Introduction

Cancer has become a major threat to human beings due to its high incidence, severe mortality, and low survival rate. Besides traditional surgical operation, chemotherapy plays an important role as a primary and supportive care for cancer treatment. However, current chemotherapy still faces severe challenges including side effects and drug-resistance. Multiple drug resistance (MDR) is one of the major limitations in cancer therapy. MDR decreases the accumulation of drug within the cell and increases the repairing mechanism of DNA damage therapy. MDR decreases the accumulation of drug within the cell and increases the repairing mechanism of DNA damage leading to low therapeutic efficiency of single agents. The efflux pump mediated drug resistance is predominantly attributed to the over-expression of P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP) which may pump the anticancer drugs out of the cancer cells. RNA interference with small interfering RNA (siRNA) that mediates gene-silencing phenomena has shown great potential as tactics for a number of diseases. Contemporaneously, delivering siRNA and drug could achieve a combination effect of chemotherapy and gene therapy. Nevertheless, the clinical use of siRNA is hindered by three major problems: (1) rapid enzymatic degradation-induced short blood circulation; (2) poor cellular uptake; (3) insufficient tissue bioavailability. It is difficult for naked siRNA to reach tumour sites and successfully enter cells, which means that it needs a carrier system to exert the biological activity of siRNA, especially, in vivo application. Therefore, to stabilize the siRNA in systemic circulation and to ensure its maximum accumulation in cancer tissue, an effective drug delivery carrier is urgently desired.

To date, various codelivery platforms for siRNA and chemotherapy agent have been established to resolve the problem of MDR in cancer cells. As a naturally nontoxic biomineral, calcium phosphate (CAP) featuring acid-degradability, biocompatibility, and simple chemical composition, shows great potential for biomedical applications, such as serving as a vehicle for gene delivery. CAP formed complexes with the nucleic acid backbone and imparted a stabilizing function to certain DNA structures. Then the complexes passed across the cell membrane via ion channel mediated endocytosis. Usually, the CAP/DNA complex was prepared via co-precipitation. However, the rapid growth of calcium phosphate crystal was hard to control, and resulted in the formation of large agglomerates, which drastically reduced the complex quality and transfection efficiency, inspiring us to develop new strategy to prepare CAP-based composite materials.

In this paper, a novel drug delivery system (DDS) with good biocompatibility and pH sensitivity was developed as depicted in Scheme 1. Colloid CAP was embedded into the net structure of SiO2, forming CAP/SiO2 composite through reverse microemulsion method. Anticancer drug doxorubicin (DOX) was then
encapsulated into the CAP/SiO₂ composite in the form of Ca²⁺–DOX complex. Furthermore, P-pg-targeted small interfering RNA (siRNA) was absorbed into the CAP/SiO₂ composite via electrostatic interaction. The prepared CAP/SiO₂ composite could protect siRNA due to the high affinity of Ca²⁺ ion to the phosphate groups in the nucleic acids. The prepared nanocarrier exhibited an excellent pH-responsive release property, for the CAP component in CAP/SiO₂ was easily dissolved in lower pH environment. As a result, drug and siRNA could be released from the carrier in a weak acid condition. This codelivery system has been tested in human chronic myelogenous leukaemia K562/ADR cell line, which is drug-resistance cell strain. Cell experimental results suggested that DOX and siRNA codelivery by this nanocarrier could have synergistic effect in treatment in K562/ADR cell line. A greater in vitro cytotoxic effect was observed with the siRNA/DOX codelivery compared to free-DOX in K562/ADR cell line. This proposed codelivery system might be a promising approach in MDR tumour cell lines in the future.

