Optimization of Poly (D-L-lactide-co-glycolide) Microsphere Production for Oral Delivery To Promote Adenovirus Stability and Intestinal Gene Transfer

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Optimization of Poly (D-L-lactide-co-glycolide) Microsphere Production for Oral Delivery to Promote Adenovirus Stability and Intestinal Gene Transfer

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Dedication

For my parents, Willard and Roberta

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Abstract

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Adenovirus vectors have demonstrated many of the characteristics necessary to be successfully used as a carrier in a vaccine delivery system. Adenovirus is a good choice for a vaccine carrier because it generates vigorous T and B cell responses to its transgene products. Although current vaccination strategies using this vector have had some success, its use has been constrained by the presence of pre-existing immunity to human serotypes in about 50% of the population. Recently, it has been demonstrated that the induction of these adenovirus-specific neutralizing antibodies that reduce efficacy of initial treatment and booster immunizations does not apply to oral delivery.

Adenovirus is a good candidate for oral vaccine delivery because it is capable of inducing antibody responses against an encoded transgene product at mucosal surfaces, which may result in complete systemic and mucosal immunity. Nevertheless, there are some limitations associated with the oral delivery of protein, peptide, and virus-based vaccines. Virus-based vaccines are sensitive to the low pH and presence of proteases in the gut limiting their activity and cellular uptake is also hindered by the rapid transit time of compounds through the intestine.

In general, there are two primary strategies for enhancing mucosal immunity: mucosal adjuvants and encapsulation in microparticles. Although several groups have successfully encapsulated adenovirus in polymeric formulations, it has been found that the encapsulation process drastically reduces viral function. However, the best performance has been reported with formulations containing poly(lactide-co-glycolide) (PLGA).

Thus, the primary objective of this study was to optimize the encapsulation process of a recombinant adenovirus vector in PLGA microspheres to maximize virus stability and promote intestinal gene transfer. Production parameters were systematically adjusted to find the best formulation for virus release and stability. Optimization of the production process increased viral release from 7 to 15 days and resulted in a 200-fold increase in the total number of infectious virus particles released compared to the original formulation.

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Introduction

1. Current Status of Gene Therapy

As of January 2007, there are over 1260 gene therapy clinical trials that have taken place in more than 28 countries worldwide, with 85 approved in 2006 alone, according to statistics published online by The Journal of Gene Medicine (Wiley Database, 2007). The gene therapy products tested in these studies are designed to battle a variety of illnesses including cancer, vascular disease, monogenic disease, and infectious disease. Although the majority of these studies are still in phase I testing (67%), there are 40 trials at or beyond the phase II / III level of the clinical trial process (Wiley Database, 2007).

The drugs tested in these studies include a wide range of delivery vehicles including viruses, naked DNA, plasmid DNA, liposomes, and RNA transfer. Adenovirus vectors are involved in more studies (26%) than any other type of vector used in all clinical trials and are the vectors used in the only gene therapy product approved by a governmental regulatory agency (Wiley Database 2007; Majhen *et al.*, 2006). Gendicine, which uses a recombinant human adenovirus-p53 for tumor suppression, was developed by Shenzhen SiBiono GeneTech for the treatment of squamous cell carcinoma of the head and neck and was approved by the State Food and Drug Administration of China in October 2003 (Peng, 2005).

2. The Structure and Function of Adenoviruses

Adenovirus was discovered by W.P. Rowe while isolating human adenoid cells. Since then, adenovirus has been found in over 100 different mammalian species consisting of 87 serotypes, 50 of which are proven to infect and replicate in an assortment of human cells from tissues of the liver, respiratory tract, urinary bladder, eye, and gastrointestinal tract (Rux *et al*., 1999; Giamcomo *et al*., 2003; Verma *et al*., 2005). Of the six subgroups of adenoviruses classified, Ad2 and Ad5 from subgroup C are the most widely studied for clinical application because of their nominal pathogenesis and typically mild disease states (Vecil *et al*., 2003).

Adenoviruses are iscosahedral shaped particles with an outer diameter between 70 and 90 nm. The outer protein shell, called the capsid, is composed of 240 hexons (polypeptide II), 12 penton bases (polypeptide (III), and knob fiber (polypeptide IV) located at each of the vertices. Encased within the capsid is a 36 kb segment of linear double-stranded DNA which contains an inverted terminal repeat at both ends providing the cis-acting signal essential for viral replication (Majhen *et al*., 2006; Rux *et al*., 1999; Vecil *et al*., 2003; Volpers *et al*., 2004).

The adenovirus genome is divided into three different transcriptional unit categories according to there time of expression during the viral replication process. The early transcriptional units include E1A, E1B, E2, E3, and E4. E1A, E1B, and E4 are involved in manipulating the cell to facilitate a favorable

environment for virus replication, aiding survival of infected cells, and controlling the cell cycle. The E2 segment is responsible for DNA replication and E3 helps in undermining the host's immune response against the virus. After viral replication, the four intermediate and single late transcriptional units are expressed and provide for the structure of the capsid proteins (Giamcomo *et al*., 2003; Majhen *et al*., 2006).

The entry of adenovirus into the cell takes place in four steps: receptor interaction, endosomal lysis, microtubule movement, and nuclear entry. The fiber knob and penton base of the virus interact and bind with the cell's primary receptor CAR (coxsackie and adenovirus receptor) and secondary receptors, integrins $\alpha v\beta 3$ and $\alpha v\beta 5$. These three receptors are found on many cell types including epithelial, muscle, fibroblast, and neural cells. Within 5 minutes of binding, adenovirus can be found inside the endosome allowing the penton bases to breakdown the endosomal membranes facilitating endosomal escape. The hexons of the virus maneuver the particle along the microtubules towards the cell's nucleus. Along the way, the viral capsid proteins surrounding the DNA are degraded and released allowing the DNA entry into the nucleus to begin the processes of gene expression and virus replication (Majhen *et al*., 2006; Seth, 1999; Volpers *et al*., 2004).

After integrating with the nucleus, early virus proteins are expressed and force the cell to enter S-phase. During S-phase, the virus's DNA is synthesized using the host cells natural functions. Self-induced cell death is attenuated with

the expression of products produced by E1A preventing both p53-independent and dependent apoptosis. As the infection progresses, other genes encoding the structure of the virus are expressed until the cell produces approximately 10^4 to 10^5 particles. When the cell dies, the virus is spread to other nearby cells to produce more progeny infectious virions (Branton, 1999; Volpers *et al*., 2004).

