**Vibrio cholerae**-loaded poly(DL lactide-co-glycolide) microparticles

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**Vibrio cholerae** (VC)-loaded microparticles were prepared using poly(DL lactide-co-glycolide) with a water-in-oil-in-water emulsion/solvent extraction technique. Particle characteristics including size distribution, VC-loading efficiencies, and *in-vitro* release pattern were investigated. The dispersed phase was PLG dissolved in dichloromethane, and the continuous phase was water containing PVP as a stabilizer with varied sodium chloride concentrations. VC was successfully entrapped in the microparticles with trapping efficiencies up to 97.8%, a loading level of 55.4 g/mg, and particle size of 3.8 μm. Using 10% w/v PVP with 5% w/v NaCl in the continuous phase resulted in a higher loading level (55.4±6.9 g/mg), loading efficiency (97.8%), core region content (25.7±1.9 g/mg) and lower surface content (6.2±0.9 g/mg) than without NaCl (loading content: 40.7±5.1 g/mg; loading efficiency 52.1%; core region content: 8.3±0.5 g/mg; surface content: 19.5±1.1 g/mg). A linear release profile from VC-loaded microparticles was found. A preliminary animal oral administration study indicated that the VC-loaded microparticles, as an oral delivery system, have shown effective immunogenicity in rats for 2 months. The VC incorporation and physicochemical characterization data obtained in this study may be relevant in optimizing the vaccine incorporation and delivery properties of these potential vaccine targeting carriers.

**Keywords**: Vibrio cholerae, biodegradable, microparticle, vaccination.

**Introduction**

Cholera remains an important cause of illness and death in many parts of the world, especially in Asia but also in Africa and the Middle East and, since 1991, in South and Central America as well. It has been estimated that more than 150,000 people, both children and adults, die from cholera each year. It also causes significant economical problems arising from an embargo of contaminated food when marine food is suspected to be contaminated by VC. The available options, past and present, of cholera vaccines have been summarized by Finkelstein (1995). However, the parenteral whole-cell cholera vaccines that have existed for almost a century to prevent cholera are no longer regarded useful from a public health standpoint, mainly because of the short duration period of protection they provide.
Several field trials since 1960 have shown that these vaccines usually protect older people for only a few months and fail to protect many young children at all (Levine and Noriega 1995).

Another important area of vaccine research has focused on the gut mucosal immune system for improving the stimulation of immunogens. Several particulate antigens, including both live and killed micro-organisms, have proved to be effective oral immunogens (Holmgren et al. 1993). Studies by Bloom and Boedeker (1996) have defined the importance of locally produced IgA antibodies and IgA immunological memory for protection against cholera, and demonstrated that the oral route of vaccine administration is usually superior to the parenteral route in both priming and boosting the mucosal immune system.

The emulsion solvent evaporation technique is commonly used to prepare microspheres containing hydrophilic proteins or polypeptides for vaccination applications. The water-in-oil-in water (w/o/w) double emulsion method (wherein an aqueous solution of antigen is dispersed in a polymer solution to form a primary emulsion, prior to mixing with an aqueous surfactant phase) has been widely used to microencapsulate macromolecules. However, this approach generally results in low antigen loading levels (usually much less than 10% w/w) and entrapment efficiencies (usually much less than 70%) due to hydrophilic proteins partitioning into the aqueous continuous phase. In addition, non-uniform antigen release and a low delivery capacity of 1 μg/mg microparticles/day is frequently encountered in the w/o/w prepared microparticles (Yeh et al. 1995a). The antigen delivery capacity may be increased to a level of at least 5 μg/mg microparticles/day and uniform release profiles have been measured over 1 month in vitro (Yeh et al. 1995b). Importantly, protein-loading levels in excess of 25% could be obtained in small 2 μm particles. This offers opportunities for oral delivery of vaccines to the gut associated lymphoid tissue (Peyer’s Patches).

