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Increasing entrapment of peptides within poly(alkyl cyanoacrylate) nanoparticles prepared from water-in-oil microemulsions by copolymerization

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ABSTRACT

Low molecular hydrophilic actives such as peptides are typically poorly encapsulated within poly(alkyl cyanoacrylate) nanoparticles when prepared from micellar or microemulsion templates. The aim of the present study was to investigate whether the entrapment of peptides within poly(alkyl cyanoacrylate) nanoparticles could be increased by functionalizing the peptide so that it could copolymerize with the alkyl cyanoacrylate monomer. Peptide and acryloyl functionalized peptide representing the antigenic epitope of the lymphocytic choriomeningitis virus glycoprotein ($LCMV_{33–41}$) were synthesized using solid-phase peptide synthesis. Poly(alkyl cyanoacrylate) nanoparticles were prepared to encapsulate either peptide or functionalized peptide using both an aqueous micellar and a water-in-oil microemulsion polymerization template. Using the micellar template, nanoparticles could not be produced in the presence of acryloyl peptide. Rather an agglomerated mass formed on the stirrer. In contrast, nanoparticles could be prepared using both acryloyl and parent peptide using the water-in-oil microemulsion template. Encapsulation efficiency was more than twofold greater for functionalized peptide, being greater than 90%. Encapsulation efficiency of functionalized peptide was also observed to increase with increasing the amount of alkyl cyanoacrylate monomer used for polymerization. A biphasic release profile was observed for the nanoparticles entrapping the non-functionalized peptide with greater than 50% of peptide being released during the first 10 min and with around 90% being released at 6 h. In contrast, less than 10% of the total amount of acryloyl $LCMV_{33–41}$ entrapped within the nanoparticles was detected in the release media following the initial 10 min, and no further release of peptide was observed up to the termination of the release study at 360 min. The difference in entrapment and release kinetics between the parent and functionalized peptide strongly supports the presumption that most of the acryloyl peptide actually intervened in the copolymerization with alkyl cyanoacrylate monomer and was covalently bound within the nanoparticles instead of being physically entrapped or adsorbed which appeared to be the case for the parent peptide. Thus, functionalizing a peptide so that it can copolymerize with the alkyl cyanoacrylate monomer is a strategy which can be used to increase the entrapment efficiency of peptides within poly(alkyl cyanoacrylate) nanoparticles and also maintain the peptide associated with nanoparticles so that the benefits of nanoparticulate delivery can be exploited.

1. Introduction

Polymeric nanoparticles have gained extensive interests as drug carriers particularly for targeted delivery to sites of inflammation including tumours, exploiting the "enhanced permeation and retention" effect, and in delivery of vaccine antigens, exploiting the inherent adjuvant activity of particles (Grislain et al., 1983; O'Hagan et al., 1989; Lu and He, 2001; Singh et al., 2007). Polymeric nanoparticles have also been used to promote the oral absorption of proteins and peptides, which is accomplished by their entrapment protecting them from proteolytic enzymes and particle translocation across the intestinal mucosa facilitating absorption (Florence et al., 1995; Damge et al., 1997; Jung et al., 2000; Watnasirichaikul et al., 2002a). Among such polymeric nanoparticles, those fabricated from poly(alkyl cyanoacrylate) (PACA) have been studied extensively over the past two decades as they can be prepared under mild conditions, are biodegradable and their rate of degradation can be manipulated by the choice of monomer used (Vauthier et al., 2003).

PACAs are formed by anionic chain polymerization of the respective alkylcyanoacrylate monomer following nucleophilic attack on

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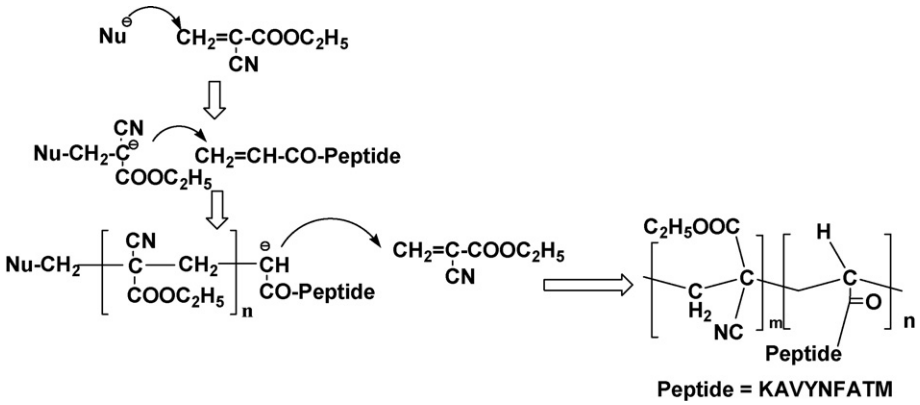