3. Adenovirus Vectors for Gene Therapy

Adenovirus vectors, usually serotype 2 or 5, are specifically designed to minimize pathogenicity yet efficiently infect cells across a wide range of tissues. First generation vectors deleted the E1 portion of the virus to inhibit virus replication and replaced it with a transgene up to 4.9 kb. Since the E1 section of the vector was removed, the virus had to be propagated in *trans* by using a cell line containing the E1 gene product such as the human kidney 293 cell line. Further development of this vector also removed the E3 segment allowing a transgene capacity of up to 8.3 kb; however these vectors still displayed high toxicity and immunogenicity (Katayose *et al*., 1999; Verma *et al*., 2005; Volpers *et al*., 2004).

To decrease the immune response generated by the first generation adenovirus vectors and increase the loading capacity, second generation vectors deleted or inactivated the E2 and / or E4 segments. However, these vectors still produced high immunogenicity, which led to the development of “gutted” vectors. These third generation vectors allow incorporation of transgenes up to 36 kb

because they do not carry any viral sequences except the inverted terminal repeats and packaging signal. The drawback associated with gutless adenovirus vectors is that they are much more difficult to produce requiring a helper-dependent vector to provide the E1 functions in *trans* (Verma *et al*., 2005; Volpers *et al*., 2004).

4. Toxicity and Immunogenicity of Adenovirus Vectors

The host immune response associated with many gene delivery systems, including both non-viral and viral vectors, has been one of the major impediments facing current gene therapy treatment. Therefore, understanding and limiting this response is crucial to the development of viable gene therapy strategies. Recombinant adenoviral vectors have been one of the most widely studied vectors in gene therapy research because of their efficiency in transducing a wide variety of cells. However, adenovirus vectors are known to stimulate both the innate and adaptive immune system when given *in vivo* (Murvue, 2004).

The strength of the host's immune response to adenovirus vectors is greatly influenced not only by the dose of particles administered, but also by the route of administration, which inherently can alter localized uptake (Zhou *et al*., 2004). Intravenous adenoviral administration results mostly in hepatic and splenic uptake, while other routes of administration result in more localized viral distribution patterns. Rats dosed with adenovirus by other routes of administration, such as intraperitoneal, subcutaneous, and mucosal, show limited

signs of toxicity compared to animals dosed intravenously (Morrissey *et al*., 2002).

The initial innate response triggered against the adenovirus capsid takes place within hours and is characterized by the induction of cytokines and chemokines including tumor necrosis factor α, interleukin-6, interleukin-1β, interferon γ, macrophage inflammatory protein 2 (MIP-2), MIP-1α, and MIP-1β. These agents act to localize the infection and recruit effector cells such as macrophages, monocytes, natural killer cells, and granulocytes to the site for additional support. This first phase immune response takes place within 24 hours and is produced by all generations of adenovirus vectors, including those inactivated, indicating that viral genes do not provoke the innate response (Murvue, 2004).

The second phase of the immune response occurs 4 to 5 days after administration and is mounted by the adaptive immune system. This response is characterized by helper $CD4^+$ T cells activated by MHC I complexes on the surface of antigen presenting cells and the formation of cytotoxic $CD8^+$ T cells by the binding of $CD8^+$ cells to the MHC I complex. $CD4^+$ T cells release interleukin-2, interleukin-4, and interferon-γ to enhance the development of the immune response against the virus. Cytotoxic $CD8^+$ T cells are produced specifically against the virus itself or the transgene product (Kaplan, 1999). This response against the virus is not observed with gutless or inactivated vectors indicating the need for gene transcription for significant adaptive recognition (Murvue, 2004).

5. Adenovirus Vectors as Vaccines

Despite the presence of over 400 discrete viruses with the ability to infect humans, vaccines are available for less than 20 of these pathogens (Xiang *et al*., 2003). Historically, traditional means of vaccination involved the use of inactivated pathogens introduced systemically to provide immunity. This approach has limited ability in protecting against diseases such as HIV, herpes, or Ebola and has yet to produce any approved vaccines against parasitic or fungal human diseases (Souza *et al*., 2005). With recent advances in molecular biology, many approaches using DNA and recombinant viruses as vaccine carriers are under investigation and have demonstrated some success in a number of preclinical and clinical trials (Bangari et al., 2006). One of the most successful vehicles in this field thus far has been the adenovirus vector.

Previous use of adenovirus vectors by the US military has demonstrated safety and efficacy using this virus as a vaccine for respiratory disease (Souza *et al*., 2005). Adenovirus vectors are a good choice for a vaccine carrier because it generates vigorous T and B cell responses to its transgene products (Kobinger *et al*., 2005). They have also demonstrated the ability to generate strong immunity using many different routes of administration including subcutaneous, intravenous, intramuscular, and mucosal (Bangari et al., 2006).

The majority of adenovirus vector vaccine work has centered on human adenovirus serotype 5 (HAd5). HAd5 is currently undergoing pre-clinical trials as a vaccine vector against Ebola virus, HIV-1, malaria, anthrax, and severe acute

respiratory syndrome. Non-human primates in these studies were protected against lethal challenge after immunization with the HAd5 expressing specific transgenes (Bangari et al., 2006; Brave *et al*., 2006). Although current vaccination strategies using this vector have had some success, its use has been constrained by the presence of pre-existing immunity to human serotypes in about 50% of the population (Nwanegbo *et al.*, 2004; Xiang *et al*., 2003). Recently, it has been demonstrated that the induction of these adenovirus-specific neutralizing antibodies that reduce efficacy of initial treatment and booster immunizations does not apply to oral delivery (Xiang *et al*., 2003).

6. Oral Administration of Adenovirus Vectors

Adenovirus is a good candidate for oral vaccine delivery because it is capable of inducing antibody responses against an encoded transgene product at mucosal surfaces, which may result in both complete systemic and mucosal immunity (Boyer et al. 2005). Mucosal immunization is highly desirable for vaccination because many infectious pathogens naturally access the host through mucosal membranes. This non-invasive means of delivery increases practicality and compliance by reducing injection site reactions and the risk of disease transmission through biohazzardous waste, which can occur through the more commonly used intramuscular method of administration.

