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Resveratrol liposomes and lipid nanocarriers: Comparison of characteristics and inducing browning of white adipocytes

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Abstract

Trans-resveratrol (R) has a potential to increase energy expenditure via inducing browning in white adipose tissue. However, its low levels of aqueous solubility, stability, and poor bioavailability limit its application. We have successfully synthesized biocompatible, and biodegradable R encapsulated lipid nanocarriers (R-nano), and R encapsulated liposomes (R-lipo). The mean particle size of R-nano and R-lipo were 140 nm and 110 nm, respectively, and their polydispersity index values were less than 0.2. Nanoencapsulation significantly increased aqueous solubility and enhanced chemical stability of R, especially at 37 °C. R-lipo had higher physical and chemical stability than R-nano while R-nano had more prolonged release than R-lipo. Both R-nano and R-lipo increased cellular R content in 3T3-L1 cells. Both R-nano and R-lipo dose-dependently induced uncoupling protein 1 (UCP1) mRNA expression and decreased white specific marker insulin growth factor binding protein 3 expression under isoproterenol (ISO)-stimulated conditions. At the low dose (5 μM), nanoencapsulated compared to native R enhanced UCP1 and beige marker CD137 expression under ISO-stimulated conditions. Compared to R-nano, R-lipo had better biological activity, possibly due to its higher physical and chemical stability at the room and body temperature. Taken together, our study demonstrates that nanoencapsulation increased R's aqueous solubility and stability, which led to enhanced browning of white adipocytes. Even though both R-lipo and R-nano increased R's browning activities, their differential characteristics need to be considered in obesity treatment.

Keywords

Trans-resveratrol; Obesity; Liposome; Nanocarrier; Nanomedicine; White adipocyte; Beige cell; Brown adipocyte

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1. Introduction

Obesity remains to be the major public health issue in the United States and worldwide, paralleled by rising rates of co-morbidities such as metabolic syndrome, diabetes, coronary heart disease, and certain types of cancer [1].

Two different adipose tissues are found in mammals: white adipose tissue (WAT), which is responsible for energy storage; and brown adipose tissue (BAT), which is responsible for thermogenic energy expenditure [2]. BAT has been positively associated with energy expenditure and negatively associated with adiposity in animal models and humans. Uncoupling protein 1 (UCP1) found in the inner mitochondrial membrane of brown adipocytes in the BAT can dissipate the proton electrochemical gradient generated by oxidative phosphorylation in the form of heat [3]. Emerging data have demonstrated that UCP1 is expressed not only in classical brown adipocytes but also in “brown-like” or beige adipocytes within WAT upon stimulations, such as a chronic cold challenge or pharmacological or bioactive compounds [3]. To increase adipocyte UCP1 expression and induce WAT “browning” might result in enhanced thermogenic and fat-burning activities, which subsequently lead to weight loss.

Trans-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, R) is a polyphenolic compound, abundant in the skin of grapes and red wine. Many *in vitro* studies have demonstrated that R at concentrations between 10–100 μM exhibits anti-obesity activities by modulating adipocyte differentiation, lipolysis, fatty acid oxidation, and mitochondria biogenesis and activities [4]. R activates NAD-dependent deacetylase sirtuin 1 (SIRT1), which can deacetylate peroxisome-proliferator-activated receptor γ (PPAR γ). This modification is essential for enhancing PPAR γ -binding activity, recruiting transcription factor PR-domain-containing 16 (PRDM16) to PPAR γ , and activating PPAR γ co-activator 1 α (PGC1 α), which subsequently enhance UCP1 expression and initiate browning of WAT [4]. Native R (at 10 μM) enhanced mRNA expression of UCP1 and beige marker CD137 and Tmem26 during brown-like differentiation of primary stromal cells derived from inguinal WAT (iWAT) of CD1 mice [5]. Consistently, *in vivo* feeding of R promoted the appearance of multilocular adipocytes and increased UCP1 expression in iWAT in the mice fed with a high-fat diet [5]. These results suggest that browning WAT may be a new anti-obesity target for R.

