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Quercetin conjugated poly(β-amino esters) nanogels for the treatment of cellular oxidative stress

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ABSTRACT

PβAE polymers have emerged as highly promising candidates for biomedical and drug delivery applications owing to their tunable, degradable and pH sensitive properties. These polymeric systems can serve as prodrug carriers for the delivery of bioactive compounds which suffer from poor aqueous solubility, low bioavailability and are biologically unstable, such as the antioxidant, quercetin. Using acrylate functionalized quercetin, it is possible to incorporate the polyphenol into the backbone of the polymer matrix, permitting slow release of the intact molecule which is perfectly timed with the polymer degradation. While formulating these quercetin conjugated PβAE matrix into nanocarriers would allow for multiple delivery routes (oral, intravenous, inhalation etc.), well known oil-water nano-emulsion formulation methods are not amenable to the crosslinked hydrolytically sensitive nanoparticle/nanogel. In this work, a single-phase reaction–precipitation method was developed to formulate quercetin conjugated PβAE nanogels (QNG) via reaction of acrylated quercetin (4–5 acrylate groups) with a secondary diamine under dilute conditions using acetonitrile as the reaction medium, resulting in a self-stabilized suspension. The proposed approach permits the post synthesis modification of the spherical nanogels with a PEGylated coating, enhancing their aqueous stability and stealth characteristics. Nanogel size was controlled by varying feed reactant concentrations, achieving drug loadings of 25–38 wt%. Uniform release of quercetin over 45–48 h was observed upon PβAE ester hydrolysis under physiological conditions with its retained antioxidant activity over the extended times.

Statement of Significance

Here we present the first demonstration of using poly(beta amino ester) chemistry to form nanogels composed of a bioactive polyphenol for the control of cellular oxidative stress. Previous nanogel and nanoparticle approaches, which use a water phase, are not readily amenable to PBAE chemistry due to their hydrolytic sensitivity. Here we demonstrate a simple approach to control particle size, modify surface chemistry and achieve highly regulated controlled release of active antioxidants, which can protect cells against external oxidative stress signals. This work has importance in the area of controlling material biocompatibility through augmenting the antioxidant status of cells.

1. Introduction

Oxidative stress is a pathophysiological condition, where endogenous antioxidants are unable to counteract the production of oxidants, leading to cellular dysfunction. This overproduction of the reactive oxygen and nitrogen species (ROS/RNS) (e.g., hydroxyl radicals, singlet oxygen, hydrogen peroxide, peroxyl radicals) can be caused by both endogenous sources and exogenous sources. Examples of endogenous routes include ROS generating enzymes such as nitric oxide synthase, xanthine oxidase, amplified mitochondrial metabolism especially in aging cells resulting in mitochondrial dysfunction, damaged membrane and hence leakage of ROS into intracellular environment [1–4]. Some of the exogenous

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate); DCF-DA, dichlorofluorocein-diacetate; DEGDA, diethylene glycol diacrylate; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; EBM, endothelial basal medium; HCl, hydrogen chloride; HPLC, high pressure liquid chromatography; HUVEC, Human Umbilical Vein Endothelial Cell; IgG, imunnoglobin G (antibody); NNDA, N,N′ dimethyl 1,3-propane diamine; PBAE, poly(β-amino esters); PBS, phosphate buffer saline; PEG, polyethylene glycol; PEGDA, polyethylene glycol diacrylate; PEGME5000, polyethylene glycol methylether, Mn ~ 5000; PEGMEMA4000, polyethylene glycol methylether methacrylate, Mn ~ 4000; PLA, poly(lactic acid); PLGA, poly(lactic-co-glycolic acid); QMA, quercetin multiacrylate; QNG (x), quercetin conjugated PBAE nanogels (feed QMA concentration); RNS, reactive nitrogen species; ROS, reactive oxygen species; SLN, solid lipid nanoparticles; TCA, trichloroacetic acid; TEAC, trolox equivalent antioxidant capacity; THF, tetrahydrofuran; TNF-α, tumor necrosis factor-α.

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sources include exposure to ozone, UV, γ-irradiation, air pollutants penetrating through skin or via inhalation, intake of various drugs, xenobiotic and many more [4–9]. At a systemic level, oxidative stress has been shown to play a role in the development and acceleration of many diseases, including diabetes, cardiovascular diseases, Alzheimer's and Parkinson's disease, acute renal failure, acute lung injury, radiation injury, etc. [10,11].

Rationally, one would expect that the supplementation of dietary antioxidants would be sufficient to reduce excess ROS into non-reactive stable molecules, resolving the oxidative stress and thereby treating many diseases. In fact, this beneficial property of antioxidants, including polyphenol flavonoids, has been successfully demonstrated *in vitro* multiple times [12,13]. But despite these *in vitro* demonstrations, nearly all clinical trials with antioxidants have failed to demonstrate substantial benefit [14,15]. As a result of these outcomes, it has been inferred that a major limitation of dietary antioxidants was their inability to impact the oxidative stress levels in the patients. Given the highly unstable nature of these antioxidants, their typically poor aqueous solubility and lack of natural accumulation in tissues of interest, it is unsurprising that these molecules were unable to perform their intended function. For instance, quercetin has been shown *in vitro* and *in vivo* studies to possess anti-inflammatory [16], anti-hypertensive [17], anti-allergic [18] properties and an ability to control metabolic syndrome [19]. Yet, it has not been used therapeutically for pharmaceutical applications due to its low bioavailability, which is likely due to its poor aqueous solubility, structural instability and extensive first pass metabolism [20,21]. It has been reported that the oral bioavailability of quercetin is 17% in rats and merely 1% in humans [21,22]. Moreover, upon intravenous injection, a 100 mg of dose resulted in a 12 μM plasma concentration after 5 min and 1 μM after 3 h, demonstrating the relatively short half-life of the compound [23].

