DOI: 10.55415/deep-2022-0051.v1

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

Xiaoyan Xu[a,b#], Renjie Li[a,b#], Runqi Dong[c], Yanfang Yang[a,b], Hongliang Wang[a,b], Jialing Cheng[a,b], Yuling Liu[a,b*], and Jun Ye[a,b*]

[a]State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, P.R. China

[b]Beijing Key Laboratory of Drug Delivery Technology and Novel Formulation, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, P.R. China

[c]Capital Normal University High School, Beijing 100048, P.R. China

Correspondence: Professor Yuling Liu and Jun Ye, State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, 1 Xiannongtan Street, Beijing 100050, P.R. China, Tel (86)10-89285188, Fax (86)10-89285190, Email ylliu@imm.ac.cn (Yuling Liu) and yelinghao@imm.ac.cn (Jun Ye).

DOI: 10.55415/deep-2022-0051.v1

Abstract

Tumor-associated macrophages (TAMs) are one of the 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 much attention in targeted nano delivery. In this study, CCN was used as the matrix material to develop a UNO peptide-modified lipid CCN for targeted immunomodulation of TAMs by using the mannose receptor overexpressed on the surface of TAMs as targets. The preparation of CCN was optimized by the single-factor test using 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 could enter TAMs via actively targeted uptake mediated by mannose receptors. These 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 TAMs immunomodulatory agents.

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

DOI: 10.55415/deep-2022-0051.v1

1. Introduction

The immunosuppressive network within the tumor microenvironment is one of the key factors that promote tumor development and limit immunotherapy [1, 2]. Among the immunosuppressive microenvironment, tumor-associated macrophages (TAMs) are an extremely important class of immunosuppressive cells that are involved in almost the entire process of tumor development [3, 4]. TAMs are also the most infiltrated cells within the tumor microenvironment, occupying almost 50% of the total cell weight of the tumor in some malignant tissues [5-7]. With high plasticity and heterogeneity, TAMs are mainly classified into two types: M1 type with anti-tumor effect and M2 type with pro-tumor effect [8, 9]. It has been shown that TAMs infiltrating the tumor microenvironment mainly exhibit M2-type characteristics, recruit immunosuppressive cells through the production of immunosuppressive cytokines and chemokines, and induce tumor cells to express PD-L1, which together form an immunosuppressive network and ultimately promote tumor immune escape [8, 10, 11].

The critical role of TAMs in the tumor microenvironment makes them a high potential target for tumor immunotherapy [5, 12]. At present, immunotherapy strategies for TAMs mainly focus on the use of immunomodulatory agents, such as chlorogenic acid and sorafenib, etc, to induce the polarization of M2 type to M1 type in the tumor microenvironment and achieve victory without fighting [5, 13]. Nevertheless, most immunomodulators have difficulties in targeting the tumor microenvironment over multiple physiological barriers in vivo, which in turn leads to a weak polarization effect of TAMs and is prone to toxic side effects. Hence, designing nano-delivery carriers with active targeting of TAMs is an advantageous strategy to improve their efficiency in tumor immunotherapy [12, 14-16].

Calcium carbonate is a type of bio-mineralized material with superior biocompatibility and biodegradability, in which 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 triple unique advantages: 1) easy preparation and drug loading: soluble calcium salts can be used to form CCN by co-precipitation with CO_2 diffused in the gas