Fig. 1. Copolymerization mechanism of alkyl cyanoacrylate and acryloyl peptide.

the β-carbon resulting in a reactive carbanion which is involved in chain propagation (Fig. 1). The initiating nucleophile is often the hydroxyl ion of water and PACA nanoparticles can thus be formed in systems containing water in the presence of a nanostructured template, such as micelles or submicron emulsions. Polymerization of alkylcyanoacrylate monomers on a micellar template was first introduced by Couvreur et al. in 1979. In micellar polymerization, droplets of liquid monomer are typically added to an acidic aqueous solution containing surfactant. The monomer, which has poor aqueous solubility, localizes within the micelles where polymerization takes place. This approach is more suitable for entrapment of lipophilic actives as these molecules are also localized in the micelles where polymerization occurs, while entrapment of hydrophilic actives such as peptides is usually low due to the aqueous nature of the continuous phase. Reasonable entrapment of some peptides has been achieved by adsorbing them onto preformed nanoparticles (Fresta and Puglisi, 1994). However, surface adsorbed peptide is not physically protected from proteolytic enzymes and hence is more likely to be degraded than encapsulated peptide (Peula et al., 1998). For this reason, and also to increase entrapment efficiency of hydrophilic molecules, researchers have investigated the possibility of using w/o submicron emulsions or microemulsions as templates to prepare PACA nanocapsules where polymerization is believed to occur at the interface and hence any water-soluble active may be efficiently encapsulated (Lambert et al., 2000; Watnasirichaikul et al., 2000). Microemulsions offer several advantages over the use of submicron coarse emulsions in that they form spontaneously, are thermodynamically stable and have small and uniform dispersed aqueous droplets, often described as swollen micelles. All these size characteristics are reflected in the characteristics of the nanocapsules prepared from such a template (Watnasirichaikul et al., 2002b).

Despite being able to efficiently entrap large proteins, interfacial polymerization of microemulsions has proved less effective for the entrapment of small molecules with entrapment efficiency apparently being a function of molecular weight (Pitaksuteepong et al., 2002). It was reported that the entrapment efficiency of bioactives having a molecular weight of less than 1000 was less than 20% as compared to 97% for a protein having a molecular weight of 45,000. Strategies to increase the encapsulation efficiency of small bioactive molecules such as low molecular weight peptides are therefore required. Several authors have previously reported that molecules added to the polymerization template before or early on in the polymerization may intervene in the reaction and undergo copolymerization and consequently be entrapped (Guise et al., 1990; Grangier et al., 1991). Hillery et al. (1996) investigated the possibility of copolymerization as a strategy to increase entrapment of peptides within PACA nanoparticles prepared by micellar polymerization. The luteinizing hormone releasing hormone (LHRH) peptide was functionalized by conjugating with a range of unsaturated acids with the premise that the conjugate by virtue of its unsaturation would intervene in the polymerization process and form a constituent of the oligomeric alkylcyanoacrylate chain. In their study however, an aggregated copolymer of vinyl LHRH and *n*-butyl cyanoacrylate resulted and this aggregate had to be re-dissolved and subsequently formulated into nanoparticles by precipitation.

The objective of this work was to investigate whether the aggregation noted in the copolymerization from the use of a micellar template as reported by Hillery et al. (1996) could be overcome by the use of a w/o microemulsion template. A further objective of the work was to extend the findings to prepare PACA nanocapsules encapsulating a model peptide antigen, being the antigenic epitope of the lymphocytic choriomeningitis virus glycoprotein ($LCMV_{33–41}$). $LCMV_{33–41}$ was chosen as the model antigen as this peptide is expressed by the Lewis lung carcinoma cell line and as such would subsequently allow assessment of the system in terms of its ability to stimulate an immune response (Hermans et al., 1997).