Results and discussion

Fabrication and characterization the CAP/SiO₂ composite

CAP/SiO₂ composite were prepared using a reverse microemulsion method and further characterized by TEM, XRD and IR. The CAP colloid was prepared with one-step method in water–ethanol liquid, as shown in Fig. 1a, generating CAP/SiO₂ composite with coarse surface and uniform size around 100 nm in Fig. 1b. Dynamic light scattering (DLS) was used to determine the size distribution of particles. The particles have narrow size distribution with mean size of 125 nm, and the poly dispersity index (PDI) less than 0.2, indicating that the particles were homogeneous. To investigate the chemical composition, XRD patterns of pure CAP and CAP/SiO₂ composite at room temperature were recorded in Fig. 1c. The generated sharp peaks ranging from 15 degree to 40 degree were attributed to the hexagonal hydroxyapatite (JCPD 09-0432). CAP/SiO₂ showed only one hump peak and a small peak located at about 31 degree, indicating that CAP has been incorporated into the skeleton of SiO₂ during the synthesis process. The fabrication mechanism of the CAP/SiO₂ composite was also investigated. In this work, a reverse microemulsion (water/oil) method was adopted to embed CAP into SiO₂ skeleton to form CAP/SiO₂ nanohybrids. Cyclohexane served as a continuous phase in which tetraethyl orthosilicate (TEOS) was dissolved. Microscale water pools containing CAP colloid were stabilized by Triton X-100 and hexyl alcohol. Under the catalysis of ammonia, the TEOS underwent hydrolysis at the oil/water interface, forming Si–O–Si–O continuous network structure to encapsulate CAP. It was found that the amount of CAP colloid was crucial to the preparation of homogenous CAP/SiO₂ composite. Pure silicon sphere-like CAP/SiO₂ composite with smooth surface were obtained (Fig. S1a†) when a small amount of CAP was added. As the amount of CAP increased, the surface became coarse (Fig. S1b†). However, if excessive CAP was added,

| Atom  | Si 2p | Ca 2p | P 2p | O 1s | N 1s |
|-------|-------|-------|------|------|------|
|       | 25.78 | 11.80 | 8.79 | 52.78 | 1.39 |

Table 1  Element containing of CAP/SiO₂ by XPS
separated CAP and silica would be observed as shown in Fig. S1c and d.† The reason might be that: as the amount of negatively charged CAP colloid in water phase increased, more CAP were pushed out of the silica intermediate due to the electrostatic repulsion. The surface charge is another important factor that influences the heterogeneous nucleation of CAP/SiO2 composite. Here, positively charged aminopropyltriethoxysilane (APTES) was used to reduce the negatively charge density of the silica intermediates and promote the stable incorporation of more CAP into the composite.26 Furthermore, the amino group in APTES molecule also catalysed the hydrolysis reaction, accelerated their reaction process and rapidly increased the viscosity of the SiO2 spheres, which also helped to trap the CAP colloid within the microspheres. As shown in Fig. S2,† mono-dispersed CAP/SiO2 composite particles were prepared when the volume ratio TEOs to APTES was 1 : 1 (Fig. S2a†). However, excessive APTES increased the viscosity and caused agglomeration (Fig. S2b and c†).

**Drug loading and pH controlling release study**

CAP/SiO2 composite was proved to be an ideal nanocarrier for drug delivery that CAP on the surface provided a large number of anchoring points large for drug molecule, wherein Ca2+ ion was chelated with DOX to form Ca2+-DOX complex.24 Ca2+ ion had a high affinity with DOX than ordinary static electricity, which avoided non-specific adsorption and early drug release. The supernatant of CAP/SiO2 was detected by UV-vis (Fig. S3†), and the decreasing absorbance indicated the successful formation of the CAP/SiO2/DOX composite. The proposed CAP/SiO2 composite had good loading capability that encapsulation of DOX was up to 90% and the amount of DOX loaded into the CAP/SiO2 was determined to be 180 mg g−1.

