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Poly(rutin) Micro/nanogels for Biomedical Applications

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ABSTRACT

Flavonoids are natural phenolic compounds, which are active molecules commonly found in woody and herbaceous plants used in the natural defense mechanisms of plants against harmful microorganisms. In this research, the rutin (RUT) molecule, which is a natural flavonoid, was bonded with epoxy groups of poly(ethylene glycol) diglycidyl ether crosslinker with the micro emulsion crosslinking technique to synthesize poly(rutin) (p(RUT)) micro/nanogels. These p(RUT) micro/nanogels had spherical morphologic structure with 0.3 nm-2 µm dry size range based on scanning electron microscope images. Furthermore, p(RUT) micro/nanogels were found to have injectable size range with 548±8.9 nm average size distribution in aqueous solution according to dynamic light scattering (DLS) measurements. In addition, the zeta potential measurements performed in different pH conditions and potentiometric titration of the prepared p(RUT) micro/nanogels were also determined and the isoelectronic point and pKa values of these micro/nanogels were estimated as pH 2.85 and 2.16, respectively. The effects of RUT and p(RUT) micro/nanogels on α-glucosidase (AG) (EC 3.2.1.20) enzyme activity were investigated and RUT and p(RUT) micro/nanogels stimulate this enzyme. Moreover, fibrinogen interaction results showed that p(RUT) micro/nanogels were more compatible with the vascular system than RUT, with less interaction ability between fibrinogen and p(RUT) micro/nanogels. In addition, p(RUT) micro/nanogels had the highest antioxidant scavenging ability with 251.3±20.2 gallic acid equivalency total flavonoid content (TFC) at 250 µg/mL and even 5 µg p(RUT) microgel/nanogels reduced 1.75±0.27 µmol Fe (III). Furthermore, no Fe (II) chelating activity was obtained for RUT monomer, but p(RUT) micro/nanogels had significant Fe (II) chelating activity of 43.11±17.4%.

Keywords:
Rutin; Microgel; Nanogel; Alpha-Glucosidase Enzyme; Antioxidant.

INTRODUCTION

Interest in flavonoids, which are phenolic compounds, has grown with the French paradox. Interest in bioflavonoids has increased based on Mediterranean culture, which includes a diet rich in phenol directly proportional to high fat with an inverse relationship with the risk of heart attack. Flavonoids are widely used in the food industry due to antioxidant and antimicrobial properties. Flavonoids are known to affect many biofunctions such as lowering blood pressure and having anti-inflammatory, hypocholesterolemic, anticancer platelet stabilizer, antiallergic, antidepressant and blood clotting properties [1,2]. Rutin is a known flavonoid and has two sugar molecules in its structure. Rutin's chemical name is quercetin-3-remnosyl glucoside [3]. It is present in many fruits and vegetables such as onion, tea, apple, parsley, citrus, sage, and grape [4]. It is used in the food, cosmetics, and even pharmacology industries [5,6], especially due to its antioxidant and antimicrobial properties [7]. In addition, it has many benefits for the human body, which makes rutin pharmacologically important. While it displays antioxidant properties by destroying free radicals, it has positive effects on the nervous system as well as cardiovascular health [8–10]. There are studies about its use as an anti-inflammatory and antidepressant and for patients with Alzheimer's and stroke [11]. In addition, flavonoids are known to have an inhibitory effect on cancer cells [12,13].

Alpha-glucosidase is a well-known enzyme that hydrolyzes 1,6 glycoside bonds of disaccharides, such as sucrose and maltose. Disaccharides cannot pass through the cell membrane from the digestive tract in diabetes, but due to enzymes that break down sugar with alpha-glucosidase, different disaccharide molecules are

broken down into monosaccharide units and glucose is directly transferred into the cells. However, glycogen storage diseases obstruct the body's ability to convert glycogen into glucose [14,15]. One of these diseases, Pompe disease, is a genetic deficiency of acid alpha-glycosides in patients. These enzymes trigger biochemical reactions in the body. In a healthy person with normal enzyme activity, the function of this enzyme is to destroy complex sugar molecules stored in lysosomes in cells. However, the destruction of complex sugar molecules cannot occur due to this innately deficient or insufficient enzyme, and storage begins. Enzyme replacement therapy is one of the treatment methods applied [16,17]. Cross-linked particle forms of RUT were synthesized in our previous study [9]. In this study, enzyme interaction, fibrinogen interaction and various antioxidant tests were investigated for RUT and p(RUT) microgel/nanogels to show the biomedical potential of p(RUT) microgel/nanogels.