Human studies indicate that R can maintain metabolic health, but the evidence is inconclusive [4]. The major problems are its low aqueous solubility and bioavailability, and high metabolism in humans [6]. The solubility of R in water and physiological fluid is very low, less than 0.1 mg/mL [7]. When orally administering around 0.3 mg/kg body weight of R to healthy adult males, blood peak concentrations of R appeared at 0.5 h, and the peak plasma concentrations were less than 1 μM [8]. Even when R was given a single dose of 5 g, the peak plasma concentrations were still less than 10 μM [9]. Moreover, R stability is further reduced by various metabolic transformations, including methylation, glucuronidation and others, primarily in the liver *in vivo* [4].

Nanoencapsulation has been proved effective in increasing aqueous solubility, chemical stability, and bioavailability of many bioactive compounds in combating obesity and

associated metabolic disorders [7]. Recent animal and human studies indicate that encapsulating R into nanocarriers can increase R's aqueous solubility, protect R from metabolic degradation, and enhance its transport across the plasma membrane, with ultimately augmented absorption and bioavailability [10].

In this study, we synthesized R encapsulated lipid nanocarriers (R-nano) and R encapsulated liposomes (R-lipo), two biodegradable and biocompatible nanocarrier delivery systems, and compared their physical and physicochemical characteristics and browning activities with native R in 3T3-L1 white adipocytes. This study provides a foundation for developing innovative anti-obesity approaches using natural bioactive compounds and nanocarrier delivery systems.

2. Materials and methods

2.1. Chemicals and reagents

R was purchased from Cayman Chemical Co. (+)-Alpha (α)-tocopherol acetate (α TA), cholesterol, dexamethasone (Dex), 3-isobutyl-L-methylxanthine (IBMX), insulin, isoproterenol (ISO) and rosiglitazone (Rosi) were purchased from Sigma-Aldrich Chemical Co. Soy L- α -phosphatidylcholine (PC) was purchased from Avanti Polar Lipids Inc. Kolliphor[®] HS15 was given as a gift from BASF Chemical. All organic solvents were the high-performance liquid chromatography (HPLC) grade.

2.2. Preparation of R-nano and R-lipo

R-nano was prepared using a mixture containing 1 mg of R, 70 mg of soy PC, 17.6 mg of Kolliphor[®] HS15 and 18 mg of α TA. The mixture was dissolved in ethanol and completely dried under nitrogen gas. After suspending the mixture with 76 °C deionized water, the suspension was homogenized for 1 min followed by sonication for 1 min. The R-nano tube was put on ice immediately. After ultrafiltration to remove free R, the R-nano was resuspended in 1 $\times$ phosphate-buffered saline (1 $\times$ PBS). R-lipo was prepared using 1 mg of R, 20 mg of soy PC and 2 mg of cholesterol by a film dispersion method followed by a membrane extrusion method. The void nanocarriers (V-nano) and void liposomes (V-lipo) were prepared using the above methods without adding R.

2.3. Particle size, zeta potential, and morphology

The particle size and polydispersity index (PI) values of R-nano, R-lipo and their void counterparts were measured using a Brookhaven BI-MAS particle size analyzer, and the zeta potential was measured using a Zeta PALS analyzer. The morphology and size of nanocarriers and liposomes were determined using a 200 kV Hitachi H-8100 transmission electron microscope (TEM) as described [11].

2.4. Encapsulation efficiency and loading capacity

They were measured as described [11] (Supplemental methods).

2.5. Physical and chemical stability

The freshly made R-nano and R-lipo were aliquoted into transparent or black tubes and stored at 4 °C, 22 °C, and 37 °C for 7 days, and their physical and chemical stability was measured during this period. The mean particle size, PI and zeta potential were measured every 2 h for the first 10 h, and every 24 h for 7 days. The chemical stability of R-nano, R-lipo and native R was measured using the HPLC system every day for 7 days.

2.6. Physicochemical characterization

2.6.1. *In vitro* release study—Before the *in vitro* release study, the stability of R-nano, R-lipo and native R in the dissolution medium were measured at 37 °C for 24 h. The dissolution medium was composed of 1 × PBS and methanol (80:20, v/v). The *in vitro* release behaviors of R-nano, R-lipo and native R containing 0.5 mg of R were performed in the dissolution medium using a dialysis method as described [11].

