Solvent Evaporation Processes for the Production of Controlled Release Biodegradable Microsphere Formulations for Therapeutics and Vaccines

Jeffrey L. Cleland*

Genentech, Inc., 1 DNA Way, S. San Francisco, California 94080

Novel drug delivery technologies have now evolved to allow the clinical production of new dosage forms. One new form, biodegradable microspheres, may have utility as a second-generation formulation for parenterally administered proteins and peptides or may be required for the success of some new chemical entities. Here, we have focused on the use of poly(lactic-*co*-glycolic acid) (PLGA) microspheres to provide a continuous delivery of therapeutic proteins or a pulsatile delivery of protein-based vaccines. To date, our success with solvent evaporation processes has been primarily in the production of microspheres with a triphasic protein release pattern instead of a continuous release. However, with continued development efforts, this method may be used to produce continuous release protein formulations. While this review draws from our own work, there is a great deal of excellent research in this area at both universities and industrial laboratories. This work combined with our studies indicates that this technology holds much promise for new protein formulations that will provide improvements in patient care and, perhaps, increased efficacy.

Introduction

The biotechnology industry has evolved to allow the large-scale production of recombinant proteins for commercial use. However, many of these therapeutic proteins and vaccines present a unique challenge for drug delivery. The successful clinical application or commercial use of many proteins requires the use of a drug delivery system to reduce the frequency of administrations, provide a lower toxicity through a reduced peak serum concentration, localize the drug at the site of action, or yield a steady-state level of the drug to achieve the desired effect. Prior to developing a delivery system, both the in vitro and in vivo behaviors of the protein must be well-understood (*1*; Figure 1). With an understanding of the clinical indication, biological effect of the protein, and physicochemical properties of the protein, a delivery system may be designed to improve the convenience of administration and, possibly, the efficacy and safety of a protein drug.

One approach to addressing these drug delivery requirements is the use of biodegradable microspheres. Biodegradable microspheres may be produced from a variety of synthetic and natural polymers and may provide a continuous or pulsatile release of the protein over a period of days, weeks, or months (*2*; Figure 2). A biodegradable microsphere formulation, Lupron Depot, has been commercially successful for several years (*3*). This formulation consists of leuprolide acetate, a decapeptide, encapsulated in biodegradable microspheres composed of poly(lactic-*co*-glycolic acid) (PLGA) for the treatment of prostate cancer, endometriosis, and precocious puberty. The drug is released continuously over either 1 or 3 months depending upon the formulation (*4*).

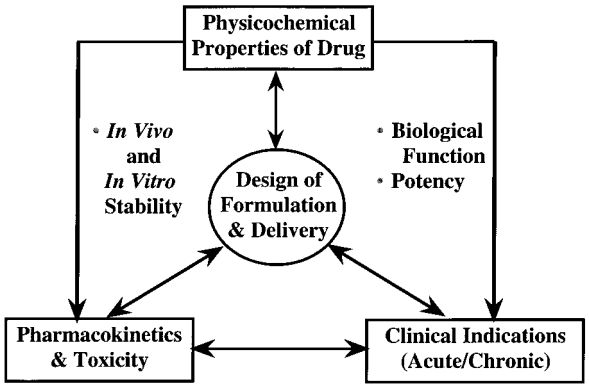

Figure 1. Design of drug formulations and delivery systems is influenced by several factors. A protein's physicochemical properties often affects its pharmacokinetics and toxicity and may have an impact on its clinical use. Upon administration, the clearance and metabolism of the protein depends upon in vitro and in vivo stability. All of the factors shown in this figure have interdependencies that must be considering when designing a formulation or delivery system. Reprinted from ref 1. Copyright 1994 American Chemical Society.

PLGA has also been used for over 20 years as resorbable suture material (*5*). With the prior success of a PLGA depot formulation and the safety of PLGA, protein-controlled release formulations have been developed using PLGA microspheres. A great deal of research has been published with PLGA microspheres (for reviews, see refs 6−9), and this review will focus on the work done primarily in our laboratories over the past 6 years.

Production of Protein-Loaded PLGA Microspheres

The traditional process of producing drug-loaded PLGA microspheres involves the use of a solvent evaporation

* Telephone: 650-225-3921. Fax: 650-225-3979. E-mail: cleland@gene.com.