2. Materials and methods

2.1. Materials

9-Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids were obtained from Novabiochem (Läufelfingen, Switzerland). Trifluoroacetic acid (TFA), *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU), piperidine and *N*,*N*′-dimethylformamide (DMF) were obtained from Auspep (Victoria, Australia). Diisopropylcarbodiimide (DIC), 1-hydroxy-7-azabenzotriazole (HOAt), *N*,*N*′-diisopropylethylamine (DIPEA), triisopropylsilane (TIPS) and all other chemicals were purchased from Sigma–Aldrich (Missouri, USA). Ethyl oleate (Crodamol EO™), polyoxyethylene 20 sorbitan mono-oleate (Crillet 4 super™), and sorbitan monolaurate (Crill 1™) was obtained from Croda Oleochemicals (Auckland, New Zealand). Ethyl-2-cyanoarylate and *n*-butyl-2-cyanoacrylate were gifts from Henkel Loctite Ltd (Australia). De-ionized water was used in all studies.

2.2. Peptide synthesis and characterization

$LCMV_{33–41}$ having the peptide sequence KAVYNFATM was assembled manually using Fmoc chemistry on Rink amide resins from the C to the N terminus. After the assembly of the peptide

was complete, the resin was split and part used for the synthesis of the functionalized acryloyl derivative. *N*-Acylation was carried out by removal of the terminal Fmoc protecting group and subsequent addition of equimolar amounts of acryloyl chloride and DIPEA. All couplings throughout the synthesis of the peptide and its functionalized derivative were monitored by quantitative ninhydrin test. The peptide or acryloyl derivative was cleaved from the resin using a cleavage cocktail of 88% TFA, 5% phenol, 5% water, and 2% TIPS for 2 h. The crude peptide was purified by preparative RP-HPLC on a Vydac C18 22 mm × 250 mm column, using a linear solvent gradient from 100% solvent A to 80% solvent B (solvent A: 0.1% TFA in water, solvent B: 90% acetonitrile, water and 0.1% TFA) over 45 min at a flow rate of 5 mL/min. The presence of desired peptide in fractions was confirmed by electrospray mass spectrometry, and the purity of preparative HPLC fractions was determined by analytical RP-HPLC using a Vydac C18 4.6 mm × 250 mm column with a flow rate of 1 mL/min and a linear gradient from 100% solvent A to 80% solvent B over 30 min. The fractions containing pure peptide were combined and lyophilized to yield a white powder.

2.3. Preparation of PACA nanoparticles

PACA nanoparticles were prepared by two methods using a micellar dispersion and a w/o microemulsion according to the methods previously described by Hillery et al. (1996) and Watnasirichaikul et al. (2000), respectively. For studies using micellar polymerization, the typical polymerization medium was an aqueous solution of 0.1% Pluronic F68 and 0.1% Dextran 70 adjusted to pH 3.0 with HCl. 1 mg of acryloyl peptide dissolved in 1 mL of distilled water and alkyl cyanoacrylate monomer (20, 40 or 100 mg) dissolved in 1 mL of ethanol were injected separately and simultaneously into the polymerization medium. In another procedure, 1 mg of acryloyl peptide was first dissolved in 10 mL of the polymerization medium and the polymerization was initiated by the addition of alkyl cyanoacrylate monomer. For studies using the microemulsion template, a w/o microemulsion was formed by mixing 1.1 g of ethyl oleate, 0.7 g of surfactant mixture (Crillet 4 super: Crill 1 6:4, w/w) and 0.2 g of water. Alkyl cyanoacrylate monomer (10, 20, or 40 mg) dissolved in three times of chloroform (v/v) was added slowly to the microemulsion under mechanical stirring, and the mixture was left for at least 4 h for polymerization. The nanoparticles were separated from the polymerization media by centrifugation at 30,000 × *g* for 1 h at 10 °C (Jouan KR22i centrifuge, AK16.20 rotor). For preparation of peptide-loaded nanocapsules, 1 mg peptide was dissolved in the aqueous phase prior to addition of oil and surfactant mixture.

