In vitro characterization and cellular uptake profiles of TAMs-targeted lipid calcium carbonate nanoparticles for cancer immunotherapy

Graphical abstract

Highlights

• The optimal reaction for preparation of calcium carbonate nanoparticles was determined through a single-factor investigation.

• The surfaces of calcium carbonate nanoparticles were modified with UNO peptide targeting TAMs.

• UNO-peptide-modified calcium carbonate nanoparticles entered tumor-associated macrophages via actively targeted uptake mediated by mannose receptors.

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In brief
UNO-peptide-modified calcium carbonate nanoparticles not only exhibited ideal particle size and good stability, but also entered tumor-associated macrophages via actively targeted uptake mediated by mannose receptors. These findings demonstrate the potential of UNO-peptide-modified calcium carbonate nanoparticles in a delivery system that promotes the tumor cancer immunotherapy effects of immunomodulators targeting tumor-associated macrophages.
In vitro characterization and cellular uptake profiles of TAMs-targeted lipid calcium carbonate nanoparticles for cancer immunotherapy

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ABSTRACT

Tumor-associated macrophages (TAMs) are key contributors to tumor development, accelerated tumor invasion and metastasis, and induction of immunosuppression. Targeted delivery of immunomodulatory agents to promote polarization of TAMs may alleviate the immunosuppressive tumor microenvironment. Calcium carbonate nanoparticles (CCN), which exhibit excellent biocompatibility, pH sensitivity, and easy surface modification, have attracted substantial attention in targeted nano delivery. In this study, CCN were used as a matrix material to develop UNO-peptide-modified lipid CCN for targeted immunomodulation of TAMs by using the mannose receptor overexpressed on the surfaces of TAMs as targets. The preparation of CCN was optimized through single-factor testing with the gas diffusion method with the particle size as the index. The surface modification of CCN with UNO-peptide-modified phospholipids was performed, and its targeting effect on TAMs was investigated. The average particle size of the CCN and UNO-peptide-modified CCN was 144.5 ± 3.8 nm and 167.0 ± 1.3 nm, respectively. UNO-peptide-modified CCN entered TAMs via actively targeted uptake mediated by mannose receptors. Our results demonstrated that the developed UNO-peptide-modified CCN with controlled nano-size and excellent TAMs-targeting properties is a highly promising nanocarrier for targeted delivery of TAM immunomodulatory agents.

Keywords: Calcium carbonate nanoparticles, tumor-associated macrophages, cancer immunotherapy, targeted delivery

1. INTRODUCTION

The immunosuppressive network within the tumor microenvironment is a key factor promoting tumor development and limiting immunotherapy [1, 2]. In the immunosuppressive microenvironment, tumor-associated macrophages (TAMs) are an extremely important class of immunosuppressive cells, which are involved in nearly the entire process of tumor development [3, 4]. TAMs are also the most infiltrated cells within the tumor microenvironment, accounting for almost 50% of the total cell weight of the tumors in some malignant tissues [5-7]. Given their high plasticity and heterogeneity, TAMs are classified into primarily two types: M1 type, with anti-tumor effects, and M2 type, with pro-tumor effects [8, 9]. TAMs infiltrating into the tumor
microenvironment exhibit primarily M2-type characteristics, recruit immunosuppressive cells through the production of immunosuppressive cytokines and chemokines, and induce tumor cells to express PD-L1, thereby forming an immunosuppressive network and ultimately promoting tumor immune escape [8, 10, 11].

The critical role of TAMs in the tumor microenvironment makes them a promising potential target for tumor immunotherapy [5, 12]. Current immunotherapy strategies for TAMs focus on the use of immunomodulatory agents, such as chlorogenic acid and sorafenib, to induce the polarization of M2 type to M1 type in the tumor microenvironment [5, 13]. Nevertheless, most immunomodulators pose difficulties in crossing multiple physiological barriers in vivo to target the tumor microenvironment, thereby resulting in weak polarization of TAMs and toxic adverse effects. Hence, designing nano-delivery carriers with active targeting of TAMs is an advantageous strategy to improve the efficiency of tumor immunotherapy [12, 14-16].

