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The stability and controlled release of l-ascorbic acid encapsulated in poly (ethyl-2-cyanoacrylate) nanocapsules prepared by interfacial polymerization of water-in-oil microemulsions

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Synopsis

The L-ascorbic acid (AA) was encapsulated into biodegradable and biocompatible poly(ethyl-2-cyanoacrylate) (PECA) nanocapsules by interfacial polymerization of water-in-oil (W/O) microemulsions. The influences of surfactant concentration, pH value of the dispersed aqueous phase, and W/O ratio on nanocapsule size were discussed. The stability and *in vitro* release of encapsulated AA were also investigated. The results show that nanocapsules could be obtained under the conditions with low pH value, high fraction of aqueous phase, and appropriate surfactant concentration. The encapsulated AA was protected by nanocapsules from oxidation and presented superior storage stability in aqueous medium than pure AA. Releasing AA from the inner core of nanocapsules could be controlled by adjusting the enzyme hydrolysis extent of the PECA wall.

INTRODUCTION

L-Ascorbic acid (AA), an antioxidant and free radical scavenger, is one of the most popular agents used in various fields such as biological, pharmaceutical, dermatological, and cosmetic. It provides photoprotection to protect tissues and cells from oxidative damage by free radicals and reactive oxygen–derived species, boosts collagen biosynthesis to improve the elasticity of the skin and resist wrinkle, restrains skin pigmentation and reduces melanin to keep skin white, and enhances the immunity (antivirus effect) (1–5).

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Unfortunately, the practical use of AA in skin care is limited because of some difficulties. One is due to it's instability. As AA exists in the enol form of α-ketolactone, the enediol group at carbons 2 and 3 can easily be oxidized to yield diketone lactone structure and to produce biologically inactive compounds such as dehydroascorbic acid, oxalic acid, L-threonic acid, L-xylonic acid, and L-lyxonic acid, which are not only ineffective but also potentially harmful to the human body (6). Such decomposition is usually caused by air, moisture, light, heat, trace metal ions, oxygen, and base, and occurs in just a few days after being produced. Another difficulty for practical use is the low skin penetration ability of AA arising from its hydrophilic character (7,8).

To improve the stability and lipophilicity of AA, many efforts have been done in recent years. One method to suppress the decomposition is to derive AA as a salt, for example, ascorbyl phosphate or ascorbyl palmitate (9–11). Morisaki *et al*. investigated the thermal stability and reducing activity against free radicals of ascorbic acid phosphodiesters and found that the phosphodiesters exhibited high thermal stabilities but low antioxidant activities *in vitro* (9). The stabilities of ascorbyl palmitate were found less than those of ascorbyl phosphates (10,11). Another method used to overcome the instability and hydrophilicity was to encapsulate and immobilize AA by using microemulsions (W/O, or oil-in-water, O/W) (5,11–13). Gallarate *et al*. investigated the stability against oxidation of AA in O/W microemulsions, O/W and W/O emulsions, and a W/O/W multiple emulsion at different pH values (5). They found all emulsified systems provided protection for AA and slowed its degradation rate. The W/O/W multiple emulsion can provide better stability of AA over time than aW/O emulsion does. Although the use of microemulsions seems to be suitable for cosmetic products, it should be noted that they are usually a low-viscosity Newtonian fluid, and the suitable thickening agents, which may have drawbacks for people with sensitive skin, are needed before they can be utilized directly (13).

More recently, nanoparticles that can deliver the nutrition substance without damaging the sensitive active ingredient of cosmetic products have attracted much attention (14–17). Two types of nanoparticles, i.e., nanospheres and nanocapsules, have been defined according to their different preparation method. Nanospheres have a matrix-type structure in which the drug is dispersed, whereas nanocapsules are vesicular systems with a drug confined cavity surrounded by a polymeric membrane or layered inorganic matrix. For instance, Choy *et al*. used the inorganic nanocapsule to encapsulate AA and found the inorganic nanocapsule could enhance the storage stability and sustained release of AA, which was helpful in delivering AA into skin through stratum corneum (18).