2.6.2. Raman spectroscopy, X-ray diffractometry (XRD) and differential scanning calorimetry (DSC) analysis—The native R, R-nano, R-lipo, V-nano, and V-lipo were frozen and then lyophilized using a vacuum freeze-drying system before taking Raman spectra, XRD and DSC as described [11] (Supplemental methods).

2.7. Cytotoxicity, cellular R content and browning activities in 3T3-L1 fibroblasts

2.7.1. Cell culture and viability—Murine 3T3-L1 fibroblasts purchased from ATCC were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum following a standard protocol, and cell viability was measured by the colorimetric 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assays (Supplemental methods).

2.7.2. Cellular R content studies—R content in 3T3-L1 cells was using the HPLC system. Quercetin was used as an internal standard. Total cellular R content was expressed as µg of R per mg of protein (Supplemental methods).

2.7.3. Real time-PCR—Total RNA was extracted, cDNA was synthesized, and real-time PCR was performed using an ABI 7300HT instrument (Supplemental methods). Primer sequences of target genes are listed in Supplemental Table 1.

2.7.4. Transfection and reporter gene assays—3T3-L1 cells were transiently transfected with a peroxisome proliferator response element (PPRE)-driven luciferase reporter (PPRE-Luc) and β-galactosidase (β-gal) control plasmid with Lipo-fectamine 3000 and PLUS Reagent for 24 h. The cells were then treated with nanoparticles and the controls for an additional 15–18 h. The luciferase activities were measured by a Promega GloMax-Multi Detection System and normalized by the β-gal activities.

2.8. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) and SigmaPlot 13 (Systat Software, Inc). One-way ANOVA was performed followed by multiple comparison tests with Student-Newman-Keuls method or Holm-Sidak method to

compare with controls. Each experiment was conducted independently at least three times; each measurement was performed in triplicates within each experiment. The level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Characteristics of R-nano and R-lipo

R-nano and R-lipo were successfully synthesized. Traditional nanostructured lipid carriers (NLCs) as drug carriers usually contain a large amount of triglyceride [12]. In our laboratory, we have successfully replaced triglyceride with α TA, consequently eliminating exogenous triglyceride and increasing the anti-oxidative capacity of nanocarriers [11,13], making them more functional and beneficial. Fig. 1A shows that 1 mg of native R was hardly dissolved in 1 mL of $1 \times$ PBS and precipitated from the suspension immediately; however, 1 mg of nanoencapsulated R in both R-nano and R-lipo was dissolved in the same volume of $1 \times$ PBS, which had 25-fold higher aqueous solubility than native R. Both R-nano and R-lipo were translucent and opalescent (Fig. 1A). Several studies have demonstrated that entrapment of R in nanoparticles increases R's aqueous solubility and further bioavailability [12]. TEM images indicated that both R-nano and R-lipo were spherical (Fig. 1A). The average particle size of R-nano and R-lipo was around 140 nm and 110 nm, respectively (Fig. 1A). Recently research data indicate that tissue distribution of nanocarriers was size-dependent [14], and nanocarriers close to 100 nm was found to be delivered effectively into adipose tissues of obese mice. The PI values of R-nano and R-lipo were 0.084 and 0.140, respectively. The low PI values indicated a high level of size homogeneity of R-nano and R-lipo. While higher PI values (>0.3) indicate higher levels of heterogeneity [12]. Surface charges of nanocarriers play vital roles in cellular uptake, biodistribution, and bioavailability of nanocarriers [12]. The Zeta potentials of freshly made R-nano and R-lipo were around -19 and -28 mV, respectively. Soy PC, the major surface component of R-nano and R-lipo, rendered negative charges on the surface of particles [12]. In general, they can be dispersed stably due to the electric repulsion of negative charges among particles.