In order to overcome this difficulty of low systemic bioavailability, one potential solution is to deliver quercetin through encapsulation into nanoparticles, which can be administered via a number of routes (e.g., *i.v.*, *s.c.*, inhalation, etc.) with an objective of extended drug release and control the rate of first pass metabolism [24]. Several studies have demonstrated the ability to encapsulate quercetin into nanoparticles composed of poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) PLGA, and solid lipid nanoparticles (SLN). These approaches, while potentially useful, possessed significant burst release and low overall drug loading, ranging from only 0.05 to 2 wt% of the total particle weight [25,26]. Incorporating antioxidants into the backbone of a polymer system could be an alternative towards the effective antioxidant delivery as demonstrated on enzymatically biodegradable poly(trolox) nanoparticle system to deliver trolox, a water soluble analog of vitamin E [27].

Building upon this prior work, it may be possible to overcome drug stability, solubility and drug release limitations by conjugating the drug into the backbone of a hydrolytically biodegradable polymer, such as poly(β-amino esters) (PβAEs), to form pro-drug nanoparticles/nanogels [28,29,30]. We have shown previously that antioxidants like curcumin and quercetin can be loaded into crosslinked PBAEs and subsequently released in their original structure upon hydrolysis [31]. But due to the fast reaction kinetics and degradation properties of PβAE, the bulk crosslinking approach is not amendable towards typical nanoparticle synthesis methods (e.g., o/w emulsion polymerization), which use water as a dispersing media. To overcome this problem, we have synthesized quercetin conjugated PBAE gel nanoparticles/nanogels using a single phase reaction–precipitation method in an organic solvent under dilute conditions giving a stable suspension of uniformly sized particles. Covalently reacting available amine groups with poly(ethylene glycol) monomethacrylate resulted in a coating that minimizes post purification instability, expects to reduce the rate of first pass metabolism and serves as an alternative to conventional surfactant based stabilization of nanoparticles. Uniform degradation of these nanogels over 48 h successfully demonstrated release of active quercetin with negligible burst release. Particle sizes were easy to control with resulting quercetin loading capacities of 25–38 wt%, which possessed biocompatibility equivalent to pure quercetin and were able to suppress cellular oxidative stress over 48 h.

2. Materials and methods

2.1. Reagents

Quercetin was purchased from Cayman Chemicals, Michigan, USA. Acryloyl chloride, potassium carbonate, N,N′-dimethyl 1-3-propane diamine, polyethylene glycol methyl ether methacrylate (Mn = 4000) (PEGMEMA4000), potassium persulfate, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid), hydrogen peroxide were purchased from Sigma Aldrich. IgG antibody was purchased from Jackson Immuno Research Laboratory Inc. For cell culture studies, EBM basal medium (phenol red free), EGM-2 growth factors, and Human Umbilical Vein Endothelial Cells were purchased from Lonza. Calcein-AM red-orange and 2′,7 dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Life Technologies. Iodogen® iodination reagent (1,3,4,6-tetrachloro-3α-6α-diphenylglycouril) was bought from Thermo Scientific, Rockford, IL.

2.2. Quercetin functionalization to quercetin multiacrylate (QMA) monomer

Quercetin multiacrylate (4–5 acrylate groups per molecule) (QMA) was prepared in accordance with the protocol described by Wattamwar et al. [31] with slight change of using potassium carbonates as the acid capturing agent instead of triethylamine. Briefly, reaction between quercetin (20 g in 200 ml of THF) and acryloyl chloride was carried out in anhydrous THF. Potassium carbonate was added to the reaction system to capture the hydrogen chloride forming its salt as the reaction byproduct. System was purged with nitrogen for 30 min initially and then was further allowed to react overnight at room temperature to functionalize quercetin phenolic groups into acrylate. Acryloyl chloride and potassium carbonate, both were added in the molar ratio of 1:1.2 with respect to the phenolic groups present in quercetin. Quercetin has four phenolic groups, which can be actively functionalized. Therefore, for every mole of quercetin, five moles of acryloyl chloride and potassium carbonate were added to the reaction medium. Acrylated quercetin solubilizes in THF as the reaction proceeds. After the reaction, solubilized product was first filtered out to remove the salts. Subsequently, dissolved product was vacuum dried to remove all the THF and was re-dissolved in DCM in order to carry out aqueous-organic solvent extraction process to remove any unreacted acryloyl chloride/acrylic acid and quercetin. A basic solution of 0.1 M potassium carbonate in DI water was used as the extraction medium for acrylic acid and quercetin both. After extraction, magnesium sulfate was added to the product solubilized in DCM to remove any remains of water. Finally the pure QMA was obtained as a dry product after evaporation of DCM overnight. Characterization of the functionalized quercetin was completed using ^{1}H-NMR and mass spectroscopy to identify the number of acrylate groups per molecule of quercetin (Figs. 2 and 3 and Table 1 in Supplement information).

2.3. Quercetin PBAE nanogel (QNG) synthesis

Quercetin PBAE nanogels were prepared using a single-pot dilute synthesis approach. QMA was dissolved in anhydrous