Preparation of Perfluorocarbon Emulsion and Its Application in Cells Reoxygenation

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Perfluorotripropylamine (FC-3283), a kind of perfluorocarbon as oxygen carrier, is encapsulated in a copolymer using polyethylene glycol (PEG) to modify poly lactic-*co*-glycolic acid (PLGA) to prepare a PLGA-PEG/FC-3283 emulsion for highly efficient reoxygenation to cell. ¹H nuclear magnetic resonance (¹H NMR) spectra, Fourier transform infrared spectrum (FT-IR), transmission electron microscopy (TEM), dynamic light scattering (DLS) and high performance ion chromatography proved the formation of the PLGA-PEG and PLGA-PEG/FC-3283 emulsion. High performance ion chromatography revealed that FC-3283 content in the emulsion is 9.4%. Furthermore, CCK-8 assay was used to detect the cytotoxicity of the PLGA-PEG/FC-3283 emulsion to HCT 116 cells. Finally, the ability of oxygen supply and release of PLGA-PEG/FC-3283 emulsion was evaluated by CCK-8 assay and fluorophotometry based on establishing hypoxia/reoxygenation model of HCT 116 cells with liquid paraffin. By detecting the cells increment rate and the content changes of reactive oxygen species (ROS) before and after reoxygenation, we explored the oxygen carry and release ability of PLGA-PEG/FC-3283 emulsion. The results showed that the cell viability increased significantly through PLGA-PEG/FC-3283 emulsion administration after the cells were treated with hypoxia.

Keywords perfluorocarbon emulsion, hypoxia/reoxygenation, cytotoxicity, reactive oxygen species

Introduction

Oxygen is a key parameter in maintaining cell viability and functionality, and cellular necrosis follows inevitably extended periods of anoxia or severe hypoxia. In the past years, artificial oxygen carriers have attracted increasing attention due to their ability of dissolving and delivering oxygen. Perfluorocarbon (PFC), a kind of artificial oxygen carrier, has excellent dissolution of non-polar gas, such as O₂ and CO₂. Therefore, PFC has been applied for gas exchange medium of partial liquid ventilation treatment of acute respiratory distress syndrome.^[1,2] However, PFC has nice stability and is insoluble in water, plasma and cell culture medium, so it can't be used for biological environment in the body until modified.^[3] Among them, PFC emulsion has become the main application form of PFC materials in the biomedicine and biotechnology, such as injectable oxygen carrier, contrast agents, and drug delivery system.^[4-6] For example, Yao *et al.*^[7] has reported a inclusion complex of perfluorocarbon compound with

β -cyclodextrin for an ultrasound contrast agent. Meanwhile, our groups^[8] have developed an emulsion as enhancement agents for highly efficient reoxygenation to cell and organism. The reports focused on some PFCs, such as perfluoroalkane mixture with perfluorocyclic ether (FC-77) and 2',7'-dichlorofluorescein diacetate and perfluorooctyl bromide (PFOB). The application of other PFCs is still an interest and active task.

Poly lactic-*co*-glycolic acid (PLGA) micro-/nano-structure, has generated tremendous research interest due to its excellent biocompatibility, efficient transport to the target and the changeable biodegradability, molecular weight and chemical structure by varying composition (lactide/glycolide ratio).^[9,10] Therefore, they have been widely used in pharmaceutical and medical engineering materials, such as skin transplantation, wound closure, drug carrier, and so on. PLGA/PFC micro-/nanostructure can be used as an oxygen carrier because PLGA shell has similar mechanical properties and permeability to the red blood cells which has the excellent ability to keep oxygen combination and release.