The encapsulation efficiency of R-nano and R-lipo was 96.5% and 96.0%, respectively; and R's loading capacity in R-nano and R-lipo was 28.5% and 25.3%, respectively. Since a high proportion of hydrophobic α TA on R-nano, we predict that R-nano might have a monolayer of PC and Kolliphor[®] HS15 on the surface and a hydrophobic α TA core (Fig. 1A). The high encapsulation efficiency and loading capacity of R-nano were partially due to the hydrophobicity of α TA, which accommodates more R in the hydrophobic core. R-lipo might have multiple PC bilayers and a hydrophilic core. Due to the biphasic characteristics of PC, R can be embedded into the sections of hydrophobic fatty acid tails of PC bilayer [15]. Multiple PC bilayers on liposomes also accommodate a good amount of R.

3.2. Physical and chemical stability

Storage of both R-nano and R-lipo at 4°C for 4 days, their diameters and zeta potentials did not change significantly, and the PI values remained under 0.2, indicating relatively good homogeneity and physical stability (Fig. 1B). Storage of R-nano at room temperature (22°C) for 4 days, its diameters, and PI values were slightly increased (Fig. 1B). At 37°C ,

R-nano can only maintain its original size for 8 h, and its PI values were gradually increased (Fig. 1B). There have two reasons contribute to the poor physical stability of R-nano at 37 °C. First, the hydrophobic lipid core of R-nano was composed of α TA. The melting point of α TA was around 25 °C (Sigma T3001) indicated on the product sheet, and the solid core would turn to liquid resulting in the fragile and unstable structure of R-nano. Second, a high temperature may break the hydrogen bonds of Kolliphor® HS15 [16], the surfactant incorporated on the surface of R-nano, leading to a reduced stability of R-nano at 37 °C. The diameter and zeta potential of R-lipo did not change significantly at 22 °C and 37 °C for 7 days, and the PI values remained under 0.2 (Fig. 1B), indicating its higher levels of physical stability and homogeneity than R-nano. It should be emphasized that the cholesterol was incorporated into R-lipo to increase its physical stability. Even though R-lipo is more stable than R-nano, other characteristics and anti-obesity bioactivities should be considered and measured.

R-nano and R-lipo also enhanced the chemical stability of R that is sensitive to light. In a neutral pH 7.4 condition, native R was degraded by 40% at 4 °C, 60% at 22 °C and 96% at 37 °C under the light after 7 days (Fig. 2A). Storage of native R in the dark decreased the R degradation rates to 17% at 4 °C, 48% at 22 °C, and 52% at 37 °C after 7 days (Fig. 2B). After incubation at 37 °C for 6 days under the light, R degradation rates were 90%, 80% and 20% in native R, R-nano, and R-lipo, respectively (Fig. 2A). Both lipo and nanostructures protected R from degradation no matter under dark or light, and their protective capability was similar at 4 °C and 22 °C (Fig. 2). R-nano might be solid at the room temperature because the melting temperatures of α TA (Sigma T3001), soy PC (Avanti 441601) and Kolliphor® HS15 are 25 °C and above. The hydrophobic R can be encapsulated into the solid core, which can enhance its stability and prolong its release [17]. R-lipo had better protective capacity than R-nano at 37 °C. The higher R degradation rate in R-nano could be partially due to lack of a solid phase of R-nano at 37 °C. Additionally, many research studies have indicated the nanoencapsulation can increase R chemical stability via protecting it from light degradation [18,19].

3.3. Physicochemical characterization

3.3.1. Raman spectroscopy analysis—Raman spectroscopy is a proved method to quickly and effectively identify the encapsulated molecules [20]. Raman spectra of native R, R-nano, V-nano, R-lipo, V-lipo, were presented in Fig. 3. The spectrum of native R shows three major characteristic bands, including olefinic bands at 980 – 1022 cm^{-1} , C–O stretching at 1132 – 1188 cm^{-1} and C C aromatic double-bond stretching at 1570 – 1655 cm^{-1} , in addition to several other weak features, which are consistent to the reference [21]. The Raman spectra of V-lipo and V-nano are very similar, dominated by characteristic peaks of PC, the common component of these two nanocarriers. Since the signatures for native R and V-nano/V-lipo have no overlap, it becomes unambiguous to differentiate the compositions in the encapsulated particles and void nanocarriers. As observed from the spectra, the major characteristic peaks of native R were present in R-nano and R-lipo, but absent in V-nano and V-lipo, while those peaks of V-nano and V-lipo were present in their corresponding R-nano and R-lipo, but not in native R. These clearly indicate the encapsulation of R into R-nano and R-lipo.