Microparticle carriers made from synthetic biodegradable polyesters such as PLG have been widely investigated. These have elicited impressive immune responses to a large number of antigens, including tetanus toxoid and cholera toxoid (Singh et al. 1991, Men et al. 1995, O’Hagan et al. 1995). Antigens have been adsorbed onto PLG microparticles and encapsulated in a polymer matrix for parenteral administration (O’Hagan et al. 1993). The ability to control the degradation rate of PLG microparticles by variation of the molecular weight and molar ratio of the copolymer potentially allows release of entrapped antigens over time. PLG microparticles, therefore, show considerable promise as single-dose, controlled release antigen delivery systems for inducing long-term immune responses. The goal of this study is to develop a single dose VC vaccine, which might overcome the dropout problem of multiple vaccine administration and enhance the efficacy of the vaccine (adjuvant effect) while conferring long term protection.

This study applied the water-in-oil-in-water (w/o/w) technique for the preparation of Vibrio cholerae (VC)-loaded microparticles, and formulation factors on the encapsulation of VC into PLG microparticles were evaluated. The two goals of the study were to develop an optimal formulation and to show the future use of VC or related non-toxic binding derivatives as vehicles to facilitate induction of mucosal immune responses to a broad range of antigens for human vaccination purposes.
Methods and materials

Bacterial strains

*Vibrio cholerae* biotype classical, serotype Inaba, strain ATCC 25870, which is a fimbriated mutant, was kindly provided by Dr Yu-Tien Liu, Department of Microbiology, National Defence Medical Center, Taiwan.

Materials

Bicinchoninic acid solution (BCA), copper (II) sulphate, sodium dodecyl sulphate (SDS), polyvinylpyrrolidone (PVP; MW 40000), sodium chloride (NaCl), sodium hydroxide (NaOH), urea and phosphate buffered saline (PBS) were obtained from Sigma (St. Louis, MO). Poly(dL-lactide-co-glycolide) (50:50, PLG, MW 9000, RG503), was from Boehringer Ingelheim (Ingelheim, Germany). Dichloromethane (DCM), acetonitrile, tetrahydrofuran and methanol were supplied by BDH (Poole, UK). All materials were used as supplied.

Preparation of *Vibrio cholerae*-loaded microparticles

The preparation of microparticles was adapted from previous papers using the w/o/w solvent evaporation method (Yeh et al. 1995a, b). Briefly, 1 ml of VC aqueous solution (4.8 × 10^5 CFU/ml) was added to 5 mL of dichloromethane (DCM) containing 300 mg of PLG, and mixed by a homogenizer (8000 rpm for 2 min) to obtain the primary emulsion. The resulting emulsion was then mixed at high speed with 30 ml of continuous phase solution containing either 5 or 10% (w/v) PVP as an emulsion stabilizer and sodium chloride (0, 3 and 5% w/v) concentrations. The osmotic pressure was adjusted with urea to keep it constant. The resulting w/o/w multiple emulsion was stirred with a magnetic stirrer for 18 h under ambient conditions to extract DCM. The microparticles were cleaned by resuspending in distilled water and centrifuging three times to remove PVP. After lyophilization, the final product was stored in a desiccator below 4°C.

Microparticle characterization

Six parameters were examined to assess the characteristics of VC microencapsulation: zeta potential, morphology, size distribution, encapsulation efficiency (particle formed yields, VC-loading, entrapped efficiency, and VC distribution in microparticles), distribution, release, and degradation pattern.

Determination of total *Vibrio cholerae* loading of microparticles: Freeze dried microparticles (8–10 mg), accurately weighed, were treated with 3.0 ml, 0.1 M NaOH containing 5% w/v sodium lauryl sulphate (SDS) by shaking overnight. The sample was centrifuged and a BCA protein microassay was used to determine the VC concentration in the supernatant against a series of VC standards prepared in 0.1 M NaOH containing 5% w/v SDS. Each sample was assayed in triplicate.

