An Intelligent Stealth Andrographolide Imprinted Drug Carrier of Poly (2-ethyl-2-oxazoline) with Multi Stimuli Response and Lung Cancer A549 Cytotoxicity

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2 Experimental

2.1 Materials and instruments

2.1.1 Materials

Iodine (I_2, 99.8%), sodium methoxide (CH_3ONa, 98%), acetic anhydride ($C_4H_6O_3$, analytical pure), 4-dimethylaminopyridine (DMAP, 99%), pyridine (C_5H_5N, analytical pure), andrographolide (ADR, 98%), methacryloyl chloride ($CH_2C(CH_3)$ COCl, 95%), acetonitrile (ACN, analytical pure), acryloyl chloride (C_3H_3ClO, 96%), p-nitroaniline ($C_6H_6N_2O_2$, analytical pure), 3-glycidylethoxypropyltrimethoxysilane (GLYMO, 97%) and sodium hydride (NaH, 60%) were purchased from Shanghai McLean Biochemical Technology Co., Ltd. 2-ethyl-2-oxazoline (ETOX, 98%) and triethylamine (98%) were got from Ron reagent. PMDETA (98%), ethylene glycol dimethacrylate (EGDMA, 98%), polyacrylamide (PAM, analytical grade), and tetraethyl orthosilicate (TEOS, analytical grade) were purchased from Shanghai Aladdin Biotechnology Co., Ltd. Dichloromethane (CH_2Cl_2, analytical pure), sodium sulfide ($Na_2S·9H_2O$, analytical pure), ammonium persulfate ($(NH_4)_2S_2O_8$, analytical pure), N, N-dimethylformamide (DMF, analytical pure), methanol (CH_3OH, analytical pure), potassium hydroxide (KOH, analytical pure), ammonium nitrate (NH_4NO_3, analytical pure), anhydrous ethanol (CH_3CH_2OH, analytical pure), and trisodium citrate ($Na_3C_6H_5O_7·2H_2O$, analytical pure) were bought from Hunan Huihong Reagent Co., Ltd. Concentrated sulfuric acid (H_2SO_4, analytical grade) and

ammonia water ($NH_3 \cdot H_2O$, analytical grade) were purchased from Tianjin Damao Chemical Reagent Factory. Acetic acid (HAC, analytical grade) was from BASF Chemical Co., Ltd. in Tianjin. β-cyclodextrin (β-CD, analytical pure) was got from Beijing Aobo Xing Biotechnology Co., Ltd. Acetone (CH_3COCH_3, analytical pure) was purchased from Chengdu Cologne Chemical Co., Ltd. Anhydrous ferric chloride ($FeCl_3$, analytical pure) was bought from China National Pharmaceutical Group Chemical Reagent Co., Ltd. Urea (NH_2CONH_2, analytical grade) was purchased from Tianjin Shentai Chemical Reagent Co., Ltd. Hexadecyltrimethylammonium bromide (CTAB, analytical grade) was from Tianjin Guangfu Fine Chemical Research Institute.

2.1.2 Instruments

The concentration of ADR was detected through a UV vis spectrophotometer (UV-8500, Shanghai tianmei scientific instrument Co., Ltd. China). Fourier transform infrared spectroscopy system (FTIR, IRTracer-100, Shimadzu, Japan) was used to describe the functional group information in materials. The magnetic properties of the materials were analyzed by Model 7404 vibrating sample magnetometer (VSM, Lake Shore, USA). A 90 plus pals high sensitivity zeta potential and particle size analyzer was used for zeta potential analysis. Transmission electron microscope (TEM, JEOL-JEM 1400, apan) was used to analyze the morphology and internal microstructure of materials. The crystal formation of magnetic particles were assessed through X-ray diffractometer (XRD, Brooke D8 adbance, Germany), and the pore volume and specific surface of materials were conducted through beishide BSD-PS2 instrument by Brunauer Emmett teller (BET) method. The tests of cytotoxicity and immune escape were respectively performed in the related laboratories of School of Medicine and School of Pharmacy, University of South China.

2.3 Characterization experiments

2.3.1 FT-IR

The infrared spectroscopy experiment was carried out by the potassium bromide

pellet method. Firstly, placing the pressed well sample pellet in sample cell and ensuring that it was perpendicular to the infrared light, then scanning the blank background and the sample, finally performing a Fourier transform to obtain the infrared spectra.

2.3.2 XRD

Place the prepared dry sample on the sample table of X-ray diffractometer to ensure that the surface of the sample was smooth and flat. According to the property of the sample, the copper X-ray source was selected with the scanning range of X-ray diffractometer 5-90°, the scanning angle range 10-90°, and the scanning speed $5°\cdot min^{-1}$. After the instrument was calibrated, X-ray diffraction started to scan, and the diffraction patterns of samples at different angles were recorded.