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Furthermore, PEGylated poly(lactic-co-glycolic acid) (PEG-PLGA) copolymer was always used to prepare the stealth nanoparticles or long-circulating nanoparticles for drug, protein and peptide delivery.^[11,12]

In this work, perfluorotripropylamine (FC-3283), used as a model medical PFC, is chosen as an object because of its widely application in clinic. We report preparation and characterization of a new type of emulsion formed from PLGA-PEG and FC-3283 with an aim to improve the stability and bioavailability of PFC. The obtained PLGA-PEG/FC-3283 emulsion can be used in hypoxia/reoxygenation of HCT 116 cells, which is potential for oxygen carry in future. PFC encapsulated in PLGA as an oxygen carrier used for cells reoxygenation was rarely reported and it's an important and attractive subject. The research provides a potential chance to prepare artificial oxygen carriers in biomedical applications.

Experimental

Materials

Poly(lactide-co-glycolide) Resomer RG502 (PLGA-COOH, MW 20,000) was purchased from Ji'nan Daigang Biomaterial Co., Ltd. Amine-PEG-Carboxymethyl (NH₂-PEG-COOH, MW 3,400) was purchased from Shanghai Seebio Biotech. Perfluorotripropylamine was purchased from Sigma. All chemical reagents were analytical grade or above and purchased from Sino-pharm Chemical Reagent Co., Ltd. Triethylamine and dichloromethane were dried with calcium hydride before use. HCT116 cell line was purchased from cell bank of Chinese Academy of Sciences, and the cultivate reagents were purchased from Life Technologies (Gibco, USA). Cell counting kit-8 (CCK-8) was purchased from Beijing Fanbo Biochemicals Co., Ltd.

Preparation of PEG-PLGA copolymer

Preparation of PEG-PLGA copolymer is similar to a previous report.^[11] Carboxylate-functionalized copolymer PLGA-PEG was synthesized by the conjugation of PLGA-COOH to NH₂-PEG-COOH. A total of 1 g of PLGA-COOH, 9 mg of *N*-hydroxysuccinimide (NHS), 14.5 mg of 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), and 5 mg of 4-dimethylaminopyridine (DMAP) were dissolved in 10 mL of dichloromethane and stirred for 24 h at room temperature to convert PLGA-COOH to PLGA-NHS. Then, 170 mg of NH₂-PEGCOOH and 43 μ L of triethylamine were added into the solution. After further stirring for 8 h, dichloromethane was removed through a rotary evaporator and 5 mL of DMF was added to dissolve the product. Then, the solution was added to a dialysis bag (the width is 44 mm, and the molecular weight cutoff is 8,000–14,000) and unreacted PEG and other small molecules were removed by dialyzing for 3 d. Then, the product was freeze-dried for 24–48 h. The samples were measured by ¹H NMR to verify the re-

sponse rate.

Preparation of PLGA-PEG/FC-3283 emulsion

10 mg of PLGA-PEG was dissolved into 200 μ L of dichloromethane along with the desired amount of FC-3283 and 200 μ L 15% Span80@dichloromethane (*w/V*). After well blended, the organic solution was then emulsified into 2 mL using an Ultra-turrax T10 (IKA) to form a pre-emulsion, and emulsification was performed in a 15 mL centrifuge tube placed over ice for 1 min. Then, the pre-emulsion was sonicated with a vibrating metallic tip, JY 92-IIN (SCIENTZ, China), for 3 min over ice. It works for 3 s, then stops for 10 s, and repeats the process under 50% power (300 W). After further stirring with Ultra-turrax T10 (IKA) for 10 min (works for 1 min and stops for 1 min) over ice, organic solvents were then evaporated by magnetic stirring for about 3 h at room temperature.

Characterization

Particle size was measured by a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK) based on dynamic light scattering (DLS). The ¹H NMR spectra were recorded on a Bruker AM-400 spectrometer in CDCl₃ solution at room temperature. Transmission electron microscopy (TEM) images were recorded on a JEOL-2100F instrument using an accelerating voltage of 200 kV. Fluorine content was determined by high performance ion chromatography (Dionex 500, USA) with conductivity detection through oxygen flask combustion (separating column, IonPac AG14-AS14; eluent, 0.0010 M NaHCO₃ + 0.0035 M Na₂CO₃).