Calcium carbonate is a bio-mineralized material with superior biocompatibility and biodegradability. Ca\(^{2+}\) and CO\(_3^{2-}\) are common components in human tissues and blood. Compared with other nano-delivery carriers, calcium carbonate nanoparticles (CCN) have three unique advantages: 1) easy preparation and drug loading, in which soluble calcium salts can be used to form CCN by co-precipitation with CO\(_2\) diffused in the gas phase, thus encapsulating the drug in the nanoparticle core during nanoparticle formation; 2) inherent pH sensitivity, in which its nanostructure remains stable in a neutral physiological environment and decomposes in an acidic environment, such as the tumor microenvironment, thus achieving controlled release of loaded drugs within the tumor microenvironment; and 3) easy surface modification, such that all types of polymers or lipids can be physically adsorbed or chemically bonded to Ca\(^{2+}\) on the surface of calcium carbonate, thereby forming a composite core-shell structure of CCN [17]. Notably, the surface modification of CCN by lipid bilayers not only significantly enhances the stability of the CCN core in the blood circulation, but also modifies functional peptides or inserts specific targeting groups in the surface lipid layer through self-assembly, thus conferring therapeutic functions or receptor-mediated active targeting properties. These advantages have led to increased interest in calcium carbonate-based nanocarriers in recent years in the field of tumor-targeted nano delivery, such as tumor chemotherapy, gene therapy, and tumor photodynamic therapy [18]. However, few studies have focused on the targeted delivery of TAM modulators by lipid-modified CCN for cancer immunotherapy.

Given the unique advantages of CCN in targeted delivery, we used pH-sensitive CCN as a matrix material to develop UNO-peptide-modified CCN (UNO@CCN) for targeted immunomodulation of TAMs by using the mannose receptor overexpressed on the surfaces of M2-type TAMs as the target site (Scheme 1). The UNO peptide (sequence: CSPGAKVRC) was screened through in vivo phage display to target TAMs via a specific mannose

![Scheme 1](image_url)
binding receptor (CD206). A previous study has reported that UNO targets TAMs across a spectrum of solid tumors of different origins and can guide drug-encapsulated nanocarriers to TAMs. The ability of the UNO peptide to guide the coupled nanocarriers into TAMs indicates that the peptide has the potential to be used to modify nanocarriers for efficient delivery of immunomodulatory agents into TAMs [19]. The therapeutic advantages of polarized TAMs can ultimately lead to dismantling of the immunosuppressive tumor microenvironment and successful cancer immunotherapy. First, we optimized the preparation conditions through the gas diffusion method by performing single-factor investigation to obtain CCN with controllable particle size and good stability. To form lipid CCN capable of targeting TAMs, we subsequently modified the CCN surface with phospholipids and the UNO peptide, which specifically targets TAMs. After entering the blood circulation, the CCN achieved passive target accumulation in tumor tissues through the enhanced permeability and retention effect, then entered TAMs through receptor-mediated active targeting and released immunomodulators promoting TAMs polarization. To our knowledge, this is the first report of the use of UNO@CCN in TAMs-targeted nanocarriers for cancer immunotherapy. This study provides theoretical guidance for the future design of targeting carriers for anti-tumor immunotherapeutic agents targeting TAMs.

2. MATERIAL AND METHODS

2.1 Materials
Calcium chloride dihydrate (CaCl₂·2H₂O) and ammonium hydrogen carbonate (NH₄HCO₃) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ethyl alcohol was purchased from Tong Guang Fine Chemicals Company (Beijing, China). Soybean lecithin (Lipoic S100) was obtained from Lipoide (Ludwigshafen, Germany). DSPE-PEG2000 and DSPE-PEG2000-Mal were purchased from AVT (Shanghai) Pharmaceutical Tech Co., Ltd. (Shanghai, China). UNO peptide (sequence: CSPGAKVRC) was purchased from GL Biochem (Shanghai) Ltd. (Shanghai, China). Coumarin-6 (cou-6) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Recombinant murine interleukin-4 (IL-4) was supplied by PeproTech (Rocky Hill, NJ, USA). Dulbecco’s modified Eagle’s medium and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

