

A NOVEL STRATEGY FOR DETERMINING THE AMOUNT OF PROTEIN ENCAPSULATED IN POLYMERIC MICROPARTICLES

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PURPOSE

Several different methods have been reported for determination of protein loading of polymeric microparticles (MP):

- (i) extraction techniques using e.g. alkaline SDS solution or methylene chloride,
- (ii) solubilization of polymer(s) and protein in a single phase, followed by spectrophotometric determination of total protein,
- (iii) complete hydrolysis of MP with 6N hydrochloric acid (HCl) at 110°C for 20h, followed by amino acid analysis (1,2).

For a number of proteins extraction methods were shown to give variable results which depend on the physical characteristics of the MP, the type of polymer(s), the distribution of protein inside the MP and *in vitro* release profiles (1). Spectrophotometric methods are a good choice provided that a solvent or solvent/co-solvent mixture is known which completely dissolves both the polymer(s) and the protein.

Hydrolysis followed by amino acid analysis has proven to yield accurate values for protein loading. Amino acid analysis is however relatively difficult and expensive to perform, and consequently is only available in a small number of specialized labs.

To overcome these limitations, we developed a novel easier-to-perform acid hydrolysis method for quantitating the protein content of polymeric MP. This new hydrolysis method is based on complete digestion of MP with HCl followed by quantitation of L-glutamic acid with a commercially available glutamic acid quantitation kit. The results obtained by this novel hydrolysis method were compared with those obtained with a spectrophotometric technique.

METHODS

Preparation of microparticles containing ovalbumin (OVA):

50mg of poly(lactide-co-glycolide) (PLGA) 50:50 (Resomer RG503H, Boehringer Ingelheim) and 50mg of a copolymer of acrylic and methacrylic acid esters were dissolved in 2ml of ethyl acetate, whereas the OVA (5mg for microparticle preparation 1 and 10mg for microparticle preparation 2) was solubilized in 0.4ml of water. OVA-containing MP were prepared using a classical w/o/w double emulsion solvent evaporation method. The resulting OVA-containing MP were filtered, washed with deionized water and air-dried at atmospheric pressure at room temperature for 18h.

Complete acid hydrolysis of the ovalbumin (OVA) entrapped in the MP, followed by L-glutamic acid quantitation:

Samples of the OVA-loaded MP (dry weight ca. 5mg) were resuspended in 0.8ml 6N HCl and subsequently incubated for 15h at 110°C. The HCl was then completely evaporated from the samples under a stream of nitrogen gas at 60°C. The dry residue containing free amino acids and the degradation products of PLGA and copolymer was reconstituted with water and L-glutamic acid was quantitated using a commercially available L-glutamic acid quantitation kit (Boehringer Mannheim/R-Biopharm). The amount of OVA entrapped in the MP was determined from an OVA standard curve generated by hydrolyzing known amounts of free OVA with 6N HCl. OVA standard curves generated in the presence and absence of 2.5mg/ml PLGA and 2.5mg/ml copolymer were found to be identical (data not shown).

Spectroscopic determination of the OVA entrapped in the MP:

Samples of OVA-loaded MP (dry weight ca. 5mg) were completely solubilized in a dimethylformamide (DMF)/8M aqueous guanidine hydrochloride (GnHCl) (9/1,v/v)

mixture. The spectra were corrected for (i) the absorbance of DMF/8M aqueous GnHCl (9/1) mixture and (ii) the light scattering (by extrapolation of the absorbance between 320 and 400nm in a log-log form and subtraction of the scattering component). The amount of OVA entrapped in the MP was determined from an OVA standard curve generated by dissolving known amounts of free OVA in a DMF/8M aqueous GnHCl (9/1) mixture and by plotting the absorbance at 280nm against the OVA concentration. OVA standard curves generated in the presence and in the absence of 2.5mg/ml PLGA and 2.5mg/ml copolymer were found to be identical (data not shown).