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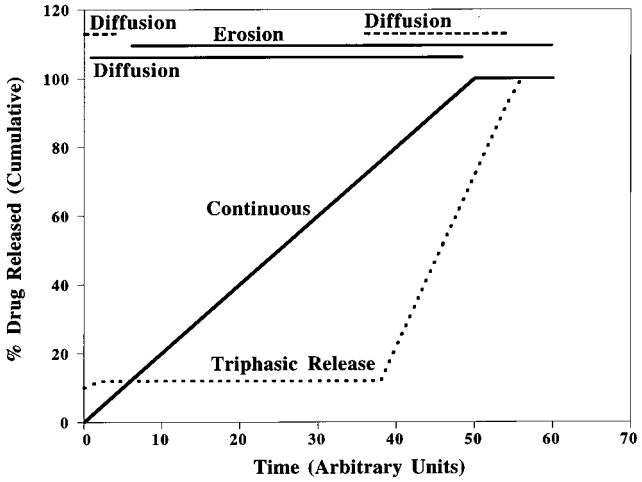

Figure 2. Two primary types of release patterns for proteins encapsulated in PLGA microspheres. A continuous release is achieved by the continuous diffusion of the protein out of the microspheres with initial diffusion from the microsphere surface and subsequent diffusion through the internal pores of the microspheres. The rate of drug release is primarily controlled by the protein's diffusivity. For triphasic release (S release pattern), the protein initially diffuses from the surface of microspheres. After this initial diffusive phase, there is a lag phase where the PLGA undergoes hydrolysis (bulk erosion), resulting in a significant increase in pores or channels for diffusion. The remaining drug may then diffuse out of the more porous polymer matrix. The time scale listed for each type of release is directly dependent upon the processing conditions, polymer characteristics, and drug properties as discussed in the text. Reprinted with permission from ref 2. Copyright 1997 Plenum Publishing.

process to remove the organic solvent that is required to solubilize the PLGA (*10*; Figure 3). Typically, PLGA is dissolved in methylene chloride or ethyl acetate and a liquid or solid form of the protein is then mixed with the PLGA solution to produce an emulsion (liquid protein) or suspension (solid protein). This mixture is then added to a solution of water and an emulsifying agent such as poly(vinyl alcohol), resulting in the formation of PLGA microspheres. The nascent microspheres are next added to an excess of water to facilitate the removal of the organic solvent. The final microspheres are then washed with additional water and filtered to remove small (<20 μm) and large (>100 μm) particles. The microspheres are then lyophilized or air-dried to produce a homogeneous free-flowing powder (Figure 4). The dry microspheres are resuspended in an aqueous medium (usually a viscous solution to keep them suspended during administration) and then injected subcutaneously or intramuscularly.

As shown in Table 1, the solvent evaporation process for producing PLGA microspheres included several process parameters (*2*). Several key process parameters were evaluated for their impact on the efficiency of protein encapsulation (actual/theoretical loading), initial protein release phase, and the type of release pattern, continuous or pulsatile (*11, 12*). Critical parameters included the viscosity of the polymer solution, which is affected by the polymer molecular weight, solubility and concentration, the volume of the aqueous protein phase, and the rate of polymer solvent extraction (too rapid, lack of microsphere formation; too slow, lower encapsulation efficiency and higher burst). As the viscosity of the polymer solution increased, the encapsulation efficiency increased to allow complete encapsulation of the protein (*11*). A small volume of the aqueous protein per unit mass of polymer (≤0.1 mL/g PLGA) added to the polymer solution resulted in a pulsatile release pattern: initial release, lag phase with little or no release, and a second release phase. As the aqueous protein volume is increased, the initial release increases and the encapsulation efficiency decreases. However, a more continuous protein release pattern is observed with a higher aqueous protein volume added to the polymer solution. This result is likely caused by the generation of additional pores and channels within the microspheres.

Development of Continuous-Release Microsphere Formulations

Recombinant human growth hormone (rhGH) has been used for several years for the treatment of growth disorders in children. Currently, patients receiving growth hormone therapy must take daily injections of rhGH. A depot form of rhGH would allow patients to reduce the frequency of administration from daily to once or twice per month. Previous work in children has shown that a continuous administration of rhGH provides comparable safety and efficacy to daily administration (*13*). In addition, clinical trials are currently underway to evaluate the administration of rhGH in a PLGA formulation for the treatment of children with growth hormone deficiency.