2.3.3 VSM

The LakeShore 7404 VSM was powered on and subjected to a two - hour preheating process. A certain quality of dry powder sample was weighed by electronic analytical balance. In order to prevent the sample cup from being polluted, the powder was wrapped with non-magnetic plastic skin and then put into the sample cup, then compacted and put on the sample rod to start the measurement.

2.3.4 TEM

Firstly ensure that the indicator light of sample table was normal, and check the working condition of air conditioner, cooling water machine, air compressors and other equipments. Then add liquid nitrogen and wait for 15 minutes, place the sample on the sample rod, check the pressure displayed by the software, and confirm that the objective diaphragm and the selected diaphragm were set correctly. Finally use and adjust the focusing system of TEM to get the clear images.

2.3.5 BET

Firstly put the sample into the sample tube for degassing. When installing the sample tube, it was necessary to align with the port and tighten the screw to ensure the sealing safety. Then put the heating package on the sample tube, set the file information

and degassing temperature and other parameters, turn on the vacuum pump, and start heating and vacuum degassing of sample to remove the gas adsorbed on its surface. After degassing, turn off the heating power supply, and backfill helium when the sample was cooled to room temperature. After helium was filled to normal pressure, pull down the sample tube and immediately cover the rubber stopper, and record the weight of the helium-filled sample tube, stopper and filling rod. After that, load the weighed sample tube into the analysis station, add liquid nitrogen to the Dewar bottle and input the sample quality into the analysis file, and set the test parameters and start the adsorption-desorption test process. Finally, take out the sample for analysis from the sample tube, wash and dry it for later use.

2.3.6 Zeta potential

Firstly start Brooke 90 plus zeta potential and particle size analyzer, then use the sampler to slowly push the sample into the sample pool in instrument, finally select measurement mothod by the Measure Menu and enter the sample name for test.

2.4 Adsorption experiments

2.4.1 Solid-liquid ratio optimization

1 mg, 3 mg, 5 mg, 7 mg and 9 mg of ADR/PEOX-MMIP and ADR/PEOX-MNIP were respectively added to 5 mL 0.08 mg·mL $^{-1}$ of ADR methanol solution, and then shaken to attain adsorption equilibrium in a water bath at 25 ℃. After the external magnetic field was used for solid-liquid separation, 3 mL of supernatant was taken to determine the absorbance of ADR at 224 nm by UV spectrophotometer, and then the ARD concentration at adsorption equilibrium was calculated. The adsorption capacities of ADR/PEOX-MMIP and ADR/PEOX-MNIP to ADR at different solid liquid ratios were calculated according to the formula (S1) as below. Each group of experiment was repeated three times, and the results were averaged.

$$Q_e = (C_0 - C_e)V \Big/ M \tag{S1}$$

Where Q_e (mg·g^{-1}) denotes the adsorption capacity of ADR/PEOX-MMIP and

ADR/PEOX-MNIP at different solid-liquid ratios; C_0 and C_e ($mg \cdot mL^{-1}$) are the initial and the equilibrium concentration of ADR, respectively; V (mL) is the solution volume; M (mg) is the mass of added polymer.

2.4.2. Thermodynamic adsorption

3 mg of ADR/PEOX-MMIP and ADR/PEOX-MNIP were respectively added to 5 mL of ADR methanol solution with a concentration gradient of 0.005~0.110 $mg \cdot mL^{-1}$, and then shaken to attain adsorption equilibrium in a water bath at 25℃. The external magnetic field was used for solid-liquid separation, and 3 mL of supernatant was taken to determine the absorbance of ADR at 224 nm by UV spectrophotometer. The adsorption capacities of ADR/PEOX-MMIP and ADR/PEOX-MNIP to ADR at adsorption equilibrium were calculated according to the formula (S1). Each group of experiment was repeated three times, and the results were averaged. The isothermal adsorption curve of the adsorption amount with the initial ADR concentration was plotted.

2.4.3.Kinetic adsorption

3 mg of ADR/PEOX-MMIP and ADR/PEOX-MNIP were respectively added to 5 mL 0.08 $mg \cdot mL^{-1}$ of ADR methanol solution, and then shaken for 1 min, 3 min, 5 min, 7 min, 10 min, 15 min, 20 min, 30 min and 60 min in a water bath at 25 ℃. After the external magnetic field was used for solid-liquid separation, 3 mL of supernatant was taken to determine the absorbance of ADR at 224 nm by UV spectrophotometer. The adsorption capacities of ADR/PEOX-MMIP and ADR/PEOX-MNIP to ADR at different adsorption time were calculated according to the formula (S1), and then the kinetic adsorption curve of the adsorption capacity changing with the adsorption time was plotted. Each group of experiment was repeated three times, and the results were averaged.