Although the oral route is generally considered the pinnacle route of administration of many common medicinal agents, there are limitations

associated with the oral delivery of vaccines. Protein and virus-based vaccines are sensitive to the low pH and presence of proteases in the gut limiting their activity *in vivo*. Cellular uptake is also hindered by the rapid transit time of compounds through the intestine and the limited ability of intestinal epithelial cells to readily absorb large molecules (Delgado *et al*., 1999; Singh *et al*., 1998).

In general, there are two primary strategies for enhancing mucosal immunity: mucosal adjuvants and encapsulation in microparticles (Lameiro *et al*., 2006). Although several groups have successfully encapsulated adenovirus vectors into polymeric formulations, it has been found that the encapsulation process drastically reduces viral function (Barrio *et al*., 2004; Beer *et al*., 1998; Davison *et al*., 1997). However, the best performance has been reported with poly(lactide-co-glycolide) (PLGA) based preparations (Lameiro *et al*., 2006).

7. PLGA for Microsphere Production

PLGA is an FDA approved, amorphous, non-toxic, biodegradable polymer (molecular weight between 10 to 100 kDa) composed of lactide and glycolide monomers of varying ratios (50:50 to 100:0) linked together by ester bonds. Degradation of PLGA occurs by hydrolytic cleavage of the ester bonds in aqueous fluids producing lactic and glycolic acids which are metabolized into carbon dioxide and water (Tamber *et al*., 2005). The degradation rate of the polymer can be adjusted by changing the monomer ratio, the molecular weight, or hydrophilicity of the polymer (Kang *et al*., 2001). In recent years, the use of

this polymer to entrap antigens into polymeric microspheres for controlled release drug delivery systems has been extensively studied.

The most common technique to encapsulate antigens into polymeric microspheres is solvent evaporation. This method involves dissolving the polymer in an organic solvent and dispersing the antigen by homogenization to form a water-in-oil emulsion. This primary emulsion is further dispersed in a larger aqueous volume forming a water-in-oil-in-water (W / O / W) emulsion producing the antigen loaded microspheres. Many factors such as polymeric properties, organic solvents, stirring rates, emulsion stabilizers, and concentrations have been found to significantly alter antigen stability during encapsulation (Beer *et al*., 1997; Singh *et al*., 1998; Tamber *et al*.; 2005).

Dichloromethane and ethyl acetate are the two most commonly used organic solvents for solvent evaporation with PLGA. Some important factors that must be considered when choosing the organic solvent to use for this process include toxicity, volatility, and aqueous solubility. Dichloromethane has a median lethal dose (LD_{50}) in rats of 5.62 g / kg, a boiling point of 40°C, and an aqueous solubility of 2% v / v. Although ethyl acetate demonstrates improved tolerance (LD_{50} of 1.6 g / kg in rat) and has been used orally in humans to improve flavor in foods, it has a much higher boiling point of 77°C and increased water solubility of 10% v / v (Sah *et al*., 1997; Singh *et al*., 1998). The higher boiling point increases the amount of residual solvent in the system after the evaporation time under vacuum. This is especially important for protein or virus-based systems which

would be sensitive to the elevated temperatures required to remove all of the solvent.

Microsphere size can be influenced by both the concentration of polymer used to form the primary emulsion and the stirring rate used to form the emulsion (Singh *et al*., 1998). Particle size is of particular importance for oral vaccine delivery with PLGA because microspheres less than 10 μm have been found to be naturally taken up by the Peyer's patches, where they are presented to antigen presenting cells (Desai *et al*., 1997; Shakweh *et al*., 2005). Considering that the polymer concentration is directly proportional to particle size, lower polymer concentrations between 2 to 5 % are often used to achieve small particles sizes. The stirring rate is inversely proportional to particle size with faster rates producing a decrease in microsphere diameter; however the increased stirring rates can denature protein and virus-based drugs due to the shear stress on the compounds (Stivaktakis *et al*., 2004).

Another important factor to consider during microsphere formation is the choice and concentration of stabilizer used in both the primary and secondary emulsion steps. The concentration of stabilizers found in most microshere preparations usually range from 0.1% to 10% with higher concentrations yielding increased loading levels and more regular size distributions. Polyvinyl alcohol (PVA) is the most commonly used stabilizer in W / O / W emulsions because it demonstrates high solubility in water and has a low toxicity (Singh *et al*., 1998).

8. Summary of Objectives

Because of the many factors influencing the stability and release of adenovirus vectors from PLGA microsphere formulations, we propose that optimizing each parameter in the emulsion process will enhance virus stability and maximize release over time. An initial preparation was prepared with 1 ml of 5×10^{12} virus particles (vp) / ml of a first generation adenovirus vector expressing the beta-galactosidase transgene using a PLGA concentration of 100 mg / ml, a polymer lactide to glycolide ratio of 50:50, dichloromethane, and 1% w / v PVA. This microsphere preparation released 1.44×10^{6} infectious virus particles (ivp) over a period of 10 days resulting in over a 4-log drop from the number of infectious virus particles incorporated into the system. Therefore, the first objective of this study is to increase the total number of infectious virus particles released over time from the system.

The second objective of this study is to investigate the basis for this loss in infectious virus released after encapsulation, as well as, determine the influence of microsphere storage conditions on virus release. Studies will be performed to determine the stability of adenovirus upon exposure to organic solvents and release buffer. The influence of different storage conditions, length of storage, and drying microspheres after collection will also be investigated.

Materials and Methods

1. Chemicals and Reagents

PLGA polymers with monomer ratios of 50:50, 65:35, and 75:25, polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), polyethyleneimine (PEI), phosphate buffered saline (PBS), pepsin, Hanks' Balanced Salt solution (HBBS), non essential amino acid solution (NEAA), sodium pyruvate, sodium caprate, sodium laurate, sodium taurocholate, linoleic acid, ethylene glycol tetraacetic acid (EGTA), squalene, DEAE dextran, Tween® 80, and Span® 85 were purchased from Sigma Chemicals (St. Louis, MO). Ethyl acetate, dichloromethane, L-glutamine, sucrose, sodium chloride, sodium bicarbonate, and sodium citrate were obtained from EMD Chemicals (Gibbstown, NJ). X-gal was ordered from Gold Biotechnology Inc. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM, Cellgro, Mediatech, Herndon, VA) containing 10% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD) and 1% penicillin-streptomycin (PCN, 100 U/ml) was used for all cell culture work unless otherwise stated.