Using an emulsion process, a PLGA microsphere formulation was developed for recombinant human growth hormone (rhGH) (*12, 14*−*16*). Screening of several excipients to stabilize rhGH during encapsulation revealed that excipients which caused preferential hydration of the protein provided stabilization of rhGH (*14*). From

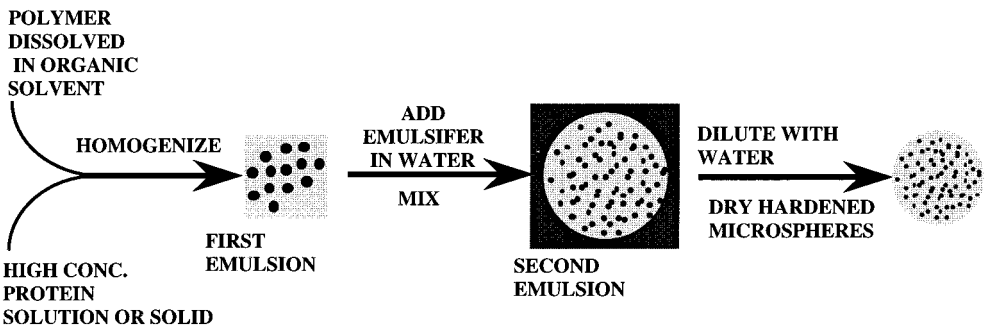

Figure 3. Solvent evaporation method for production of PLGA microspheres. The polymer (PLGA or PLA) is dissolved in organic solvent (e.g., methylene chloride or ethyl acetate). The aqueous or solid protein is then added, and the solution is mixed by sonication or homogenization to form the first emulsion (solid or water in-oil). This emulsion is then transferred to water containing an emulsifying agent (e.g., poly(vinyl alcohol)). Mixing of the first emulsion in the water phase produces the microspheres resulting in a second emulsion (solid or water in-oil-in-water). The final emulsion is diluted with excess water to facilitate removal of the organic solvent in the oil phase. The microspheres are then dried. Reprinted with permission from ref 10. Copyright 1995 Plenum Publishing.

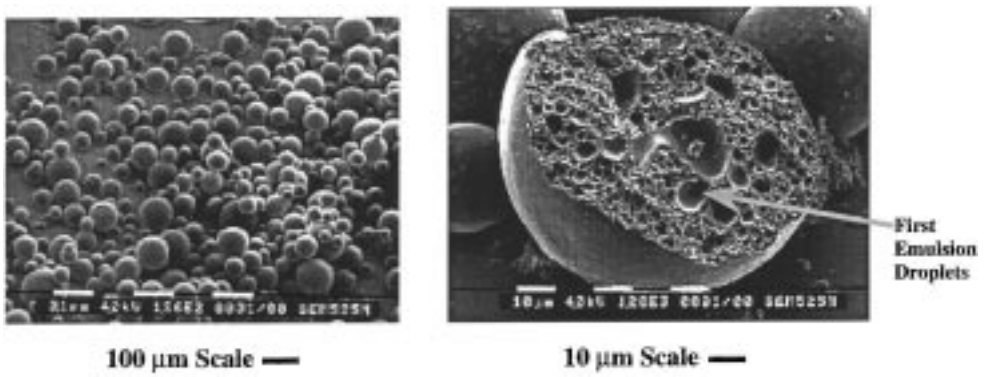

Figure 4. Scanning electron microscope image of PLGA microspheres made using the process shown in Figure 3.

Table 1. Main Process Variables for Double-Emulsion Method in Production of Protein-Loaded PLGA Microspheres[a]

process step	variables
primary emulsion	polymer concentration in organic solvent
	polymer composition and molecular weight
	organic solvent (e.g., methylene chloride or ethyl acetate)
	oil phase volume
	concentration of protein in aqueous solution
	protein solution volume
	mixing rate
	mixing device (e.g., homogenization or sonication)
	rate of protein addition to oil phase
	duration of mixing
	temperature and pressure
secondary emulsion	water phase volume
	concentration of emulsifying agent in water phase
	rate of oil phase addition to water phase
	mixing rate
	mixing device (e.g., stirred tank)
	duration of mixing
	temperature and pressure
hardening bath	water volume
	presence of additives or stabilizers
	incubation time
	mixing rate
	mixing device (e.g., stirred tank)
	temperature and pressure
filtration and washing	filtration device (e.g., stirred cell)
	rate of filtration
	filtration mesh size
	wash volume
	wash composition
	temperature and pressure
drying	method of drying (e.g., lyophilization)
	facilitated mass transfer (e.g., fluidized bed)
	drying time
	amount of residual moisture
	addition of excipients

