MCNCs composed of relatively small primary Fe3O4 nanoparticles, thus affording high surface areas of 207 m² g⁻¹ and accessible mesopores of 6.3 nm. Casein and PGA were similarly used to obtain nanoporous MCNCs, albeit with reduced surface areas. Their structures feature branched packing and their saturation magnetizations are considerably enhanced due to the increased crystallite sizes of the primary magnetite nanoparticles. Meanwhile, the effect of reaction conditions on the evolution of the porous structure was investigated. Combined with analysis by various characterizations, it seems most likely that, upon the formation of biopolymer-embedded MCNCs, PGA, soybean and casein serving as sacrificial templates are amenable to degradation for creating the high surface area of MCNCs under solvothermal conditions. Dependent on the comprehensive properties of the MCNCs synthesized with the various biopolymers, the PGA-stabilized MCNCs were applied as drug delivery carriers to encapsulate the two drugs. CER in the composite system successfully alters resistance to DOC in the PC-3 cells. As such, a significant enhancement of the inhibitory and apoptotic effects in PC-3 cells was found. In addition, in view of the apoptosis study, the synthesized MCNCs are demonstrated to be of superior biocompatibility and are a promising delivery vehicle for chemotherapy for cancers.

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Notes and references