Cell cytotoxicity

Cells were cultured in 37.5 cm² flasks in dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics at a humidified, 37 °C and 5% CO₂ incubator. The cytotoxic effect of PLGA-PEG/FC-3283 emulsion was determined with the CCK 8 assay. Briefly, HCT 116 cells were seeded at a density of 5000 cells per well in 96-well plates. After 48 h, the medium was replaced with 100 μ L medium with different concentration of PLGA-PEG/FC-3283 emulsion, but control cells were incubated with DMEM medium alone. After incubation for 4, 8 and 12 h, the supernatant was removed and 10 μ L CCK-8 solutions were added to each well of the plate (total medium 100 μ L/well). Then cells were incubated with at 37 °C. After 4 h incubation, absorbance was measured using iMark Microplate Reader (Bio-Rad, USA) at 450 nm as measure wavelength.

Cell viability during hypoxia-reoxygenation treatment

HCT 116 cells were seeded at a density of 5000 cells per well in 96-well plates. After 48 h, the medium was replaced with 100 μ L DMEM without FBS, then 100 μ L liquid paraffin was added to each well of the plate

except the control group. Because of its low density and highly hydrophobic nature, liquid paraffin formed a film over the medium. As a consequence, the medium was separated from the normoxic atmosphere by the overlying liquid paraffin film, which provided a hypoxic environment to the cell culture. After incubation under hypoxia condition for 4 and 8 h, liquid paraffin and DMEM were removed and 100 μ L medium with 10% FBS and different concentration of PLGA-PEG/FC-3283 emulsion was added for 2 h reoxygenation. Finally, cell viability was determined with the CCK-8 assay.

Measurement of reactive oxygen species

Intracellular reactive oxygen species (ROS) generation was measured by 2',7'-dichlorofluorescein diacetate (DCFH-DA). After cellular uptake of DCFH-DA, it was cleaved by cellular esterases to 2',7'-dichlorofluorescein (DCFH), and then the intracellular ROS would cause the oxidation of DCFH to the fluorescent product of 2',7'-dichlorofluorescein (DCF). Cells plated on 6-well cell culture plates were maintained under hypoxic conditions for 12 h, then DMEM were replaced with the medium containing different concentrations of PLGA-PEG/FC-3283 emulsion for 2 h reoxygenation, after which cells were washed and incubated with DCFH-DA (10 μ M) in serum-free culture medium under normoxic condition for additional 30 min. The medium was removed and cells were washed three times with phosphate buffer saline (PBS). The 2',7'-dichlorofluorescein (DCF) fluorescence intensity was measured using fluorescence microscopy excited by blue light, and it was also detected by flow cytometer with an excitation wavelength of 488 nm after cells should be digested with trypsin enzyme first and washed three times with PBS through centrifuged (1000 rpm, 3 min, 4 $^{\circ}$ C).

Statistical analysis

Results are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was done using Student's *t*-test. Differences were considered significant at *P* value < 0.05 .

Results and Discussion

PLGA-PEG/FC-3283 emulsion is prepared by emulsification of a copolymer of PEGylated PLGA and applied as an oxygen carrier to supply oxygen during a model hypoxia/reoxygenation of HCT 116 cells with liquid paraffin. The emulsion and the interaction with cell are schematically illustrated in Figure 1. To evaluate the effect of PLGA-PEG/FC-3283 emulsion on cells, the cell viability and intracellular ROS production are detected on the condition of hypoxia, reoxygenation, and reoxygenation with the emulsion.

Figure 1 Schematic illustration of PLGA-PEG/FC-3283 emulsion preparation and its interaction with HCT 116 cell on the condition of hypoxia and reoxygenation.