MATERIAL AND METHODS

Materials

Rutin dihydrate (RUT, >95%, Sigma), L-alpha lecithin (granular, 98%, Acros Organic), poly (ethylene glycol) diglycidyl ether (PEGGE, Mn: 500, Aldrich), triethylamine (TEA, 99.5%, Sigma Aldrich), sodium hydroxide (Sigma-Aldrich), gasoline (95 octane, Total), cyclohexane (99.5%, Sigma-Aldrich), and ethyl alcohol (99%, Birkim) were used as received. Ultra-pure distilled water was obtained from GFL, 2108 and Millipore Direct-Q3 UV (18,2 MΩ.cm). Sodium nitrite (Merck, extra pure) and aluminum chloride (Merck, anhydrous powder sublimed from synthesis) were used for total flavonoid content (TFC) assay. Sodium acetate anhydrous (Fisher, 99%) and hydrochloric acid (Sigma, 37%) were used as received. Fe (III) chloride hexahydrate (Acros, 99%) and Fe (II) chloride tetrahydrate (Sigma Aldrich, 99%) were used for ferric reducing antioxidant power (FRAP) assay. Fe (II) sulfate heptahydrate (Merck, 99.5 %) and 5,6-Diphenyl-3-(2-pyridyl)-1,2,4-triazine-4,4-disulfonic acid disodium salt hydrate (Alfa Aesor) were used for Fe (II) chelating test. Bovine fibrinogen (Alfa Aesar) was used for fibrinogen interaction. alpha-glucosidase (obtained from Saccharomyces cerevisiae, 10 unit/mg protein) as enzyme and p-nitrophenyl-α-D-glucopyronidase as substrate were obtained from Sigma Aldrich.

Synthesis of p(RUT) Micro/nanogels

To synthesize p(RUT) micro/nanogels, RUT was crosslinked with PEGGE via water-in-oil microemulsion system in lecithin-gasoline reverse micelle medium in accordance with the previously published process [9,18]. Briefly, 2 mL of 60 mg/mL concentration RUT in 0.5 M NaOH solution was suspended in 150 mL of 0.1 M lecithin-gasoline emulsion medium with 750 rpm mixing rate at 50 °C reaction temperature. Ten minutes later, PEGGE crosslinker at 300% mole ratio of RUT and 20 µL TEA accelerator were added into the reaction medium and the same reaction conditions were maintained for 12 h. Cross-linked p(RUT) micro/nanogels were separated from the reaction medium by precipitation of the micro/nanogels using a centrifuge at 10,000 rpm for 10 min. Then, the precipitated micro/nanogels were washed with gasoline one time, cyclohexane two times, ethanol: water (80:20, v:v) mixture three times, and ethanol two times by centrifugation at 10,000 rpm for 10 min. The p(RUT) micro/nanogels were dried in an oven at 50 °C and stored in a closed container.

Characterization of p(RUT) Micro/nanogels

A scanning electron microscope (SEM, Jeol JSM-5600 LV) was used to visualize the morphological structure and for size analysis of p(RUT) micro/nanogels. SEM images were taken at 20 kV operating voltage after coating with gold/palladium for 30 seconds. Dynamic light scattering (DLS) and zeta potential (ZP) measurements of p(RUT) micro/nanogels were determined by using 90Plus/BI-MAS and ZetaPlus analyzer (Brookhaven Ins. Corp.) instruments, respectively. These measurements were repeated 10 times and the results are given with standard deviations. For the zeta analysis, 10 mg of p(RUT) micro/nanogels was suspended in 50 mL of 10-3 KCl solution and ZP values of p(RUT) micro/nanogels were measured at different pH conditions between pH 2 and pH 12. The pH value was adjusted with 0.1 M HCl and 0.1 M NaOH solutions. The isoelectric point of p(RUT) micro/nanogels was evaluated as the pH value with zero mV zeta potential. Furthermore, 50 mg of p(RUT) micro/nanogels was suspended in 10-3 KCl solution to measure the equivalent point and pKa values by potentiometric titration. In the titration, the pH value of micro/nanogel suspension was decreased to pH 2 using 0.01 M HCl solution and titrated up to pH 12 by 0.01 M NaOH solution. The chemical structures of RUT and p(RUT) micro/nanogels were characterized by FTIR spectra (PerkinElmer Spectrum 100) using attenuated total reflectance (ATR) in the range of 2000-650 cm^{-1} with a resolution of 4 cm^{-1}. Thermal stability of PEGGE-crosslinked p(RUT) micro/nanogels was investigated with thermogravimetric analysis (TGA, SII TG/DTA 6300, Japan) from 50 to 1000 °C with 10 °C/mL heating rate under nitrogen atmosphere with 100 mL/min flow rate.

Alpha-glucosidase Enzyme Activity Assay

P(RUT) micro/nanogels in 3000 µg/mL suspended solution were prepared in 67 mM PBS at pH 6.9. P(RUT)

suspended solution was diluted to 2250, 1500, 750, 375 and 187 μg/mL concentrations with pH 6.9 PBS. The RUT molecule has a suppressed enzyme peak due to high absorbance in theUV Vis spectrophotometer, so the RUT molecule (750-30 μg/mL) was studied at low concentrations. The effect of RUT or p(RUT) micro/nanogel on α-glucosidase (EC 3.2.1.20) was examined using the colorimetric substrate p-nitrophenyl-α-D-glucoside according to the literature [19]. Briefly, 70 μL containing different concentrations of RUT or p(RUT) micro/nanogel was put into the well plate with PBS as control. Enzyme solution of 0.03 unit/mL 70 μL was placed into the RUT and p(RUT) micro/nanogel solution. The mixed solution was read at 405 nm with Thermo Scientific Multiscan Go microplate reader and incubated for 10 minutes. Finally, 10 Mm substrate solution was placed into the mixed solution. After 20 minutes, the absorbance of the well plate was measured. The sample was compared to a control which had 70 μL of buffer solution. The effect of RUT or p(RUT) microgel/nanogel on α-glucosidase activity was calculated as the fraction of activity compared to the sample without added RUT or p(RUT) microgel/nanogel eluate, using Equation (1).