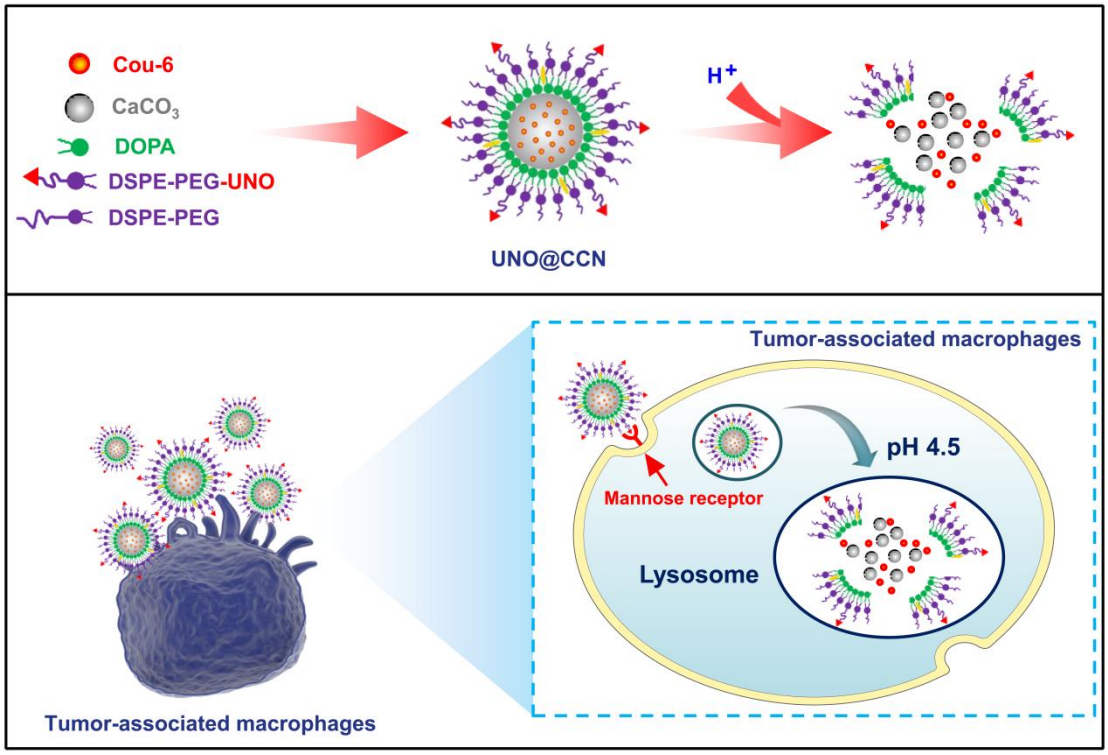
DOI: 10.55415/deep-2022-0051.v1

phase, encapsulating the drug in the core of the nanoparticles while forming the nanoparticles; 2) inherent pH sensitivity: its nanostructure remains stable in a neutral physiological environment and decomposes in an acidic environment such as the tumor microenvironment to achieve controlled release of its loaded drug within the tumor microenvironment; 3) easy surface modification: all kinds of polymers or lipids can be physically adsorbed or chemically bonded to Ca^{2+} on the surface of calcium carbonate to form a composite core-shell structure of CCN [17]. It is worth to be mentioned that the surface modification of CCN by lipid bilayer not only can significantly enhance the stability of CCN core in blood circulation but also can modify functional peptides or insert specific targeting groups in the surface lipid layer by self-assembly to confer therapeutic functions or receptor-mediated active targeting properties. These advantages have led to the interest in calcium carbonate-based nanocarriers in recent years for research in the field of tumor-targeted nano delivery, such as tumor chemotherapy, gene therapy, and tumor photodynamic therapy [18]. However, few studies are focusing on the targeted delivery of TAMs modulators by lipid-modified CCN for cancer immunotherapy.

Based on the unique advantages of CCN in targeted delivery, a pH-sensitive CCN was used as a matrix material to develop a UNO peptide-modified CCN for targeted immunomodulation of TAMs using the mannose receptor overexpressed on the surface of M2-type TAMs as the target site (Scheme 1). UNO peptide (sequence: CSPGAKVRC) was screened by in vivo phage display to target TAMs by specific binding to mannose receptors (CD206). A previous study reported that UNO targeted TAMs across a spectrum of solid tumors of different origins and guided drug-encapsulated nanocarriers to TAMs. The ability of the UNO peptide to guide the coupled nanocarriers into the TAMs demonstrated that the peptide had the potential to be used to modify nanocarriers to efficiently deliver immunomodulatory agents into TAMs [19]. The therapeutic advantages of polarized TAMs were used to dismantle the immunosuppressive tumor microenvironment and finally achieve cancer immunotherapy. First of all, we optimized the preparation conditions based on the gas diffusion method by single-factor investigation to obtain CCN with controllable particle size and good stability. The CCN were subsequently

DOI: 10.55415/deep-2022-0051.v1

surface modified with phospholipids and UNO peptide that specifically targets TAMs to form lipid CCN that can target TAMs. After entering the blood circulation, the CCN achieves passive target accumulation in tumor tissues through the enhanced permeability and retention (EPR) effect first and then enters into TAMs through receptor-mediated active targeting and releases immunomodulators to promote TAMs polarization. To the best of our knowledge, this is the first time that UNO peptide-modified CCN has been used in TAMs-targeted nanocarriers for cancer immunotherapy. This study provides theoretical guidance for the design of targeting carriers for anti-tumor immunotherapeutic agents targeting TAMs.



Scheme 1 The schematic diagram of the preparation of UNO peptide-modified CCN and its active targeting to mannose receptor-expressing TAMs.

2. Material and methods

2.1 Materials

Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) and ammonium hydrogen carbonate (NH_4HCO_3) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ethyl alcohol was bought from Tong Guang Fine Chemicals Company (Beijing, China). Soybean lecithin (Lipoid S100) was obtained from Lipoid (Ludwigshafen, Germany). DSPE-PEG2000 and DSPE-PEG2000-Mal were purchased from AVT (Shanghai)

DOI: 10.55415/deep-2022-0051.v1

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 (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

2.2 Preparation of calcium carbonate nanoparticles (CCN)

Calcium carbonate nanoparticles (CCN) were prepared by the gas diffusion method using $CaCl_2 \cdot 2H_2O$ ethanol aqueous solution and NH_4HCO_3 powder as raw materials through four main steps [20, 21]. The first step was accurately weighing an appropriate amount of $CaCl_2 \cdot 2H_2O$ in a conical flask containing 50 mL of anhydrous ethanol, adding a small amount of water, mixing well, then wrapping the bottle mouth with tin foil and leaving several holes for gas exchange. Subsequently, the above conical flask and another conical flask containing an appropriate amount of NH_4HCO_3 were placed in a fresh box of sufficient size and covered to create an airtight environment for the reaction, and transferred the fresh box to 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 calcium carbonate nanoparticles solution was centrifuged (H1850, Xiangyi, China) and dispersed by ultrasound (2101TH, Anpel Laboratory Technologies (Shanghai) Inc, China) to obtain the product, then placed at 4°C for standby.

2.3 Optimization of preparation conditions of CCN

Different factors such as Ca^{2+} concentration, water content, reaction temperature, as well as reaction time were suspected to possibly influence the particle size, stability, and morphology of CCN. A full factorial study of the above four factors with three levels each ($CaCl_2 \cdot 2H_2O$: 0.5, 1.0, 1.5 mg/mL; water content: 100, 200, 300 μL; reaction temperature: 20, 25, 35°C; reaction time: 24, 36, 48 h, respectively) was carried out as a way to optimize the best preparation process parameters.

2.4 Synthesis of DSPE-PEG2000-UNO

DOI: 10.55415/deep-2022-0051.v1

DSPE-PEG2000-UNO was synthesized in a reaction by 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, followed by the addition of DSPE-PEG2000-Mal and stirring at 25°C for 24 h. The reaction mixture was purified by dialysis using a membrane with a molecular weight cutoff (MWCO) 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 by ^{1}H-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. The phospholipids S100 were dissolved in absolute ethanol, and a dosed 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, then stirred at 37°C for 7 h. The mixed solution was transferred to a round-bottom 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 carried out 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 peptide-modified CCN (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 using the same procedure, except that DSPE-PEG2000 was added.

2.6 In vitro characterization of nanoparticles

DOI: 10.55415/deep-2022-0051.v1

CCN and UNO@CCN were diluted 20–40 times with absolute ethyl alcohol and ultra-pure water, respectively. Then, the average particle size and polydispersity index (PDI) of the nanoparticles were determined by the dynamic light scattering method using Zeta Potential/Particle Sizer NICOMP 380 ZLS (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 by transmission electron microscope (TEM) by 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 using a Turbiscan Tower® (Formulaction, L'Union, France) by multiple light scattering [22].