2.4. Characterization of nanoparticles

The size and size distribution of nanoparticles were measured by photon correlation spectroscopy (Zetasizer 3000, Malvern Instruments Ltd.). For nanoparticles prepared by micellar polymerization, samples were dispersed in water to measure particle size. For nanoparticles prepared by microemulsion polymerization, residual surfactant and oil were removed by washing twice in ethanol and centrifugation (15,000 × *g* for 10 min at 10 °C). The nanoparticles were then dispersed in ethanol containing 0.2% (w/w) polysorbate 80 for particle size analysis (Watnasirichaikul et al., 2000). Measurements were carried out at room temperature. The morphological structure of the nanoparticles was visualized by scanning electron microscopy (JEOL-6400, Tokyo, Japan). A drop of nanoparticle suspension in ethanol was placed on a 400-mesh carbon-coated copper grid. After drying/evaporation of the ethanol, the samples were sputter-coated with a gold–palladium alloy and analyzed at an accelerating voltage of 10 kV.

2.5. Determination of entrapment efficiency

Determination of entrapment efficiency of the parent and functionalized peptides in the nanoparticles was achieved by complete hydrolysis of the polymer and peptides using hydrochloric acid and subsequent quantitation of amino acids using a modified calorimetric ninhydrin assay (Gupta et al., 1997). Briefly, after isolation from the polymerization medium by centrifugation and washed twice with ethanol, samples were dried and hydrolysed with 2 mL of 6N HCl at 110 °C for 24 h in sealed glass tubes. After hydrolysis, the samples were freeze-dried and reconstituted in 1 mL de-ionized water. For the ninhydrin assay, 100 μL of sample was mixed with 150 μL 0.2 M citrate buffer (pH 5.0) containing 0.16% (w/v) stannous chloride and 150 μL 2-methoxyethanol with 4% (w/v) ninhydrin. The mixture was heated at 95 °C for 25 min. After the sample was cooled, 500 μL of 50% (w/v) *n*-propanol was added to each sample and the UV absorbance was measured at 570 nm. The amount of peptide entrapped within nanoparticles was determined from a standard curve generated by hydrolysing known amounts of free peptide in the presence of empty nanoparticles.

2.6. In vitro release study

Nanoparticles were collected from the polymerization medium by centrifugation, and were dispersed in 10 mL phosphate buffer solution (PBS, pH 8) in a thermostated beaker (37 °C). The sample was continuously stirred using a magnetic bar at a rate of 200 rpm. At selected times, the sample was transferred into a tube and centrifuged at 30,000 × *g* for 10 min. The supernatant was removed and the precipitated nanoparticles were redispersed in 10 mL fresh PBS and placed back in the thermostated beaker for further monitoring of drug release. The concentration of peptide in the supernatant was determined by quantitative RP-HPLC analysis using a Vydac C18 4.6 mm × 250 mm column with a flow rate of 1 mL/min and a linear gradient from 100% solvent A to 80% solvent B over 30 min.

3. Results and discussion

3.1. Synthesis of acryloyl peptide

In preliminary studies, functionalization of the peptide was attempted by coupling acrylic acid to the N-terminus of peptide using HBTU or DIC/HOAt as coupling/activation reagents according to the methods reported by Hillery et al. (1996). Both methods gave very poor yields of *N*-acryloyl peptide which were contaminated with many un-identified side-products as observed by mass spectroscopy. However, consistent and high yield of *N*-acryloyl peptides was achieved by acylation of the peptide N-terminal amino group with acryloyl chloride. The crude acryloylated peptide was purified by preparative HPLC and the purity of the peptide was confirmed to be more than 95% by analytical HPLC. The acryloyl peptide showed an increased retention time compared to the non-acryloylated, parent peptide, with the retention time of 17.2 min compared to 15.5 min. The successful synthesis of the parent and functionalized $LCMV_{33–41}$ were confirmed by LC–MS with the purified products showing characteristic *m*/*z* peaks at the expected values of 1044 and 1099 $[M+H]^+$, respectively.

3.2. Preparation and characterization of nanoparticles

In the preliminary investigations for micellar polymerization, acryloyl peptide in water and alkyl cyanoacrylate monomer in ethanol was added separately and simultaneously into the polymerization medium under vigorous stirring. The formed copolymer