2. Adenovirus Purification.

A replication deficient adenovirus vector expressing the *E. coli* beta-galactosidase transgene (AdlacZ) was amplified in human embryonic kidney 293T cells and purified as previously described (Callahan *et al.*, 2006). Briefly, we purified the viral vector by cesium chloride density ultracentrifugation. After the first centrifugation, bands were collected and layered on a second gradient

for further purification. After final centrifugation, bands were collected and desalted on an Econo-Pac 10DG disposable chromatography column (BioRad, Hercules, CA). The viral fractions were collected and the concentrations were adjusted to a final concentration of 5×10^{12} virus particles (vp) / ml after determination by UV spectrophotometric analysis at 260nm (Beckman Du 530 UV/Vis, Fullerton, CA). We calculated the final number of vp / ml by the following equation:

Virus Concentration (vp/ml) = (Absorbance at 260nm) x dilution x 1.1 x 10^{12} (Maizel *et al.,* 1968).

Endotoxin tests were performed on all reagents used for adenovirus preparations with a QCL-1000 Chromogenic LAL Endpoint assay kit (Cambrex Bioscience, Walkersville, MD). Final adenovirus preparations used contained less than 0.2 E.U./ml. The sterility of all adenovirus preps used in this study was tested in accordance with the guidelines established for biotechnology-derived drug substances by the U.S. Pharmacopeia (2005).

Replication Competent Adenovirus Detection Assay (RCA) bioassay was performed on adenoviral preparations used in this study. Using the method of Murakami *et al.,* approximately one RCA event was detected for every 3×10^{12} virus particles tested (Murakami et al., 2002).

3. Microencapsulation of Virus – General Protocol

PLGA polymers (100 mg/ml) of varying monomer ratios were dissolved in 5 ml of organic solvent, either dichloromethane or ethyl acetate, to form a polymer solution. A primary emulsion was prepared by homogenizing 1 ml of AdlacZ (5×10^{12} vp / ml) in the polymer solution for 2 minutes. The resulting emulsion was then added to a 10 ml aqueous solution containing 1% (w/v) PVA, or other substituted surfactants at varying concentrations, and homogenized for an additional 2 minutes. The consequent water-in-oil-in-water (W/O/W) emulsion was added to a 100 ml aqueous solution containing 0.1% (w/v) PVA and stirred under vacuum for 2.5 hours to facilitate solvent evaporation. The formed microspheres were then collected by gentle centrifugation, washed three times with PBS, air-dried, weighed and either directly resuspended in 1 ml of PBS for *in vitro* release studies at 37°C, or stored at 24°C, 4°C, -20°C, or -80°C for stability release studies.

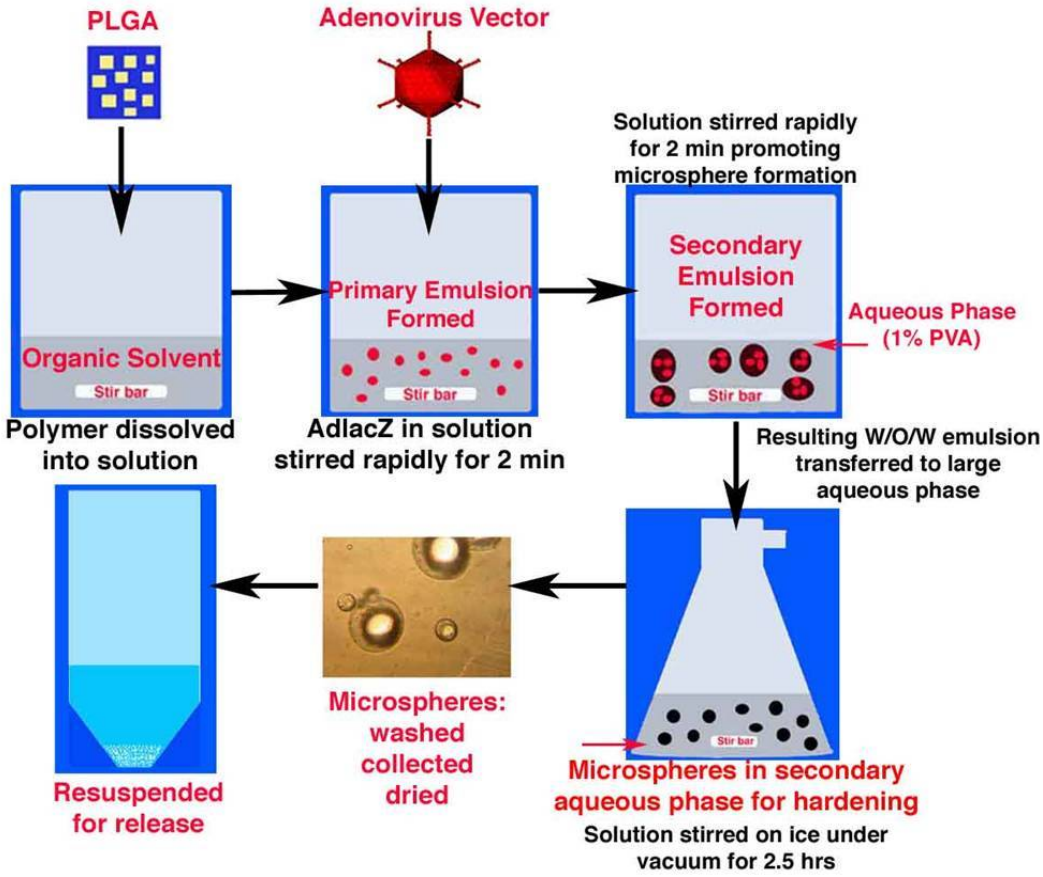

Figure 1: Microsphere Production Process. A primary emulsion is formed by rapidly stirring a high concentration of adenovirus with a solution of PLGA previously dissolved by homogenization in organic solvent. The primary emulsion is placed into an aqueous phase containing PVA to facilitate the formation of a W/O/W emulsion. The emulsion is transferred to a large aqueous volume containing a surfactant and stirred under vacuum for 2.5 hours to enhance solvent evaporation and allow microsphere hardening. Microspheres are then washed, collected, and air-dried. After drying, the microspheres can be either resuspended for release or stored for later use.