2.7 In vitro cellular uptake profile of UNO@CCN

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 DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

The cellular uptake profiles of the PEG@CCN and UNO@CCN loaded with coumarin-6 in M2 macrophages were measured by flow cytometry [23]. Briefly, RAW264.7 cells were seeded into 12-well plates at a density of 2×10^5 cells per well and incubated for 24 h. Then, the cells were pretreated with IL-4 (20 ng/mL) for 24 h to induce the polarization of RAW264.7 cells to M2 macrophages, followed by incubation with coumarin-6-encapsulated nanoparticles at 37°C. After incubation for 1 h, the cells were harvested, washed three times with cold PBS, and then analyzed using 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, M2 macrophages were treated with UNO@CCN and excess UNO peptide (1 mg/mL), individually or in combination, followed by flow cytometer analysis.

2.8 Statistical analysis

All data subjected to statistical analysis were obtained from at least three parallel experiments. The statistical analysis was performed by one-way ANOVA for multiple

DOI: 10.55415/deep-2022-0051.v1

groups using 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 was prepared by a facile gas diffusion method using calcium chloride ($CaCl_2 \cdot 2H_2O$) and ammonium bicarbonate (NH_4HCO_3) powder as raw materials. The decomposition of ammonium bicarbonate powder produces carbon dioxide (CO_2), ammonia (NH_3), and water (H_2O), and these decomposition products gradually diffuse into the calcium chloride solution. Subsequently, carbon dioxide (CO_2) hydrolyzes to form carbonate ions (CO_3^{2-}) and reacts with calcium ions (Ca^{2+}) to produce calcium carbonate nanoparticles (CCN, $CaCO_3$). In this reaction process, hydrogen ions (H^+) were consumed in reaction with ammonia water, which provided a weak alkaline environment for the whole solution system to ensure stability and promote the further growth of CCN.

The effects of 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 polydispersity index (PDI) of CCN were analyzed by four single-factor models and optimized at three levels.

3.1.1 The effect of calcium chloride concentration

Calcium chloride is one of the reactants in the formation of CCN. Its concentration directly affects the reaction speed and plays a crucial role in the particle size of CCN. The effect of calcium chloride concentration on the particle size of CCN was shown in Figure 1. The calcium chloride concentration (0.5, 1.0, and 2.0 mg/mL) in the prescriptions was investigated as a single factor of the conditions of the fixed water volume of 100 μL, reaction temperature of 25°C, and reaction time of 24 h. The particle size of CCN first showed an enhancement and then a diminution with the increase of 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). Comparatively, with further increment in concentration (from 1.0 mg/mL to

DOI: 10.55415/deep-2022-0051.v1

2.0 mg/mL), the particle size of CCN appeared to decrease (from 139.9 ± 1.6 nm to 131.9 ± 0.8 nm). It may be that when the concentration of $CaCl_2$ was low, CO_3^{2-} was saturated in the system, crystal growth was the dominant process, and the particle size continuously increased. However, when the concentration of $CaCl_2$ increased to a certain level, the CO_3^{2-} in the system was rapidly used up, the reaction rate decreased, the crystal size grew slowly instead, and the particle size decreased.

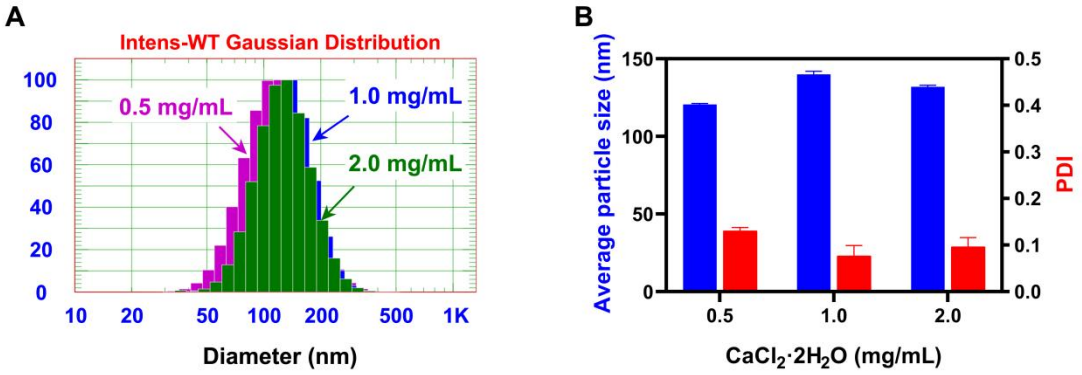


Figure 1 The effect of calcium chloride concentration on the particle size of CCN. (A) Particle size distribution of CCN at different calcium chloride concentrations. (B) The average particle size and PDI of CCN at different calcium chloride concentrations. Each value represents the mean ± standard deviation (SD) (n = 3).

3.1.2 The effect of water content

A univariate investigation was conducted on the water content of the prescription, setting the $CaCl_2 \cdot 2H_2O$ content at 1.0 mg/mL, the reaction temperature at 25°C, the reaction time at 24 h, and the water content variables of the system as 100, 200, and 300 μL. The relationship between the water content and the particle size of CCN was shown in Figure 2. As shown in Figure 2, there was a positive correlation between water content and the particle size of CCN. With the increase in 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. Since CCN are insoluble in anhydrous ethanol, the reaction is carried out in anhydrous ethanol containing trace amounts of water, which can effectively prevent spontaneous aggregation and deposition of nanoparticles [24]. The solubility of NH_3 and CO_2 in water is much higher than that in ethanol. Therefore, the increasing water content may accelerate the formation of CCN by promoting the hydrolysis of CO_2 to CO_3^{2-}, thus enhancing its particle size.

