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Stabilization of resveratrol immobilized in monodisperse cyano-functionalized porous polymeric microspheres

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Abstract

To preserve the high anti-oxidant activity of resveratrol for a long period of time, porous particles were utilized. The porosity of these substrate particles was controlled by DVB content and toluene/heptane ratios in the seeded polymerization. The influence of porosity on the anti-oxidant activity of the immobilized resveratrol was investigated. From SEM and CLSM analysis, it was confirmed that the resveratrol was stabilized in the porous particles after the immobilization process. The resveratrol has a crystalline structure, which was maintained even after immobilization; however, the resveratrol exhibited an amorphous form in the case of the smallest pore size and the highest specific surface area. Although the loading capacity of resveratrol was largely affected by the hydrophobicity and the specific surface area of porous particles, the anti-oxidant activity of immobilized resveratrol was preserved in over 93% in comparison to the raw resveratrol. In addition, the bioactivity of resveratrol immobilized in the porous particles was sustained for 5 weeks.

Keywords: Resveratrol; Cyano-functionalized porous particles; Long-term stability; Porosity

1. Introduction

Several non-flavonoid classes of phenolic compounds are synthesized by plants in response to environmental stress, or attacks by non-pathogenic or avirulent bacteria, viruses, or fungi. Among them, 3,4′,5-trihydroxystilbene, named resveratrol, has been identified as the major bioactive compound of stilbene phytoalexins, and is presumed to be beneficial for human health [1–3]. Generally, resveratrol is composed of the mixture of two geometrical isomers; E- (*trans*-) and Z- (*cis*-). *trans*-Resveratrol is the predominant form [4] as first discovered by Langcake and Pryce in grapevines [1]. Since this discovery, several useful properties of resveratrol have been reported such as anti-oxidizing effects [5,6], anti-atherosclerotic effects, inhibition of platelet aggregation [7], effects on the cardiovascular system [8], anti-mutagenic effects [9], and chemoprotective advantage against cancer proliferation [10]. According to the free radical theory of aging postulate, free radicals in the atmosphere oxidize with proteins, cell membranes, or DNA, resulting in great damage like aging in the human body [11,12]. Therefore, considerable interest has been focused on the anti-oxidizing effect of resveratrol since Frankel et al. [13] demonstrated that the resveratrol prevented the oxidation of low-density lipoproteins (LDL).

In spite of the high anti-oxidizing effect of resveratrol, it has some drawbacks for commercialization; it is easily degraded by sunlight, only soluble in alcoholic solvents, and difficult to stabilize in the liquid phase. Breemen et al. [14] reported that the concentration of resveratrol in methanol was decreased to 30% within 48 h when it was exposed to sunlight. Although considerable research has been devoted to investigate the biological activity and synthesis routes of resveratrol [15–17], less attention has been devoted to stabilizing the resveratrol, especially using a polymer matrix. Therefore, the main purpose of the research reported here was to stabilize the resveratrol using functionalized porous polymeric microspheres for long-term storage. Because the polymer particles, having micron size range, completely scatter light, the resveratrol incorporated into

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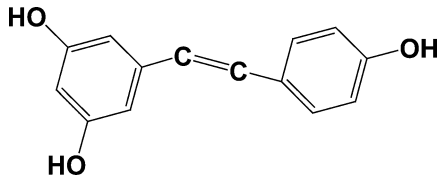

Scheme 1. The molecular structure of resveratrol.

porous particles was efficiently stabilized for a long time. In addition, the resveratrol was immobilized using hydrogen bonding between hydroxyl groups of the resveratrol and cyano-functional groups in the porous particles to prevent the release of resveratrol from the porous particles.

In the present study, monodisperse functionalized porous polymeric microspheres were applied to a support material for the stabilization and preservation of the resveratrol. Furthermore, the porosity of these particles was controlled, and the influence of porosity on the stability of the resveratrol was investigated. Monodisperse cyano-functionalized porous polymeric microspheres were prepared by seeded polymerization, and their morphology and porosity were monitored using a scanning electron microscope and BET measurements, respectively. The presence of resveratrol immobilized in the porous particles was confirmed using X-ray diffraction analysis and confocal laser scanning microscopy. The concentrations of resveratrol were obtained by a high-performance liquid chromatography. In addition, the anti-oxidizing effects of the resveratrol stabilized in the porous polymer particles were measured as a function of storage time in sunlight.