4. Modifications of General Protocol for Optimization

The general protocol formulation used for encapsulation was systematically adjusted to determine the influence of each parameter on AdlacZ stability and overall release profile. Parameters subject to optimization were:

a.) Polymer lactide:glycolide ratio
- 75 :25
- 65 :35
- 50 :50

b.) Polymer concentration in organic solvent
- 50 mg/ml
- 100 mg/ml

c.) Virus concentration in infectious virus particles (ivp) / ml
- 8.7×10^{10} ivp / ml
- 1.74×10^{11} ivp /ml

d.) Organic solvent
- Dichloromethane
- Ethyl acetate

e.) Osmotic balance of emulsion
- Aqueous solution - 1% PVA
- Aqueous solution - 1% PVA / 2% $NaHCO_3$ /10% sucrose

5. Microsphere Release Sampling Procedure

After suspension in PBS for release studies, samples were collected at 2 and 4 hours after suspension and then daily until virus release was no longer detected. At each sampling time point, microspheres were gently centrifuged to the bottom of the tube and the entire volume of supernatant was removed. After

removal, the microspheres were resuspended in 1 ml of PBS and returned to 37°C until the next sampling time. This process was repeated for every time point during the study.

6. Determination of Infectious Viral Particles

Supernatants from microspheres suspended in PBS for *in vitro* release studies were collected at various time points and frozen at -80°C in a 1:1 dilution with DMEM supplemented with 2% FBS and 1% PCN (2% media). Aliquots from frozen supernatants were serially diluted in 2% media and added to 293 cell monolayers for 2 hours at 37°C. After this time, 1ml of DMEM (10% FBS, 1% PCN) was added to each well and infection continued for 24 hours. Cells were stained histochemically with 5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside (X-gal) for beta-galactosidase expression (Croyle *et al*., 2002.). Cells expressing the transgene were counted and the number of infectious virus particles released was determined by the following formula:
(Average number of positive cells) x dilution x 845* x 10 = Number of ivp / ml
*This number is a magnification factor valid for 12-well culture plates using a 20x objective. Each aliquot from microsphere supernatant was assayed three separate times in three different wells. The averages of these three concentrations were used for the number of infectious virus particles released per time point.

7. X-gal Staining of Cells

Infected cells were washed with PBS, fixed with 0.5% glutaraldehyde for 10 minutes, and washed with a PBS solution of 1mM $MgCl_2$. The infected cells were subsequently stained with X-gal by incubating in the dark for at least 4 hours at 37°C. After the reaction, the stain was removed and the cells were washed with PBS to facilitate the counting of blue-stained positive cells. Ten fields per well were counted to determine the number of infectious virus particles released.

8. Simulated Gastric Fluid Release Studies

Simulated gastric fluid (SGF) containing 7 ml of 1N HCL, 2 g NaCl, and 3.2 g pepsin / L was prepared and used *in vitro* to simulate microsphere exposure to the the gut. A known quantity (0.45 g) of microspheres was exposed to SGF for 1 hour at 37°C and samples were taken at 15, 30, and 60 minutes. After the final sampling time, microspheres were collected by gentle centrifugation and the SGF was removed. The microspheres were washed three times with 10 ml of PBS and resuspended in buffer for release at 37°C according to the previously stated microsphere release sampling procedure. Washes were kept and analyzed for virus presence using the same method.

9. Particle Size

Particle size of microsphere preparations was determined at 0, 1, 7, and 14 days after suspension in buffer from release studies. An aliquot of microspheres were removed from the tube and particle size was measured by dynamic light scattering (Protein Solutions DynaPro, Wyatt Technlogy Co.).

10. Stability of Virus

Stability of AdlacZ was assessed to determine the effect of exposure to organic solvent during the encapsulation process and to incubation in the release buffer following release at 37°C. To determine stability of the virus in organic solvent, AdlacZ was diluted to 1 x 10^{11} vp / ml in either dichloromethane or ethyl acetate. Samples were collected at 0, 1, 2, 5, 10, 20, 30, and 60 minutes. To assess the stability of the virus in release buffer, AdlacZ was diluted in PBS and samples were collected at various time points. All samples were assayed immediately for quantifying the change in the number of ivp / ml.

11. Effect of Storage Conditions on Microsphere Release

After air-drying, collection, and weighing, equal quantities of microspheres were either resuspended immediately for release, stored short-term, or stored long-term. Short-term stability was performed on microspheres stored at 24°C and 4°C for 1 day and -20°C and -80°C for 1 week. Long-term stability was performed on microspheres stored at -80°C for 1 month. When the specified

time was reached, microspheres were removed from storage and suspended in PBS for *in vitro* release according to the microsphere release sampling procedure.

13. Validation of Sample Titer and Microsphere Prep Reproducibility

To determine the variability of intra-sample titers, four random samples were chosen from three identically prepared microsphere batches. Three aliquots from each sample were tittered in different wells of 293 cells and X-gal stained. The average number of infectious virus particles was assessed for each individual well. The variability of titers from each sample was reported as the relative standard deviation (RSD), where RSD equals the standard deviation divided by the means of the three samples.

For the determination of inter-prep variability of infectious virus particles released at specific time points, the means of three preps at each corresponding sampling time was assessed. The variability of infectious virus particles released at each time point for all three preps were assessed by the RSD. The variability in the total number of infectious virus particles released was determined by randomly picking four time points from three different preps and reporting the RSD.

Results

1. Inter-prep Variability of Release from Single Batch of Microspheres

For determination of inter-prep variability, three separate batches of microspheres were prepared following the same protocol on three different days. A virus concentration of 5×10^{12} vp / ml of AdlacZ, previously frozen in 1 ml aliquots from the same virus preparation, was used for each microsphere preparation for this study. The release profile of total infectious virus particles released is shown in Figure 2. There was no significant difference in the number of infectious virus particles released at any time during the course of the study. One day after resuspension, prep 1 released 9.42×10^6 ivp, prep 2 released 9.63×10^6 ivp, and prep 3 released 1.02×10^7 ivp. The total number of infectious virus particles released by the three preps remained comparable through 11 days. At that time, preps 1 and 2 both released a total of 1.1×10^7 ivp, whereas prep 3 released 1.6×10^7 ivp.