In general, the biodegradable nanocapsules can be obtained through two main processes: interfacial dispersion of preformed polymers including poly(D,L-lactide), poly(D,L-glycolide), poly(lactide-co-glycolide), poly(lactic acid), and poly(cyanoacrylate) (19), and interfacial polymerization of dispersed alkyl cyanoacrylate monomers (20,21). Poly(alkyl cyanoacrylate) (PACA) interfacial polymerization of microemulsions was first introduced by Gasco and Trotta (22) and has been developed as a simple one-step process without further isolation of nanocapsules from the reaction matrix by using biocompatible microemulsions (23). Poly(ethyl-2-cyanoacrylate) (PECA), one type of PACA polymerized from ethyl-2-cyanoacrylate (ECA), has produced very promising results as polymeric substrates in the nanoparticle delivery system for its mechanical properties, biodegradability, high biocompatibility, drug compatibility, and permeability (24). Much research on drug delivery properties of PECA nanocapsules has been done in O/W microemulsions for lipophilic drug such as 5,5-diphenylhydantoin, carbamazepine, ethosuximide, idebenone,

and doxorubicin (25–27), and in W/O microemulsions for hydrophilic proteins and peptides such as insulin (23,28–30). Even though, to our knowledge, the applications of PECA nanocapsules for entrapping hydrophilic drug are still limited, especially in the dermatological and cosmetic field.

In this work, AA was encapsulated into PECA nanocapsules by interfacial polymerization of W/O microemulsions. The influences of surfactants concentration, pH value of the dispersed aqueous phase, and W/O ratio on nanocapsules size were discussed. After optimizing the interfacial polymerization conditions, AA was incorporated into the nanocapsules. The stability and the release profiles of AA from nanocapsules by enzyme esterase hydrolysis of PECA were also investigated.

EXPERIMENTAL

MATERIALS

The nonionic surfactants, Span 80 and Tween 80, were purchased from Fluka (St. Louis, MO). ECA was supplied by courtesy of Zhejiang Jinpeng Chemical Co., Ltd. (Zhejiang, China). AA dry powder was received as a gift from DSM Vitamins Trading Co. (Shanghai, China), Ltd. Enzyme esterase (10 U/mg) was purchased from Sigma (Santa Clara, CA). Ethanol, *n*-hexane, *N*,*N*-dimethylformamide (DMF), and other solvents and agents were all AR grade and used as received without further purification. Deionized water was used except where specifically indicated.

PREPARATION OF NANOCAPSULES

For preparing the microemulsion, oil mixtures were prepared at room temperature by dissolving certain amount of nonionic surfactant mix (Span 80 and Tween 80, 3:2 weight ratio) in *n*-hexane. Subsequently, adequate deoxidized water was dropped into the oil mixture under ultrasonic dispersion in nitrogen atmosphere. The pH value of the aqueous phase was adjusted by using hydrochloric acid (HCl). The solution was then mixed by magnetic stirring, and a stable W/O microemulsion was achieved. ECA monomer (20 mg) was slowly added to the microemulsion system under continuous stirring. The interfacial polymerization was then performed at room temperature for 15 h. Finally, the white PECA nanocapsules were separated from the colloidal suspension by ultracentrifugation at 1677*g* for 10 min and dried in vacuum at room temperature.

For the preparation of AA-encapsulated PECA nanocapsules, an aqueous solution of AA with a concentration of 3 mg/ml and pH 2.0 was used as aqueous component of microemulsions.

CHARACTERIZATION OF NANOCAPSULES

Before characterization, the residual surfactants were removed by repeated washing of at least twice in *n*-hexane and then centrifuged and dried to a constant weight by the method described above. AA-encapsulated nanocapsules were further freeze-dried for *in vitro* release studies. Before investigating the size and the morphology, the nanocapsules were redispersed by ultrasonication in water.

The average size and its distribution of nanocapsules with and without AA loaded were measured by dynamic light scattering (DLS) (Zetasizer Nano ZS; Malvern Instruments, Worcestershire, UK) with a He–Ne laser beam at a wavelength of 633 nm at 25°C. The scattering angle used is 175°. The results are expressed in volume-averaged scales as unimode.

The morphology and the structure of AA-encapsulated PECA nanocapsules were visualized by transmission electron microscope (TEM) (JSM-2100F, JEOL, Tokyo, Japan) after negative staining with phosphotungstic acid. Drops of the suspensions were dripped on a carbon film–coated copper grid and dried under room temperature. The TEM bright field imaging was performed with 120 kV accelerating voltage.