2. Experimental

2.1. Materials

Styrene (St, Kanto), acrylonitrile (AN, Aldrich), polyvinylpyrrolidone (PVP, $M_w = 4.0 \times 10^4$ g/mol, Sigma), aerosol-OT (AOT, Sigma) and ethanol (Carlo) were all reagent grades. 1-Chlorododecane (CD, TCI), divinylbenzene (DVB, Fluka), sodium lauryl sulfate (SLS, Yakuri) and Tween 20 (polysorbate 20, Uniquema Americas) were also used without further purification. The structure of resveratrol (above 95% degree of purity, Kaden Biochemicals) is shown in Scheme 1. Azobis(isobutylronitrile) (AIBN, Junsei) and benzoyl peroxide (BPO, Junsei) were recrystallized in methanol before use. Polyvinyl alcohol (PVA, $M_w = 8.8 \times 10^4 - 9.2 \times 10^4$ g/mol, 87–89% degree of saponification) was supplied by Kuraray Co. 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma) was used in the anti-oxidizing effect measurements.

Table 1
Standard recipe of the seeded polymerization

Stage	Ingredient	Quantity (g)
Seed dispersion	PS seed particles	0.50
	SE solution[a]	40
CD swelling	1-CD	0.5
	SE solution	10
The second monomer swelling	St	7/5/2
	DVB	1/3/6
	AN	2
	Diluent[b]	10
	BPO	0.1
	SE solution	40
Stabilization	PVA solution	50

Polymerization condition: 80 °C, 12 h.
[a] 0.25 wt% of SLS in EtOH/water (1/5, w/w) solution.
[b] Toluene/heptane ratio (porogen): 0/100, 30/70, 60/40, 100/0 by wt%.

2.2. Monodisperse cyano-functionalized porous poly(St-co-DVB-co-AN) microspheres

Monodisperse porous polymer particles containing cyano groups were prepared by seeded polymerization in the presence of porogen [18]. Firstly, polystyrene (PS) seed particles, prepared by dispersion polymerization [19], were dispersed in 0.25 wt% SLS water/EtOH (5/1, g/g) solution (SE solution) using a 250 ml four-necked round bottom flask equipped with a reflux condenser, nitrogen inlet apparatus and a mechanical stirrer. The CD emulsions in the SE solution were poured into the seed dispersion. The stirring speed and temperature were fixed at 200 rpm and 30 °C throughout the swelling process. After complete disappearance of the CD droplets, the second monomer mixture (St/DVB/AN), BPO and diluent (toluene/heptane) were emulsified and added into the reactor for another 8 h of swelling. The swollen particles were stabilized with a 5% PVA aqueous solution, and then polymerized at 80 °C for 12 h. The resulting particles were washed repeatedly using water and ethanol. Subsequently, the soxhlet extraction was followed by methylene chloride for 24 h to eliminate the seed particle and porogen. The standard recipe for seeded polymerization of cyano-functionalized porous polymeric microspheres is shown in Table 1.

2.3. Immobilization of resveratrol

The immobilization of resveratrol into the monodisperse porous poly(St-*co*-DVB-*co*-AN) particles was carried out. Four grams of cyano-functionalized porous particles were weighed in a beaker, and 0.2 wt% of Tween 20 aqueous solution (86 g) was added. Vigorous stirring for 24 h was performed before the immobilization procedure to achieve sufficient wetting of the porous particles. Ethanol (10 g) containing 0.2 g of resveratrol was then slowly dropped into the beaker. After the reaction, Tween 20 and other reactants were removed by filtering with DDI water repeatedly.

2.4. Characterization

The morphology and porosity of the porous particles

Table 2
Characteristics of the functionalized porous particles

Symbol[a]	St/DVB/AN/diluent[b] (g/g/g/g)	Toluene/heptane ratio (w/w)	D_n (μm)	PSD[c]	BET analysis		
					A_p[d] (m^2/g)	V_p[e] (cm^3/g)	D_p[f] (nm)
POR1-0	7/1/2/10	100/0	7.38	1.01	11.27	0.04	13.58
POR3-0	5/3/2/10	100/0	7.33	1.01	49.98	0.06	8.88
POR6-0	2/6/2/10	100/0	7.26	1.01	349.79	0.34	3.86
POR3-30	5/3/2/10	70/30	7.42	1.01	35.55	0.11	10.61
POR3-60	5/3/2/10	40/60	7.97	1.01	20.17	0.14	13.41
POR3-100	5/3/2/10	0/100	8.39	1.03	3.85	0.0053	5.37

[a] PORα-β: α and β correspond to the concentrations of DVB used in 2nd monomer, and wt% of heptane in the diluent mixture, respectively.
[b] Weights of 2nd monomers used in the seeded polymerization: Total 40 folds based on the weight of the seed particles.
[c] Particle size distribution, D_w/D_n.
[d] Specific surface area from BET measurement.
[e] Total pore volume.
[f] Average pore diameter.