2.4.4. Selective adsorption

In order to study the selective performance of imprinted materials for ADR, dehydroandrographolide, a structural analog of ADR, was selected as the competitive

substrate in this experiment, and its structure was shown in Figure S3. 3 mg of ADR/PEOX-MMIP and ADR/PEOX-MNIP were respectively added to 5 mL 0.08 $mg \cdot mL^{-1}$ of ADR and dehydroandrographolide methanol solution, and then shaken to attain adsorption equilibrium in a water bath at 25 ℃. The external magnetic field was used for solid-liquid separation, and 3 mL of supernatant was taken to determine the absorbance of ADR at 224 nm by UV spectrophotometer. The adsorption capacities and their corresponding selective adsorption of ADR/PEOX-MMIP and ADR/PEOX-MNIP to ADR/dehydroandrographolide were respectively calculated according to the formula (S2-S5).

$$K_d = Q_e / C_e \quad \text{(S2)}$$

$$\alpha = K_{d1} / K_{d2} \quad \text{(S3)}$$

$$\beta = \alpha_1 / \alpha_2 \quad \text{(S4)}$$

$$\gamma = Q_M / Q_N \quad \text{(S5)}$$

Where K_d is the partition coefficient; C_e ($mg \cdot mL^{-1}$) denotes the concentration of ADR/dehydroandrographolide in the solution when the adsorption equilibrium is attained; α is the selection coefficient; β shows the relative selectivity coefficient of ADR/PEOX-MMIP; K_{d1} and K_{d2} represent the partition coefficients of ADR and dehydroandrographolide, respectively, and α_1 and α_2 are the selection coefficients of ADR/PEOX-MMIP and ADR/PEOX-MNIP, respectively.

Where γ denotes the imprinted factor of imprinted materials; Q_M and Q_N represent the adsorption capacity of ADR by ADR/PEOX-MMIP and ADR/PEOX-MNIP, respectively.

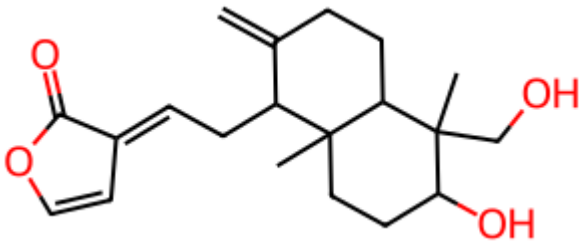

Figure S1. Dehydroandrographolide

2.4.5. Regeneration and reusability

3 mg of ADR/PEOX-MMIP was added to 5 mL 0.08 mg·mL^{-1} of ADR methanol solution, and then shaken to attain adsorption equilibrium in a water bath at 25 ℃. After the external magnetic field was used for solid-liquid separation, 3 mL of supernatant was taken to determine the absorbance of ADR at 224 nm by UV spectrophotometer, and the adsorption capacities of ADR/PEOX-MMIP to ADR were calculated according to the formula (S1). The above separated adsorbent was then turned into a Soxhlet extractor with the template ADR completely washed out by the eluent of methanol-acetic acid (9:1, v/v). Finally, the eluted adsorbent was dried and reused for the next adsorption experiment. In the reusability experiment, the above operation was repeated for five times at least.

2.5. Swelling behavior

5 mg of dried ADR/PEOX-MMIP was put into phosphate buffer solution (PBS) with pH values of 5.8, 7.4 and 8.2, respectively, and then they were removed and wiped the surface moisture at some preset intervals and weighed. Swelling rate ratios (SR) were calculated by formula (S6).

$$SR(\%) = {W_t - W_0}/{W_0} \times 100\% \quad \text{(S6)}$$

Where W_0 (mg) and W_t (mg) are the weights of dried/wet samples, respectively.

2.6. In vitro drug release

$$\mathrm{Re}lease(\%) = {\left[C_{n-1} \times V_1 + \sum (C_{n-1} \times V_{n-1})\right]}/{M} \times 100\% \quad \text{(S7)}$$

Where C_n (mg·mL^{-1}) is ADR concentration in the solution at the nth sampling; V_1 (mL) denotes the total volume of buffer solution; V (mL) represents the volume of displacement solution; M (mg) refers to the drug-loaded amount of the polymer; and V_{n-1} (mL) is the volume of the (n-1)th replacement solution.

