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Chrysin-nanoencapsulated PLGA-PEG for macrophage repolarization: Possible application in tissue regeneration

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ABSTRACT

The purpose of this study was to investigate the efficiency of a natural flavonoid, Chrysin (Chr), encapsulated in PLGA-PEG nanoparticles (NPs) for the modulation of macrophage polarity from the pro-inflammatory M1 to anti-inflammatory M2 phenotype.

The synthetized NPs were characterized using FTIR, DLS and FE-SEM. MTT assay was used to assess the toxicity of different concentration of Chr-encapsulated NPs on LPS/IFN-γ stimulated peritoneal exudate macrophages. To investigate the repolarization efficiency of Chr-encapsulated NPs, real-time PCR was applied to measure M1 (iNOS and SOCS3) and M2 (Arg1 and Fizz) markers expression. Also, the relative mRNA and protein expression levels of pro-inflammatory cytokines including IL-6, IL-1β and TNF-α were investigated in M1 macrophages treated with Chr-encapsulated NPs.

Findings revealed that the Chr-encapsulated NPs with spherical shape and an average diameter of 235 nm were considerably less toxic to the macrophages. Additionally, the nano-formulated Chr efficiently showed a reduction in M1 markers and an increase in M2 markers levels than free Chr. Furthermore, macrophage phenotype switching by PLGA-PEG encapsulated Chr NPs significantly suppressed LPS/IFN-γ induced inflammation by a remarkable reduction in pro-inflammatory cytokine levels, TNF-α, IL-1β, and IL-6.

Convincingly, the results revealed that PLGA-PEG encapsulated Chr based drug delivery system might be introduced into biomaterials to fabricate bioactive smart multifunctional nanocomposites with macrophage repolarization activities for regenerative medicine purposes.

1. Introduction

Macrophages are critical mediators in organizing the innate and adaptive immunity. Predominantly, they are purposefully distributed throughout the body and involved in host defense, initiation and resolution of inflammation, cell proliferation, and tissue repair and regeneration [1]. Macrophages which are enormously plastic and versatile cells can be fully polarized in response to the local environmental cues and obtain specific phenotype like pro-inflammatory M1 or anti-inflammatory M2 [2]. M1 macrophage phenotypes are induced by inflammatory stimuli such as Lipopolysaccharide (LPS), Interferon-γ (IFN-γ) and Tumor necrosis factor (TNF-α). These macrophages enhance secretion of many pro-inflammatory cytokines like IL-6, IL-1β, TNF-α and production of nitric oxide (NO) and reactive oxygen intermediates to guarantee effective microbial eradication; and upregulate CD80, CD86 and MHC-I/II expression levels [3,4]. Nevertheless, an endless activation of M1 phenotype may prompt tissue injury and inflammation [5]. On the contrary, in the process of wound healing or in the presence of various anti-inflammatory cytokines, including IL-13, IL-10 and IL-4, M1 polarized cells shift to M2 phenotypes and produce

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high amounts of anti-inflammatory cytokines, including TGF-β, IL-10 and IL-4 for tissue restoration and reconstruction. Also, Up-regulated surface expression of macrophage mannose receptor (MMR) and enhanced Arginase 1 (Arg-1) activity are involved in the remodeling and anti-inflammatory effects of M2 macrophages [6]. Hence, M2 macrophages support tissue repair and reconstruction by producing anti-inflammatory cytokines that mediate angiogenesis, cell replacement, and matrix remodeling.

The application of nanotechnology to improve current methods in tissue regeneration has received extensive attention over the years [7–10]. Especially, nanoparticles (NPs) propose interesting characteristics to progress the field of regenerative medicine. NPs are tiny materials with dimensions usually between 1 and 100 nm and propose great variety in terms of size, surface topography and components [11,12]. Owning to their size and surface chemistries, NPs can be applied as theranostic agents or as carriers for the delivery of various therapeutic agents, growth factors and genetic materials [13–16]., Also, NPs can be used as drug delivery systems to introduce novel aspects within biomaterials and create smart multifunctional nanocomposites due to their unique chemical properties and plasticity in design [17].

Recently, there has been growing interest to explore a suitable strategy for shifting macrophage type from an M1 to M2 phenotype and achieve a short pro-inflammatory period in which M1 macrophages are recruited to the site, followed by an anti-inflammatory stage where the M2 phenotype dominates [6,18]. Among some strategies used to achieve this aim, the release of natural immunomodulatory agents from NP-based structures may be an effective strategy to control inflammation, manipulate the M1–M2 responses and stimulate tissue regeneration [19].

Chrysin (Chr) (5,7-di-OH-flavone), a natural flavonoid and biologically active immunomodulatory agent found in plants, has been well evidenced to possess wide spectrum of biological activities such as anti-inflammatory, antioxidant, antidiabetic, anti-allergic, antibacterial, antiestrogenic, and anticancer activities [20–23]. However, low bioavailability, highly instability in physiological pH and extremely low water solubility hamper its application for therapeutic purposes [24–26].