Particle size: Particles were sized by a Coulter LS 230 counter (Coulter, Miami, USA) after dispersing microparticles in deionized water using an ultrasonicater. Average particle size was expressed as volume mean diameter (vmd) in μm. Each sample was analysed in triplicate.
Zeta potential: Microparticles were dispersed in deionized water by ultrasonication. The particle zeta potential was recorded using a Zeta Plus (Brookhaven Ins. Co., NY, USA). Each sample was analysed at least five times to obtain an average value and a standard deviation.

Determination of Vibrio cholerae distribution on microparticles: PLG microparticles containing VC were analysed by modifying a previous reported method (Coombes et al. 1998). Briefly, 10 mg of microparticles were dispersed completely in 3 ml of modified phosphate buffered saline and the pH was adjusted to 8 using sodium hydroxide solution. The mixture was maintained at 40°C for 1 h. Then, microparticles were collected by centrifugation at 1000 g for 1 min, the aqueous phase in the supernatant was taken for determining protein content by the BCA method (surface VC on microparticles). The remaining microparticles were treated with 3 ml of BCA solution at room temperature for 10 min. After that, the BCA solution was centrifuged at 1000 g for 1 min and withdrawn for measuring protein content in the BCA solution by BCA assay (strong binding VC on microparticle). The remaining microparticles were washed three times in 5 ml of deionized water to remove the remained BCA reagent on the surface of the microparticles. The remaining microparticles were resuspended in 5 ml of 0.1 M sodium hydroxide containing 5% sodium lauryl sulphate and shaken at 37°C for 14 h. The sample was centrifuged and a BCA protein microassay was used to determine the VC concentration in the supernatant against a series of VC standards prepared in 0.1 M NaOH containing 5% w/v SDS (microparticle core VC). Each sample was run in triplicate.

In-vitro release of Vibrio cholerae from microparticles: A series of tubes, each containing ~30 mg freeze-dried microparticles dispersed in 2.0 ml of pH 7.4 phosphate buffered saline releasing medium, containing 0.02% sodium azide as a bacteriostatic agent, were retained in a water-bath at 37°C and continuously stirred by a magnetic stirrer bar. At 2, 6, 9, 13, 17, 21 and 24 days, tubes containing microparticles were centrifuged (2010 g, 5 min), the supernatant was removed and the protein content of the supernatant was analysed using a BCA microprotein assay. Fresh PBS was added to the microparticles and incubation was continued. Release profiles were calculated in terms of cumulative release percentage and release amount (μg VC/mg microparticles) versus incubation time.

In-vitro degradation of VC-loaded PLG microparticles: A series of tubes, each containing ~10 mg of freeze-dried microparticles dispersed in 2.0 ml of PBS with 0.02% sodium azide, were retained in a water-bath at 37°C with a shaking rate of 200 rpm. Periodically, the microparticle samples were centrifuged (2010 g, 5 min) and the supernatants were removed. The microparticles were cleaned three times using distilled water by mixing and centrifugation. Finally, the microparticles were lyophilized and the microparticles were examined by scanning electron microscopy.
The influencing factors of encapsulation (i.e. concentrations of PVP and sodium chloride in the continuous phase) on the prepared microparticles (i.e. VC-loading, encapsulation efficiency, zeta potential, particle size and yields) are summarized in Table 1. The PVP and sodium chloride concentrations in the continuous phase were important factors affecting the VC entrapment efficiency (%), and yield. The VC entrapment efficiency (%) increased with increasing sodium chloride concentration in the continuous phase from 52.1% (without NaCl) to 97.8% (5% NaCl) at 10% PVP and from 51.7% (without NaCl) to 87.9% (5% NaCl) at 5% PVP with the same isotonic condition in the continuous phase, and also increased with increasing PVP concentration in the continuous phase from 49.6% (5% PVP) to 52.1% (10% PVP) at 0% NaCl, and from 87.9% (5% PVP) to 97.8% (10% PVP) at 5% NaCl. The microparticle yield increased with increasing sodium chloride concentration in the continuous phase from 34% (without NaCl) to 71% (5% NaCl) at 5% PVP with the same isotonic condition in the continuous phase, and also increased with increasing PVP concentration in the continuous phase from 44.2% (5% PVP) to 56.7% (10% PVP) without NaCl and from 70.8% (5% PVP) to 78.3% (10% PVP) at 5% NaCl. The VC content (%w/w) was not much affected by the PVP levels, e.g. found for 5% PVP and 10% PVP to be 4.0~5.5%(w/w), respectively. The Zeta potential of PLG microparticles without VC and VC-loaded microparticles were $-54.8 \text{ mV}$ and $-24\approx-29 \text{ mV}$, respectively. Zeta potentials were not affected by PVP or sodium chloride concentrations, but increased with increasing urea concentration in the continuous phase from $-25$ (without urea) to $-45 \text{ mV}$ ($0.1g/ml$ urea). In this study, the mean particle size varied between 3.1~5.0 μm, which might be useful for VC-loaded microparticles as an oral delivery system.