DETERMINATION OF ENCAPSULATION EFFICIENCY OF AA

The encapsulation efficiency of AA in PECA nanocapsules, i.e., the ratio of the weight of AA encapsulated in nanocapsules to the initial weight of added AA was determined according to an indirect fluorimetry method as described in Reference 31. In brief, first, 0.1 mg freeze-dried AA-encapsulated nanocapsules were redispersed into 10 ml deionized water under ultrasonic generator. Then 1.0 ml of such solution was mixed with a prefab mixture containing 1.0 ml DMF and 1.0 ml cerium (IV) ion standard solution (3.0×10^{-4} M), followed by adding 1.0 ml sodium hexametaphosphate standard solution (1.0×10^{-3} M). The final mixture was diluted to 10 ml by deionized water and equilibrated for 30 min before fluorescence detection. The fluorescence spectra were recorded at room temperature on fluorescence spectrophotometer (F-4500, Hitachi, Tokyo, Japan) with the excitation and emission wavelengths at 303 nm and 340 nm, respectively. The excitation bandwidth was 10 nm. The fluorescence intensity of cerium (IV)-AA at 340 nm in emission spectra was recorded and compared with a standard curve generated from the corresponding solution. A reference sample of empty nanocapsules with no encapsulated AA was prepared by the same procedure.

AA STABILITY TEST

For the stability evaluation, 1 mg freeze-dried AA-encapsulated nanocapsules (containing 0.116 mg of AA calculated from the encapsulating efficiency) and corresponding weight of pure AA (as a reference) were separately dispersed or dissolved in 100 ml deionized water, 10 ml of each solution was sealed carefully with the caps and stored in an oven with the constant temperature of 40° and 80°C, respectively. Then 1.0 ml of such solution was treated with DMF, cerium (IV) solution, and sodium hexametaphosphate solution as described in the section Determination of Encapsulation Efficiency of AA. The retention of AA was analyzed by the fluorescence method mentioned above at different storage periods.

DEGRADATION OF PECA AND *IN VITR0* RELEASE OF AA

AA can be released from nanocapsules when PECA wall of nanocapsules is decomposed by enzyme esterase hydrolysis. The yielding amount of ethanol produced in hydrolysis can be

used to estimate the degradation extent of PECA (32–34). For the hydrolysis extent test, 50 mg AA entrapped nanocapsules was ultrasonically dispersed to 25 ml phosphate buffer solution (pH 7.0) followed by adding 3.6 mg enzyme esterase (closes to its saturation value in current system). The solution was placed in a shaking incubator (60 cycles/min) at 37°C for equilibration. Then a 4 ml suspension was taken out at times of 0.5 h, 1.5 h, and 3–9 h at 2-h intervals and 2 g ammonium sulfate was added to salt out the enzyme. After centrifuged at 12,300*g* for 10 min, 3 ml of supernatant liquor was taken out to detect the ethanol yielded from PECA degradation by gas chromatography test. The capillary column used was HP-INNOWAX (Agilent, Santa Clara, CA) with nitrogen as the carrier gas chromatography. The oven temperature was isothermal for 3 min at 30°C, then ramped to 180°C at a rate of 10°C/min. A series of 5 ml phosphate buffer solutions (pH 7.0) with various amount of ethanol (0.25–2.5 mg) were prepared. The ethanol was extracted by adding 1.0 ml methyl tertiary-butyl ether. A calibration curve of ethanol concentration (mg/ml phosphate buffer solution) was plotted against the relative response to ethanol. The concentration of ethanol in supernatant liquor was determined from the calibration curve.

For its instability, AA released from decomposed nanocapsules would be oxidized soon, which may cause errors in determining concentration. To minimize the influence of oxidization, the release profiles were performed by detecting the content of residual AA in the decomposed nanocapsules through the fluorescence method described above.