$$Fraction\ of\ activity = \frac{\varnothing A_{405}^{sample}}{\varnothing A_{405}^{control}} \quad (1)$$

Fibrinogen Interaction of RUT and p(RUT) Microgel/nanogel

The effects of RUT and p(RUT) microgel/nanogel on the fluorescence properties of fibrinogen were demonstrated with fluorescence spectroscopy (Thermo Scientific Lumina Spectrophotometer) according to the literature [20]. Different concentrations of RUT or p(RUT) micro/nanogel, 16-250 μg/mL in PBS, were mixed with fibrinogen solution in 1:1 ratio by volume. The width of excitation and emission slit was set as 5 nm and the excitation wavelength of 280 nm was used. The scanning range was set between 280-420 nm. The interaction of RUT and p(RUT) micro/nanogel with fibrinogen was determined in terms of the reduction in the fluorescence intensity.

Antioxidant Properties of p(RUT) Micro/nanogel

Total flavonoid content (TFC), ferric reducing antioxidant powder (FRAP) and iron chelating assays were carried out to determine the antioxidant properties of p(RUT) micro/nanogels.

The total flavonoid content (TFC) test for RUT and p(RUT) micro/nanogels was done using a UV-Vis spectrophotometer at 405 nm in accordance with the literature with some modifications [21]. RUT solution or p(RUT) micro/nanogel suspension at 500, 250, 125, and 62.5 μg/mL concentration was prepared in DI water and 0.5 mL volume RUT or p(RUT) suspension was placed in 10 mL tubes. Distilled water, 2 ml, was added into the tubes. Then, 5 minutes later 0.15 mL of 5% NaNO2 was added. After 5 minutes, 0.15 mL of 10% AlCl3.6H20 was added to this medium. After 5 minutes, 1 M 1 mL of NaOH was added to this mixture. Keeping this solution still for 15 minutes, the UV-Vis spectra of this solution was read at 405 nm wavelength. NaNO2 and AlCl3.6H20 mixture solution in DI water was used as blank. Gallic acid was used as standard.

The ferric reducing antioxidant potential (FRAP) of RUT and p(RUT) micro/nanogels was examined with a UV-Vis spectrophotometer at 595 nm according to the literature [22,23]. Briefly, 0.3 M acetate buffer was prepared at pH 3.6. Tripyridyl triazine (TPTZ) solution at 10 mM concentration was prepared using 2.5 mL 40 mM HCl. Acetate buffer at 25 mL volume was mixed with 2.5 mL TPTZ solution, and 2.5 mL 20 mM FeCl3.H2O (in acetate buffer) was mixed to achieve Fe(III)- TPTZ complex. The FRAP test was done using 3 mL of the prepared Fe-TPTZ complex solution. First, the UV-Visible spectra of Fe-TPTZ complex were measured at 595 nm. RUT solution and suspended p(RUT) micro/nanogels at 500 μg/mL concentration were prepared in acetate buffer and 5-80 μL volumes of RUT and/or p(RUT) micro/nanogel suspension was placed into the Fe-TPTZ complex solution and stirred for 4 minutes with plastic pipette tips. Then, the UV-Vis spectra were recorded and the difference between the absorbance values was calculated as μmol Fe (II) reduced. FeSO4.7H20 was used as Fe (II) source to complex with TPTZ as standard to generate a calibration curve for the determination of Fe (II) reduced by RUT and/or p(RUT) micro/nanogels. Gallic acid (GA) was taken as reference material.

Fe (II) chelating activity was studied according to the literature [24]. RUT and p(RUT) micro/nanogel were prepared at 500 μg/mL concentration in DI water and diluted to 250, 125, 62.5, and 31.125 μg/mL concentrations. Different concentrations of RUT and p(RUT) micro/nanogel, at 140 μL, were added into a 96 well plate and 20 μL of 1 mM of Fe (II) aqueous solution was added to each well. The plate was measured at 562 nm by using a microplate reader (Thermo Multiskan Go). After the measurement, 40 μL of 2.5 mM ferrozine solution in DI water was put to each well. After 5 minutes, the plate was measured at 562 nm once again.