DOI: 10.55415/deep-2022-0051.v1

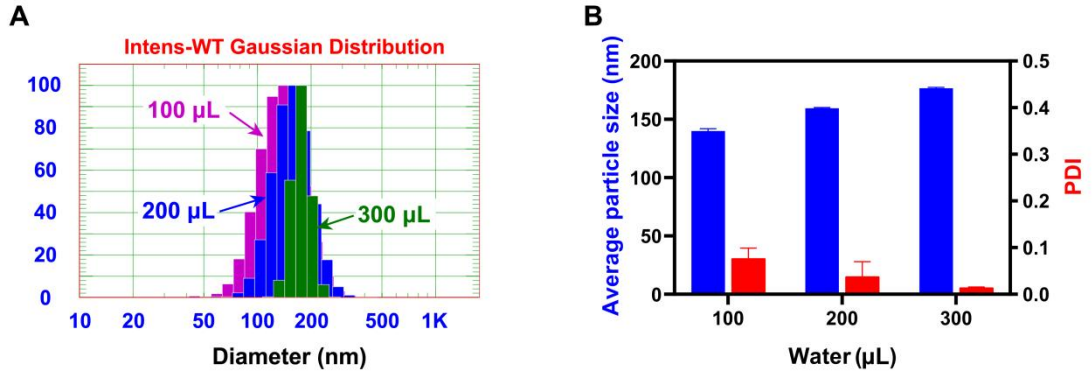


Figure 2 The effect of water content on the particle size of CCN. (A) Particle size distribution of CCN at different water content. (B) The average particle size and PDI of CCN at different water content. Each value represents the mean ± SD (n = 3).

3.1.3 The effect of reaction temperature

The reaction temperature (20, 25, and 35°C) was also studied as a single factor with other conditions fixed: the $CaCl_2$ concentration was 1.0 mg/mL, reaction time was 24 h, and the water content of the system was 100 µL. The relationship between the reaction temperature and the particle size of CCN was shown in Figure 3. The average particle size of 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. It was observed that as the temperature increased, the average particle size of obtained CCN also increased, which may be attributed to the fast decomposition rate of NH_4HCO_3 powder, enhanced solubility of CO_2 in the system of ethanol-water, and improved reaction rate of Ca^{2+} and CO_3^{2-} with the higher temperature.

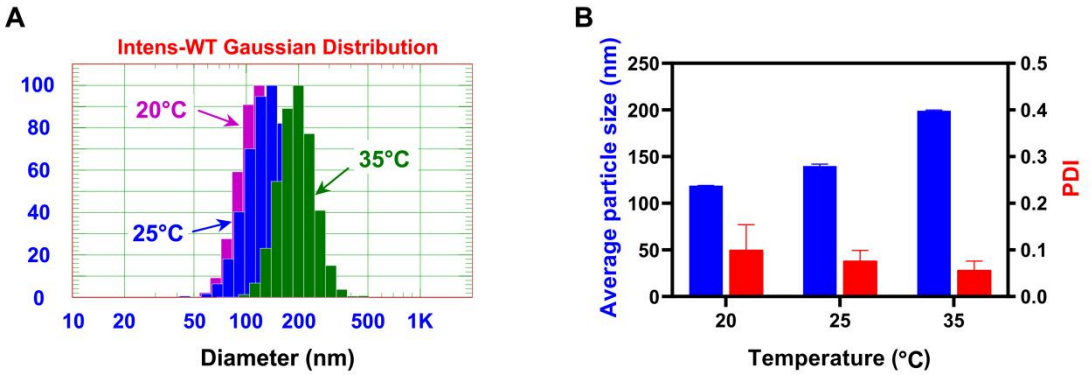


Figure 3 The effect of reaction temperature on the particle size of CCN. (A) Particle size distribution of CCN at the different reaction temperatures. (B) The average particle size and PDI of CCN at the different reaction temperatures. Each value represents the mean ± SD (n = 3).

3.1.4 The effect of reaction time

Since CCN grows gradually by reacting with CO_3^{2-} and Ca^{2+} in the outer layer of the

DOI: 10.55415/deep-2022-0051.v1

nucleus, the particle size of CCN can be controlled by the length of reaction time. To screen the appropriate reaction time, the particle size was used as an indicator for the single-factor investigation into different reaction times (24, 36, and 48 h). The effect of reaction time on the average particle size were shown in Figure 4. During the experiment, the average particle size was 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 time was, the larger the particle size was: at 36 h, the average particle size increased to 218.9 ± 1.3 nm; at 48 h, the average particle size showed an increase to 280.7 ± 0.7 nm. The average particle size of CCN gradually increased with the reaction time, mainly because the extension of the reaction time could increase 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 for around 24 h.

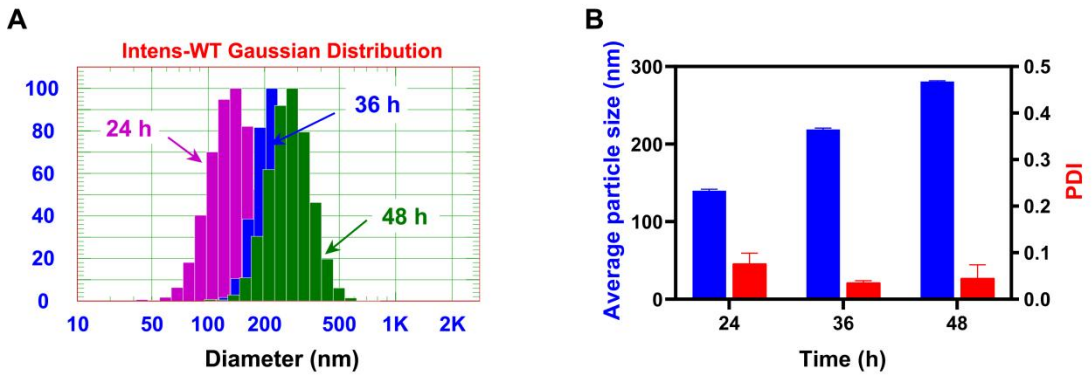


Figure 4 The effect of reaction time on the particle size of CCN. (A) Particle size distribution of CCN at different reaction times. (B) The average particle size and PDI of CCN at different reaction times. Each value represents the mean ± SD (n = 3).