Inter-prep variability of the total number of infectious virus particles released is shown in Table 1. Four random time points (1, 4, and 7 days, and total release) were investigated for all three preparations and the RSD were reported. RSD for the earliest time point chosen was 4.14%; however the RSD climbed to 24.87% after all three preps completed their release at 11 days.

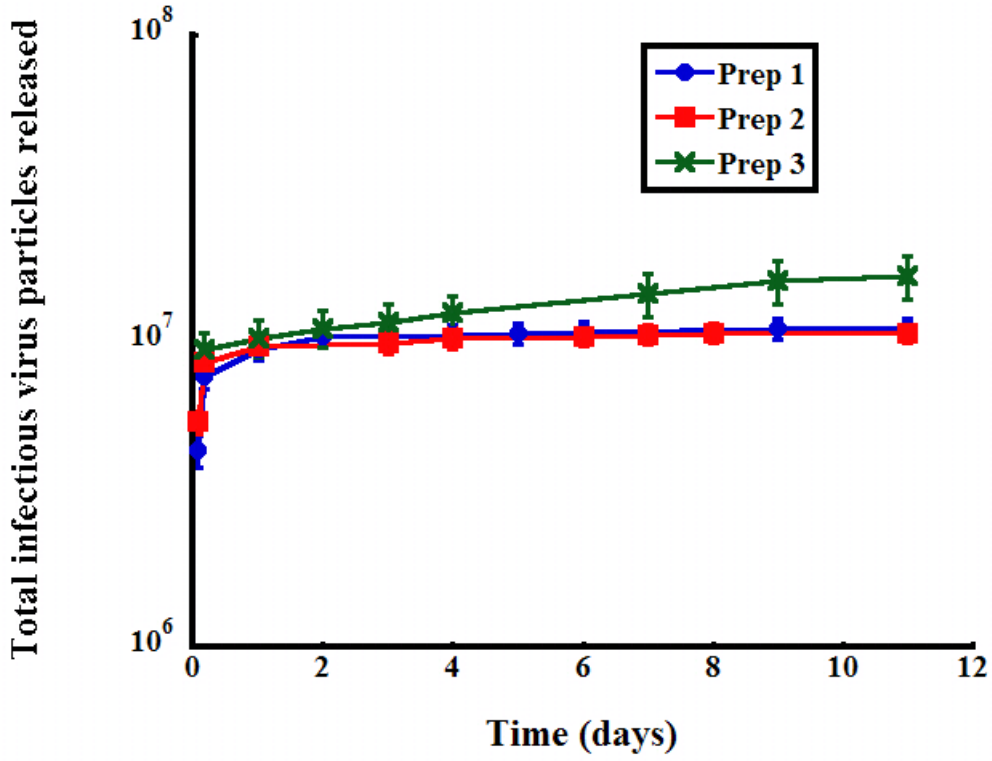

Figure 2: Inter-prep Release Variability. The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Three microsphere batches were prepared using general protocol with dichloromethane as the organic solvent. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant.

Table 1: Inter-prep Variability of Total Infectious Virus Particles

Sample Time	Prep #1	Prep #2	Prep #3	Mean	SD	RSD
1 day	9.42×10^6	9.63×10^6	1.02×10^7	9.75×10^6	4.04×10^5	4.14%
4 days	1.05×10^7	1.04×10^7	1.24×10^7	1.11×10^7	1.13×10^6	10.15%
7 days	1.08×10^7	1.04×10^7	1.43×10^7	1.18×10^7	2.15×10^6	18.13%
Total	1.10×10^7	1.07×10^7	1.63×10^7	1.27×10^7	3.15×10^6	24.87%

2. Validation of Intra-sample Titers

Intra-sample variability was performed using 3 aliquots from microsphere samples at each specified time point. Each aliquot was tested on a different well of 293 cells and X-gal stained for the determination of infectious virus particles released. This assay was performed on three different microsphere preparations and the means, standard deviation (SD), and relative standard deviations (RSD) were calculated for each and for all three preps at each randomly chosen time point (Table 2). At 4 hrs, the RSD for preps 1- 3 ranged from 5.68% to 16.79% with an overall RSD of 7.60%. The 1 day release showed somewhat more variability with the RSD ranging from 15.23% to 21.84%. The overall RSD for all three preps at 1 day was 24.34%. At 4 days, the RSD for the individual preps ranged from 7.38% to 19.99% with the overall RSD equaling 20.07%.

Table 2: Validation of Intra-sample Titers of Infectious Virus Particles

Sample Time	Prep #	Well 1	Well 2	Well 3	Mean	SD	RSD
4 hrs	1	$3.47x10^6$	$3.13x10^6$	$3.17x10^6$	$3.25x10^6$	$1.85x10^5$	5.68%
	2	$3.38x10^6$	$3.21x10^6$	$2.66x10^6$	$3.08x10^6$	$3.77x10^5$	12.21%
	3	$3.55x10^6$	$4.98x10^6$	$4.25x10^6$	$4.26x10^6$	$7.15x10^5$	16.79%
	All				$3.53x10^6$	$2.68x10^5$	7.60%
1 day	1	$2.20x10^6$	$1.69x10^6$	$1.44x10^6$	$1.77x10^6$	$3.87x10^5$	21.84%
	2	$1.18x10^6$	$1.26x10^6$	$9.30x10^5$	$1.12x10^6$	$1.72x10^5$	15.23%
	3	$1.32x10^6$	$1.01x10^6$	$1.52x10^6$	$1.28x10^6$	$2.57x10^5$	20.02%
	All				$1.39x10^6$	$3.39x10^5$	24.34%
4 day	1	$1.77x10^5$	$1.44x10^5$	$1.61x10^5$	$1.60x10^5$	$1.67x10^4$	10.41%
	2	$3.30x10^5$	$2.87x10^5$	$2.96x10^5$	$3.40x10^5$	$2.25x10^4$	7.38%
	3	$6.76x10^5$	$9.30x10^5$	$1.01x10^6$	$8.72x10^5$	$1.74x10^5$	19.99%
	All				$4.45x10^5$	$8.94x10^4$	20.07%