Pure DI water without RUT or p(RUT) microgel/nanogel was used as a blank. Each concentration was tested in triplicate. The results were calculated according to Equation 2 and given as μmol Fe (II) chelating activity %.

$$\text{Fe(II) chelating activity\%} = \left(\left[1 - \frac{\Delta A_{562}^{sample}}{\Delta A_{562}^{control}} \right] \right) x100 \quad (2)$$

RESULTS AND DISCUSSION

RUT flavonoid, which also known as vitamin P, has a wide range of pharmaceutical activities in various types of diseases related to its sedative, antidepressant, anticonvulsant, anti-Alzheimer, antiarthritic, antidiabetic, anti-hypercholesterolemic, antiulcer, anti-asthmatic, anti-osteoporotic, anticancer, and antimicrobial effects [5]. RUT monomer can be crosslinked with glycerol diglycidyl ether or poly (ethylene glycol) diglycidyl ether (PEGGE) crosslinkers to synthesize p(RUT) micro/nanogels in reverse micelle microemulsion medium as reported earlier [9,18]. According to these studies, p(RUT) micro/nanogels provide sustainable and long-term RUT release as an active agent through the degradable crosslinker structure. Furthermore, antimicrobial and antioxidant effects, blood compatibility, and cytotoxicity against healthy and cancer cells were studied in the research. In this study, the effects of PEGGE-crosslinked p(RUT) micro/nanogels on enzyme activity, chelating therapy, and blood clotting mechanism by fibrinogen interaction were investigated to show the bioactivity of degradable p(RUT) micro/nanogels for different biological functions. The schematic representation of the reaction between RUT monomer and PEGGE crosslinker to prepare p(RUT) micro/nanogels is illustrated in Fig. 1a. Hydroxyl groups of RUT reacted with opened epoxy groups of the PEGGE crosslinker in basic conditions in the presence of TEA accelerator at 50 °C reaction temperature. Thus, RUT monomer crosslinked with PEGGE to synthesize p(RUT) micro/nanogels in a water-in-oil reverse micelle microemulsion medium. Also, SEM images and hydrolytic size distribution of the prepared p(RUT) micro/nanogels are illustrated in Fig. 1b and 1c.

As can be seen in the SEM image, p(RUT) micro/nanogels had spherical shapes in the range of 0.3 nm to 2 μm. The hydrolytic size distribution of these micro/nanogels was 548±8.9 nm average particle size according to DLS measurement. In addition, p(RUT) micro/nanogels have polydispersed size range with 0.452±0.041 polydispersity index value. Several studies indicated that polymeric particles in the range of hundred nanometer to few micrometer sizes can be used in a wide range of biomedical applications. Zhang et al. reported that the 1 to 10 μm size range of chitosan/peptide

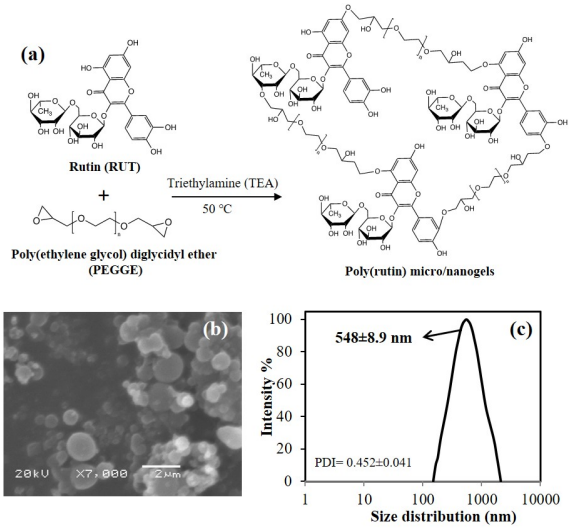

Figure 1. (a) Reaction schema for p(RUT) micro/nanogels. (b) SEM image of p(RUT) micro/nanogels and (c) hydrolytic size distribution graph measured by dynamic light scattering (DLS).

microspheres made it a promising biomaterial for wound healing applications [25]. In another study, Anderson et al. also observed that polymeric microparticles with nearly 3 nm particle size could be used as a DNA delivery system [26]. Furthermore, Decuzzi et al. proposed that the spherical shape of particles with sizes ranging between 0.7 and 3 μm is within the injectable range with good biodistribution [27]. These results indicate that p(RUT) micro/nanogels have injectable size range and can be used for in vivo applications.

The chemical structures of RUT and p(RUT) micro/nanogels were analyzed by FT-IR spectroscopy, as seen in Fig. 2a. Characteristic peaks of RUT monomer in the region between 1600 and 1562 cm^{-1} are assigned to the stretching bands of C-C aromatic groups of the flavonoid. In addition, the peaks at 1183, 1078 and 1016 cm^{-1} correspond to the stretching vibrations for the substituted benzene ring of RUT. Among these peaks, p(RUT) micro/nanogels have a ridge between 1096-1060 cm^{-1} attributed to ether linkages due to possible binding of RUT monomer with opened epoxy groups on the PEGGE crosslinker. These results support that p(RUT) micro/nanogels were successfully prepared via a crosslinking reaction of RUT with the degradable crosslinker of PEGGE. Moreover, thermal degradation of p(RUT) micro/nanogels from 50 to 1000 °C was measured by thermogravimetric analysis (TGA), as seen in Fig. 2b.

Fig. 2. (a) FT-IR spectra of RUT and p(RUT) micro/nanogels. (b) Thermal degradation profiles of p(RUT) micro/nanogels.