In summary, CCN was prepared by 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 by a single-factor study. All the factors have a significant influence on 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. Combining the changing of particle size during the single-factor study, the optimal reaction conditions, which were set as $CaCl_2$ concentration of 1.0 mg/mL, the water content of 100

DOI: 10.55415/deep-2022-0051.v1

μL, reaction temperature of 25°C, and reaction time of 24 h, were established as the final conditions for 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). The MALDI-TOF and ^{1}H-NMR analysis 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, which was in agreement with the calculated molecular weight of the UNO peptide (920). The result of MALDI-TOF provides preliminary evidence that the UNO peptide has been successfully modified at one end of the polymer of DSPE-PEG2000-Mal. As shown in Figure 5C, the peaks of the ^{1}H-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]. Following UNO modification of DSPE-PEG2000-Mal, the characteristic peak from the maleimide group at ~6.9 ppm disappeared in the ^{1}H-NMR spectrum of DSPE-PEG2000-UNO, suggesting that the free sulfhydryl group of UNO had reacted with the maleimide group of DSPE-PEG-Mal.

DOI: 10.55415/deep-2022-0051.v1

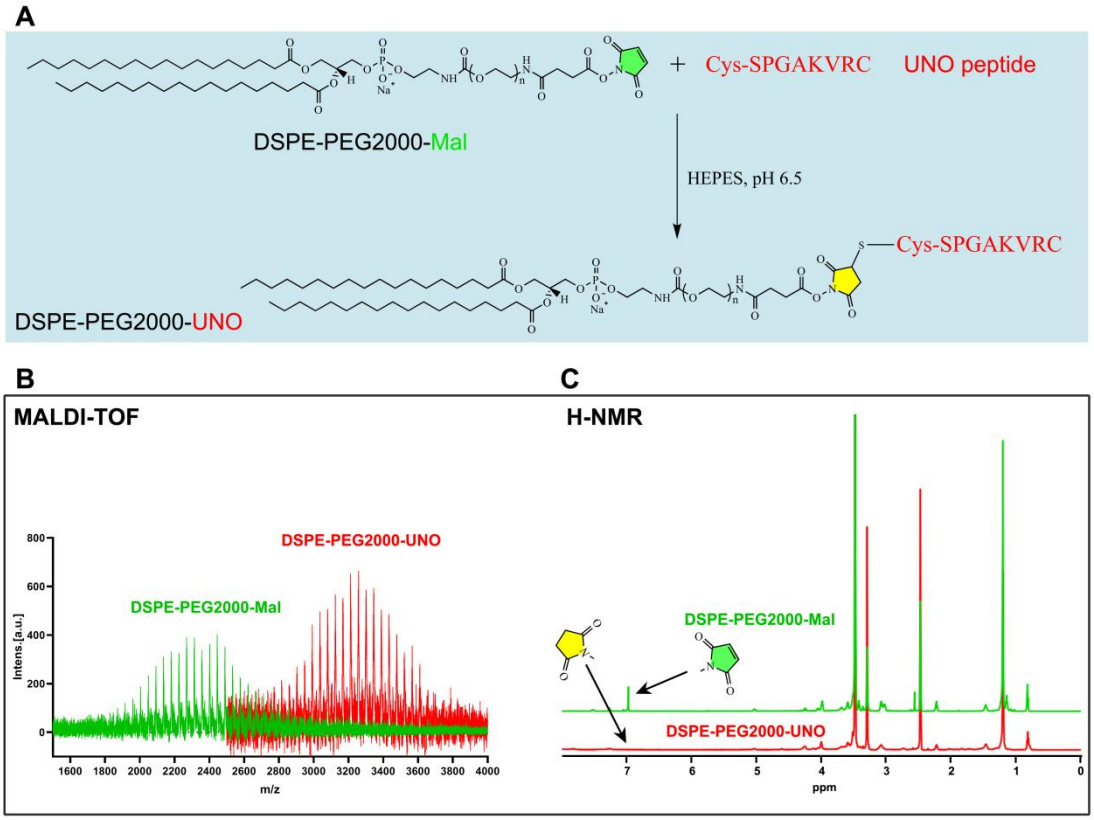


Figure 5 Synthesis and structure confirmation of DSPE-PEG2000-UNO. (A) Schematic of synthetic DSPE-PEG2000-UNO. (B) The MALDI-TOF analysis of DSPE-PEG2000-Mal and DSPE-PEG2000-UNO. (C) The ^{1}H-NMR spectrum of DSPE-PEG2000-Mal and DSPE-PEG2000-UNO.

3.3 In vitro characterization of UNO@CCN

CCN has properties such as high porosity, high loading, and instability. When 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, which could address the problem of instability of amorphous CCN in water effectively [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 increases rapidly when exposed to water, indicating that it is unstable in an aqueous solution. The change in particle size of CCN before and after lipid modification was shown in Figure 6. Following surface modification with lipid and DSPE-PEG-UNO, the average particle size of the resulting UNO@CCN increased from 144.5 ± 3.8 nm to 167.0 ± 1.3 nm, indicating the successful deposition of lipids on the surfaces of CCN. TEM image revealed a roughly

DOI: 10.55415/deep-2022-0051.v1

spherical morphology of CCN and the particle size of CCN was consistent with that determined by the dynamic light scattering method (Figure S2). The change in the zeta potential of CCN before and after lipid modification was 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. The result of the colloid stability study showed that the variations of transmission profiles (ΔT) and the turbiscan stability index (TSI) value of lipid-modified CCN were less than 5% and 1, respectively, 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, which laid the foundation for the following experiments. However, one limitation of this study is that the pH sensitivity of CCN and lipid-modified CCN was not investigated.