RESULTS AND DISCUSSION

CHARACTERIZATION OF PECA NANOCAPSULES

Contrary to the standard emulsion, W/O microemulsions are spontaneously formed and thermodynamically stable, requiring only minimal input of energy to obtain small and uniform dispersed droplets. Therefore, once the monomer inventory rating, which affects the size and its polydispersity (30), is fixed, the droplet size may be mainly controlled by

Table I
Size of Nanocapsules Prepared Under Different Conditions

Surfactant concentration (mg/ml)	Water volume (ml)	n-Hexane volume (ml)	pH	Mean diameter (nm)
72	1	50	2.0	1081
84	1	50	2.0	984
96	1	50	2.0	570
114	1	50	2.0	316
120	1	50	2.0	733
108	1	50	2.0	256
108	1	50	3.0	380
108	1	50	4.0	450
108	1	50	5.0	910
108	1	100	2.0	670
108	1	120	2.0	753

three variables including the surfactant concentration, pH value of the dispersed aqueous phase, and the aqueous fraction of the microemulsion. The mean diameters of the empty PECA nanocapsules prepared under different conditions are listed in Table I.

As shown in Table I, a significant decrease in particle diameter from 1081 nm to 256 nm could be observed when the surfactant concentration was increased from 72 to 108 mg/ml at pH 2.0, ECA 20 mg, and W/O 1/50 (v/v). At low surfactant concentration, when the amount of oil and aqueous phases are fixed, the surfactants trend to form large emulsified droplets with smaller total surface area to ensure that there are sufficient surfactants adsorbed on the oil–water interface for emulsification. As a result, polymeric particles with large size are formed. Increasing the surfactant concentration in a certain range, the number of emulsified droplets increases whereas the size decreases, resulting in small polymeric nanocapsules. When the surfactant concentration increased to 108 mg/ml, the smallest nanocapsules with about a 256 nm diameter were obtained. However, large particles emerged again by further increasing the surfactant concentration more than 108 mg/ml. It's well known, above the critical micelle concentration, increasing surfactant concentration not only varies the morphology of emulsified particles from spherical micelles to cubic, hexagonal, and even lamellar phase, but also increases the aggregation number, accompanied with the size expanding. The volume expansion of the emulsified particles finally gives rise to a rapid increase in polymeric particle size.

The effects of original pH value of the dispersed aqueous phase on the particle size are also shown in Table I. The surfactant concentration and W/O ratio were maintained at 108 mg/ml and 1/50 (v/v), respectively. Raising original pH value of dispersed aqueous phase from 2.0 to 5.0 brought about a five times increase in size and formed large particles with diameter of 910 nm. PECAs were prepared based on anionic polymerization mechanism, which was initiated by nucleophilic attack on β carbon of the monomer resulting in a reactive carbanion. The reaction is initiated by hydroxyl ions and terminated by protons (35–37). Therefore, at higher pH of the dispersed aqueous phase, more hydroxyl ions are generated to encourage the chain initiation. Meanwhile, the lowered proton concentration at higher pH reduces the probability of chain termination. As a result, the polymers with higher molecular weight were produced, leading to the thickening of the capsule wall. Because the size of the aqueous core in capsule was fixed, the size of the whole particle was then correspondingly increased. The investigation on current system shows that the original low pH value of dispersed aqueous phase is more suitable for preparing small-sized nanocapsules.

Another variable effect on the particle size is the aqueous fraction in the system. The results listed in Table I show that the particle size decreased from 753 to 256 nm as the W/O volume ratio varied from 1/120 to 1/50 with constant surfactant concentration at pH 2.0, indicating a size decrease with increasing aqueous fraction. This is in agreement with Reference 30 in which the same surfactant mix with different weight fraction was applied (30). The larger fraction of aqueous media was used, the smaller capsules could be formed. Increasing water fraction would form more emulsified droplets (38) and decrease the mass of available monomer per unit interfacial area, resulting in nanocapsules with a thinner polymer wall and hence a smaller size.

According to the above discussion, the optimized polymerization condition for preparing the particle with minimal size was determined at pH = 2.0, W/O = 1/50 (v/v), and 108 mg/ml surfactant mix.