It is clearly seen that p(RUT) micro/nanogels had one main degradation at about 103-380 °C with 39.0% weight loss and one slight degradation step at about 400-600 °C with 47.2% weight loss. Finally, 50% of the total remaining

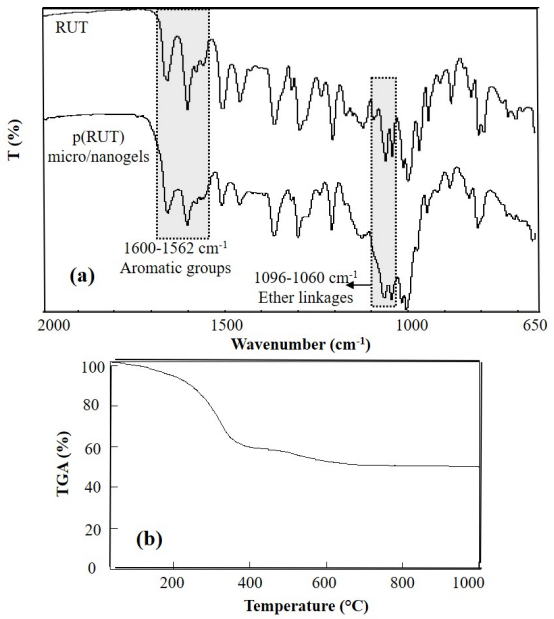

Figure 2. FT-IR spectra of RUT and p(RUT) micro/nanogels. (b) Thermal degradation profiles of p(RUT) micro/nanogels.

material was obtained at 1000 °C because of the high ratio of crosslinker in the polymeric structure. Sahiner reported that thermal degradation of RUT monomer, which has more degradable behavior than p(RUT) micro/nanogels, had a similar degradation pattern [18]. These results indicate that the crosslinker PEGGE in the micro/nanogel network increased the thermal stability of RUT.

The isoelectric point and surface charge of biomaterials should be known to understand the interaction of the materials with biomacromolecules. Therefore, zeta potential values and potentiometric titration of p(RUT) micro/nanogels in a wide range of pH conditions between pH 2 and pH 12 are demonstrated in Fig. 3a and 3b.

Zeta potential of p(RUT) micro/nanogels was +9.69±2.61 mV at pH 2, whereas negative zeta potential values were determined at conditions of pH 3 and above pH between -3.1 and -32.35 mV. The isoelectric point is the pH value at which p(RUT) micro/nanogels contain the same amount of positive and negative charges on the material surface. The isoelectric point with neutral charge on p(RUT) micro/nanogels was calculated at about pH 2.85 according to zeta potential analysis. Furthermore, potentiometric titration of p(RUT) micro/nanogels was monitored via pH analysis by titration with NaOH solution. These results indicated that pKa values of p(RUT) micro/nanogels have pH 7.06 equivalent point and 2.16 pKa value. No significant differences in isoelectric point and the pKa value of p(RUT) micro/nanogels were found depending on the unbound phenolic hydroxyl groups in RUT. Furthermore, negatively-charged p(RUT) micro/nanogels had a tendency to interact with positively-charged molecules in physiological conditions at

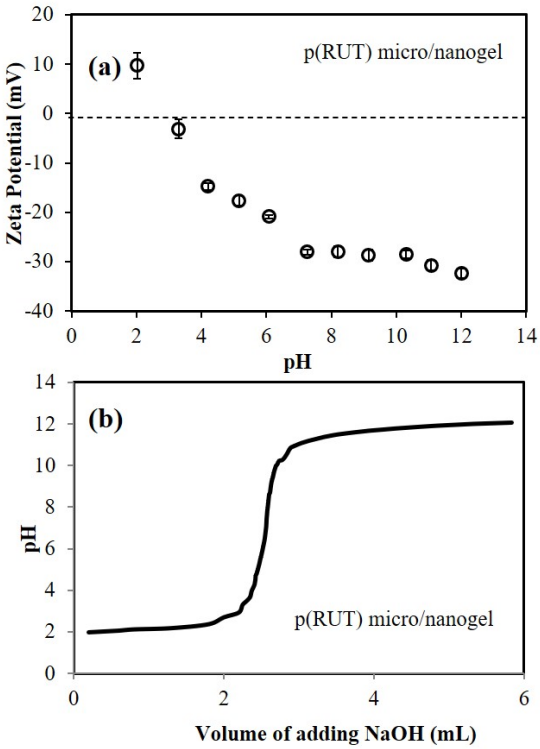

Figure 3. (a) Zeta potential values and (b) potentiometric titration of p(RUT) micro/nanogels in the range of pH 2 and pH 12. pH values of p(RUT) micro/nanogels suspension in 10-3 KCl solution was adjusted with 0.1 M NaOH and 0.1 M HCl solutions.

pH 7.4. Therefore, these p(RUT) micro/nanogels could be effective for transportation or activation/inactivation mechanisms of various positively-charged amino acids, proteins, enzymes, and vitamins as well as metal ions during biological reactions.

Enzyme activity of RUT and p(RUT) micro/nanogels was investigated on α-glucosidase enzyme, which regulates starch hydrolysis and some disaccharide to the monosaccharide units in the body. It was concluded that RUT and p(RUT) micro/nanogels stimulated the enzyme α-glucosidase. Hence, alpha-glucosidase fraction activities of RUT and p(RUT) micro/nanogels are shown in Fig. 4a and 4b.