2.2 Preparation of calcium carbonate nanoparticles
CCN were prepared through the gas diffusion method with CaCl₂·2H₂O ethanol aqueous solution and NH₄HCO₃ powder as raw materials through four main steps [20, 21]. In the first step, an appropriate amount of CaCl₂·2H₂O was accurately weighed in a conical flask containing 50 mL of anhydrous ethanol, a small amount of water was added and mixed well, and the bottle mouth was wrapped with tin foil containing several holes for gas exchange. Subsequently, the above conical flask and another conical flask containing an appropriate amount of NH₄HCO₃ were placed in a fresh box of sufficient size and covered to create an airtight environment for the reaction, and the fresh box was transferred to an incubator with a thermostat (FYL-YS-138L, Beijing Fuyilian Medical Equipment Co., Ltd., China) to keep the reaction at a constant temperature. After a predetermined time interval for the vapor-diffusion reaction, the CCN solution was centrifuged (H1850, Xiangyi, China) and dispersed by ultrasound (2101TH, Anpel Laboratory Technologies (Shanghai) Inc, China) to obtain the product, which was then stored at 4°C until further use.

2.3 Optimization of preparation conditions for CCN
Different factors such as Ca²⁺ concentration, water content, reaction temperature, and reaction time were suspected to potentially influence the particle size, stability, and morphology of CCN. A full factorial study of the above four factors with three levels each (CaCl₂·2H₂O: 0.5, 1.0, 1.5 mg/mL; water content: 100, 200, 300 mL; reaction temperature: 20, 25, 35°C; and reaction time: 24, 36, 48 h) was performed to optimize the preparation process parameters.

2.4 Synthesis of DSPE-PEG2000-UNO
DSPE-PEG2000-UNO was synthesized in a reaction through covalent binding with DSPE-PEG2000-Mal and UNO short peptide (sequence: CSPGAKVRC). Briefly, DSPE-PEG2000-Mal was dissolved in chloroform, and the organic solvent was removed by rotary evaporation under a vacuum at 40°C to form a thin film layer. The lipid film was hydrated in HEPES buffered solution (pH 6.5) to obtain an active lipid micelle system. UNO short peptide was dissolved in HEPES buffered solution; subsequently, DSPE-PEG2000-Mal was added and stirred at 25°C for 24 h. The reaction mixture was purified through dialysis with a membrane with a molecular-weight cutoff of 1000 Da (Spectrum Laboratories, Rancho Dominguez, CA, USA) for 24 h. The product was freeze-dried, and the chemical structure of DSPE-PEG2000-UNO was characterized with ¹H-nuclear magnetic resonance (NMR) (AVANCE-III HD 600, Bruker) and MALDI-TOF (ultrafleXtreme, Bruker).

2.5 Surface modification of CCN
CCN with an average particle size of ~140 nm was selected for surface modification. S100 phospholipids were dissolved in absolute ethanol, and the proper amount of CCN (S100:CCN=1:1, w:w) was dissolved in anhydrous ethanol containing phospholipids and chloroform (anhydrous ethanol:chloroform=1:1, v:v), ultrasonically dispersed, and then stirred at 37°C for 7 h. The mixed solution was transferred to a round-bottomed flask and subjected to rotary evaporation under a vacuum at 45°C for 30 min, to form a dried film. The film was dissolved in...
absolute ethanol, to which 20% (w/w to S100) of DSPE-PEG2000 and DSPE-PEG2000-UNO (1:1, w/w) were added. The dispersion was performed by probe sonication (100 W, 10 min, on 3 s, off 3 s). Finally, the above solution was injected into 18 mL of water through a syringe at a constant rate and stirred continuously. The obtained UNO@CCN was stirred for 4 h at room temperature to evaporate the ethanol in the system, and then the particle size was measured. The DSPE-PEG2000 modified CCN (PEG@CCN) was prepared through the same procedure, except that DSPE-PEG2000 was added.