3. PLGA Monomer Ratio Influence on Virus Release

Microsphere batches were prepared using three different lactide:glycolide monomer ratios (75:25, 65:35, and 50:50) to determine the influence of each on infectious virus particles released over time. PLGA composed of a lactide:glycoldie ratio of 65:35 released the highest quantity of infectious virus particles, 2.45 x 10^7 ivp, over the course of the 24 hour period following resuspension (Figure 3). This value was a 23-fold increase in infectious virus particles released at that time point compared to that observed in microspheres prepared using a 50:50 polymer ratio (1.06 x 10^6 ivp). This 23-fold increase in total infectious virus particles released was maintained throughout the remainder of the study. PLGA composed of a lactide:glycolide ratio of 75:25 released 4 times more infectious virus particles (4.36 x 10^6 ivp) than the 50:50 polymer ratio 24 hours after suspension in release buffer. Even though the total amount released with this ratio is less than that released by the 65:35 polymer ratio, the rate of release of total infectious virus particles increased over the course of the next four days. The increase in rate can be observed by the 7-fold increase in total infectious virus particles seen at 4 days (9.2 x 10^6 ivp) compared to the 50:50 polymer ratio (1.3 x 10^6 ivp). The 7-fold increase in total infectious virus particles released was maintained throughout the remainer of the study. The 50:50 polymer ratio released a total of 1.44 x 10^6 ivp over 10 days. The 75:25 polymer ratio released a total of 1.02 x 10^7 ivp over 14 days and the 65:35 ratio released 3.07 x 10^7 ivp over 12 days.

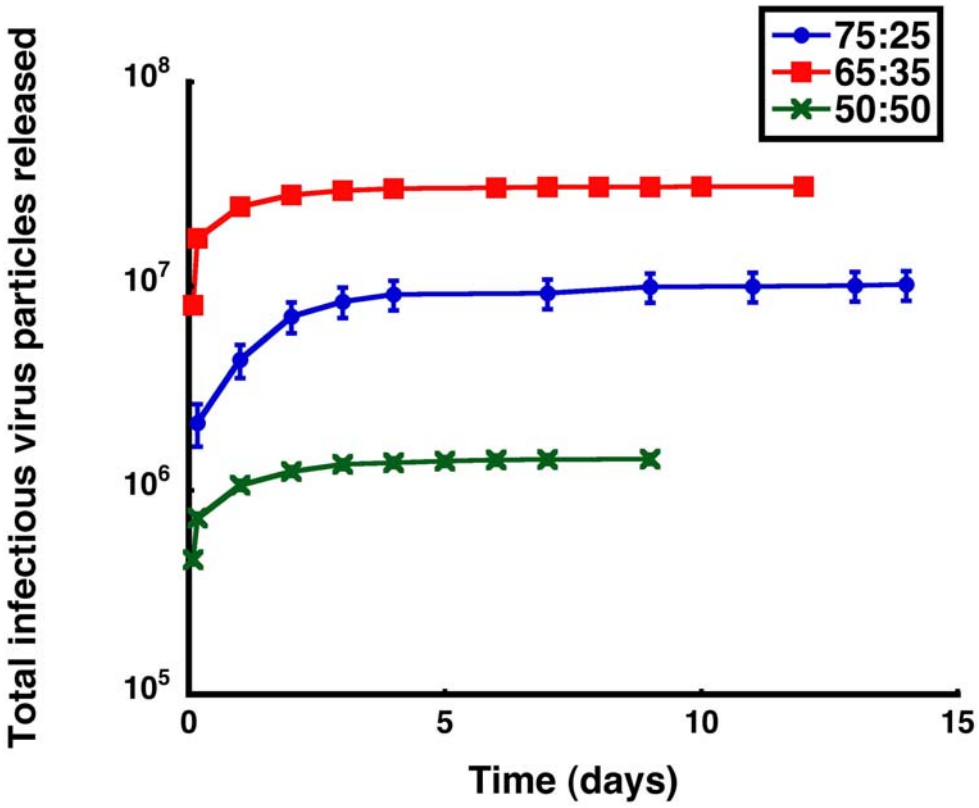

Figure 3: Effect of Lactide:Glycolide Ratio on Active Virus Release. The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Microspheres were prepared using general protocol with dichloromethane as the organic solvent. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant.

4. Influence of PLGA Concentration on Virus Release

To determine the influence of PLGA concentration used in the microsphere formulation on the infectious virus particles released over time, two different polymer concentrations, 50 mg/ml and 100 mg/ml, were employed. Over the course of 24 hours following suspension in release buffer, the microspheres prepared with 100 mg/ml PLGA released a total of 9.47×10^6 ivp, over 44 times more than that released from microspheres prepared using a 50 mg/ml concentration (2.14×10^5 ivp). The rate of release from the 50 mg/ml PLGA microspheres over the following 6 days increased compared to that of the 100 mg/ml (Figure 4). At 7-days, a total of 6.47×10^5 ivp were released from the 50 mg/ml PLGA, whereas 1.15×10^7 ivp were released from microspheres prepared with 100 mg/ml PLGA. This 18-fold increase in total infectious virus particles released was observed throughout the remained of the study. The 50 mg/ml polymer concentration released a total of 6.52×10^5 ivp for 8 days and the 100 mg/ml preparation resulted in 1.19×10^7 ivp released over a 10-day period.