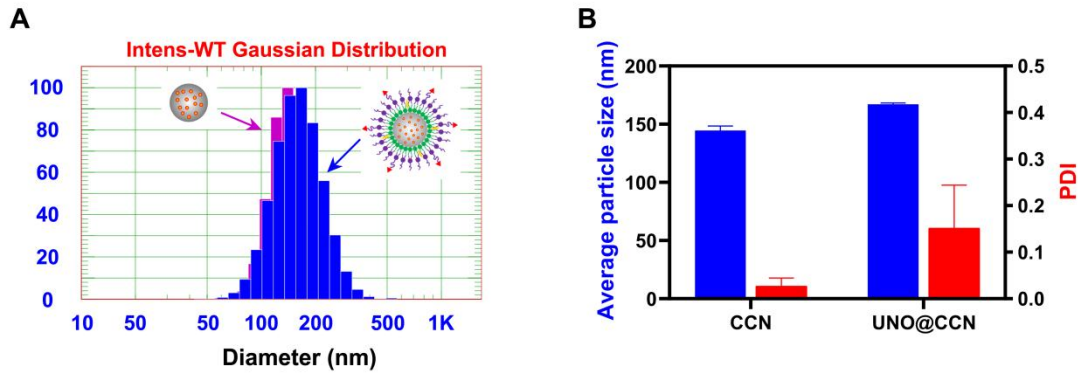


Figure 6 In vitro characterization of CCN and UNO@CCN. (A) Particle size distribution of CCN and UNO@CCN. (B) The average particle size and PDI of CCN and UNO@CCN. Each value represents the mean ± SD (n = 3).

3.4 In vitro cellular uptake profiles of nanoparticles

Extensive studies have shown that TAMs are a class of extremely important immunosuppressive cells, which are involved in almost 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 mainly exhibit M2-type macrophage characteristics, including overexpression of mannose receptors (named CD206) on the surface [22, 23]. The peptide codenamed UNO has been identified to target mannose receptors on TAMs across a spectrum of solid tumors of different types [19, 27, 28]. It is

DOI: 10.55415/deep-2022-0051.v1

worth mentioning that UNO does not only act as a cellular membrane-docking ligand but is also robustly internalized in mannose receptors-expressing macrophages [19]. Thus, a pH-sensitive CCN was used as a matrix material to develop a UNO peptide-modified CCN for targeted immunomodulation of TAMs using the mannose receptor overexpressed on the surface of M2-type TAMs as the target site.

To investigate the UNO peptide-mediated TAMs-targeting efficiency of UNO@CCN on M2-type macrophages, the in vitro cellular uptake profiles of cou-6-encapsulated CCN in IL-4-induced M2-type macrophages were determined quantitatively by flow cytometry. As shown in Figure 7A and C, UNO@CCN exhibited the highest mean fluorescence intensity in M2-type macrophages compared to DSPE-PEG2000-modified CCN (PEG@CCN) and free cou-6. To confirm whether this improved uptake by M2-type macrophages was mediated by the UNO peptide, RAW264.7 macrophages were firstly pretreated with IL-4 (20 ng/mL) to stimulate the expression of mannose receptor and were then incubated with excess UNO peptide to saturate the mannose receptors. As shown in Figure 7B and D, the cellular uptake of UNO@CCN significantly increased in RAW264.7 macrophages pretreated with IL-4 (M2-type) compared to the IL-4-free group (M0-type). Additionally, pre-incubation with excess UNO peptide significantly reduced the uptake of UNO@CCN in IL-4-treated RAW264.7 macrophages (M2-type). These results demonstrate that UNO modification on the surface of CCN effectively facilitates in vitro cellular uptake by M2-type macrophages and that the expression profiles of mannose receptors regulate the cellular uptake of UNO@CCN before and after polarization.

Accumulated evidence indicated that hydrophilic agents, including doxorubicin hydrochloride and mitoxantrone, could be efficiently encapsulated in CCN [21, 29, 30]. Chlorogenic acid (CHA), a phenolic acid derived from traditional Chinese medicine herbs, possesses multiple beneficial pharmacological activities, especially, antitumor activity mediated by immunomodulatory pathways. In our previous studies, CHA functions as an antitumor immunomodulator that promotes the polarization of TAMs from the M2 to M1 phenotype via the promotion of STAT1 activation and the inhibition of STAT6 activation, thereby modulating the tumor microenvironment and inhibiting the growth of glioblastoma

DOI: 10.55415/deep-2022-0051.v1

[22, 31, 32]. CHA formulated as a lyophilized powder for injection has completed the phase I clinical trial and is presently in clinical Phase II studies. However, because CHA is a hydrophilic polyphenol compound, it will be quickly cleared in vivo after injection and cannot be effectively transported through multiple barriers to TAMs. Therefore, the targeted delivery of CHA into TAMs by UNO peptide-modified CCN has great potential to promote the TAMs polarization of CHA.

After entering the blood circulation, the CHA-encapsulated UNO peptide-modified CCN achieves passive target accumulation in tumor tissues through the EPR effect first. Although CCN has intrinsic pH sensitivity, the acid-sensitive nature of lipid-modified CCN might be suppressed due to the blocking effect of the lipid layer, leading to slower decomposition in the acidic tumor microenvironment (~pH 6.5) [21]. After entering into TAMs through receptor-mediated active targeting, most lipid-modified CCN are concentrated in the lysosomes and decompose rapidly into Ca^{2+} and CO_2 under acidic conditions (~pH 5.5) [29]. The higher internal pressure leads to the destruction of lysosomes, after which the released CHA promoted TAMs polarization via the promotion of STAT1 activation and the inhibition of STAT6 activation.