2.6 In vitro characterization of nanoparticles

CCN and UNO@CCN were diluted 20–40 times with absolute ethanol and ultra-pure water, respectively. Subsequently, the average particle size and polydispersity index (PDI) of the nanoparticles were determined through the dynamic light scattering method with a Zeta Potential/Particle Sizer NICOMP 380 ZLS instrument (PSS NICOMP, Santa Barbara, CA, USA) at room temperature. Each sample was measured in triplicate. The average particle size and size distribution of CCN and UNO@CCN were comparatively determined. Morphological examination of CCN was performed through transmission electron microscopy (TEM) (H-7650; Hitachi, Tokyo, Japan). The colloidal stability of lipid-modified CCN in 10% FBS at 37°C was qualitatively determined with a Turbiscan Tower® instrument (Formulaction, L’Union, France) with multiple light scattering [22].

2.7 In vitro cellular uptake profile of UNO@CCN

The RAW264.7 murine macrophage cell line was obtained from the Cell Resource Center, Peking Union Medical College (Beijing, China). RAW264.7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO2 at 37°C.

The cellular uptake profiles of the PEG@CCN and UNO@CCN loaded with cou-6 in M2 macrophages were measured with flow cytometry [23]. Briefly, RAW264.7 cells were seeded into 12-well plates at a density of 2 × 105 cells per well and incubated for 24 h. Subsequently, the cells were pretreated with IL-4 (20 ng/mL) for 24 h to induce the polarization of RAW264.7 cells into M2 macrophages; this was followed by incubation with cou-6-encapsulated nanoparticles at 37°C. After incubation for 1 h, the cells were harvested, washed three times with cold PBS, and then analyzed with a flow cytometer (Accuri C6; BD Biosciences, San Jose, CA, USA). To further confirm the potential of mannose receptors to mediate the uptake of UNO@PCC, we treated M2 macrophages with UNO@CCN and excess UNO peptide (1 mg/mL), individually or in combination, then performed flow cytometry analysis.

2.8 Statistical analysis

All data subjected to statistical analysis were obtained from at least three parallel experiments. The statistical analysis was performed with one-way ANOVA for multiple groups in GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). A p-value ≤ 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Preparation and formulation optimization of CCN

CCN were prepared through a facile gas diffusion method with calcium chloride (CaCl2·2H2O) and ammonium bicarbonate (NH4HCO3) powder as raw materials. The decomposition of ammonium bicarbonate powder produced carbon dioxide (CO2), ammonia (NH3), and water (H2O), and these decomposition products gradually diffused into the calcium chloride solution. Subsequently, CO2 was hydrolyzed, thereby forming carbonate ions (CO32-), and reacted with calcium ions (Ca2+), thus producing calcium carbonate nanoparticles (CCN, CaCO3). In this reaction process, hydrogen ions (H+) were consumed in the reaction with ammonia water, thus providing a weak alkaline environment for the entire solution system to ensure stability and promote further growth of CCN.

The effects of the reaction components (calcium chloride concentration: 0.5, 1.0, and 2.0 mg/mL; water content: 100, 200, and 300 μL), and reaction conditions (reaction temperature: 20, 25, and 35°C; reaction time 24, 36, and 48 h) on the average particle size, size distribution, and PDI of CCN were analyzed with four single-factor models and optimized at three levels.