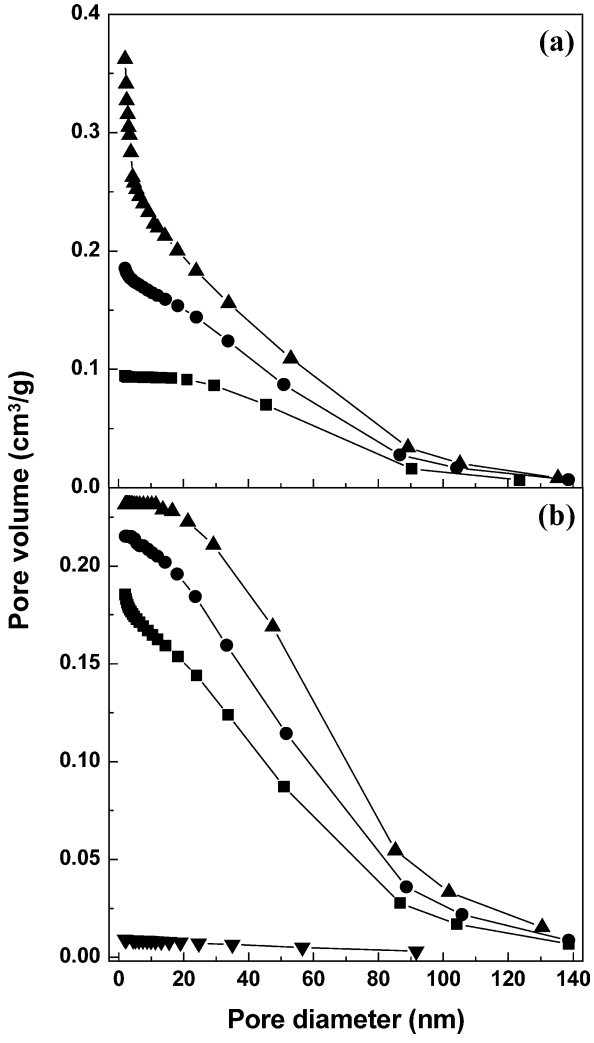

Fig. 1. Pore size distributions of the porous particles controlling the DVB contents (a); POR1-0 (-■-), POR3-0 (-●-), POR6-0 (-▲-), and heptane ratios (b); POR3-0 (-■-), POR3-30 (-●-), POR3-60 (-▲-), POR3-100 (-▼-).

were characterized using a scanning electron microscope (SEM, JSM-6340F JEOL), and Brunauer–Emmett–Teller (BET, ASAP2010 Micromeritics) isotherm of sorption/desorption of nitrogen, respectively. The structural states of the resveratrol immobilized in the porous particles were verified using a confocal laser scanning microscope (CLSM, MRC-1024, Biorad), and X-ray diffractometer (XRD, Bruker) in the 2θ range. Cu Kα radiation ($\lambda = 1.542$ Å) was used in the XRD measurements. The concentration of resveratrol loaded in the porous particle was determined from a high performance liquid chromatograph (HPLC, Waters) equipped with a solvent delivery module, a UV detector, and an autosampler. The chromatographic separation was achieved using a C-18 reverse phase column (Mightysil, RP-18 GP 250–4.6, 5 μm) at 310 nm. The carrier solvent for HPLC analysis was a mixture of 10 mM of phosphoric acid (65%) and acetonitrile (35%). The flow rate was set at 1.0 ml/min.

2.5. Free radical scavenging method

Anti-oxidant activity of the immobilized resveratrol was measured as a function of time according to the method described by Brand-Williams et al. [20] using a UV–vis spectrophotometer (UV–vis, Simadzu 1601). Activity of pure resveratrol was also observed as a reference sample. A solution containing 100 ppm of resveratrol immobilized in porous particles was poured into 2000 μl of ethanol with sonification, and the same quantity of ethanol solution containing DPPH (0.15 mM) was prepared. Both solutions were mixed and vigorously shaken for 30 min at room temperature in a dark box. The DPPH radical scavenging activity was evaluated from the difference in the peak-area of the DPPH radical detected at 517 nm between a blank solution and a sample solution. The data were calculated using the formula:

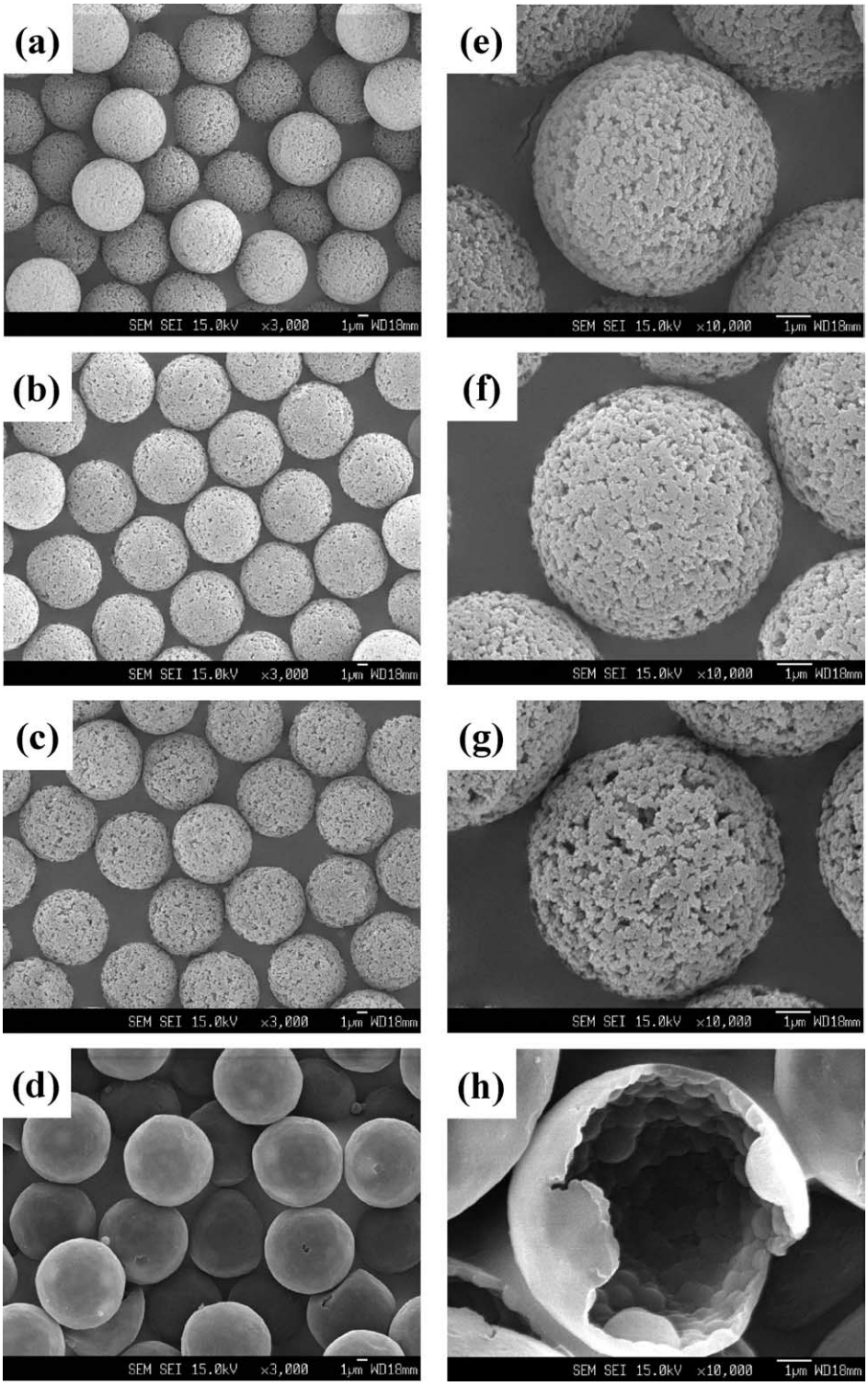

Fig. 2. SEM images of porous particles prepared with varying heptane ratio (a) and (e) POR3-0, (b) and (f) POR3-30, (c) and (g) POR3-60, (d) and (h) POR3-100; (e)–(h) are high magnification of (a)–(d).

$$\text{DPPH radical scavenging activity} = \frac{(A-B)}{A} \times 100(\%)$$

where A is the peak area of the blank, and B is the peak area of the sample corresponding to DPPH. All samples were tested three times to ensure repeatability of the measurements.