[a] Adapted from ref 10.

these studies, the rhGH was encapsulated as a solution at 400 mg/mL protein, 100 mg/mL trehalose, and 100 mM phosphate, pH 8 (*14*). The sugar, trehalose, was required to stabilize the rhGH against denaturation during processing. Studies of rhGH stability in the PLGA microspheres during incubation at 37 °C demonstrated that the microspheres did not alter the stability of rhGH as measured by a variety of chemical, physical, and biological assays (*15*). Biologically active rhGH was released from the PLGA microspheres in vitro over 30 days (*15*; Figure 5). Animal studies also demonstrated the release

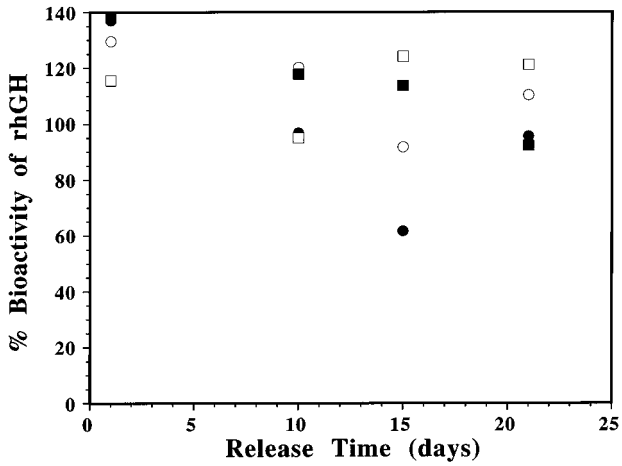

Figure 5. rhGH released from the PLGA microspheres incubated at physiological conditions was measured by the in vitro cell-based bioassay. The protein concentration measured by the bioassay with the appropriate rhGH standards was compared to the protein concentration measured by the bicinchoninic (BCA) assay (% bioactivity = [rhGH] by bioassay/[rhGH] by BCA × 100%). rhGH released from batches 1 (closed circles), 2 (open circles), 3 (closed squares), and 4 (open squares) was assayed after a given incubation time. The bioassay results varied by ±20% due to the variability of the biological system. Reprinted with permission from ref 15. Copyright 1997 Plenum Publishing.

of rhGH from the microspheres and indicated that the released protein was bioactive and not immunogenic (*12*; Figure 6). Unfortunately, a pulsatile release of rhGH (10-day lag phase) was observed in the animal experiments and these results did not correlate with the continuous release observed from the initial in vitro studies (*15*; Figure 6). The lag phase may have been caused by the low aqueous volume or high polymer concentration, both of which may generate a less porous structure requiring polymer erosion prior to diffusion of the rhGH out of the microspheres. Therefore, further development efforts were required to achieve a continuous release of rhGH from PLGA microspheres. These development efforts resulted in the use of novel microencapsulation process (spray freezing of microspheres into ethanol and liquid nitrogen), resulting in a continuous-release rhGH PLGA formulation (*16*). The current literature lacks data demonstrating a continuous in vivo release of a protein from PLGA microspheres prepared with the double-emulsion process, whereas there are several examples of continuous in vivo release of peptides from PLGA microspheres produced with this method (cf. ref 3).