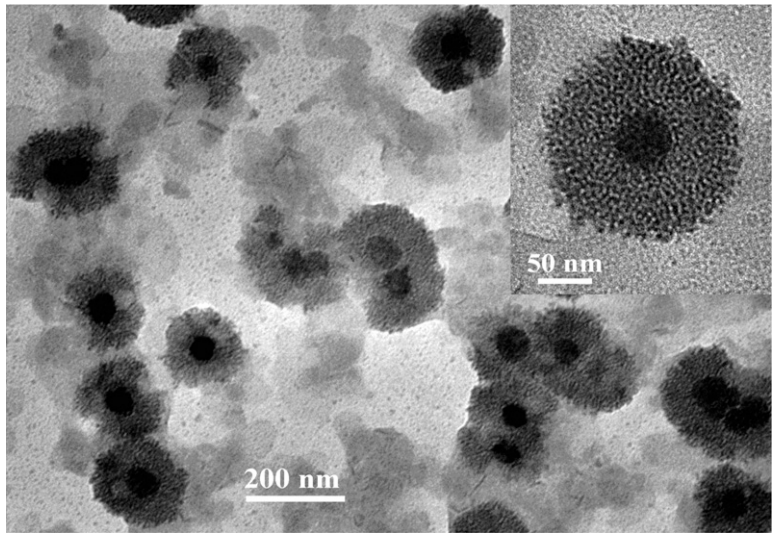

Figure 1. TEM photograph of AA entrapped PECA nanocapsules prepared under optimized polymerization condition with pH = 2.0, W/O = 1/50 (v/v), and 108 mg/ml surfactant mix. Inserted is an amplified picture of the typical AA loaded nanocapsules.

ENCAPSULATION OF AA

Figure 1 shows a TEM photograph of AA-encapsulated PECA nanocapsules prepared under the optimized polymerization condition. Inserted is an amplified picture of AA entrapped nanocapsules. As it can be seen, AA-encapsulated PECA nanocapsules were homogeneous core-shell-like spherical particles with the size ranged from 100 to 300 nm. The hyperchromatic core about 50 nm was the entrapped water soluble AA, while the light contrast shell was PECA wall with thickness about 70 nm.

Figure 2 represents the typical size distribution of AA loaded nanocapsules detected by DLS. The average size of nanocapsules was about 280 nm with the polydispersity index of 0.153.

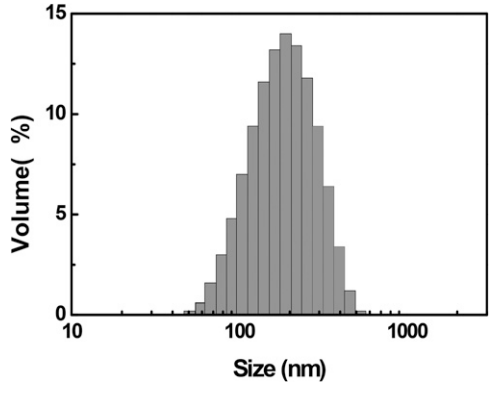

Figure 2. Size distribution of AA entrapped PECA nanocapsules prepared under optimized polymerization condition.

The encapsulation efficiency of AA was detected by an indirect fluorimetry method. AA has the ability to deoxidize the cerium (IV) ion with no fluorescence emitting to cerium (III) ion with characteristic fluorescence emitting in water solution. The addition of sodium hexametaphosphate could greatly enhance the fluorescence intensity of cerium (III) (31). According to the standard curve obtained from corresponding solution, the fluorescence intensity of cerium (IV)-AA at 340 nm in emission spectra was converted to the mass amount, and the entrapped AA was detected to be 0.116 mg per milligram nanocapsules prepared under the optimized polymerization condition. The corresponding encapsulation efficiency of AA in nanocapsules was estimated to be 87.5%.

STABILITY OF ENCAPSULATED AA

The stability of AA in aqueous solution was evaluated by monitoring the retention of AA in different storage periods at various temperatures. Figure 3 compares the retention stability of pure AA and encapsulated AA in PECA nanocapsules in aqueous solution at 40°C and 80°C, respectively. Curves (a) and (b) show that there were two periods for the degradation of pure AA at each temperature. In the first 4–6 h, a rapid decrease of active AA content took place, followed by degradation with relatively slow rate. The oxidation of AA proceeded faster at 80°C than at 40°C so that the active AA almost disappeared after being heated for 24 h at 80°C. Figure 4 shows the plots of $1/[AA]_t$ (the reciprocal of remaining AA concentration at time t) versus time for pure AA in aqueous solution at 40° and 80°C, respectively. Both curves show a good linear relationship. These results coincide with those reported in the literature (39–41) and imply that the degradation of AA under this condition was a second-order mechanism for which the oxidation rate was proportional to the square of the AA concentration. The degradation rate constants were calculated to be 5.28×10^{-4} $\%^{-1}\cdot h^{-1}$ and 6.32×10^{-3} $\%^{-1}\cdot h^{-1}$ for 40° and 80°C, respectively.