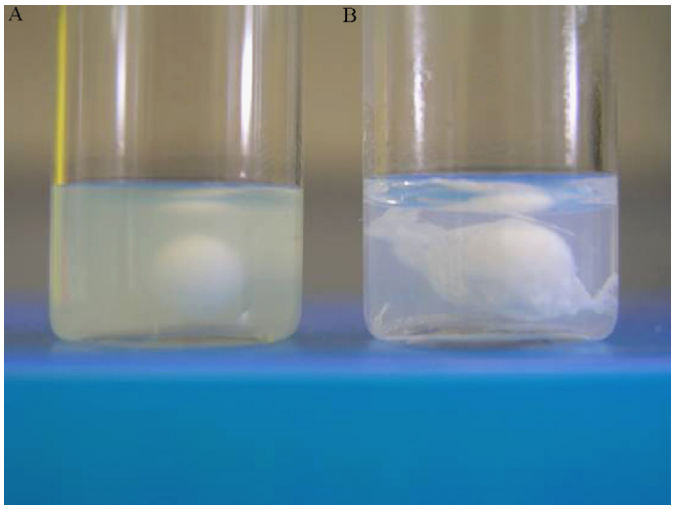

Fig. 2. Polymerization media following copolymerizing of acryloyl $LCMV_{33-41}$ with alkyl cyanoacrylate by using a w/o microemulsion (A) and a micellar template (B).

precipitated out from the reaction media and produced large aggregate around the magnetic stirring bar, regardless of the ratio of functionalized peptide to monomer (Fig. 2). This is in agreement with the previous report as described by Hillery et al. using a similar procedure for the preparation of PACA nanoparticles (Hillery et al., 1996). This could possibly be explained by the fact that acryloyl peptide is hydrophilic and as such, would not be expected to localize within the hydrophobic domains of the micellar template. Rather, the functionalized peptide would be expected to disperse within the aqueous continuous phase. Therefore, the alkyl cyanoacrylate monomer may be able to copolymerize with the acryloyl peptide in the aqueous continuous phase which cannot act as a template for nanoparticle formation. This leads to the formation of an aggregated mass instead of forming discrete nanoparticles as is obtained when polymerization occurs within the micelle. A second procedure was also investigated in which acryloyl peptide was first dispersed homogeneously throughout the polymerization media and polymerization was subsequently initiated by adding alkyl cyanoacrylate monomer. Using this procedure, nanoparticles were successfully prepared both in the absence of peptide as well as with unmodified peptide, with the size of nanoparticles being around 250 nm. However, similar aggregation behaviour was again observed when functionalized acryloyl peptide was used with a polymer mass forming around the magnetic stirrer (Fig. 2).

As a result of the aggregation observed and failure to produce nanoparticles using micellar polymerization, a second procedure for the preparation of PACA nanoparticles was investigated using a w/o microemulsion as a polymerization template. Microemulsions are transparent and thermodynamically stable colloidal systems which form spontaneously and reproducibly. Microemulsions can have a droplet (oil in water, o/w, or water in oil, w/o) or a bicontinuous structure and have been used as templates for the formation of nanoparticles including PACA nanoparticles. In w/o microemulsions, small and uniform water-swollen micelles of surfactants are considered to be dispersed in an oil continuous matrix. Upon addition of alkyl cyanoacrylate monomer to the w/o microemulsion, polymerization is believed to occur at water–oil interface and initiated by the hydroxyl ions present in the aqueous domains (Gasco and Trotta, 1986). The polymer thus formed around the swollen aqueous micelle to produce nanocapsules whose wall thickness can be controlled by varying the ratio of monomer to the mass of water contained in the microemulsion (Watnasirichaikul et al., 2002b). In the present study, the w/o microemulsion used was

Table 1
Particle size of nanoparticles prepared by the interfacial polymerization of w/o microemulsion templates

Formulation	Mass of ethyl cyanoacrylate monomer		
	10 mg	20 mg	40 mg
No peptide (nm)	–	–	182 ± 8
$LCMV_{33-41}$ (nm)	–	–	186 ± 11
Acryloyl $LCMV_{33-41}$ (nm)	158 ± 12	174 ± 14	206 ± 15