3.1.1 Effect of calcium chloride concentration.

Calcium chloride was one of the reactants in the formation of CCN. Its concentration directly affected the reaction speed and played a crucial role in the particle size of CCN. The effect of calcium chloride concentration on the particle size of CCN is shown in Figure 1. The calcium chloride concentration (0.5, 1.0, and 2.0 mg/mL) in the reactions was investigated as a single factor under conditions of a fixed water volume of 100 μL, reaction temperature of 25°C, and reaction time of 24 h. The particle size of CCN first increased and then decreased with increasing calcium chloride concentration. In the first stage, the particle size of CCN increased (from 120.5 ± 0.6 nm to 139.9 ± 1.6 nm) with an increase in calcium chloride concentration (from 0.5 mg/mL to 1.0 mg/mL). With a further concentration increase (from 1.0 mg/mL to 2.0 mg/mL), the particle size of CCN appeared to decrease (from 139.9 ± 1.6 nm to 131.9 ± 0.8 nm). When the concentration of CaCl2 was low, CO32- might have been saturated in the system, crystal growth might have been the dominant process, and the particle size continuously increased. However, when the concentration of CaCl2 increased beyond a certain level, the CO32- in the system was rapidly consumed, the reaction rate decreased, the crystal size grew slowly, and the particle size decreased.
3.1.2 Effect of water content. A univariate investigation was conducted on the water content of the reaction, with a CaCl₂·2H₂O content of 1.0 mg/mL; reaction temperature of 25°C; reaction time of 24 h; and system water content of 100, 200, and 300 μL. The water content and the particle size of CCN (Figure 2) showed a positive correlation. With increasing water content (from 100 μL to 300 μL), the particle size of the formed nanoparticles also increased from 139.9 ± 1.6 nm to 176.6 ± 0.7 nm. Because CCN are insoluble in anhydrous ethanol, the reaction was performed in anhydrous ethanol containing trace amounts of water, which effectively prevents spontaneous aggregation and deposition of nanoparticles [24]. The solubility of NH₃ and CO₂ in water is much higher than that in ethanol. Therefore, the increasing water content might have accelerated the formation of CCN by promoting the hydrolysis of CO₂ to CO₃²⁻, thus increasing the particle size.

3.1.3 Effect of reaction temperature. The reaction temperature (20, 25, and 35°C) was also studied as a single factor with other conditions fixed (CaCl₂ concentration of 1.0 mg/mL, reaction time of 24 h, and water content of the system of 100 μL). The relationship between the reaction temperature and the particle size of CCN is shown in Figure 3. The average particle size of the obtained CCN was 118.8 ± 0.3 nm, 139.9 ± 1.6 nm, and 199.1 ± 0.6 nm at 20, 25, and 35°C, respectively. As the temperature increased, the average particle size of the obtained CCN also increased, a finding possibly attributable to the rapid decomposition rate of NH₄HCO₃ powder, enhanced solubility of CO₂ in the system of ethanol-water, and greater reaction rates of Ca²⁺ and CO₃²⁻ at higher temperatures.

3.1.4 Effect of reaction time. Because CCN grow gradually by reacting with CO₃²⁻ and Ca²⁺ in the outer layer of the nucleus, the particle size of CCN can be controlled by the length of the reaction time. To determine the appropriate reaction time, we used particle size as an indicator in single-factor investigation of different reaction times (24, 36, and 48 h). The effect of reaction time on the average particle size is shown in Figure 4. During the experiment, the average particle size positively correlated with the reaction time. When the reaction time was 24 h, the particle size was 139.9 ± 1.6 nm. The longer the reaction time, the larger the particle size: at 36 h, the average particle size increased to 218.9 ±
1.3 nm; at 48 h, the average particle size increased to 280.7 ± 0.7 nm. The average particle size of CCN gradually increased with reaction time, mainly because prolonged reaction time increased the growth time of the outer layer of the CCN nucleus. Given the preparation of CCN with suitable particle size, the reaction time should be controlled to approximately 24 h.

In summary, CCN were prepared through a gas diffusion reaction, and the effect of each factor, including calcium chloride concentration, water content, reaction temperature, and reaction time, on the particle size was initially investigated in a single-factor study. All factors significantly influenced the particle size of the obtained CCN. Except for the calcium chloride concentration, the other factors such as water content, reaction temperature, and reaction time were positively correlated with the particle size of CCN. On the basis of the changes in particle size observed during the single-factor study, the following optimal reaction conditions were determined: CaCl$_2$ concentration of 1.0 mg/mL, water content of 100 μL, reaction temperature of 25°C, and reaction time of 24 h. These were the final conditions used in the routine preparation of CCN for subsequent experiments.