Unlike rhGH, recombinant human interferon-γ (rhIFN-γ), a therapeutic protein used for treatment of chronic granulomatous disease, was very unstable during incubation at 37 °C. Although hIFN-γ formulated in treha-

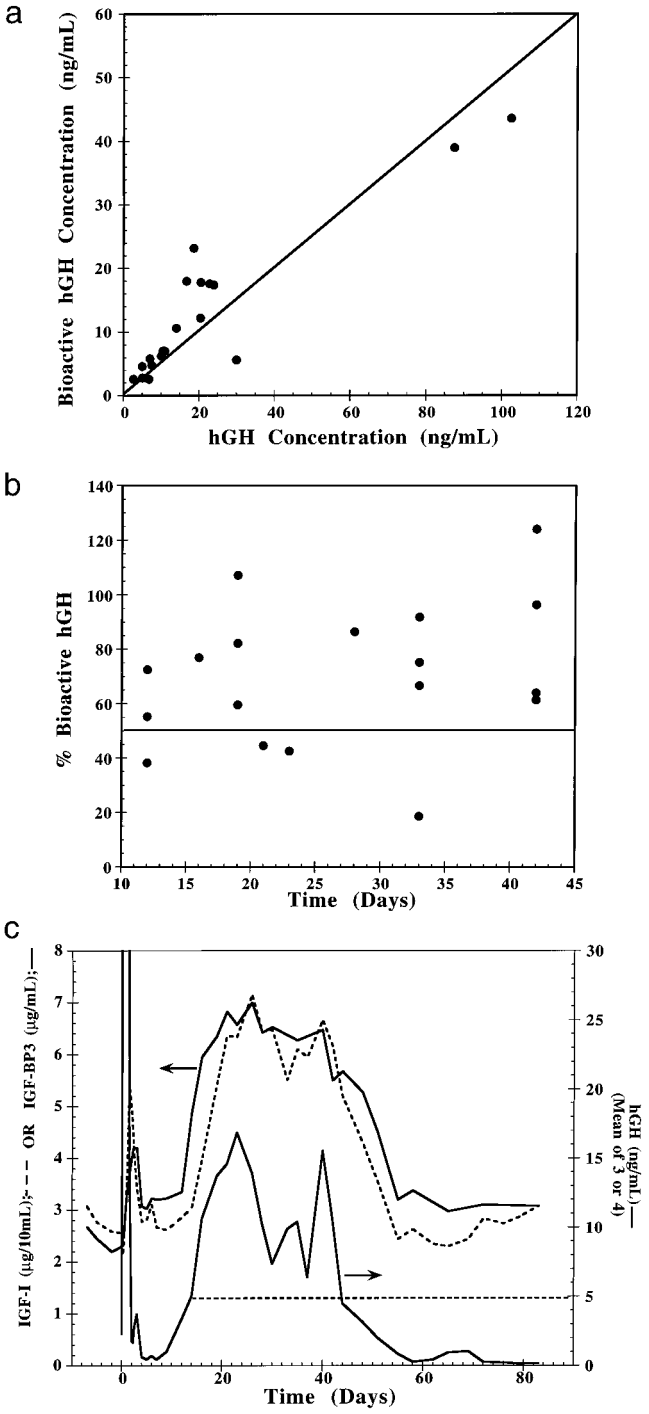

Figure 6. Bioactivity of hGH in the rhesus serum samples was determined by an in vitro cell-based assay, which provides a concentration of bioactive hGH based upon standards of known concentration. (A) The bioactive hGH concentration is shown as a function of the total hGH serum concentration (ELISA). The solid line represents the typical minimum (50%) recovery of bioactive rhGH in rhesus serum spiked with rhGH. This assay is affected by the presence of serum proteins causing variability in the measured recovery of bioactive rhGH. (B) The fraction of bioactive hGH (bioactive hGH concentration/total hGH concentration × 100%) was determined from rhesus serum taken at different time points. (C) Serum profile of human growth hormone (hGH), insulin-like growth factor-I (IGF-I), and insulin-like growth factor binding protein-3 (IGF-BP3) in rhesus monkeys (mean of three or four animals) after administration of 200 mg of rhGH PLGA microspheres (24 mg of rhGH) at day 0. All serum samples were from animals that did not have anti-hGH antibodies. Reprinted with permission from ref 12. Copyright 1997 Elsevier.