The influencing factors (i.e. concentrations of PVP, and sodium chloride in the continuous phase) on VC distribution pattern in the VC-loaded microparticles such as on the surface, in the outer layer and in the core region are summarized in Table 2. The distributions (%) of VC on the microparticle surface due to surface...
adsorption were found to decrease with increasing sodium chloride concentration in the continuous phase from 16.7% (without NaCl) to 7.4% (5% NaCl). The distributions (%) of VC in the core region were found to increase with increasing sodium chloride concentration in the continuous phase from 14.6% (without NaCl) to 24.3% (5% NaCl). The same trend was also noticed in the release profile, the less surface VC adsorption, the less the burst release in the first day.

**Morphological studies of microparticles**

Figure 1(a) is a typical scanning electron micrograph (SEM) of VC-loaded microparticles prepared from PLG. An intact outer surface of microparticles appeared spherical with a smooth surface. Figures 1(b) and (c) display the SEM of the VC-loaded PLG microparticles during release studies. After 1 week in release medium, their spherical shape and smooth surface were little changed, the smooth surface of microparticles became crenated and three or four microparticles were fused together as a unit. This suggested that the microparticles absorbed water as they were immersed in the release medium and shrank upon dehydration for SEM studies. After 2 weeks in the release medium, some microparticles appeared misshapen and lost their spherical shape. A surface structure with some troughs and small pores was seen scattered over the microparticles. Microparticles were slowly degraded by erosion, resulting in pores on the surface. The pore size increased with time, and by 3 weeks the microparticles were highly porous. After 4 weeks, the microparticles became fragile, their shape changed and they no longer maintained their regular structure.

SEM investigations of the *in vitro* microparticle degradation offered important information on surface porosity and roughness, as well as general microparticle morphology such as shape changes. These SEM photographs might be further used for interpreting the release behavior. *In vitro*, polymer degradation lagged behind drug release for 1–2 weeks, indicating that part of the drug was released by diffusion through the polymer matrix. VC release for longer than 3 weeks, due to bio-erosion or resorption of the polymer matrix, consequently formed pores (resulting from the removal of water soluble, degraded chain fragments), which could be released from the microparticle core (Yeh et al. 1995b).
In-vitro release of Vibrio cholerae microparticle

The cumulative release profiles of VC-loaded microparticles are shown in figures 2; these microparticles were prepared with various PVP and sodium chloride concentrations in the continuous phase. The surface of VC-loaded PLG microparticles is characteristically smooth (figure 1). This is probably due to embedded or adherent VC, which resists the washing process used in microparticle harvesting. The cumulative release of VC, in terms of % w/w, provided an index of delivery efficiency, it had a upper limit of ~80% of the VC load at 24 days. The initial burst release of VC microparticles was estimated from the cumulative release at 24h to be 14% for 5 and 10% PVP with 5% w/v sodium chloride in the