3. Results and discussion

3.1. Porosity control of the microspheres

In order to stabilize the resveratrol efficiently from the

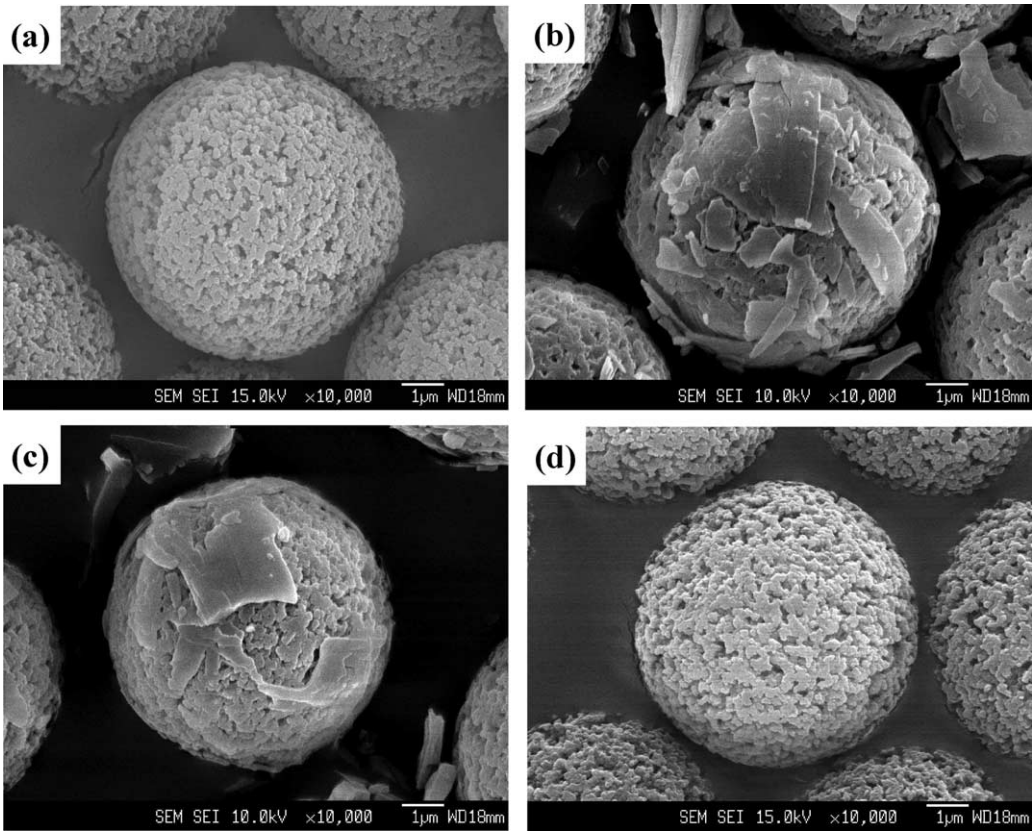

Fig. 3. SEM images of porous particles before (a), and after immobilization process with wetting for 6 h using non-functionalized (b), 24 h using non-functionalized (c), 24 h using cyano-functionalized (d) microspheres.

sunlight for long-term storage, monodisperse porous polymeric microspheres were applied to a support material. In addition, cyano-functional groups were incorporated into the porous particles to prevent release of the resveratrol by forming hydrogen bonds between hydroxyl groups of resveratrol and functional groups. In the preparation of the porous particles, the porosity was controlled using the amount of DVB in 2nd monomer mixture, and composition ratios of diluents (toluene/heptane). Characteristics including the BET analysis and the pore size distribution are summarized in Table 2 and Fig. 1, respectively. The diameter of the porous particles was slightly decreased, maintaining their high monodispersity, when DVB contents were increased. This can be explained by the fact that as the content of DVB in the second monomer mixture increases, porous structures formed by the phase separation become smaller and denser due to the highly crosslinked network [18,21,22]. Consequently, small D_p, large A_p, and high V_p could be obtained with high content of DVB as shown in Table 2 and Fig. 1(a).

It is well known that the solvency of diluent against polymer composition plays an important role in determining the pore structure and porosity of porous particles. In this study, a good and a poor solvent (toluene and heptane) for the second monomers (St and DVB) were employed as a porogen to alter the porosity by controlling the ratios in the diluent mixture, the concentration of which was fixed as shown in Table 1. It can be shown that increasing the heptane content results in an increase of the diameter, pore volume and size of porous particles, and a corresponding decrease in the specific surface area. This is similar to the trend observed in the morphology of the porous particles as shown in Fig. 2. These results directly imply that the presence of a poor solvent enhances the phase separation and structure heterogeneity during the pore generation stage [21,23,24]. Interestingly, a hollow structure (Fig. 2(d) and (h)) without pores at the surface (Fig. 1(b)) was obtained in case of POR3-100, mainly due to the complete phase separation between polymer compositions (St and DVB) and the poor solvent (heptane).