The pH-responsive degradability of the CAP/SiO2 composite was demonstrated by the following experiments. Firstly, the release rate of DOX from CAP/SiO2 composite in different pH solutions (pH 7.4 and 5.0) were measured to mimic the micro-environment of healthy tissue and cellular lysosomes, respectively. The released DOX was quantified by UV-vis absorbance at 480 nm within 48 h. As shown in Fig. 2a, the release amount reached to 67% after DOX-loaded CAP/SiO2 dispersed in pH 5.0 for 48 h compared to that of 20% in pH 7.4 buffer solution, indicating good stability in a physiological environment. Upon exposure to pH 5.0 buffer, the cumulative release of DOX from CAP/SiO2 composite was much faster than that in pure SiO2 (Fig. 2b), indicating that the degradation rate of pure silica with stable network structure was much slower than that of CAP/SiO2 composite.24 The reason was that, the embedded CAP in the CAP/SiO2 composite was dissolved to release Ca2+ ion under weak acidic condition. With the Ca2+ ion entering into solution, the DOX was also removed from the original composite, which not only facilitated the DOX release, but also promoted the degradation of the composite.24 The degradation process of CAP/SiO2 in acid buffer solution (pH 5.0) was also monitored by TEM (Fig. S4†). At first stage, the nanoparticles decomposed at the periphery with small pieces of debris shed from the surface. As time went on, the particle size became smaller and smaller until all composites were broken down into pieces, demonstrating that the prepared CAP/SiO2 had fast pH responsive behaviour and good biodegradability.

**Delivery of CAP/SiO2/DOX@siRNA complex into cells**

CAP/SiO2/DOX@siRNA complex was then prepared by adsorbing siRNA on the surface of CAP/SiO2/DOX composite via electrostatic interaction (Fig. S5†). The strong interaction between calcium ions and negatively-charged phosphate enabled the formed complex to construct a stable inner core for siRNA encapsulation.22 As shown in Fig. 3, when the mass ratio of the composite to siRNA increased to 20 : 1, almost all of the siRNA was absorbed on the complex surface. When the ratio of composite to siRNA was further increased to 25 : 1, the line of siRNA disappeared, indicating that there was no residual siRNA in supernatant.

Next, we investigated the intracellular delivery of siRNA and DOX using our nano-complex. K562 and K562/ADR cells lines were selected as DOX-sensitive and DOX-resistant cell lines, respectively. The IC50 values of free DOX towards K562 and K562/ADR were 0.5 and 40 μg mL−1, respectively. We incubated the K562/ADR cells with DOX-loaded CAP/SiO2@siRNA-FAM (containing DOX 20 μg mL−1) nanocarriers for 12 h. As shown in Fig. 4a, the green fluorescence from siRNA-FAM and the red fluorescence from DOX were all observed in the cytoplasm of K562/ADR cells, indicating that our nano-complex have delivered both the drug and siRNA into the cells.9 As shown in Fig. 4b, CAP/SiO2/DOX@siRNA were incubated with K562/ADR

![Fig. 2](image-url) Mass percentage of cumulative released DOX from (a) CAP/SiO2 in buffer solution (pH 7.4 and 5.0) (b) CAP/SiO2 and SiO2 in pH 5.0 buffer solution.

![Fig. 3](image-url) Agarose gel electrophoresis of CAP/SiO2/DOX to which siRNA was complexed at various composite to siRNA ratios. Lane 1 was for molecular weight mark, lane 5 was for siRNA only, lane 2, 3, 4, 6, 7, 8 was for the supernatant at different ratios of composite to siRNA, 25 : 1, 20 : 1, 16 : 1, 10 : 1, 8 : 1, 4 : 1, respectively.
cells for 4 h, 8 h, and 12 h, the red fluorescence intensity increased along with the incubation time, indicating gradual internalization of CAP/SiO₂/DOX@siRNA, subsequent disintegration and the following DOX release. By co-staining with DAPI molecules, it was found that CAP/SiO₂/DOX@siRNA was mostly distributed in the cytoplasm (Fig. S6†).