As shown in Fig. 4a, RUT monomer has no significant activity against the enzyme with slight stimulation of alpha-glucosidase with 1.15±0.1 fraction value at 50 μg/mL concentration, a decline in activity up to 125 μg/mL and then no effect until 250 μg/mL. The alpha-glucosidase enzyme activity in the presence of p(RUT) micro/nanogels on the other hand gradually increased between 50 and 750 μg/mL with fractional activity changing from 1.2±0.3 to 1.5±0.7 as seen in Fig. 4b.

In the literature, many studies reported that inhibition or stimulation activity of alpha-glucosidase enzyme in the

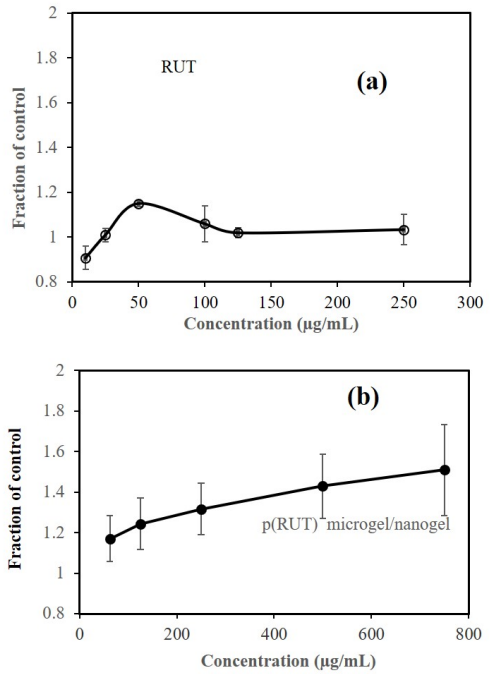

Figure 4. Fraction activity of alpha-glucosidase enzyme with (a) RUT and (b) p(RUT) micro/nanogels.

presence of phenolic materials depends on the molecular structure of flavonoids [28,29]. Alam et al. reported that the well-known flavonoid quercetin can inhibit the alpha-glucosidase enzyme prepared in butanol, ethyl acetate, hexane, and methanol solutions [29]. Another study stated that naringin molecule, a phenolic compound with two sugar units, could not inhibit the alpha-glucosidase enzyme with slight stimulating effects [19]. Very similar results were obtained for enzyme activity of RUT with naringin phenol due to the sugar unit in the chemical structure of RUT. Moreover, Dubey et al. mentioned that RUT monomer dissolved in DMSO could inhibit the alpha-amylase and alpha-glucosidase enzymes to some extent [30]. DMSO was not chosen for in vivo applications as an organic solvent because of toxicity and RUT monomer shows slight solubility in aqueous medium as buffer solutions. In addition to enzyme stimulation effects of sugar units of phenolics, lower solubility of RUT should affect the enzyme activity oppositely. Alam et al. reported that RUT is more stable against oxidation when compared with quercetin with sugar-free structure [29]. Another probability is that this stability may have been effective on its interaction with the enzyme. Our results support that p(RUT) micro/nanogels can stimulate alpha-glucosidase enzyme and this material could be used to trigger the digestion of a wide range of carbohydrates including starch, amylose, amylopectin, dextrins, maltooligosaccharides, etc. which contain alpha(1-4) or alpha(1-6) glycosidic bonds [31].

Fibrinogen is a well-known protein in the blood which plays a significant role in coagulation of blood. Phenolic compounds can interact with some proteins like fibrinogen and may affect their biological functions [20]. Therefore, the fibrinogen interaction effects of RUT and p(RUT) microgel/nanogel were investigated to find the blood compatibility of materials. As shown in Fig. 5a and 5b, RUT and p(RUT) micro/nanogels were interacted with fibrinogen at 0-250 µg/mL concentrations.

Fibrinogen has highest emission intensity at 340 nm wavelength at 0.1 mg/mL concentration in DI water with almost 64700 intensity. As can be seen in Fig. 5a, the intensity of the fibrinogen peak started to decrease after interaction with increasing concentrations of RUT. The intensity of the fibrinogen peak was nearly six-fold decreased at 250 µg/mL RUT concentration. In addition, p(RUT) micro/nanogels could slightly reduce the intensity of fibrinogen peaks up to 250 µg/mL of p(RUT) micro/nanogel concentration, as seen in Fig 5b.

It is apparent from Fig. 5c that p(RUT) micro/nanogels were not as effective as RUT molecules in interacting with fibrinogen molecules which play an important role in the coagulation of blood. These results support that p(RUT) micro/nanogels showed more blood compatibility than RUT molecule even at high concentrations with no significant effects on the biological functions of fibrinogen due to molecular interactions.

The antioxidant capacity of RUT and p(RUT) micro/nanogels was investigated by total flavonoid content (TFC) and ferric reducing antioxidant power (FRAP). TFC values of RUT and p(RUT) micro/nanogels at different concentrations are given as gallic acid equivalent (GA eq) in Fig. 6a.