3.3.2. XRD analysis—The XRD analysis can effectively distinguish between the crystalline and amorphous phases of R-nano and R-lipo. The crystallinity of nanocarriers is critical because the solubility and dissolution rate of R-nano and R-lipo in the R delivery process can be significantly affected by the degree of crystallinity [22]. The XRD patterns of native R, R-nano, R-lipo and the corresponding control forms and physical mixtures were shown in Fig. 3. The diffractogram of native R exhibited intense peaks between 5° and 35°, indicating the native R in a highly crystalline form. In the lyophilized nanocarriers, the sharp peaks from the crystalline native R were absent, suggesting less crystallinity or more amorphous state after R molecules were loaded in the soy PC shell to form an amorphous complex. More interestingly, although the Raman spectra of V-lipo and V-nano are very similar, their XRD patterns are dramatically different, suggesting their different composition and structures, as two different nanocarriers. With the absence of R crystalline peaks, XRD patterns of R-lipo and R-nano present the features of V-lipo and V-nano, respectively.

3.3.3. DSC analysis—The DSC curves of native R, R-nano, R-lipo and the corresponding control forms were presented in Fig. 3, as a second method to examine the crystallinity. The difference between V-lipo and V-nano again was notified. The DSC thermogram of native R showed the characteristic endothermic peak at 267 °C, corresponding to its melting temperature. However, R's endothermic peak was disappeared in the DSC scans of R-nano and R-lipo, suggesting its amorphous phase in these nanocarriers [23]. Interestingly, the DSC curves of R-lipo and V-lipo and those of R-nano and V-nano exhibit different features. This difference strongly suggests that R molecules were dispersed into the two nanocarriers, resulting in the amorphous phase. This conclusion is reinforced by the XRD measurements in which only the characteristic crystalline peaks of the V-nano or V-lipo were observed. All three physicochemical analyses confirm that R drug amorphization occurred in both R-nano and R-lipo nanocarriers.

3.3.4. *In vitro* release—Hydrophobic native R has a low level of aqueous solubility. Methanol was added to make the dissolution medium to dissolve released native R. Different methanol contents had been tested before conducting the release study to ensure to use the minimal amount of methanol. The dissolution medium containing 20% methanol was the optimal formula, which could dissolve almost all released native R without destroying nanostructures (data not shown). The release study was conducted in the dark to prevent light-induced R degradation. To minimize the effect of R stability, the dissolution medium was changed completely each hr for the first 2 h, and every 2 h for the rest 8 h. Native R showed a burst release phenomenon, while R-nano and R-lipo exhibited a sustained release behavior (Fig. 4). After 1 h, about 0.17 mg, 0.05 mg, and 0.02 mg of R was released from dialysis bags containing native R, R-lipo, and R-nano, respectively (Fig. 4A). At the 2–4 h period, about 0.02 mg, 0.05 mg and 0.04 mg of R was released from dialysis bags containing native R, R-lipo, and R-nano, respectively (Fig. 4A). After dialysis for 2 h, the accumulative released R mass from the native R dialysis bag reached a plateau (Fig. 4B), while for R-lipo and R-nano, R was continuously released in the 10 h period. Furthermore, R-lipo released more R than R-nano (Fig. 4B). Hydrophobic compounds like R might release faster from the membrane PC bilayers of liposomes than from the hydrophobic core of nanocarriers [12].

The data indicate R is probably distributed in the PC bilayers of R-lipo, and in the hydrophobic core of R-nano.

3.4. Cytotoxicity

None of the forms of R at tested doses negatively affected cell viability in 3T3-L1 cells after 24 or 72 h (Fig. 5A). The results suggest that two biocompatible and biodegradable nanostructures are safe to use, at least in 3T3-L1 cells.

3.5. Cellular R content

To investigate cellular bioavailability, we measured the R content in 3T3-L1 cells (Fig. 5B). The cellular R content was dose-dependently increased by all treatments. As compared to native R, R-nano and R-lipo only slightly increased R content at 10 μ M; but increased R content by more than 20% and 25% at 20 μ M, respectively. Due to the instability of native R at 37 °C, 3T3-L1 cells were only treated for 4 h in this experiment. Considering only 4 h treatment, the increase in cellular R content by R-nano and R-lipo is significant.