**Therapeutic effects of the drug delivery system in living cells**

The therapeutic efficacy of the prepared nanocarriers was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Fig. 5a, no significant cytotoxicity of CAP/SiO₂ composite was observed even at a high concentration of 100 μg mL⁻¹, indicating good biocompatibility of CAP/SiO₂ composite. K562/ADR cells were further treated with free DOX, CAP/SiO₂/DOX and CAP/SiO₂/DOX@siRNA for 24 h. All the materials contained equivalent DOX concentration. The cell viability decreased as the concentration of DOX increased, indicating a dosage-dependent cytotoxicity (Fig. 5b). The CAP/SiO₂/DOX@siRNA containing 30 μg mL⁻¹ DOX, causing 77% cell death, which might be ascribed to the fact that the siRNA knocked down the expression of P-gp protein and sensitized the drug toxicity and therefore favoured the therapy.

**Molecular biology assays**

The knockdown efficiency was assessed by measuring the levels of siRNA and protein based on the real-time polymerase chain reaction (RT-PCR) and western blot (WB), respectively. As shown in Fig. 7a and b, the levels of MDR1 mRNA and its P-gp protein in K562/ADR cells were higher than those in K562 cells.
The proposed drug/gene codelivery system has potential application and improve therapeutic efficacy of human chronic leukaemia. Such easy-to-fabricate composite nanomaterial not only featured good biocompatibility and acid biodegradability, but also showed synergistic effect of siRNA and drug in inducing multiple drug resistance cells apoptosis. We believe that the proposed drug/gene codelivery system has potential application in MDR tumour cell lines for clinical trial in future.

Conclusions

In this work, we have successfully developed novel pH-sensitive CAP/SiO2 composite nanocarrier for drug and gene codelivery. This nanomaterial exhibits narrow size distribution and good stability in physiological environment. In this system, DOX was loaded onto the CAP/SiO2 composite by forming Ca2+-DOX complex, while siRNA-targeted P-gp was absorbed on composite via electrostatic attraction. This composite material demonstrated high drug loading efficiency and protected the siRNA from being degraded by enzyme in cells. The CAP/SiO2/DOX@siRNA delivery system combining chemotherapy and gene therapy exhibited enhanced cellular uptake with higher cytotoxicity towards K562/ADR cells as compared to a single loaded system. Such easy-to-fabricate composite nanomaterial not only featured good biocompatibility and acid biodegradability, but also showed synergistic effect of siRNA and drug in inducing multiple drug resistance cells apoptosis. We believe that the proposed drug/gene codelivery system has potential application in MDR tumour cell lines for clinical trial in future.

Experimental

Synthesis CAP/SiO2 composite

Calcium phosphate (CAP) was synthesized according to a previous report.14 Briefly, 0.1388 g CaCl2 was dissolved in 32.5 mL ethanol under magnetic stirring. Then 1.5 mL 0.1 M NaOH aqueous and 3.5 mL deionized water were injected into the ethanol solution. In addition, 12.5 mL of phosphate aqueous solution containing 0.2685 g Na2HPO4·12H2O was poured into above solution. The reaction continued under magnetic stirring for 1 minute. The colloidal product was centrifuged and washed with ethanol and deionized water, then dissolved in water and kept in refrigerator at 4 °C. CAP/SiO2 composite was prepared via the synchronous modification of functional group in the water-in-oil microemulsion.19 In a typical synthesis, 7.5 mL cyclohexane, 1.8 mL hexyl alcohol, 1.77 mL Triton-X and 100 μL water was put together to get reverse microemulsion, then 380 μL calcium phosphate colloidal solution was put into the microemulsion as water phase. After half an hour later, 25 μL TEOS and 25 μL APTES were added, under stirring for another 30 minutes. The 60 μL NH3 was added another 40 min later. After stirring for another 22–24 h, the emulsion was broken by acetone. At last, the product was obtained by centrifugation, and washed by ethanol and water twice respectively.

Drug loading onto the CAP/SiO2 composite

Doxorubicin was loaded inside the CAP/SiO2 composite as follows: 1.0 mg CAP/SiO2 composite was added to the solution of DOX solution (200 μg mL−1, 1 mL), for 24 h while gently shaking at speed of 180 rpm at room temperature, followed by centrifugation to remove any free DOX. The supernatant was detected by UV-vis spectra to determine the quantity of DOX left in solution. The encapsulation efficiency of DOX (EE) was calculated as the equation:

$$EE = \frac{W}{W_0} \times 100\%$$

where $W$ was the weight of DOX in the particle after centrifugation, $W_0$ was the weight of DOX initially added in particle preparation.