The TFC value of RUT at 250 µg/mL concentration was 445.0±22.5 GA eq, but p(RUT) microgel/nanogel had a value of 251.3±20.2 GA eq TFC at the same concentration. RUT is a flavonoid used as a reference substance in TFC antioxidant tests. These results with high flavonoid content values were also obtained with the polymeric form of RUT. Furthermore, FRAP assay, known as the Fe (III) reduction test, is another antioxidant test which was performed for RUT and p(RUT) microgel/nanogel. Gallic acid (GA) was taken as reference antioxidant material for the FRAP test. As shown in Fig. 6b, 5 µg RUT and p(RUT) microgel/nanogels reduced to 19.04±3.37 and 1.75±0.27 µmol Fe (II) levels, respectively. These antioxidant tests confirm that p(RUT) microgel/nanogels have promising antioxidant ability with high TFC values and significant Fe(III) reducing ability for Fe(II) even at low concentrations. Kurisawa et al. prepared poly(rutin) by the oxidative polymerization reaction of RUT with laccase enzyme [32]. This study indicated that water soluble rutin polymer has significant superoxide scavenging capacity and high preventive ability for oxidative stress on endothelial cells related to the high antioxidant activity

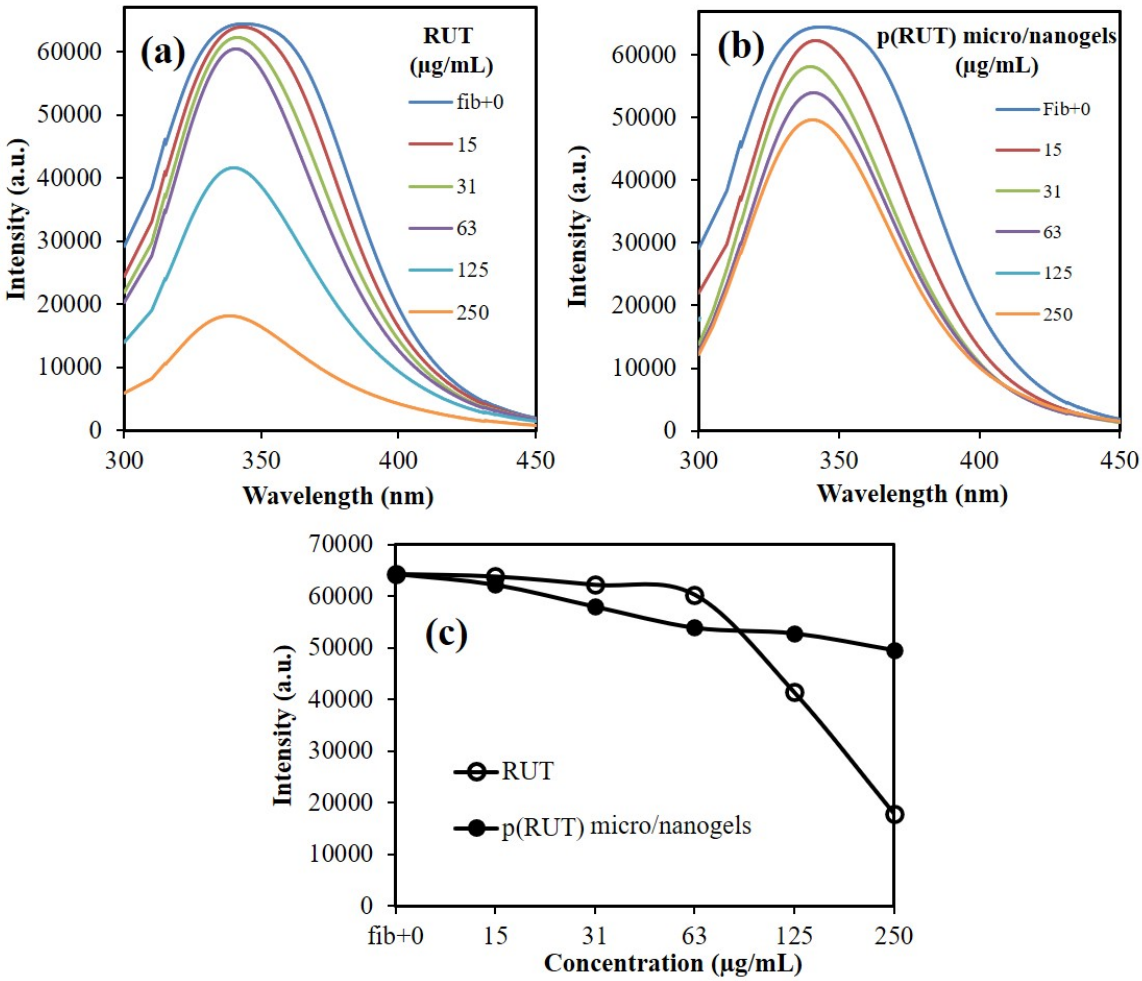

Figure 5. Fibrinogen interaction of (a) RUT and (b) p(RUT) micro/nanogels at different concentrations. (c) Emission intensity of RUT and p(RUT) micro/nanogel interactions at 340 nm wavelength.

[32]. Similarly, our results supported that p(RUT) microgel/nanogels have promising antioxidant capacity according to different antioxidant analyses.

Phenolic-based materials can used as metal chelator to regulate metal ion absorption at the required level and forms in the blood. Metal chelators are used to provide systemic iron homeostasis in the treatment of some neurodegenerative diseases such as Alzheimer and Parkinson [33]. The Fe (II) chelating capacity of RUT and p(RUT) microgel/nanogels in DI water was studied and the results are given in Fig 7.