Figure 1. Scanning electron micrographs of VC-loaded PLG microparticles. (a): before incubation in PBS at 37°C; (b): after 2 weeks incubation; (c): after 4 weeks incubation (in 0.1 M phosphate buffer pH 7.4 at 37°C).
A steady release rate was found to be 1.25 μg VC/mg microparticles/day of VC release maintained for 24 days. No significantly burst release was observed, which is in contrast to PLG based microparticle release profiles which revealed a burst phase (generally considered to result from loss of surface protein) followed by a short lag phase, which lasted for 10 days. However, the less sodium chloride in the continuous phase, the more surface VC adsorption on the microparticle surface, the more the burst release in the first day (table 2). This effect is generally considered to result from loss of weakly bound surface VC. The cumulative released amount (μg VC/mg microparticles) of VC (table 2) provides an indication of the efficiency of VC delivery from the microparticles and was limited at 24 days to approximately 30 μg/mg (10% PVP; without NaCl), 32.0 μg/mg (5% PVP; 5% NaCl), 26.2 μg/mg (5% PVP; without NaCl), 32.3 μg/mg (5% PVP; 0.1 g/ml urea; without NaCl), 33.5 μg/mg (5% PVP; 0.04 g/ml urea; 3% NaCl), and 25.0 μg/mg (10% PVP; 5% NaCl) of the VC amount, respectively.

It is known that a salting out effect can occur during the solvent evaporation process for the preparation of microparticles, resulting in differences in matrix morphology, physicochemical and drug release characteristics (Zhang et al. 1997). The presence of VC can be expected to affect the structure (fine or coarse domains) and distribution of phases comprising the PLG microparticles and, in turn, exert an influence on the extraction and release of the soluble components of VC. The development of 80–150 nm pores in the surface of PLG microparticles for example suggests a fairly uniform dispersion but a coarse structure of the soluble phase.
Porosity development and, subsequently, VC release characteristics might be influenced by entrapped VC inside the core region of the microparticles.

An oral PLG-associated cholera vaccine consisting of amphotericin B as an adjuvant was pre-tested in rats. The results show that the VC-loaded PLG microparticle treatment group compared to the VC solution as control group, resulted in significantly enhanced immune response by the VC-loaded PLG microparticles group, as measured by the appearance of specific antibody-producing cells in the blood and gut (data to be published). These biodegradable PLG microspheres represent one possible sustained delivery antigen system. Studies have shown that particulate antigens, especially when presented as viable organisms, are more effective than soluble antigens in inducing local as well as generalized secretory and systemic immune responses. At least two reasons can be given to explain this. First, the size and composition of particulate antigens may allow them to survive the environment of the gastro-intestinal tract and be more efficiently absorbed by the gut-associated lymphoid tissue (GALT), specifically through M (microfold) cells into Peyer’s patches. Secondly, soluble antigens with low molecular weight easily cross the epithelial barrier of the gut. The recognition of these molecules by lymphoid cells at sites other than in the Peyer’s patches has been proposed as the stimulus that initiates systemic tolerance after antigen feeding and may provide a negative signal to the mucosal immune system.

In designing a vaccine, besides safety and immunogenicity, several factors must also be considered, such as ease of administration and production cost. The microparticle-based refined antigen oral cholera vaccine, with no untoward reactions, has high immunogenicity and delivery simplicity. These factors would contribute to the low cost of vaccination, and prove suitable for developing countries where cholera epidemics and endemics are problems of public health concern.

Conclusion

Microparticles containing VC using poly(DL-lactide-co-glycolide) with a water-in-oil-in-water emulsion/solvent extraction technique were prepared. The dispersed phase was composed of PLG dissolved in dichloromethane, and the continuous phase was water containing PVP as stabilizer with sodium chloride and an osmotic agent of urea. VC was successfully entrapped in the microparticles with trapping efficiencies up to 97%, loading level 5.5% w/w, and mean particle size 3.8 μm. The particle size distribution was between 1–7 μm. Using NaCl in the external aqueous phase improved the VC-loading inside microparticles and decreased the release amount in the burst stage, also shortening the lag phase release period and increasing total VC release amount.

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