DOI: 10.55415/deep-2022-0051.v1

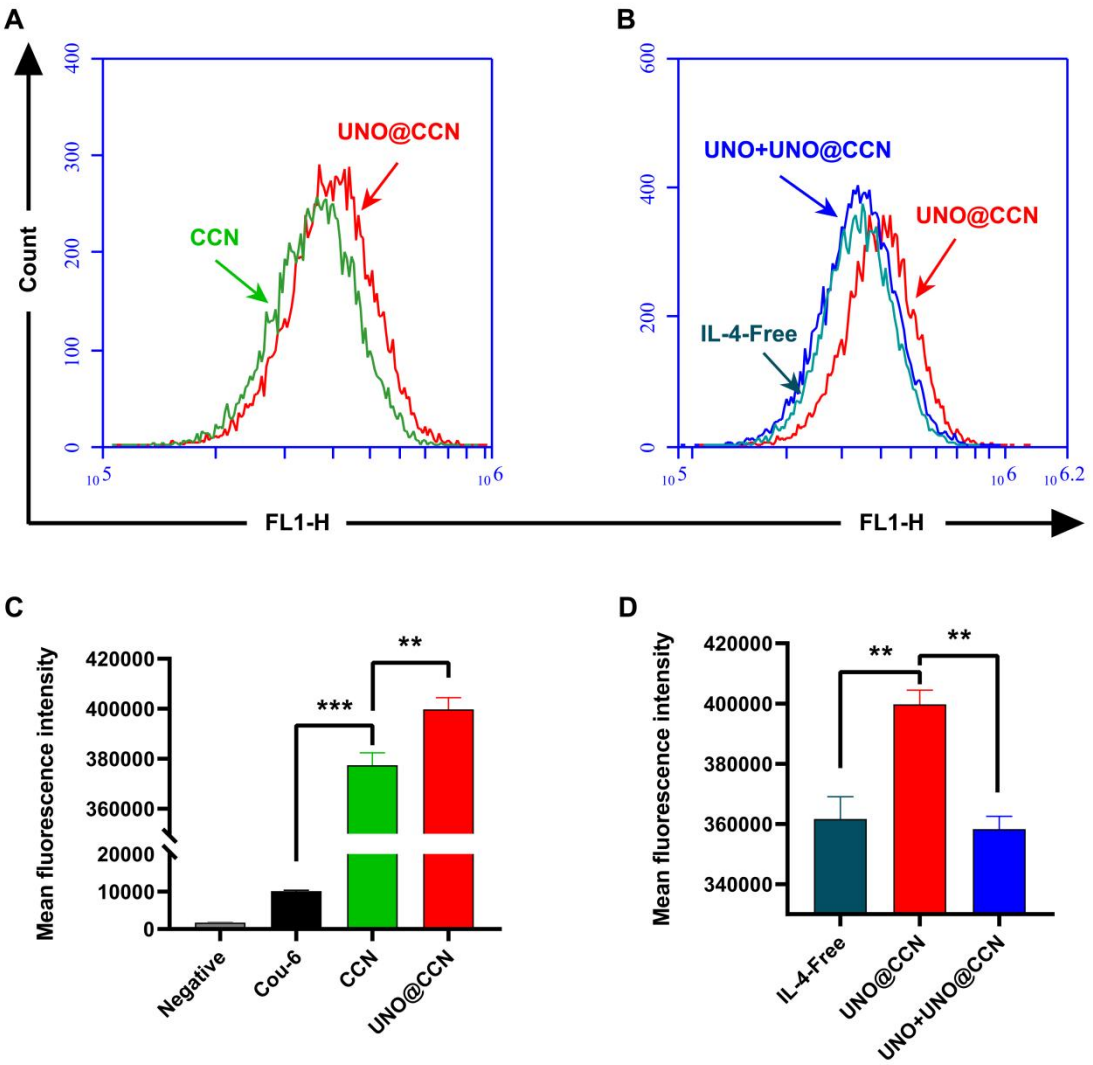


Figure 7 In vitro cellular uptake profiles of cou-6-loaded UNO@CCN in RAW264.7 macrophages or IL-4-induced RAW264.7 macrophages. (A and C) The cellular uptake profile of cou-6-loaded UNO@CCN in M2-type RAW264.7 macrophages. (B and D) The cellular uptake profile of cou-6-loaded UNO@CCN in RAW264.7 macrophages (IL-4-Free) or M2-type RAW264.7 macrophages alone (UNO@CCN) or co-incubation with excess UNO peptide (UNO+UNO@CCN). Each value represents the mean ± standard error of mean (SEM) (n = 3). **p<0.01 and ***p<0.001.

4. Conclusions

CCN is a type of 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 prescription of CCN was determined by a single factor investigation and the surface of CCN was modified with UNO peptide targeting TAMs. The obtained UNO peptide-modified CCN (UNO@CCN) not only exhibited ideal particle size and good stability but also could enter TAMs via actively targeted uptake mediated by mannose receptors. These findings demonstrate the

DOI: 10.55415/deep-2022-0051.v1

potential of UNO peptide-modified CCN in a delivery system that will promote the tumor immunotherapy effect of immunomodulators targeting TAMs.

CRediT author statement

Xiaoyan Xu and Renjie Li: conceptualization, methodology, investigation, formal analysis, writing - original draft. Runqi Dong, Yanfang Yang, and Hongliang Wang: investigation, validation. Yuling Liu and Jun Ye: supervision, project administration, funding acquisition, writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

This work was financially supported by the Fundamental Research Funds for the Central Universities (3332021101), Beijing Nova Program (Z211100002121127), and Beijing Natural Science Foundation (L212059).

References

[1] Zappasodi R, Merghoub T, Wolchok JD, Emerging Concepts for Immune Checkpoint Blockade-Based Combination Therapies, Cancer Cell. 33(2018) 581-598.

[2] Li Y, Liu J, Gao L, et al, Targeting the tumor microenvironment to overcome immune checkpoint blockade therapy resistance, Immunol Lett. 220(2020) 88-96.

[3] Beltraminelli T, De Palma M, Biology and therapeutic targeting of tumour-associated macrophages, J Pathol. 2020)

[4] Xu X, Gong X, Wang Y, et al, Reprogramming Tumor Associated Macrophages toward M1 Phenotypes with Nanomedicine for Anticancer Immunotherapy, Advanced Therapeutics. 2020)

[5] Komohara Y, Fujiwara Y, Ohnishi K, et al, Tumor-associated macrophages: Potential therapeutic targets for anti-cancer therapy, Advanced drug delivery reviews. 99(2016) 180-185.