The PLGA-PEG copolymer was synthesized by conjugating activated PLGA-COOH and NH_2 -PEG-COOH through EDC/NHS coupling chemistry. As shown in Figure 2a, the structure of the obtained product is characterized by ^1H NMR. The result shows ^1H NMR analysis of a polymer solution in CDCl_3 (δ , ppm): 5.2 (H-1 from PLGA-COOH), 4.8 (H-2 from PLGA-COOH), 3.7 (H-3 from NH_2 -PEG-COOH), and 1.6 (H-4 from PLGA-COOH), which shows successful synthesis of PLGA-PEG. On the basis of a previous report,^[11] the efficiency of the coupling reaction is calculated as follows: molecular weight of PLGA monomer is 130, and molecular weight of PLGA is about 20,000. Thus, the PLGA macromolecule includes about 154 monomers, in which there are two H-2 atoms. Similarly, the PEG macromolecule includes about 77 monomers, in which there are four H-3 atoms. Therefore, the quantity of H-2 atoms in PLGA macromolecule is equal to that of H-3 atoms in PEG macromolecule. If PLGA was conjugated with PEG completely, the ^1H NMR peak area of H-3 atoms in PEG macromolecule and H-2 atoms in PLGA macromolecule would be equal. However, on the basis of unreacted PEG being removed by dialyzing, the peak area of H-3 atoms in PEG macromolecule and H-2 atoms in PLGA macromolecule is 3.68 and 4.32, respectively, whose ratio is $3.68/4.32 \approx 85\%$. Therefore, we suggest that the coupling efficiency is about 85%. Furthermore, FT-IR spectra were performed to characterize synthesis of PLGA-PEG copolymer. As shown in Figure S1 in Supporting Information and Figure 2b, the typical peaks of PLGA are shown at bands of 1456, 1423, and 1394 cm^{-1} , which should contribute to the absorption of CH , CH_2 , and CH_3 groups, respectively. Besides the above bands in PLGA-PEG FT-IR spectra, a weak new peak at 1622 cm^{-1} can be found, which results from the absorption of amide bonds. The result indicates the successful covalent conjugation between PLGA and PEG.

Figure 2 (a) ¹H NMR of PLGA-PEG and (b) FT-IR of PLGA-PEG and PLGA.

In addition, PLGA-PEG/FC-3283 emulsions based on PLGA-PEG copolymer are characterized. Figure 3a shows that the average hydrodynamic size of the PLGA/FC-3283 emulsion in deionized water is about 225.8 nm. As shown in Figure 3b, TEM image shows PLGA-PEG/FC-3283 emulsions with core in gray and shell in dark are uniform nanospheres with an average diameter of about 200 nm, which is similar to the result of DLS. Moreover, fluorine content in the freeze-dried powder of PLGA-PEG/FC-3283 emulsion is 7.2% determined by high performance ion chromatography. After conversion, FC-3283 content in the freeze-dried powder of PLGA-PEG/FC-3283 emulsion is 9.4%. According to the solubility of oxygen in FC-3283 is about 48% (V/V), the oxygen volume in per gram PLGA-PEG/FC-3283 emulsion powder is calculated to about 0.026 mL. Additionally, we measured the zeta potential of PLGA-PEG emulsion and PLGA-PEG/FC-3283 emulsion in aqueous solution. The results show that the zeta potential is changed from -24.77 ± 0.21 mV of PLGA-PEG emulsion to -29.53 ± 0.65 mV of PLGA-PEG/FC-3283 emulsion with the addition of FC-3283 due to the strong electronegativity of perfluorocarbon. These results show that the PLGA-PEG/FC-3283 emulsion has been successfully synthesized.