Herein, we hypostasized that Chr, as a natural immunomodulatory agent, in corporation with polymeric NPs may provide a novel platform to create smart multifunctional structures for application in regenerative medicine purposes. Therefore, we loaded Chr into poly lactic acid-co-glycolic acid-polyethylene glycol (PLGA-PEG) NPs and assessed their potential to modulate functional polarity of macrophages toward anti-inflammatory M2 phenotypes. PLGA-PEG based NPs have been presented as a hopeful nano-delivery system owing to their biodegradable, biocompatible, non-immunogenic and non-toxic properties [27,28].

2. Materials and methods

2.1. Materials

Polyethylene glycol (PEG, molecular weight: 6000) Stannous octoate [Sn $(Oct)_2$], Glycolide, DL-Lactide, Polyvinyl alcohol (PVA), Dichloromethane (DCM), Chrysin, Dimethyl sulfoxide (DMSO), MTT (3, 4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide), Lipopolysaccharide (LPS), and Interferon gamma (IFN- γ) were obtained from Sigma-Aldrich (Steinheim, Germany). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), Penicillin G, Streptomycin, Trypsin-EDTA and TRIzol Reagent were purchased from Gibco (Invitrogen, Carlsbad, CA). All other reagents were of analytical grade and were used without further purification.

3. Methods

3.1. Synthesis of PLGA-PEG

PLGA-PEG tri-block copolymer was synthesized through ring opening polymerization (ROP) of DL-lactide and glycolide in presence of PEG_{6000}. As noted in melt polymerization protocol [29], PEG_{6000} and PLGA were copolymerized under vacuum using $Sn(Oct)_2$as the catalyst. The combination of DL-lactide (2.882 g), PEG_{6000} (1.44 g) and glycolide (0. 270 g) was completely melted in bottleneck flask in 140 °C under a nitrogen atmosphere. Then, 0.05% (w/w) $Sn(Oct)_2$ was added and the temperature of the reaction mixture was raised to 180 °C for 5 h. The produced copolymer was dissolved in DCM and precipitated in ice-cold diethyl ether

3.2. Fabrication of Chr-encapsulated PLGA-PEG NPs

Chr-loaded PLGA-PEG NPs were obtained using oil-in-water (O/W) emulsion-solvent evaporation technique. Briefly, 200 mg of PLGA-PEG dissolved in 5 mL of DCM-methanol co-solvent (4:1) and 2 mg of Chr were moved to a centrifuge tube, and the solution mixture was gently stirred for 15 min at room temperature and emulsified using sonication in 50 mL of PVA aqueous solution (0.5%, w/v). After vacuum vaporization of the solvent, the NPs were gathered by centrifugation at 12,000 rpm for 10 min at room temperature and washed three time using deionized water. The obtained NPs loaded suspensions were lyophilized and stored at 4 °C until further use.

3.3. Particles size and morphology determination

The averageparticle size, surface charge and polydispersity index (PDI) of PLGA-PEG NPs were determined using a Dynamic Light Scattering (DLS) method (Nano ZS, Malvern Instruments Ltd., Malvern, UK). The zeta potential of the NPs was measured in KCL 1 mM with a Malvern Nano ZS at 25 °C. Field Emission Scanning Electron Microscopy (FE-SEM) system (Hitachi S-4800 FE-SEM) was applied to determine the surface and morphology of prepared NPs. The bonding configurations of the NPs were analyzed by Fourier-transform infrared spectroscopy (FTIR) (Shimadzu 8400 S, Kyoto, Japan).

3.4. Analysis of drug encapsulation and drug loading

Following fabrication of Chr-encapsulated PLGA-PEG NPs, the supernatant was separated and used for comparing with the total amount of Chr to delineate the Chr loading efficiency of the NPs. The amount of non-entrapped Chr in aqueous phase was determined at wavelengths 348 nm (λ_{max} of Chr) using a Lambda 950 Visible-UV spectrophotometer (Shimadzu). The percentage of Chr encapsulated in the NPs (EE) (Eq. (1)) and drug loading (DL) (Eq. (2)) were determined using the following formula:

$$EE = \frac{Weight\ of\ Chr\ in\ NPs}{Weight\ of\ initial\ drug} \times 100 \quad (1)$$

$$DL = \frac{Weight\ of\ Chr\ in\ NPs}{Weight\ of\ NPs} \times 100 \quad (2)$$

3.5. In vitro drug release

The *in vitro* release of Chr from drug-loaded PLGA-PEG NPs were assessed using the dialysis method as mentioned previously [30]. Briefly, Chr-encapsulated PLGA-PEG NPs (20–30 mg) were reconstituted in 5 mL PBS (pH 7.4) and transferred to dialysis membrane tubes with a molecular weight cut-off of 3000 (Spectra/Por 7, Spectrum Laboratories Inc.). The tubes were then placed in 25 ml of PBS with stirring at 120 rpm at 37 °C. At predetermined time intervals, 1 mL of