The drug-loading capacity (DL) was calculated using the following equation

$$DL = \frac{W_{\text{drug}}}{W_{\text{total}}} \times 100\%$$

where $W_{\text{drug}}$ was the weight of DOX and $W_{\text{total}}$ was the weight of drug and particle together.

Drug release experiments

To investigate the effect of DOX release at different pH values, the loading drug particles were dispersed in 1 mL aqueous buffer solution (10 mM acetate buffer, pH = 5.0 and 10 mM PBS pH = 7.4) at room temperature. Supernatant were taken from the suspension at predetermined time intervals and other fresh 1 mL buffer solution was replenished. The concentration of released DOX was determined by measuring with UV-vis spectra.
Fabrication of CAP/SiO$_2$/DOX@siRNA codelivery system

The siRNA was captured by composite via electrostatic attraction. The complex CAP/SiO$_2$/DOX@siRNA was prepared by mixing the siRNA with different amounts of composite for 60 min at room temperature. The residual siRNA in supernatant was monitored by agarose gel electrophoresis. Briefly, 10 μL siRNA solution (20 μM) was mixed with 10–65 μg CAP/SiO$_2$/DOX in DEPC water. The mass ratios of composite to siRNA were from 4 : 1, 8 : 1, 10 : 1, 16 : 1, 20 : 1 and 25 : 1.

Cell experiments

**Confocal laser scanning microscopy imaging.** With DOX self-fluorescence, CAP/SiO$_2$/DOX@siRNA in K562/ADR cells was further investigated by confocal laser scanning microscopy (CLSM Leica TCS SP5 confocal microscope, Germany). In brief, K562/ADR cells were seeded on confocal dishes at a density of 1.2 × 10$^4$ cells per well. After overnight incubation, the medium was replaced by fresh medium, containing of CAP/SiO$_2$/DOX@siRNA (final DOX concentration was 20 μg mL$^{-1}$). After incubation for 4 h, 8 h and 12 h, the cells were washed with PBS twice to remove the away the unbound composite. Cell experiments

**Cell viability assay.** Cells (1.0 × 10$^4$ cells per well) were seeded in 96-well plates and cultured for 12 h. Then free DOX, CAP/SiO$_2$/DOX, CAP/SiO$_2$/DOX@siRNA were added into the cell culture medium respectively. Following incubation for 48 h, cells were treated with MTT solution (0.5 mg mL$^{-1}$) for 4 h at 37 °C. The supernatants were then removed, and 100 μL DMSO were added to each well. Finally, plates were measured at 570 nm on a microplate reader.

**Flow cytometry analysis.** K562/ADR cells were seeded at 2 × 10$^5$ cells per well in 6-well plate and cultured for 24 h. 25 μg mL$^{-1}$ of free DOX, CAP/SiO$_2$/DOX and CAP/SiO$_2$/DOX@siRNA were added to the cells, respectively. After incubation for 24 h, cells were washed with PBS twice, and stained with fluorescences in Annexin V-FITC/PI were added to the cells, respectively for 15 min in dark. Finally, numbers of apoptotic cells were measured by flow cytometry (Becton, Dickinson and Company, USA).

**Molecular biology assays.** Suppression on the expression of MDR1 gene in transfected K562/ADR cells was evaluated at both mRNA and protein levels. mRNA level gene was detected by real-time PCR assay. Expression of P-gp protein was evaluated by western blot analysis (WB). K562 cells and K562/ADR cells were seeded into cell culture flask overnight, incubated with 20 μg mL$^{-1}$ of CAP/SiO$_2$/DOX and CAP/SiO$_2$/DOX@siRNA for 24 h, respectively. The details were shown in ESI†.

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