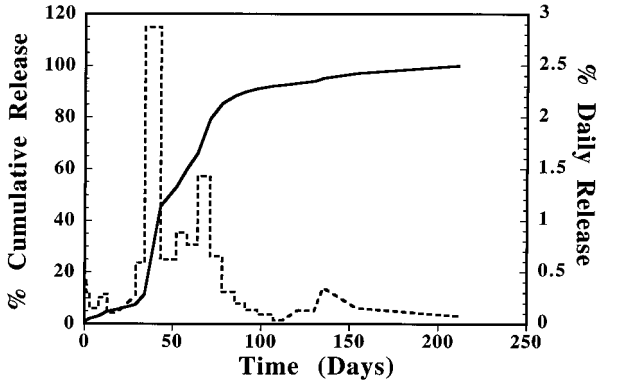

Figure 7. Example of the release profile of rgp120 PLGA microspheres produced with the double-emulsion method and incubated at 37 °C in physiological buffer. The protein was encapsulated at a 1% w/w loading in 65:35 L/G PLGA with an inherent viscosity of 0.6 dL/g. The process was performed with ethyl acetate as the solvent and low-temperature operation of the emulsions and hardening bath was employed. The preparation had a 100% encapsulation efficiency and less than 2% initial burst. The % daily release of rgp120 (dashed line) was determined by dividing the release measured at a given time point by the interval between time points. Reprinted with permission from ref 11. Copyright 1997 Elsevier.

lose was encapsulated and initially released intact and bioactive, the protein underwent significant aggregation during incubation at 37 °C (*17*). These studies also revealed that the in vitro release method affects the release rate of the protein and the stability of the protein. It was observed that different release profiles (continuous or triphasic) could be obtained depending upon the method (release system) and buffer used in the study. In addition, this work indicated that a standard assay (bicinchoninic acid, BCA) used to quantify proteins underestimated the total protein concentration when protein aggregates were present in the sample (*17*). Therefore, it is important to assess the conditions used to measure in vitro protein release and ensure that the results do not contain artifacts caused by the methodology.

Pulsatile Delivery of Proteins from PLGA Microspheres

In contrast to the therapeutic proteins discussed above, a pulsatile-release PLGA microsphere formulation was desirable for vaccine formulations because vaccines are usually administered over several injections separated in time by several months. Initial studies were performed using the MN strain of recombinant glycoprotein 120 (rgp120), the envelope glycoprotein from HIV-1 (AIDS virus), as the model protein (*11*, *18*−*20*; Figure 7). The duration of the lag phase in the triphasic release pattern was determined by the polymer used to make the microspheres (*11*). As the polymer molecular weight was increased at a constant lactide:glycolide monomer ratio, the lag phase duration increased. Increasing the lactide content of the polymer at a constant molecular weight had the same effect (*11*; Table 2). These results were expected upon the basis of the rate of degradation of these polymers (i.e., increasing the molecular weight and lactide content slows polymer hydrolysis and bulk erosion). rgp120 PLGA microspheres with lag phases of 1−6 months were developed (Table 2) and tested in guinea pigs. These studies revealed that a single immunization of soluble rgp120, an adjuvant (QS-21), and rgp120 PLGA microspheres provided high virus neutralizing titers that persisted for over 1 year after the immunization (*19*; Figure 8).

Table 2. Correlation between PLGA Properties and Second Burst[a] (Adapted from Ref 11) (Reproduced from 11, Copyright Elsevier)

inherent viscosity (dL/g; L/G; Supplier)	second burst[b] time (days)	inherent viscosity (dL/g; L/G; Supplier)	second burst[b] time (days)
Methylene Chloride			
0.21 (50:50) BI	25	0.62 (75:25) MTI	80
0.17 (75:25) BI	50	0.21, 0.76 (50:50) BI[c]	40
0.24 (50:50) MTI	35	0.17, 0.67 (75:25) BI[c]	60
0.21 (75:25) MTI	45	0.24, 0.75 (50:50) MTI[c]	40
0.75 (50:50) MTI	40		
Ethyl Acetate			
0.24, 0.75 (50:50) MTI[c,d]	30	0.71 (65:35) MTI	60
0.75 (50:50) MTI[d]	40	0.62 (75:25) MTI	70
0.61 (65:35) MTI	45	0.22 (100:0) MTI	190

[a] Microspheres were prepared as described in ref 11 (0.3 g of PLGA/mL of methylene chloride or ethyl acetate, 0.1 mL of protein solution/mL of organic solvent, reduced temperature, and no excess methylene chloride in second emulsion or 10% ethyl acetate in the second emulsion for methylene chloride and ethyl acetate processes, respectively). [b] Second burst from microspheres was usually observed over 2−4 weeks. The time listed is the start of release when the percent released was significant (>10%/w). The majority of the protein remaining in the microspheres after the initial burst is released during the second burst. [c] A 50:50 mass ratio of the low and high inherent viscosity PLGA was used to produce these microspheres. [d] Due to the insolubility of high inherent viscosity 50:50 PLGA, 20% v/v benzyl alcohol was added to the ethyl acetate phase.