3.2. Immobilization process

Fig. 3 shows SEM photographs before and after the immobilization process, with various wetting times and in the presence of cyano-functional groups for stabilizing the resveratrol in the porous substrate particles. In Fig. 3(b), a good quantity of resveratrol was deposited on the surface of the porous particles (POR3-0) or selfaggregated in the case of 6h of wetting; however, complete stabilization was obtained when the wetting time was increased to 24 h (Fig. 3(d)). It is supposed that resveratrol molecules can

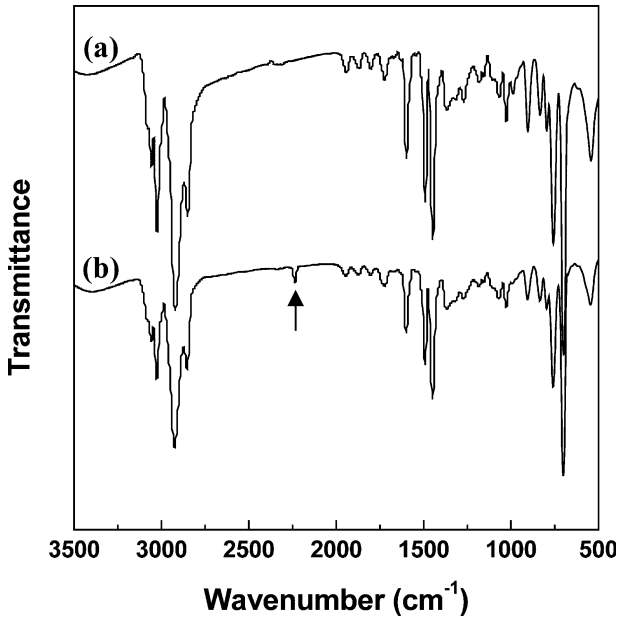

Fig. 4. FT-IR spectra of the porous particles without functional group (a), cyano-functional groups (b).

only approach the inner pore of substrate particles completely when the channels of pores were sufficiently wetted by Tween 20 aqueous solution, which acted as a driving medium for the resveratrol. At the same time, it was found that the presence of cyano-functional groups was also critical for immobilization of the resveratrol. Comparing porous particles without (Fig. 4(a)) and with cyano-functional groups (Fig. 4(b), at 2240 cm^{-1}), some resveratrol was still observed on the surface of the porous particles even after 24 h of wetting when functional groups were not present as shown in Fig. 3(c).

3.3. Resveratrol immobilized in the porous particles

The locations of resveratrol immobilized in the porous particles were evaluated using CLSM analysis. First, the resveratrol was labeled with a rhodamin B isothiocyanate (RBITC, Fluka) following the method reported by Lamprecht and Schreiber [25,26]. The labeled resveratrol was the immobilized in the porous particles. Adjusting the laser to the red fluorescence mode (excitation wavelength at 514 nm), the RBITC-labeled resveratrol can be visualized with the red channel as shown in Fig. 5. It was ascertained that the resveratrol was well-distributed in each of the porous microspheres. From the results described earlier, this leads to the general conclusion that the resveratrol was successfully introduced and stably immobilized in the inner porous particles by hydrogen bonding.

Interestingly, it was found during the SEM analysis that the resveratrol has a crystalline structure (Fig. 3(b) and (c)). To confirm the crystallinity of pure and immobilized resveratrol, XRD measurements were performed. As shown in Fig. 6, pure resveratrol had a crystalline structure

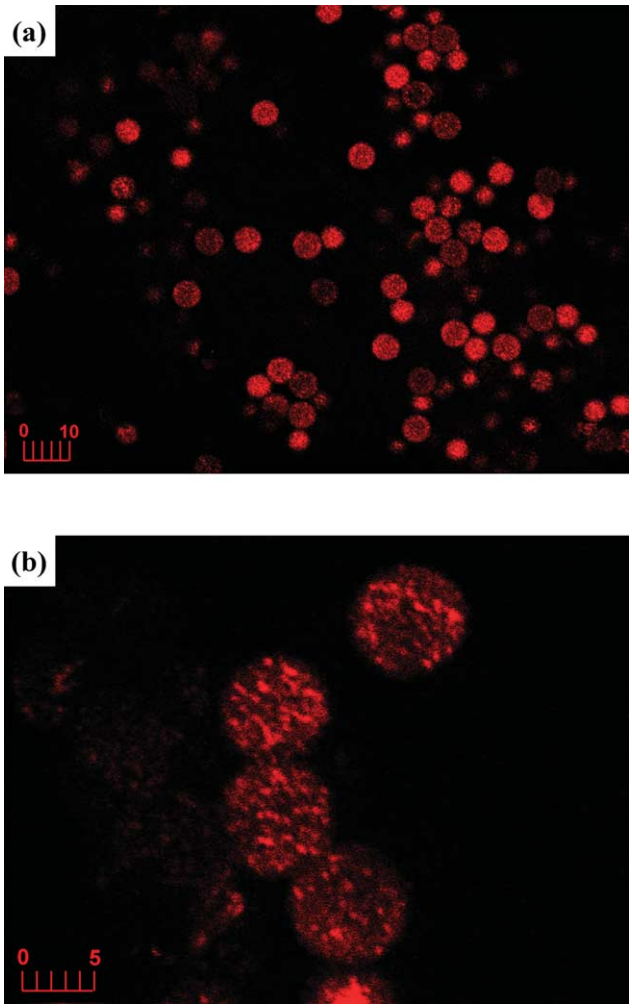

Fig. 5. CLSM images of porous particles (POR1-0) containing resveratrol labeled with RBITC; (b) is high magnification of (a).