[6] Mantovani A, Marchesi F, Malesci A, et al, Tumour-associated macrophages as treatment targets in oncology, Nature Reviews Clinical Oncology. 14(2017) 399.

[7] Solinas G, Germano G, Mantovani A, et al, Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation, Journal of leukocyte biology. 86(2009) 1065-1073.

[8] Sica A, Mantovani A, Macrophage plasticity and polarization: in vivo veritas, J. Clin. Invest. 122(2012) 787-795.

[9] Biswas SK, Mantovani A, Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm, Nat. Immunol. 11(2010) 889-896.

[10] Yagi T, Baba Y, Okadome K, et al, Tumour-associated macrophages are associated with poor prognosis and programmed death ligand 1 expression in oesophageal

DOI: 10.55415/deep-2022-0051.v1

cancer, Eur. J. Cancer. 111(2019) 38-49.

[11] Arlauckas SP, Garris CS, Kohler RH, et al, In vivo imaging reveals a tumor-associated macrophage-mediated resistance pathway in anti-PD-1 therapy, Sci. Transl. Med. 9(2017)

[12] Ovais M, Guo M, Chen C, Tailoring Nanomaterials for Targeting Tumor-Associated Macrophages, Adv. Mater. 31(2019) e1808303.

[13] Amoozgar Z, Goldberg MS, Targeting myeloid cells using nanoparticles to improve cancer immunotherapy, Adv Drug Deliv Rev. 91(2015) 38-51.

[14] Wei Z, Zhang X, Yong T, et al, Boosting anti-PD-1 therapy with metformin-loaded macrophage-derived microparticles, Nature communications. 12(2021) 440.

[15] Zhang Y, Wu L, Li Z, et al, Glycocalyx-Mimicking Nanoparticles Improve Anti-PD-L1 Cancer Immunotherapy through Reversion of Tumor-Associated Macrophages, Biomacromolecules. 19(2018) 2098-2108.

[16] Li J, Zhao M, Sun M, et al, Multifunctional Nanoparticles Boost Cancer Immunotherapy Based on Modulating the Immunosuppressive Tumor Microenvironment, ACS applied materials & interfaces. 12(2020) 50734-50747.

[17] Wang C, Chen S, Wang Y, et al, Lipase-Triggered Water-Responsive "Pandora's Box" for Cancer Therapy: Toward Induced Neighboring Effect and Enhanced Drug Penetration, Adv. Mater. 30(2018) e1706407.

[18] Maleki Dizaj S, Sharifi S, Ahmadian E, et al, An update on calcium carbonate nanoparticles as cancer drug/gene delivery system, Expert Opin Drug Deliv. 2019) 1-15.

[19] Scodeller P, Simon-Gracia L, Kopanchuk S, et al, Precision Targeting of Tumor Macrophages with a CD206 Binding Peptide, Sci. Rep. 7(2017) 14655.

[20] Wang J, Tao S, Jin X, et al, Calcium Supplement by Tetracycline guided amorphous Calcium Carbonate potentiates Osteoblast promotion for Synergetic Osteoporosis Therapy, Theranostics. 10(2020) 8591-8605.

[21] Wang C, Han M, Liu X, et al, Mitoxantrone-preloaded water-responsive phospholipid-amorphous calcium carbonate hybrid nanoparticles for targeted and effective cancer therapy, Int. J. Nanomedicine. 14(2019) 1503-1517.

[22] Ye J, Yang Y, Jin J, et al, Targeted delivery of chlorogenic acid by mannosylated liposomes to effectively promote the polarization of TAMs for the treatment of glioblastoma, Bioact. Mater. 5(2020) 694-708.

[23] Ye J, Yang Y, Dong W, et al, Drug-free mannosylated liposomes inhibit tumor growth by promoting the polarization of tumor-associated macrophages, Int. J. Nanomedicine. Volume 14(2019) 3203-3220.

[24] Chuzeville L, Boury F, Duday D, et al, Eco-friendly processes for the synthesis of amorphous calcium carbonate nanoparticles in ethanol and their stabilisation in aqueous media†, Green Chemistry. 24(2022) 1270-1284.

[25] Reginald-Opara JN, Svirskis D, O'Carroll SJ, et al, Optimisation of glutathione conjugation to liposomes quantified with a validated HPLC assay, Int. J. Pharm. 567(2019) 118451.

[26] Wang C, Liu X, Chen S, et al, Facile preparation of phospholipid-amorphous calcium carbonate hybrid nanoparticles: toward controllable burst drug release and enhanced

DOI: 10.55415/deep-2022-0051.v1

tumor penetration, Chem. Commun. (Camb.). 54(2018) 13080-13083.

[27] Lepland A, Asciutto EK, Malfanti A, et al, Targeting Pro-Tumoral Macrophages in Early Primary and Metastatic Breast Tumors with the CD206-Binding mUNO Peptide, Mol. Pharm. 17(2020) 2518-2531.

[28] Figueiredo P, Lepland A, Scodeller P, et al, Peptide-guided resiquimod-loaded lignin nanoparticles convert tumor-associated macrophages from M2 to M1 phenotype for enhanced chemotherapy, Acta Biomater. 133(2021) 231-243.

[29] Tan M, Liu W, Liu F, et al, Silk Fibroin-Coated Nanoagents for Acidic Lysosome Targeting by a Functional Preservation Strategy in Cancer Chemotherapy, Theranostics. 9(2019) 961-973.

[30] Liu X, Wang C, Ma H, et al, Water-Responsive Hybrid Nanoparticles Codelivering ICG and DOX Effectively Treat Breast Cancer via Hyperthermia-aided DOX Functionality and Drug Penetration, Adv Healthc Mater. 2019) e1801486.

[31] Xue N, Zhou Q, Ji M, et al, Chlorogenic acid inhibits glioblastoma growth through repolarizating macrophage from M2 to M1 phenotype, Sci. Rep. 7(2017) 39011.

[32] Zhang Y, Yang Y, Ye J, et al, Construction of chlorogenic acid-containing liposomes with prolonged antitumor immunity based on T cell regulation, Sci China Life Sci. 64(2021) 1097-1115.