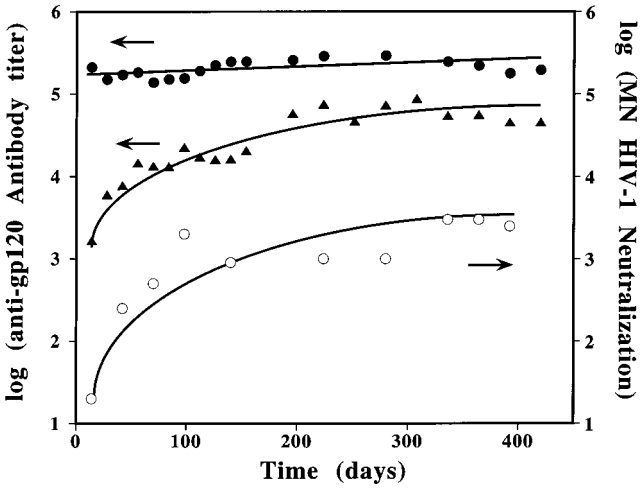

Figure 8. Effect of PLA microsphere release of rgp120 and added soluble QS-21 adjuvant on the duration of the anti-gp120 antibody titers and MN HIV-1 neutralization titers. Guinea pigs were immunized at time 0 with 30 μg of soluble gp120, either without (▲) or with (●) 50 μg of soluble QS-21 and approximately 3 mg of PLA microspheres containing 30 μg rgp120. The anti-gp120 antibody titers were determined on individual animals and the geometric mean titer values calculated. Serum from selected time points for the group with added soluble QS-21 were also assessed for MN HIV-1 neutralizing antibodies (○). Reprinted with permission from ref 19. Copyright 1996 American Chemical Society.

The PLGA microsphere systems developed for rgp120 were also applied to the antigen used in a malaria transmission-blocking vaccine, TBV25H (*21*). This vaccine was designed to block transmission of the malaria parasite via mosquitos by uptake of antibodies to the antigen during feeding on a human host. The ingested antibodies may then block the reproductive cycle of the parasite and prevent subsequent parasite transmission to a naive human host. A TBV25H PLGA microsphere formulation (0.6 dL/g, 65:35 PLGA) was developed to provide a 2-month autoboost. Administration of this formulation to mice resulted in complete blocking of malaria transmission to mosquitos after 40 days while the soluble protein given as three separate immunizations did not result in complete blocking activity (*22*). Additional animal and in vitro studies are underway at NIH to evaluate this vaccine for clinical use.

In general, these results indicate that it is possible to develop pulsatile release PLGA microsphere formulations for vaccine indications. The success of this approach for other vaccines (e.g., tetanus toxoid or hepatitis B) will depend on the ability to stabilize the antigen during encapsulation and subsequent release.

Conclusions

Overall, our studies with the solvent evaporation method have demonstrated the potential for this process in the development of pulsatile formulations for vaccines. However, additional work is required to establish the viability of this approach for continuous release of therapeutic proteins that require large doses (≥50 (μg/kg)/day) and long dosing durations (≥30 days). The key steps for development of a continuous-release PLGA formulation will be reducing the initial release phase and obtaining a continuous release without a significant lag phase. A reduction in the initial release phase may be achieved by producing protein formulations that are less soluble in water, improvements in the homogeniety of the first emulsion, and more rapid extraction of the organic solvent during the formation of the microspheres. Continuous-release formulations may be developed by increasing the porosity of the microspheres to allow diffusion-controlled release and the use of polymers that degrade more quickly, typically more hydrophilic PLGA (free carboxylic acid end groups) with a 50:50 lactide:glycolide content. While these changes may appear straightforward, significant efforts are required to achieve a continuous-release formulation with high protein loading (≥10% w/w) and a low initial release phase (<20%). The future success of solvent evaporation production methods for continuous-release PLGA formulations will depend upon this development effort, although novel process techniques such as the cryogenic process have recently shown promise for overcoming the difficulties of high protein loadings and low initial release (*12*).

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