2.7. Cytotoxicity test

The cytotoxicity assay utilized the protocol for cell countingkit-8 (CCK-8) test to assess the inhibition of ADR/PEOX-MMIP and the drug-loaded ADR/PEOX-MMIP on the activity of human non-small cell (lung cancer cell A549).[S1] DMEM was used to adjust the cell suspension concentration, and the final concentration reached 8 × 10^4 cells·mL^{-1}. 100 μL of cell solution was seeded in 96-well plates to ensure that the number of cells in each well was 8 × 10^4 CFU·mL^{-1}, and 6 multiple wells were set in each group, which incubated for 24 h in a constant temperature incubator containing 5% (V/V) CO_2 at 37 ℃. The experimental group 1 was conducted by adding 100 μL cell suspension and media containing different concentrations of ADR/PEOX-MMIP (10, 20, 40, 80, 160 μg·mL^{-1}), and the experimental group 2 was completed by replacing ADR/PEOX-MMIP with the drug-loaded ADR/PEOX-MMIP. The control group 1 was experimented by adding 90 μL DMEM medium and 10 μLCCK-8 reagent, and in the control group 2, 90 μL cell suspension and 10 μL CCK-8 reagent were added, both of which were cultured for 24 h. After 1 h of static culture, the absorbance was measured at 450 nm by microplate reader. All the above experiments were performed in triplicate and the cell viability was calculated according to the formula (S8).

$$Viability(\%) = {A_1 - A_2}\Big/{A_3 - A_2} \times 100\% \quad \text{(S8)}$$

Where A_1 is the absorbance of experimental hole; A_2 is the absorbance of control group 1; A_3 is the absorbance of control group 2.

2.8. Immune escape test

A strain of RAW264.7 mouse macrophage was cultured in DMEM medium containing 10% fetal bovine serum and 1% antibiotic solution. DMEM was used to

regulate the cell suspension concentration, and the final concentration reached 8×10^4 CFU·mL^{-1}. Then 100 μL cell suspension was inoculated into two groups of 96-well plates with 4 multiple pores in each group, and cultured under the conditions of 37 ℃ and 5%CO_2 for 24 h. Since the adsorption capacities of both ADR-MMIP and ADR/PEOX-MMIP loaded ADR were respectively 41.43 and 11.46 mg·mL^{-1}, in order to achieve the purpose of containing the same amount of ADR, DMEM solutions containing both the drug-loaded ADR-MMIP at concentrations of 0, 4.83, 12.07 and 24.14 μg·mL^{-1} and ADR at the respective concentrations of 0, 0.2, 0.5 and 1 μg·mL^{-1}, and the corresponding DMEM solutions containing both the drug-loaded ADR/PEOX-MMIP with concentrations of 0, 17.45, 43.63 and 67.26 μg·mL^{-1} and ADR with the respective concentrations of 0, 0.2, 0.5 and 1 μg·mL^{-1} were prepared in advance. ADR/PEOX-MMIP was used as the experimental group and ADR-MMIP as the control group. After 200 μL of the above solutions were added to the previous well plates containing cell suspensions and cultured for 24 h, the supernatants were removed and the cells were washed with PBS three times, and then an appropriate amount of cell lysate was added to each well and the repeated oscillation was performed. Finally, the absorbance of cell lysate was determined by UV (224 nm) to calculate the concentration of ADR. This experiment was conducted in parallel for 3 times, and the final results were the average values.

3 Results and discussion

3.2 Adsorption performance

3.2.2 Thermodynamic adsorption study

Isothermal adsorption formulas (S9-S11) of Langmuir, Freundlich and Scatchardwere as follow:[S2,S3]

$$C_e/Q_e = 1/K_L Q_m + C_e/Q_m \quad \text{(S9)}$$

$$\log Q_e = \log K_F + \log C_e/n \quad \text{(S10)}$$

$$Q_e / C_e = Q_m - K_D / K_D \quad \text{(S11)}$$

Among them, Q_e ($mg \cdot g^{-1}$) is the equilibrium adsorption capacity of adsorbent; C_e ($mg \cdot mL^{-1}$) indicates the concentration of ADR at adsorption equilibrium; Q_M ($mg \cdot g^{-1}$) denotes the maximum apparent adsorption capacity of adsorbent; K_L, K_F and K_D indicate Langmuir adsorption constant, Freundlich adsorption strength constant and Scatchard equilibrium dissociation constant, respectively; n represents Freundlich constant of adsorption capacity.

3.2.3 Kinetic adsorption study

Kinetic adsorption formulas (S12-S13) of the pseudo-first-order and pseudo-second-order [S4] kinetic models were as follow:

$$\ln(Q_e - Q_t) = \ln Q_e - K_1 t \quad \text{(S12)}$$

$$t / Q_t = 1 / K_2 Q_e^2 + 1 / Q_e \, t \quad \text{(S13)}$$

Where Q_e ($mg \cdot g^{-1}$) represents the adsorption capacity of adsorbent at equilibriumstate; Q_t ($mg \cdot g^{-1}$) is the adsorption capacity of adsorbent at time t (min); K_1 and K_2 denote the pseudo-first-order and pseudo-second-order kinetic rate constants, respectively.

Notes and references

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