While the RUT molecule did not chelate Fe (II), p(RUT) microgel/nanogels chelated Fe (II) at an increasing rate depending on the concentration. The Fe (II) chelating ability of p(RUT) micro/nanogels at 500 µg/mL concentration was 43.1±17.4 %. Pivec et al. prepared poly(rutin) by enzymatic polymerization of RUT and investigated the Fe (II) chelating ability of these materials. They found that high concentration of poly(rutin) at 5000 µg/mL concentration had nearly 70% Fe (II) chelation activity [34]. These results indicate that crosslinked p(RUT) microgel/nanogels had more Fe (II) chelating ability that poly(rutin) which was prepared by oxidative polymerization. Therefore, p(RUT) microgel/nanogels can be used as a metal chelator in the treatment of some neurological diseases.

CONCLUSION

Degradable p(RUT) micro/nanogels were synthesized as a therapeutic material to investigate their biological activity on different biological functions including enzyme activity, fibrinogen interaction, antioxidant capacity and Fe chelating ability. According to the low isoelectric point and pKa value at pH 2.85 and 2.16, respective, p(RUT) micro/nanogels have a negatively-charged surface at physiological conditions. It is stated in the literature that flavonoids inhibit α-glucosidase enzyme. However, the dissolved RUT and suspended p(RUT) micro/nanogels in the phosphate buffer did not inhibit the enzyme's activity. The effects of flavonoids containing sugar groups

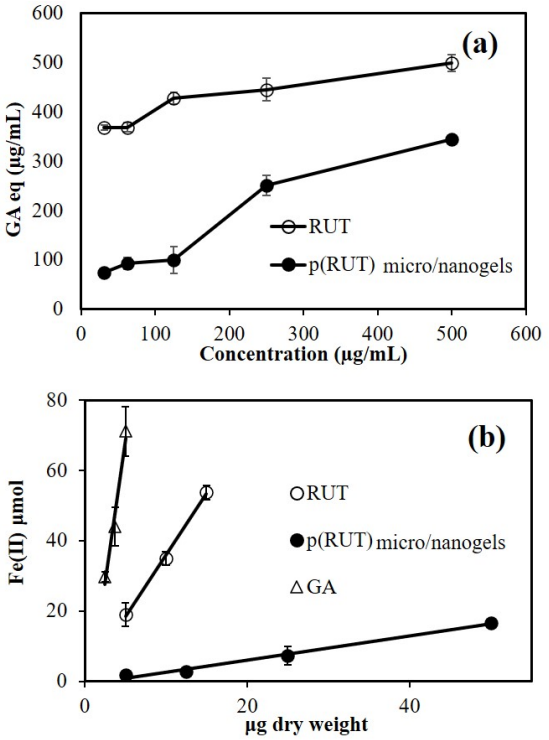

Figure 6. (a) Total flavonoid content values of RUT and p(RUT) micro/nanogels at different concentrations. (b) Ferric reducing antioxidant power (FRAP) values of RUT and p(RUT) micro/nanogels at different concentrations. (Gallic acid was used as standard antioxidant)

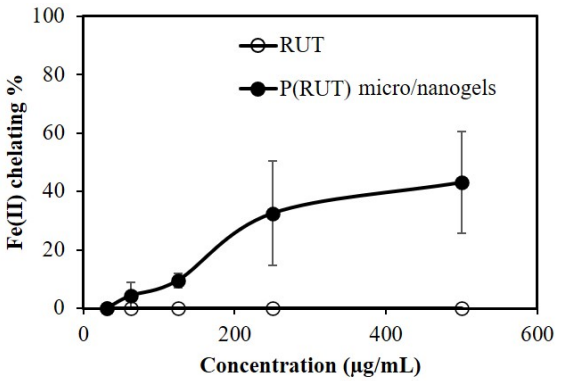

Figure 7. Fe (II) chelating activity of RUT and p(RUT) microgel/nanogels at different concentrations.

in their structure on enzymes should be investigated in more detail and even supported by in vivo studies. RUT and p(RUT) microgel/nanogel may have potential for use in the treatment of glycogen storage diseases. For such research, extensive applications should be performed. Moreover, RUT interacts extensively with fibrinogen, but p(RUT) micro/nanogels were observed to have less interaction ability with fibrinogen. By examining the interaction of fibrinogen, a blood protein, p(RUT) microgel/nanogels are more suitable for intravenous applications. Furthermore, p(RUT) microgel/nanogels had promising antioxidant ability with high total flavonoid content. In addition, p(RUT) microgel/nanogels could provide systemic iron homeostasis with their ability to reduce Fe(III) to Fe(II) and significant Fe(II) chelating activity.

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CONFLICT OF INTEREST

Authors approve that to the best of their knowledge, there is not any conflict of interest or common interest with an institution/organization or a person that may affect the review process of the paper.

AUTHOR CONTRIBUTION

Authors Mehtap Sahiner and Selin Sagbas Suner designed the study together. The experiments were performed by Mehtap Sahiner and Selin Sagbas Suner together. The results are pointed out with equal contribution. The authors have contributed to this research equally.

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