3.6. Activation of PPAR responsive reporter

It has been reported that R (native form) activates PPAR γ [24], which heterodimerizes with retinoid X receptor (RXR) to bind to PPRE sites in the promoter regions to transactivate target genes, including UCP1 [25]. All forms of R significantly activated PPRE-Luc compared to their controls ($p < 0.05$). No significant differences were detected among the various forms of R (Fig. 6). R-lipo and R-nano had similar activation abilities compared to native R (Fig. 6), a property that is associated with browning activities of Rosi [26]. The results suggest that encapsulation did not change the biological capability of R in activating PPAR γ .

3.7. Browning activities

We further studied browning effects of native R, R-nano, and R-lipo in differentiating 3T3-L1 white adipocytes, a commonly used cell model to study browning [27,28]. The hallmark of beige adipocytes is induced thermogenesis in response to stimuli, such as β -adrenergic agonist ISO. We investigated the effects of native R, R-lipo, and R-nano on the gene expression of markers of brown and white adipocytes, and mitochondrial biogenesis under either basal (non-stimulated) or ISO-stimulated conditions.

3.7.1. Effects on mRNA expression of brown adipocyte markers—Neither Rosi (positive control) nor any form of R significantly induced UCP1 mRNA expression at basal conditions. However, upon ISO stimulation, all forms of R significantly and dose-dependently induced UCP1 mRNA expression compared to their controls ($p < 0.05$), similar to Rosi ($p < 0.05$). When compared to various forms of R, there were no significant differences between R-lipo and R-nano. However, at 5 μ M (the low dose), R-lipo induced a higher UCP1 expression than native R under ISO stimulated conditions ($p < 0.05$) (Fig. 7).

PPAR γ , PGC1 α , and PRDM16 are known core regulators of browning and UCP1 mRNA expression (Reviewed in [29,30]). No forms of R affect PPAR γ mRNA under the basal

condition, in contrast to Rosi, which suppressed PPAR γ mRNA [26]; R-lipo induced higher PPAR γ mRNA levels than R-nano and native R ($p < 0.05$) (Fig. 7).

Under basal conditions, Rosi significantly induced PGC1 α mRNA expression ($p < 0.05$); however, various forms of R did not induce significant changes in PGC1 α expression at all tested doses. Under ISO stimulated conditions, R-lipo significantly induced PGC1 α mRNA expression at 20 μ M compared to its control ($p < 0.05$) and to a level that is higher than R-nano and native R at the same dose ($p < 0.05$). At 5 μ M, both R-lipo and R-nano induced higher PGC1 α mRNA than native R ($p < 0.05$) (Fig. 7).

Rosi significantly induced PRDM16 mRNA expression under both conditions ($p < 0.05$). Under the basal conditions, native R did not change PRDM16 mRNA expression. In contrast, both R-lipo and R-nano dose-dependently increased PRDM16 mRNA expression, reaching significance at 20 μ M ($p < 0.05$). Under ISO stimulated conditions, all forms of R similarly and dose-dependently increased PRDM16 mRNA expression, reaching significance at 20 μ M compared to the controls ($p < 0.05$) (Fig. 7).

3.7.2. Effects on mRNA expression of white and beige adipocyte markers—

Next, we examined various forms of R on white and beige marker mRNA expression during the browning of 3T3-L1 adipocytes. Insulin-like growth factor-binding protein 3 (IGFBP3) was identified as a white adipocyte specific marker by a transcriptome analysis of brown versus white adipocyte gene expression [31]. Rosi significantly decreased IGFBP3 mRNA expression ($p < 0.05$) under both conditions, consistent with a previous report [26]. Native R also dose-dependently decreased IGFBP3 expression compared to the controls under both conditions ($p < 0.05$ at 20 μ M under basal and $p < 0.05$ at all tested doses under ISO stimulated conditions). Comparing to native R, both R-lipo and R-nano further decreased IGFBP3 mRNA expression at 20 μ M at both conditions ($p < 0.05$) (Fig. 8A).