itself; moreover its crystallinity was mostly preserved even after stabilizing in the porous particles, which did not have a crystalline structure. But in the case of POR6-0, it did not contain any peaks associated with the crystals of the resveratrol, suggesting that the resveratrol was dispersed as an amorphous form in the polymer matrix [27]. Because it had the highest content of DVB in the preparation of porous particles, POR6-0 has the smallest pore size and the highest specific surface area. Therefore, the resveratrol immobilized in POR6-0 would exist in an amorphous form.

Fig. 7 shows the loading capacity of resveratrol incorporated in the porous particles. The porosity of particles was controlled by DVB contents and toluene/heptane ratios. The concentrations of resveratrol gradually increased with decreasing DVB contents and heptane ratios. As the specific surface area of the porous particles increased, the hydrophobicity of the porous particles also increased, which caused the insufficient wetting of Tween 20 aqueous solution during the immobilization process. Consequently, the resveratrol could not approach the inner pores efficiently through the driving medium, resulting in

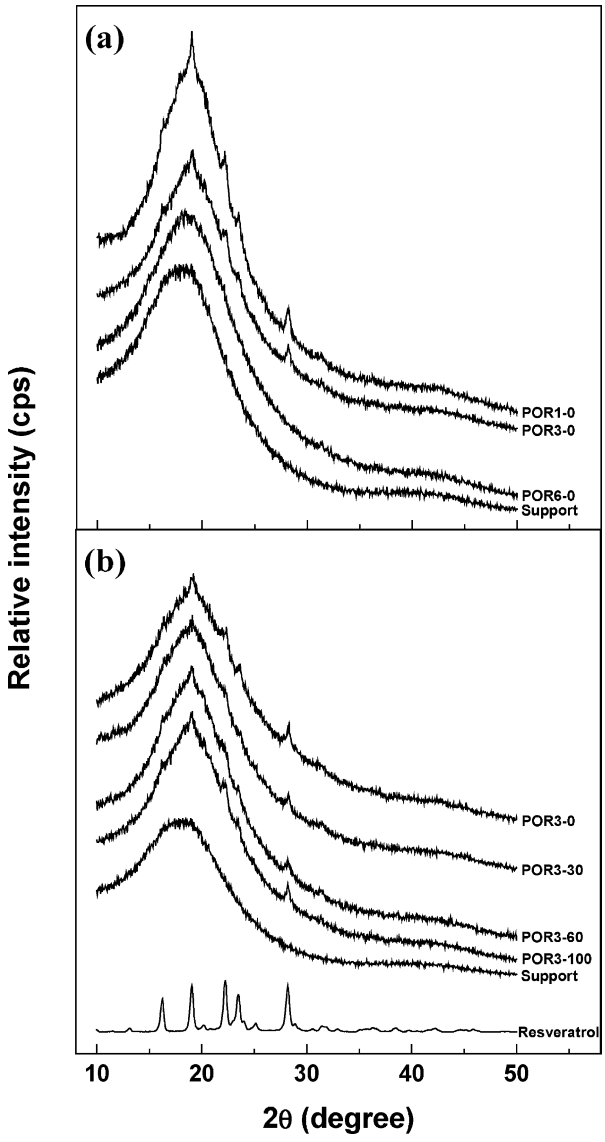

Fig. 6. XRD patterns of porous particles containing resveratrol with varying DVB contents (a), and varying heptane ratios (b).

Table 3
Anti-oxidizing activity of immobilized resveratrol

Symbol	Absorbance[a]	DPPH radical scavenging activity (%)	Relative anti-oxidizing activity[b] (%)
Resveratrol	0.1837	72.30	–
POR1-0	0.2172	67.24	93
POR3-0	0.1981	70.13	97
POR6-0	0.1841	72.22	99
POR3-30	0.1933	70.85	98
POR3-60	0.1944	70.68	98
POR3-100	0.1885	71.57	99

Absorbance value of the blank sample is 0.66304.
[a] Determined by UV–vis spectroscope.
[b] Relative anti-oxidizing activity = sample antioxidizing activity (%)/raw resveratrol antioxidizing activity (%).