RESULTS

Fig. 1A (red line) shows a representative UV absorption spectrum of free OVA dissolved in a DMF/water (9/1) mixture. Dissolution of OVA in DMF/water (9/1) induces the formation of soluble protein aggregates. Aggregate formation can be suppressed by dissolving OVA in a DMF/8M aqueous GnHCl (9/1) mixture (see black line in Fig. 1A). An OVA standard curve was generated by dissolving varying amounts of free OVA in DMF/8M aqueous GnHCl (9/1) and plotting the absorbance values at 280nm against the OVA concentration (see Fig. 1B).

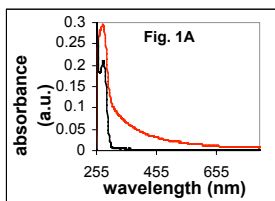


Figure 1A: Spectrum of 200µg/ml OVA dissolved in a DMF/water (9/1,v/v) mixture (red line) and spectrum of 300µg/ml OVA dissolved in a DMF/8M aqueous GnHCl (9/1,v/v) mixture (black line).

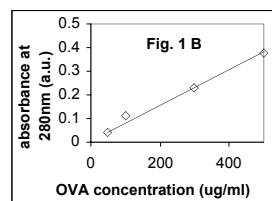


Figure 1 B: OVA standard curve obtained by dissolving varying amounts of OVA in a DMF/8M aqueous GnHCl mixture (9/1,v/v).

Fig. 2 shows a representative UV absorption spectrum of OVA-containing MP dissolved in a DMF/8M aqueous GnHCl (9/1) mixture (thick red line). Soluble protein aggregates causing considerable light scattering, appearing in the absorption spectrum at wavelengths extending well above 320nm were observed in all MP samples examined. The spectrum was corrected for light scattering by extrapolation of the absorbance between 320 and 400nm in a log-log form, and subtraction of the scattering component (see black line in Fig. 2). Fig. 2 (thin red line) displays the spectrum corrected for light scattering.

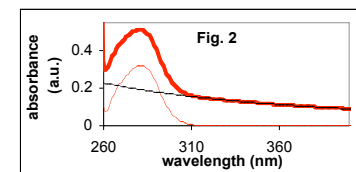


Figure 2: Spectrum of 5.3mg of OVA-containing MP dissolved in a DMF/8M aqueous GnHCl (9/1,v/v) mixture (thick red line). Scattering component of the spectrum (black line). Spectrum after subtraction of the scattering component (thin red line).

Table 1 shows the total protein loadings of two different MP formulations determined by (i) complete acid hydrolysis of the OVA entrapped in the MP, followed by L-glutamic acid quantitation as described Methods section (Method A) and (ii) dissolving the MP in a DMF/8M aqueous GnHCl (9/1,v/v) mixture and performing UV absorption spectroscopy as described in Methods section (Method B).

Preparation	Theoretical loading (µg of OVA per mg dry weight of MP)	Experimentally determined loading (µg of OVA per mg dry weight of MP)	
		Method A	Method B
Microparticle preparation 1	50	39.7±9.4	31.8 ±1.8
Microparticle preparation 2	100	78.7 ±0.9	67.8 ±8.1

Table 1: Comparison of the proteins loadings obtained by spectrophotometry (Method B) and by acid hydrolysis, followed by L-glutamic acid quantitation (Method A). Values represent ion (Method A). Values represent the mean of two independent preparations of OVA-loaded MP.

CONCLUSIONS

The novel strategy was compared to one of the spectrophotometric methods that are commonly used for determining the amount of protein incorporated into polymeric microparticles. The protein loadings determined by the two methods are very similar, indicating that the novel strategy is applicable to microparticles made up of mixtures of poly(lactide-co-glycolide) with a copolymer of acrylic and methacrylic acid esters.

REFERENCES

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- [2]. R. K. Rajesh et al., Vaccine, **15**, 672-678 (1997)