CD137 and Tmem26 have been identified as beige specific markers [32]. Rosi significantly increased CD137 mRNA expression under both conditions ($p < 0.05$). Various forms of R did not affect CD137 mRNA expression compared to the control under basal conditions. However, all forms of R dose-dependently increased CD137 mRNA expression under ISO stimulated conditions ($p < 0.05$ for native R and R-lipo at 20 μ M and $p < 0.05$ for all tested doses for R-nano) (Fig. 8B).

Rosi significantly decreased Tmem26 mRNA expression under both conditions ($p < 0.05$). Native R decreased Tmem26 mRNA expression under basal conditions ($p < 0.05$ at 5 and 10 μ M). In contrast, native R increased Tmem26 expression under ISO stimulation ($p < 0.05$ at all tested doses). There were no differences in Tmem26 mRNA expression between R-lipo and R-nano and their controls under both conditions (Fig. 8B).

Using differentiating 3T3-L1 white adipocytes coupled with ISO-induced thermogenic activation we demonstrate, for the first time, that various forms of R enhanced ISO-induced mRNA expression of UCP1 and other browning markers, such as PRDM16 and PGC1 α . In addition, various forms of R enhanced beige marker CD137 mRNA expression but suppressed white specific marker IGFBP3 mRNA expression. Our results are consistent with a previous study [5]. Of note, for the first time, we demonstrated that various R suppressed

IGFBP3 and R-nano and R-lipo had better suppression than native R at 20 μ M under ISO-stimulated conditions. Taken together, R-induced browning may contribute to the beneficial effects of R for obesity and associated metabolic dysfunction. Whether nanoencapsulated R can enhance the browning over native R *in vivo* warrant further investigation.

Compared to R-nano, R-lipo induced significantly higher levels of UCP1 mRNA than native R when both used at 5 μ M (Fig. 7). Moreover, R-lipo induced higher levels of other browning markers PPAR γ , PGC1 α than R-nano and native R under either basal and/or ISO stimulated conditions (Fig. 7). The better browning activities of R-lipo may be due to its higher physical and chemical stability compared to R-nano and native R. Moreover, the better biological activities demonstrated by R-lipo at 5 μ M is more physiological relevant since this dose is within the physiologically achievable dose range of R for human consumption [8,9]. Compared to native R, both R-lipo and R-nano had better suppression of IGFBP3, possibly due to improved overall bioavailability by nanoencapsulation.

3.7.3. Effects on mRNA expression of mitochondrial biogenesis markers—Rosi significantly increased Tfam, Nrf, Cox4a, and Uqcrrh under either basal and/or ISO stimulated conditions ($p < 0.05$). There were minimal differences among the three forms of R in any of the mitochondrial biogenesis markers in 3T3-L1 cells except for native R, which increased Cox4a mRNA at 20 μ M under ISO stimulated conditions ($p < 0.05$) (Supplemental Fig. 1).

In contrast to Rosi, all three forms of R had minimal effects on mitochondrial biogenesis genes under both basal and ISO stimulated conditions, suggesting that Rosi and R may induce browning of 3T3-L1 adipocytes via different molecular mechanisms. Lack of changes in mitochondrial biogenesis suggests that R may induce browning of 3T3-L1 by directly upregulating UCP1 expression whereas Rosi may increase mitochondrial biogenesis.

Our results suggest that various forms of R may promote browning by activating PPAR γ . Currently, we cannot rule out the possibilities that in addition to activating PPAR γ responsive promoters R-lipo and R-nano may activate other signaling pathways, such as SIRT1 [33,34], AMPK [5], or PDE [35], to induce browning. Future studies are needed to characterize potential differences in molecular mechanisms by which R-lipo and R-nano induce enhanced browning. Additionally, animal studies are required to determine the anti-obesity activity of R-lipo and R-nano *in vivo*. Oral administration is the most convenient route, but R-lipo and R-nano might be degraded in the gastrointestinal tract. Intravenous injection will ensure intact R-lipo and R-nano into the circulation system, but frequent intravenous injection faces compliance and safety issues, and clinical visits will be costly. Since animals have subcutaneous WAT, microneedles, skin patches and other trans-dermal approaches can be considered.