based on the pseudoternary phase diagram reported by Alany et al. (2001). Ethyl oleate was used as the oil component, and a 6:4 weight ratio of poly(oxyethylene 20) sorbitan monooleate and sorbitan monolaurate was used as the surfactant. The acryloyl peptide was solubilized in the aqueous phase of the microemulsions at a concentration of 5 mg/mL. The solubility of acryloyl peptide in water was measured by liquid chromatography coupled to electrospray mass spectroscopy and was determined to be 5.42 ± 0.39 mg/mL. To maximize the possible payload within nanoparticles prepared from this method, the maximum percentage of water (10%, w/w) was used for the formation of the droplet microemulsion as this allows the greatest amount of peptide to be solubilized. Increasing the percentage of water beyond 10% results in the transformation of the w/o droplet microemulsion system to a bicontinuous system (Alany et al., 2001). Therefore, for the preparation of peptide-loaded nanoparticles, 1 mg of peptide (parent or functionalized) was firstly dissolved in water. No precipitation of the peptide was observed when the aqueous solution of peptide was added to the oil components to form the microemulsions. A solution of ethyl cyanoacrylate (10, 20 or 40 mg) in chloroform (v/v, 1/3) was added slowly under magnetic stirring to initiate the polymerization. Polymerization was complete after 4 h and the reaction media was observed to become a turbid suspension (Fig. 2). After isolation from the polymerization medium, the nanoparticles were characterized by photo-correlation spectroscopy (Table 1). The polydispersity indexes were less than 0.2 in all cases, which indicates that the nanoparticles formed had a relatively narrow size distribution. A scanning electron micrograph of the nanoparticles obtained by interfacial copolymerization of acryloyl $LCMV_{33-41}$ and 40 mg ethyl cyanoacrylate using a microemulsion template is shown in Fig. 3. It can be seen from the micrograph that nanoparticles have a spherical structure with a size of around 200 nm which

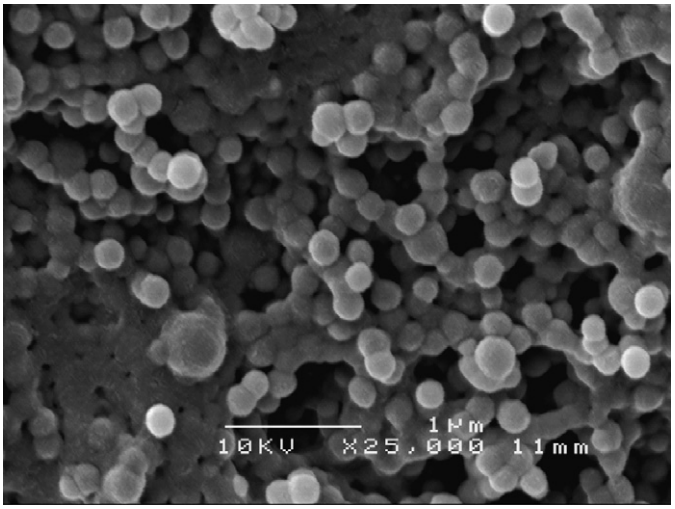

Fig. 3. Scanning electron micrograph of nanoparticles obtained by interfacial copolymerization of acryloyl $LCMV_{33-41}$ and 40 mg ethyl cyanoacrylate using w/o microemulsion template.

is in agreement with previous reports when droplet microemulsion have been used as a template for the formation of PACA nanoparticles (Watnasirichaikul et al., 2000). Interestingly, nanoparticles prepared using acryloyl peptide had a larger size than nanoparticles prepared from the parent peptide which in turn had a larger size than nanoparticles prepared from peptide-free microemulsions (Table 1). In agreement with previous studies, size of nanoparticles also increased with increasing amount of monomer used for polymerization (Watnasirichaikul et al., 2002b).

The successful preparation of nanoparticles using acryloyl functionalized peptide and a droplet w/o microemulsion template in contrast to the use of a micellar template probably is a result of the reactive, functionalized peptide being localized and confined within the aqueous domain of the w/o droplet microemulsion. Upon addition of the alkyl cyanoacrylate monomer, polymerization occurs at the water–oil interface as initiated by the hydroxyl ions of water and the acryloyl peptide can copolymerize during chain propagation. Thus, it would appear from the above results that a droplet water-in-oil template is critical for the copolymerization of acryloyl peptide and alkyl cyanoacrylate to form nanoparticles as this confines the reactive acryloyl peptide to the aqueous dispersed droplets rather than the reactive peptide being dispersed throughout the aqueous phase as in the micellar template. In contrast to when using acryloyl peptide, nanoparticles could be successfully prepared in the presence of unreactive parent peptide using both templates having a size of around 250 and 180 nm when prepared using a micellar and a microemulsion template, respectively.