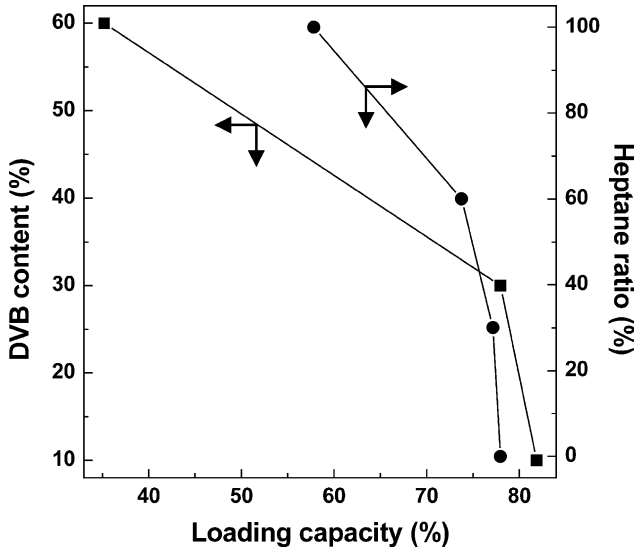

Fig. 7. Loading capacity of resveratrol in porous particles prepared with varying DVB contents (-■-), and heptane ratio (-●-).

the decrement of loading contents. Yan et al. [28] reported that the direct swelling of water in porous poly(DVB-*co*-MA) particles by suspension polymerization was limited when the DVB content was at a high level. Specifically, the loading capacity of resveratrol in POR6-0 was considerably reduced since the resveratrol was present in an amorphous form as confirmed in Fig. 6. However, the resveratrol content increased with the increase of the specific surface area when the heptane ratios were controlled at the same DVB contents.

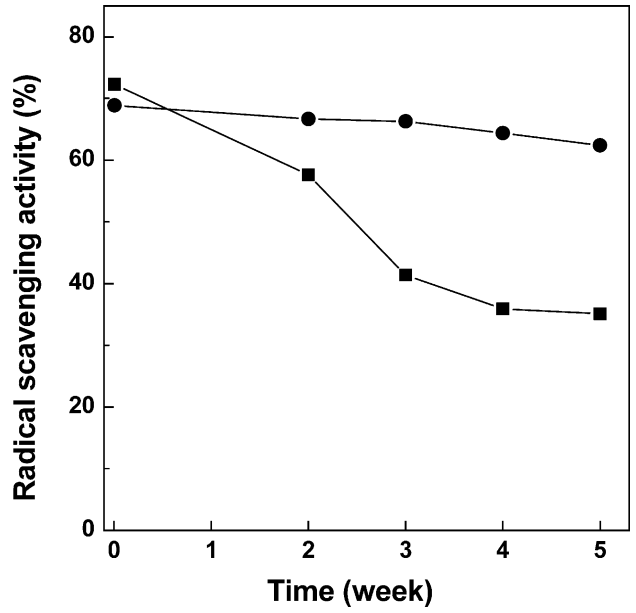

Fig. 8. Bioactivity of resveratrol immobilized in the porous particles as a function of storage time (under sunlight, in ethanol, at room temperature), immobilized resveratrol (-●-), and raw resveratrol (-■-).

3.4. Anti-oxidant activity of immobilized resveratrol

DPPH radical scavenging activities were measured in order to investigate the antioxidant effect of resveratrol stabilized in the porous particles. As shown in Table 3, overall stabilized resveratrol sustained over 93% of anti-oxidant effect compared to the pure sample. This shows that the bioactivity of resveratrol was not affected by immobilization in the porous substrate particles. Furthermore, anti-oxidant activity of stabilized resveratrol was observed as a function of time under sunlight in ethanol solution at room temperature to confirm the long term storage of resveratrol. In the case of pure resveratrol (Fig. 8), the bioactivity was dramatically diminished, which is similar to the tendency reported by Breemen [14]. However, it was confirmed that the anti-oxidant effects of the resveratrol immobilized in the porous particles were maintained for 5 weeks. These results may be attributed to the efficient protection of resveratrol in the porous microspheres from the sunlight because the substrate particles fully scattered the light.

4. Conclusion

Porous particles containing cyano-functional groups were prepared, and applied to a substrate matrix for long-term storage of resveratrol. The porosity of these substrate particles was altered by DVB contents and toluene/heptane ratios in the seeded polymerization. It was found that the wetting time during the immobilization process and existence of cyano-functional groups were important factors to stabilize the resveratrol in the porous particles. As the crosslinking density of the porous particles increased, the loading content of resveratrol decreased, due to the high hydrophobicity of those porous particles. In addition, higher contents of resveratrol were incorporated into the porous particles with higher specific surface area when the DVB content was fixed. Anti-oxidant activity of immobilized resveratrol was preserved for 5 weeks because the micron size range of the porous particles efficiently scattered the sunlight, which destroys the bioactivity of resveratrol.

Acknowledgements

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