3.2 Structure confirmation of DSPE-PEG2000-UNO

DSPE-PEG2000-UNO was synthesized through a Michael addition reaction involving the formation of a thioether bond between the free sulfhydryl group of UNO and the maleimide group of DSPE-PEG-Mal at pH 6.5 (Figure 5). MALDI-TOF and 1H-NMR analyses of DSPE-PEG2000-Mal and DSPE-PEG2000-UNO are shown in Figure 5b and c. As shown in Figure 5b, the mass-to-charge ratio (m/z) of DSPE-PEG2000-UNO at the major peak was ~920 higher than that of DSPE-PEG2000-Mal, in agreement with the calculated molecular weight of the UNO peptide (920). The MALDI-TOF results provided preliminary evidence that the UNO peptide was successfully modified at one end of the polymer of DSPE-PEG2000-Mal. As shown in Figure 5c, the peaks of the 1H-NMR spectrum were assigned to chemical structures of DSPE-PEG2000-Mal as follows: ~6.9 ppm: maleimide group, 3.5–4.0 ppm: PEG, and 1.0–1.5 ppm: DSPE [25]. After UNO modification of DSPE-PEG2000-Mal, the characteristic peak from the maleimide group at ~6.9 ppm disappeared in the 1H-NMR spectrum of DSPE-PEG2000-UNO, thus suggesting that the free sulfhydryl group of UNO had reacted with the maleimide group of DSPE-PEG-Mal.
3.3 In vitro characterization of UNO@CCN

CCN have properties such as high porosity, high loading, and instability. In contact with water, they self-dissolve and crystallize into other polycrystalline forms of calcium carbonate. In contrast, after phospholipid modification, phospholipids provide a waterproof coating for the protection of CCN particle size and morphology, thus potentially addressing the problem of instability of amorphous CCN in water [24, 26]. The average particle sizes of CCN dispersed in absolute ethanol and water were 138.6 ± 1.10 nm and 4523.7 ± 103.0 nm, respectively (Figure S1). The particle size of CCN increased rapidly after exposure to water, thus indicating instability in aqueous solutions. The change in particle size of CCN before and after lipid modification is shown in Figure 6. After surface modification with lipid and DSPE-PEG-UNO, the average particle size decreased to 138.6 ± 1.10 nm and 4523.7 ± 103.0 nm, respectively (Figure 6).

Figure 5 | Synthesis and structural confirmation of DSPE-PEG2000-UNO.
(a) Schematic of synthetic DSPE-PEG2000-UNO. (b) MALDI-TOF analysis of DSPE-PEG2000-Mal and DSPE-PEG2000-UNO. (c) $^1$H-NMR spectrum of DSPE-PEG2000-Mal and DSPE-PEG2000-UNO.

Figure 6 | In vitro characterization of CCN and UNO@CCN.
(a) Particle size distribution of CCN and UNO@CCN. (b) Average particle size and PDI of CCN and UNO@CCN. Each value represents the mean ± SD (n = 3).
size of the resulting UNO@CCN increased from 144.5 ± 3.8 nm to 167.0 ± 1.3 nm, thereby indicating the successful deposition of lipids on the surfaces of CCN. TEM images revealed a roughly spherical morphology of CCN, and the particle size of CCN was consistent with that determined with the dynamic light scattering method (Figure S2). The change in the zeta potential of CCN before and after lipid modification is shown in Figure S3. The zeta potentials of CCN and lipid-modified CCN were −16.60 ± 0.60 mV and −5.18 ± 0.39 mV, respectively. A colloid stability study showed that the variation in transmission profiles (ΔT) and the turbiscan stability index value of lipid-modified CCN were less than 5% and 1, respectively, thus indicating that the lipid-modified CCN exhibited excellent colloid stability when dispersed in 10% FBS (37°C) for 24 h (Figure S4). The lipid-modified nanoparticles successfully improved the stability of the CCN in water, thereby laying a foundation for the subsequent experiments. However, one limitation of this study is that the pH sensitivity of CCN and lipid-modified CCN was not investigated.

3.4 In vitro cellular uptake profiles of nanoparticles

Extensive studies have shown that TAMs are a class of extremely important immunosuppressive cells involved

![Figure 7](image-url)
in nearly the entire process of tumor development [3, 4]. TAMs are also the most infiltrated cells in the tumor microenvironment, accounting for almost 50% of the total tumor cell mass in some malignant tissues [5-7]. The key role of TAMs in the tumor immunosuppressive microenvironment makes them a potential target for tumor immunotherapy. TAMs exhibit primarily M2-type macrophage characteristics, including overexpression of mannose receptors (named CD206) on the surface [22, 23]. The UNO peptide has been identified to target mannose receptors on TAMs across a spectrum of solid tumors of different types [19, 27, 28]. Notably, UNO not only acts as a cellular membrane-docking ligand but also is robustly internalized in mannose-receptor-expressing macrophages [19]. Thus, we used pH-sensitive CCN as a matrix material to develop UNO@CCN for targeted immunomodulation in TAMs with the mannose receptor overexpressed on the surfaces of M2-type TAMs as the target site.