3.3. Entrapment efficiency of peptides in nanoparticles

After nanoparticles were separated from the microemulsion by ultracentrifugation, the amount of peptide within nanoparticles was determined by complete hydrolysis of the polymer and peptide with 6N HCl and subsequent quantitative amino acids analysis using a modified ninhydrin assay. The entrapment efficiency of acryloyl $LCMV_{33-41}$ and $LCMV_{33-41}$ are summarized in Fig. 4. Over 90% of acryloyl $LCMV_{33-41}$ was found to be incorporated within nanoparticles when 40 mg of ethyl cyanoacrylate monomer was used for polymerization, while only 40% entrapment was achieved for the unfunctionalized $LCMV_{33-41}$ when the same amount of monomer and conditions were used. This can be explained by the fact that acryloyl $LCMV_{33-41}$ is able to copolymerize with the alkyl cyanoacrylate monomer by virtue of its vinyl group, and thereby become incorporated into the growing polymer chain and covalently bound and associated within the nanoparticles (Fig. 1). The parent $LCMV_{33-41}$, however, is a low molecular weight hydrophilic peptide which does not contain any unsaturated groups able to participate in the chain polymerization (although it does contain functional groups able to react with the reactive carbanion to result in termination of the polymerization process). Physical entrapment of small and hydrophilic molecules within nanoparticles as prepared by interfacial polymerization of w/o microemulsions has previously been reported to be relatively inefficient. This was proposed to be due to the ability of low molecular weight hydrophilic molecules together with water to readily diffuse and escape through the forming polymeric wall (Pitaksuteepong et al., 2002). As mentioned, the possibility exists for the parent $LCMV_{33-41}$ to react with the reactive carbanion by virtue of the presence of nucleophilic groups such as amine or hydroxy groups. Intervention of bioactives to be encapsulated in the polymerization process has previously been reported and proposed as one of the mechanisms which contribute to the entrapment of low molecular weight bioactives within PACA nanoparticles as prepared by micellar polymerization (Labib et al., 1991). However, even if intervention of the parent peptide in the polymerization is possible, entrapment of the functionalized peptide was almost twice as much being over 90% as compared to 40% for the parent peptide.

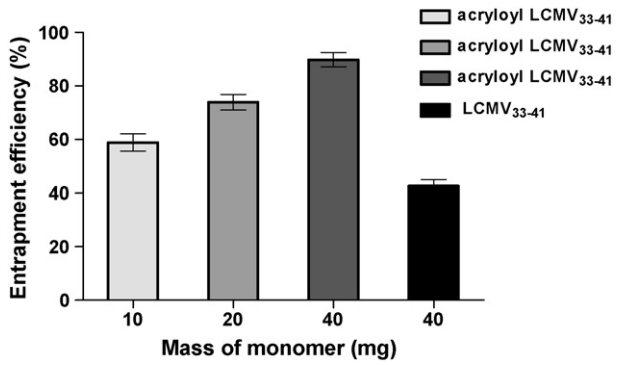

Fig. 4. Encapsulation efficiency of parent and functionalized $LCMV_{33-41}$ peptide within nanoparticles prepared using different masses of ethyl cyanoacrylate monomer ($n = 3$, mean ± S.D.).

The results also revealed that the amount of acryloyl peptide incorporated into the nanoparticles was dependent on the mass of the monomer used in the polymerization. A decrease in entrapment efficiency was observed upon using less monomer, with measured entrapment efficiencies of 70% and 60% for nanoparticles prepared using 20 and 10 mg of ethyl cyanoacrylate monomer, respectively. In a previous study by Watnasirichaikul et al., an increase in entrapment efficiency of insulin was reported upon using increasing masses of alkyl cyanoacrylate monomer for the polymerization of w/o microemulsion templates. This was considered to be a result of the formation of thicker walled capsules at higher masses of monomer which would serve to increase entrapment (Watnasirichaikul et al., 2002b). In addition to the formation of thicker walled nanocapsules using higher masses of monomer, the possibility also exists in the case of acryloyl peptide that using larger quantity of alkyl cyanoacrylate monomer allows for increased opportunity for copolymerization to occur thus increasing entrapment of the functionalized peptide. It is worth noting that although increasing monomer mass results in increased entrapment efficiency, the peptide loading within the nanoparticles, i.e. mass peptide/mass polymer actually decreases upon increasing mass of monomer used for polymerization.