Curves (c) and (d) in Figure 3 illustrate the remaining content of AA encapsulated in PECA nanocapsules after being heated at 40° and 80°C. More than 90% of AA molecules were still active even being heated for 36 h, which implies that the encapsulated AA in

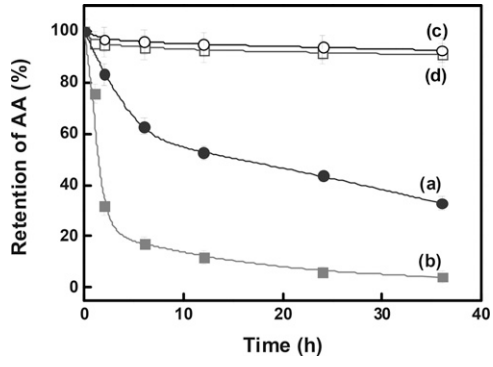

Figure 3. Retention curves of (a) pure AA in aqueous solution at 40°C, (b) pure AA in aqueous solution at 80°C, (c) encapsulated AA in nanocapsules at 40°C, and (d) encapsulated AA in nanocapsules at 80°C.

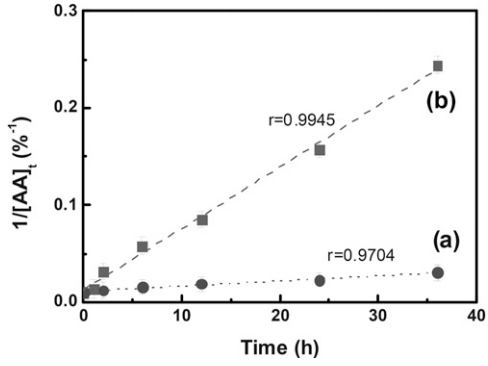

Figure 4. Second-order plots of pure AA degradation at (A) 40°C and (B) 80°C in aqueous solutions.

PECA nanocapsules was more stable than pure AA under the same conditions. The calculations give neither pseudo first-order nor second-order mechanism for degrading AA encapsulated in nanocapsules. It is not surprising since the PECA wall could act as a physical barrier to restrain the oxygen diffusing into nanocapsules. The excellent stability of AA is mainly attributed to the encapsulation of AA molecules within PECA nanocapsules that protected AA from contacting with oxygen.

DEGRADATION OF PECA AND *IN VITRO* RELEASE OF AA

By hydrolysis in aqueous phase, PECA could be degraded and release ethanol and water-soluble poly(2-cyanoacrylic) acid (42,43). The degradation rate was reported depending on both the polymer properties (e.g., molecular weight, form, and microsphere size) and the degradation medium conditions (i.e., medium pH, temperature, and initial microsphere concentration). In Figure 5, curve (a) gives the ethanol yield of enzymatic degradation of PECA as a function of hydrolysis time. The theoretical release amount of ethanol can be readily calculated, assuming that PECA could hydrolyze entirely. So the ethanol yield can be expressed as the percentage yield as shown in Figure 5. An initial burst of

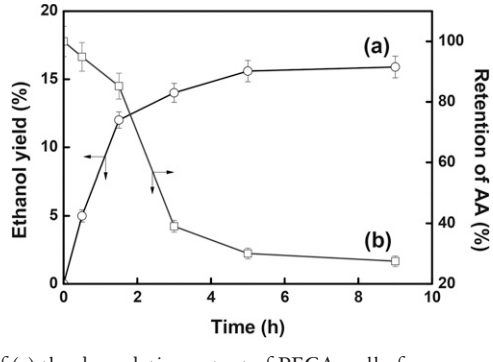

Figure 5. Curve profiles of (a) the degradation extent of PECA wall of nanocapsules and (b) the retention of AA in nanocapsules as functions of hydrolysis time in phosphate buffered saline solution.

ethanol in the first 1.5 h could be found, followed by a gradual increase of ethanol yield with time. The results are consistent with those of earlier reports in which a biphasic degradation mechanism of PACA were postulated (33,44). The initial quick release of ethanol signifies the cleavage of the ester side groups on the nanocapsules surface, while the second stage indicates the enzyme hydrolysis of the side groups of PECA within the nanocapsules matrix.