To investigate the UNO-peptide-mediated TAMs-targeting efficiency of UNO@CCN on M2-type macrophages, we quantitatively determined the in vitro cellular uptake profiles of cou-6-encapsulated CCN in IL-4-induced M2-type macrophages with flow cytometry. As shown in Figure 7a and c, UNO@CCN exhibited the highest mean fluorescence intensity in M2-type macrophages, as compared with DSPE-PEG2000-modified CCN (PEG@CCN) and free cou-6. To confirm whether the improved uptake by M2-type macrophages was mediated by the UNO peptide, we pretreated RAW264.7 macrophages with IL-4 (20 ng/mL) to stimulate the expression of mannose receptor, then incubated them with excess UNO peptide to saturate the mannose receptors. As shown in Figure 7b and d, the cellular uptake of UNO@CCN was significantly greater in RAW264.7 macrophages pretreated with IL-4 (M2-type) than the IL-4-free group (M0 type). Additionally, pre-incubation with excess UNO peptide significantly decreased the uptake of UNO@CCN in IL-4-treated RAW264.7 macrophages (M2-type). These results demonstrated that UNO modification on the surface of CCN effectively facilitated in vitro cellular uptake by M2-type macrophages and that the expression profiles of mannose receptors regulated the cellular uptake of UNO@CCN before and after polarization.

Evidence has indicated that hydrophilic agents, including doxorubicin hydrochloride and mitoxantrone, can be efficiently encapsulated in CCN [21, 29, 30]. Chlorogenic acid (CHA), a phenolic acid derived from traditional Chinese medicine herbs, has multiple beneficial pharmacological activities, particularly antitumor activity mediated by immunomodulatory pathways. In our previous studies, CHA has been found to function as an antitumor immunomodulator that promotes the polarization of TAMs from the M2 to the M1 phenotype via increasing STAT1 activation and inhibiting STAT6 activation. A phase I clinical trial of CHA formulated as a lyophilized powder for injection has been completed, and a clinical phase II study is ongoing. However, because CHA is a hydrophilic polyphenol compound, it is quickly cleared in vivo after injection and cannot be effectively transported across multiple barriers into TAMs. Therefore, the targeted delivery of CHA into TAMs by UNO@CCN has great potential to promote the TAMs polarization of CHA.

After entering the blood circulation, the CHA-encapsulated UNO@CCN achieve passive target accumulation in tumor tissues through the enhanced permeability and retention effect. Although CCN have intrinsic pH sensitivity, the acid-sensitive nature of lipid-modified CCN might be suppressed by the blocking effect of the lipid layer, thus leading to slower decomposition in the acidic tumor microenvironment (pH 6.5) [21]. After entering TAMs through receptor-mediated active targeting, most lipid-modified CCN are concentrated in the lysosomes and decompose rapidly into Ca²⁺ and CO₂ under acidic conditions (pH 5.5) [29]. The higher internal pressure leads to the destruction of lysosomes, after which the released CHA promotes TAMs polarization via increasing STAT1 activation and inhibiting STAT6 activation.

4. CONCLUSIONS

CCN are a bio-mineralized nano drug delivery system with superior biocompatibility and biodegradability, easy preparation and drug loading, inherent pH sensitivity, and easy surface modification. In the present study, the optimal preparation reaction of CCN was determined through a single-factor investigation, and the surfaces of CCN were modified with UNO peptide for targeting TAMs. The obtained UNO@CCN not only exhibited ideal particle size and good stability but also entered TAMs via actively targeted uptake mediated by mannose receptors. These findings demonstrate the potential of UNO@CCN in a delivery system that promotes the tumor immunotherapy effects of immunomodulators targeting TAMs.

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DECLARATION OF COMPETING INTERESTS

The authors declare no conflicts of interest.

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