3.4. In vitro release study

The *in vitro* release profiles of $LCMV_{33-41}$ and acryloyl $LCMV_{33-41}$ from nanoparticles prepared by the addition of 40 mg of ethyl cyanoacrylate to 2 g microemulsion containing 10% aqueous fraction are shown in Fig. 5. The concentration of peptide in the release buffer samples was determined by quantitative HPLC analysis, and release over 360 min was measured. The release of the parent $LCMV_{33-41}$ peptide from nanoparticles showed a biphasic profile. During the first 10 min, more than 50% of the total amount of $LCMV_{33-41}$ peptide entrapped within the nanoparticles was detected in the release media. This initial burst release can likely be attributed to the fast desorption of the fraction of peptide physically bound to the surface of the nanoparticles, and possible rapid diffusion of peptide located near the surface of nanoparticles. The burst release phase was followed by a second phase of slow release to around 90% release over the period 30–360 min. This second release phase can likely be attributed to release of encapsulated peptide from the inner core of the nanoparticles. As nearly complete release of peptide is observed over 6-h release study, it would seem likely that encapsulation of parent peptide is mainly a

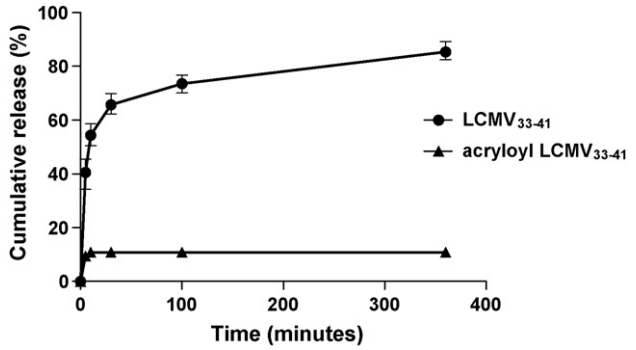

Fig. 5. *In vitro* release of $LCMV_{33–41}$ (●) and acryloyl $LCMV_{33–41}$ (▲) from nanoparticles prepared using 40 mg of ethyl cyanoacrylate monomer in phosphate buffer pH 8.0 ($n = 3$, mean ± S.D.).

result of adsorption and physical entrapment rather than as a result of the peptide being covalently entrapped due to intervention in the polymerization process. This is further confirmed by the difference in release profile observed between the parent $LCMV_{33–41}$ and the functionalized acryloyl $LCMV_{33–41}$ designed to undergo copolymerization. For the acryloyl $LCMV_{33–41}$, less than 10% of the total amount of acryloyl $LCMV_{33–41}$ entrapped into the nanoparticles was detected in the release media following the initial 10 min, and no further release of peptide was observed up to the termination of the release study at 360 min. This difference in release kinetics between the parent and functionalized peptide strongly supports our presumption that most of the acryloyl peptide actually intervened in the copolymerization with ethyl cyanoacrylate monomer and was covalently bound with the nanoparticles as shown schematically in Fig. 1.

4. Conclusion

In this study, a functionalized peptide namely acryloyl $LCMV_{33–41}$, which could intervene in chain polymerization of alkyl cyanoacrylates, was prepared with high yield and purity. The feasibility of copolymerizing acryloyl $LCMV_{33–41}$ with alkyl cyanoacrylate monomer to prepare peptide-loaded nanoparticle was then demonstrated by employing an interfacial polymerization technique using a w/o microemulsion template. Using this technique, the peptide-loaded nanoparticles were formed *in situ* in the polymerization media and no apparent polymer aggregate was observed during the process of copolymerization. More importantly, most of the peptide was covalently bound within nanoparticles and high entrapment efficiency within nanoparticles was achieved even for a low molecular weight hydrophilic peptide. The covalent attachment of peptides within nanoparticles may be beneficial in drug delivery, as it could help to retain the bioactive molecule within the nanoparticle to exploit the beneficial effects of particulate systems (enhanced permeation and retention phenomena, uptake by phagocytic cells, intestinal translocation) and avoid the burst release as can be observed when hydrophilic actives are physically adsorbed or encapsulated in nanoparticles. Further, the importance of the nature of the template used for the preparation of functionalized peptide-loaded nanoparticles has been highlighted. As the acryloyl peptide is able to copolymerize with the alkyl cyanoacrylate monomer and as a result of its hydrophilic nature, it is critical to use polymerization templates in which the reactive peptide is confined to aqueous domains which act as template for nanoparticle formation. Hence, functionalized peptide-loaded nanoparticles can be prepared from o/w microemulsions but cannot be prepared using micellar polymerization in which water is present as the continuous phase.

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