4. Conclusions

We have successfully encapsulated R into nanocarriers and liposomes, which increased R aqueous solubility and stability. R-lipo showed higher physical and chemical stability but

with a less sustained release than R-nano. Both R-nano and R-lipo increased cellular R content in 3T3-L1 cells, which led to higher expression UCP1, beige marker CD137 and other browning markers, and lower expression of white marker IGFBP3. Our study demonstrates a novel strategy of using nanoencapsulation of R to achieve improved browning efficacy with minimal side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.colsurfb.2017.12.044>.

Fig. 1.

Characteristics of R-nano and R-lipo. Visual observation of R-nano, R-lipo, and native R containing 1 mg of R suspended in 1 mL of $1 \times$ PBS, transmission electron microscope (TEM) images of R-nano and R-lipo, and predicted structures of R-nano and R-lipo (A); changes of particle size, zeta potential, and polydispersity index of R-nano and R-lipo (B) at different temperatures.

Fig. 2. Chemical stability of native R, R-nano, and R-lipo under the light (A) or the dark (B) at different temperatures.

Fig. 3. Physicochemical characterization. Raman spectra, X-ray diffraction patterns, DSC thermograms of lyophilized R-nano or R-lipo; lyophilized V-nano or V-lipo; native R.

Fig. 4. *In vitro* release profiles. Hourly (A) and accumulative (B) R release for native R, R-nano, and R-lipo;.

Fig. 5. Cytotoxicity and R content in 3T3-L1 cells. Cytotoxicity was measured by MTT assays after treating 3T3-L1 cells with native R, R-lipo, and R-nano (5, 10, 20 μM) or their respective controls for 24 h and 72 h (A); R content in 3T3-L1 cells after treating them with 10 and 20 μM of native R, R-nano and R-lipo at 37 °C for 4 h (B). Data = means ± SEM (n = 3). *, p < 0.05. E, Ethanol containing vehicle control for native R. R, rosiglitazone, a positive control.

Fig. 6.

Activation of PPAR responsive reporter by various forms of R. 3T3-L1 cells was seeded and transiently transfected with PPRE-Luc and transfection control plasmid β -gal for 24 h. The cells were then treated with native R, R-lipo, and R-nano (5, 10, 20 μ M) and their controls for 15 h. Relative luciferase activities were normalized by β -gal activities. Data = means $\pm$ SEM (n = 3). E, Ethanol containing vehicle control for native R. R, rosiglitazone, a positive control. Different letters on top of the bars indicate significant differences among various forms of R. *, p < 0.05; **, p < 0.01 compared to their controls.

Fig. 7.

Browning activities. 3T3-L1 cells were induced to undergo white adipocyte differentiation in the presence of native R, R-lipo, and R-nano (5, 10, 20 μ M) and their controls for 7 days. The cells were then stimulated with ISO for 6 h. Relative mRNA expression of UCP1, PPAR γ , PGC-1 α , and PRDM16 were analyzed by semi-quantitative Real Time-PCR. Data = means $\pm$ SEM (n = 3). E, Ethanol containing vehicle control for native R. R, rosiglitazone, a positive control. Letters of a–c or a'–c' on top of the bars define differences among various forms of R under either basal or ISO-stimulated conditions, respectively. Different letters indicate significant differences among various R. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared to their controls.

Fig. 8.

Gene expression of white and beige adipocyte markers. 3T3-L1 cells were induced to undergo white adipocyte differentiation in the presence of native R, R-lipo, and R-nano (5, 10, 20 μM) and their controls for 7 days. The cells were then stimulated with ISO for 6 h. Relative mRNA expression of white marker IGFBP3 (A) and beige marker CD137 and Tmem26 (B) were analyzed by semi-quantitative RT-PCR. Data = means ± SEM (n = 3). E, Ethanol containing vehicle control for native R. R, rosiglitazone, a positive control. Letters of a–c or a'–c' on top of the bars define differences among various forms of R under both basal and ISO-stimulated conditions, respectively. Different letters indicate significant differences among various R. *, p < 0.05; **, p < 0.01 compared to the controls.