With the enzyme hydrolysis of hydrophobic PECA, the wall of nanocapsules becomes more and more hydrophilic, which is beneficial for the release of entrapped AA from nanocapsules. In Figure 5, curve (b) is a profile of the retention of AA in nanocapsules versus enzyme hydrolysis time. In the first 3 h of hydrolysis, a burst release of about 61% of encapsulated AA took place, and then followed with a gentle release. The detailed relationship between AA release and the degradation extent of PECA is revealed in Figure 6. The release behaviors are obviously dependent on the degradation extent of PECA, i.e., the retention of AA decreases with increasing the degradation extent of PECA. When ethanol yield was less than 12%, i.e., 88% of PECA was still undegraded, only about 15% of the entrapped AA could be released from the nanocapsules. The result implies that the hydrolysis reaction in this stage mainly took place on the surface of nanocapsules and the entrapped AA in the inner core could only be released by diffusing through PECA wall. Further increases in the degradation extent from 12% to 14% in the period between 1.5 and 3 h, a burst release of AA from 15% to 61% could be observed, which followed by slow and sustained release after being degraded for 3 h. The phenomena indicate that the enzyme hydrolysis was performed not only on the surface but also in the matrix of the nanocapsules, resulting in the deformation of nanocapsules and the rapid release of AA. Almost 72.5% entrapped AA was released from nanocapsules after 16% PECA was decomposed in 9 h.

It has been reported that PECA can degrade in the absence of esterase in contact with water (45) or even in solid phase (46). The degradation rate can be accelerated by increasing pH, elevating temperature, or by introducing esterase (34,43). Usually, the average moisture content of human skin is 35–60%, the pH value is 6.5–7.4, and the temperature is around 37°C. In such a case, even the esterase amount present in human skin, which is about 3.55×10^{-4} μg/mm^2, is lower than the value we used, the degradation of PECA can take place without doubt on human skin and release entrapped AA continually. Therefore,

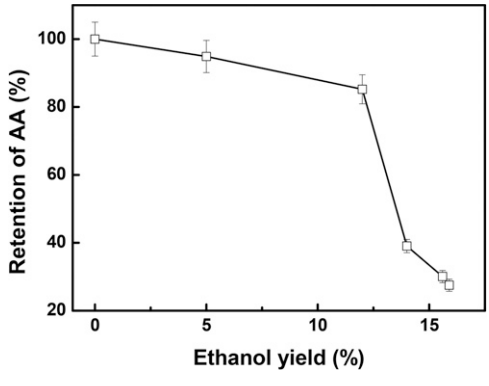

Figure 6. Relationship between AA retention and ethanol yield.

AA entrapped PECA nanocapsules are expected to be used as dermatological or cosmetic products, which can be easily decomposed to sustained release of AA for continual treatment.

CONCLUSIONS

This study demonstrates that AA could be encapsulated and protected successfully with biodegradable and biocompatible PECA nanocapsules synthesized by the interfacial polymerization of W/O microemulsions. The nanocapsule size could be controlled by manipulating surfactant concentration, pH value of the dispersed aqueous phase, and the aqueous fraction of the microemulsions. Low pH value and high fraction of aqueous phase were beneficial to getting small-sized nanocapsules, while too high or too low surfactant concentration was disadvantageous. The encapsulated AA was protected from oxidation in the core of nanocapsules and showed superior storage stability in aqueous medium compared to the pure AA. AA could be controlled released from nanocapsules by the enzyme hydrolysis of PECA wall. These findings are important factors for allowing AA-encapsulated PECA nanocapsules to be used as potential dermatological or cosmetic products for continual treatment.

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