To estimate the reoxygenation ability of PLGA-PEG/FC-3283 emulsion, a hypoxiareoxygenation treatment to HCT 116 cells was detected through the hypoxia-reoxygenation model with the liquid parafin covering method. As shown in Figure 4, cell viability is reduced after hypoxia treatment for 4 or 8 h, which is attributed to the poor cell states after hypoxia treatment,

Figure 3 (a) Size of distribution and (b) TEM image of PLGA-PEG/FC-3283 emulsion.

because cell viability cannot restore quickly after re-oxygenation with a normal medium. When PLGA-PEG/FC-3283 emulsion is added, cell viability shows a significant enhancement compared to the group reoxygenation with normal medium ($P < 0.05$). The reason for the enhancement in cell viability could lie in the high dissolved oxygen ability of FC-3283, which supplies more oxygen to help cell reoxygenation to restore from the hypoxic state.

Figure 4 Cell viability under the condition of reoxygenation with PLGA-PEG/FC-3283 emulsion for 2 h after hypoxia treatment for 4 and 8 h.

To evaluate the cell injury during the cells hypoxia and reoxygenation treatment of PLGA-PEG/FC-3283 emulsions, the production of ROS was performed. As well known, ROS has been implicated in the regulation of many important cellular events including transcription factor activation, gene expression, and cellular proliferation.^[13-15] However, excessive production of ROS

gives rise to activation of events, which could lead to cell injury or death. In our experiment, ROS in HCT116 cells was measured by DCFH-DA. As shown in Figure 5a, there is almost no DCF fluorescence in the control group, which implies that the ROS level in cells cultured under normoxia condition is very low. After 12 h hypoxia treatment, green cells from DCF fluorescence can be seen in fluorescent microscopic image (Figure 5b), which reveals that cells hypoxia can lead to their injury. As shown in Figures 5c–5f, DCF fluorescent intensity is improved slightly with the addition of PLGA-PEG/FC-3283 emulsion. However, the increasing concentration of PLGA-PEG/FC-3283 emulsion from 0.05 to 0.2 mg/mL for reoxygenation treatment hardly brings the increasing DCF fluorescent intensity. The results imply that the addition of PLGA-PEG/FC-3283 emulsion can promote the little ROS level in cells and is sublethal to HCT116 cells.

Figure 5 Fluorescent microscopic images of cellular ROS production after hypoxia and reoxygenation treatment under the condition of (a) control group cultured under normoxia condition, (b) hypoxia treatment for 8 h and reoxygenation with PLGA-PEG/FC-3283 emulsion at a concentration of (c) 0, (d) 0.05, (e) 0.1 and (f) 0.2 mg/mL after hypoxia treatment for 8 h.

The cytotoxicity was investigated to examine the feasibility of the obtained products for biomedical application. Using the viability of untreated cells for controlled, the effect of varying concentrations (0–0.5 mg/mL) of PLGA-PEG/FC-3283 emulsion on the viability of HCT 116 cells after exposure for 4, 8 and 12 h was investigated (Figure 6). It is an interesting phenomenon that there is no significant difference in cell viability with the increasing concentration of PLGA-PEG/FC-3283 emulsion. Therefore, the results demonstrate that the obtained products have a low cytotoxicity.

Figure 6 *In vitro* cell cytotoxicity of PLGA-PEG/FC-3283 emulsion.

Conclusions

PEG-PLGA copolymer is successfully prepared though conjugated NH_2 -PEG-COOH with PLGA-COOH with a grafting ratio of 85%, which further is emulsified. Furthermore, DLS and TEM show that PLGA-PEG/FC-3283 emulsion with a nanosize could be obtained, and the encapsulation efficiency of FC-3283 is about 9.4%. Cell hypoxia-reoxygenation experiment reveals that cell viability has a significant enhancement after reoxygenation with PLGA-PEG/FC-3283 emulsion because of the sufficient and timely oxygen supply. Meanwhile, little hypoxia-reoxygenation injury will happen along with cell reoxygenation, which is reflected by increasing ROS in cells. With the increasing concentration of PLGA-PEG/FC-3283 emulsion, the ROS level and cell viability imply the PLGA-PEG/FC-3283 emulsion is sublethal to HCT116 cells. The all results show that PLGA-PEG/FC-3283 emulsion can supply oxygen to HCT116 cells.

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