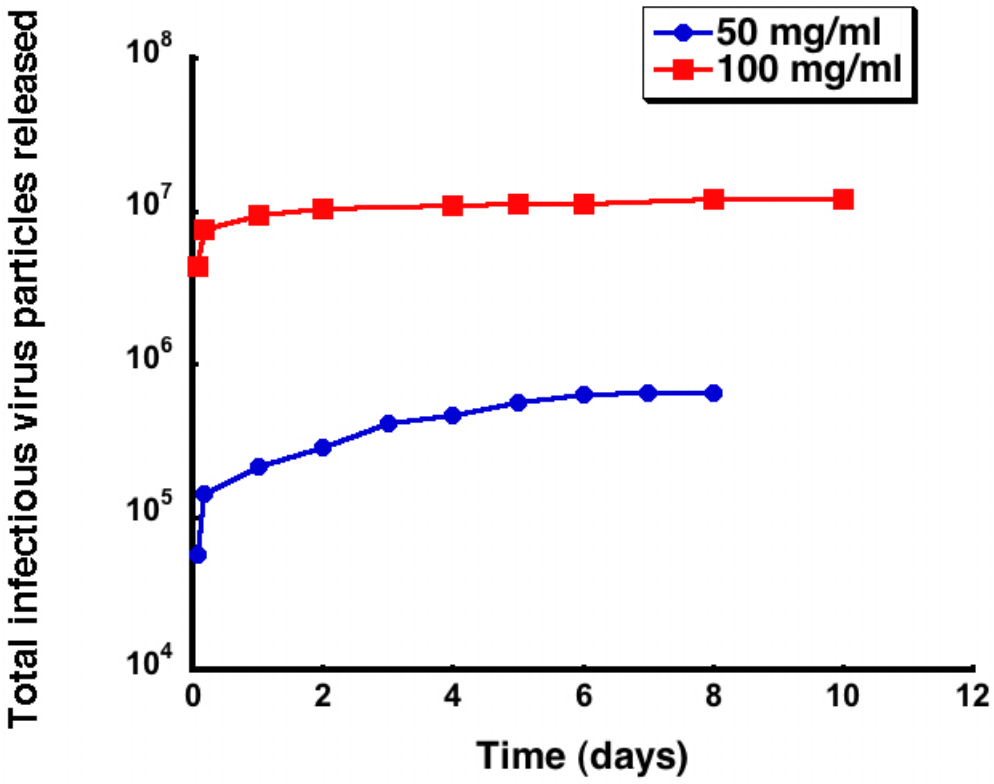

Figure 4: Effect of Polymer Concentration on Active Virus Release. The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Microspheres were prepared using general protocol with dichloromethane as the organic solvent and PLGA polymer concentrations of either 50 mg/ml or 100 mg/ml. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant.

5. Influence of the Amount of Virus Incorporated into Formulation

Microsphere preparations were made using two different volumes of virus (1 ml and 2 ml at a pre-titered concentration of 8.7×10^{10} ivp / ml) to determine the influence of each on the number of infectious virus particles released over time. Therefore, a total of 8.7×10^{10} ivp was incorporated into the microsphere preparations containing 1 ml of virus and 1.74×10^{11} ivp into the microsphere batches containing 2 ml of virus. PLGA microspheres formulated with 1 ml of virus released 6 times more infectious virus particles (1.02×10^{7} ivp) over the course of the 24 hour period following resuspension than those made with twice the amount of virus (1.67×10^{6} ivp). At 4 days, the formulations containing 1 ml of virus released 1.24×10^{7} ivp compared to 2.9×10^{6} ivp for those prepared with 2 ml (Figure 5). The 1 ml virus microsphere preparations released a total of 1.65×10^{7} ivp over the course of 15 days; whereas the batches made with twice the amount of virus only released 2.92×10^{6} ivp over an 8-day period.

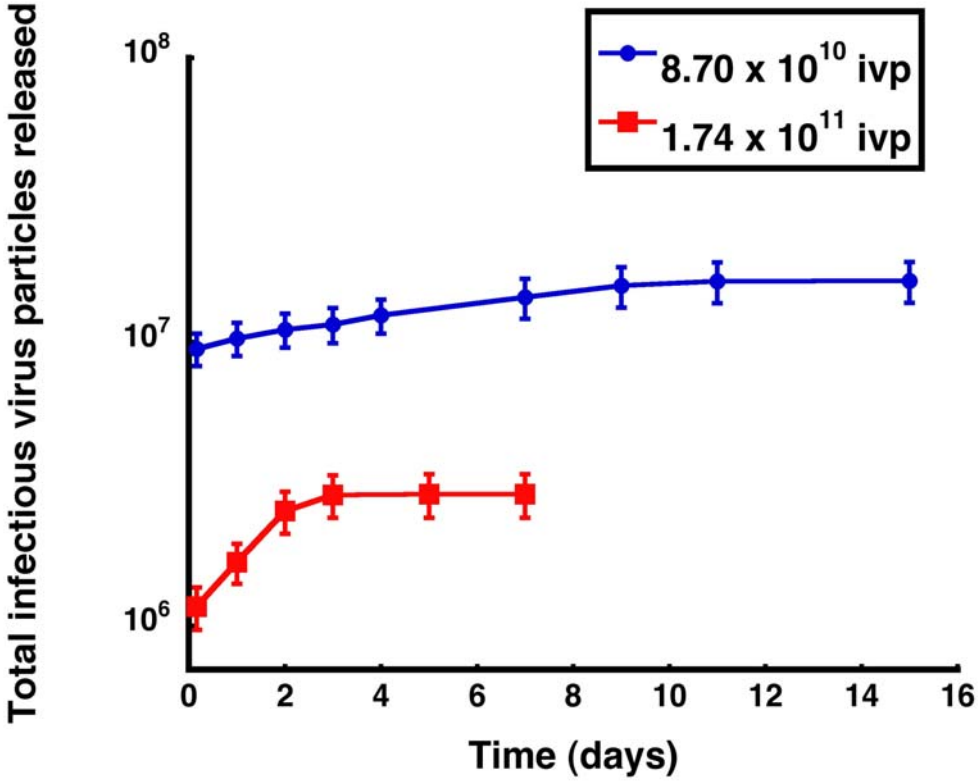

Figure 5: Effect of Increasing the Amount of Virus Loaded on Active Virus Release. The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Microspheres were prepared using general protocol with dichloromethane as the organic solvent, PLGA polymer concentration of 100 mg/ml, and lactide:glycolide ratio of 65:35. Microsphere batches containing 8.7 x 10^{10} ivp were prepared with 1 ml of virus and those containing 1.74 x 10^{11} ivp were prepared with 2 ml of virus. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant.

6. Influence of Organic Solvent on Virus Release

Microsphere preparations were prepared using two different organic solvents, dichloromethane and ethyl acetate, to determine the influence of each on the number of infectious virus particles released over time. The total number of infectious virus particles released from microspheres prepared with ethyl acetate (2.89×10^8 ivp) was approximately 10 times more than the quantity released from preparations made with dichloromethane (3.08×10^7 ivp) . This increase by a factor of 10 was seen at 1, 4, and 7 days following suspension in release buffer (Figure 6). At 24 hours, microspheres prepared with dichloromethane released only 2.45×10^7 ivp compared to the 2.54×10^8 ivp released from the ethyl acetate preparations. Similarly at 4 days, the dichloromethane and ethyl acetate batches released a total of 2.98×10^7 ivp and 2.81×10^8 ivp, respectively. Detectable infectious virus particles were released for 14 days with those prepared using ethyl acetate compared to only an 11-day period for the ones produced with dichloromethane as the solvent.

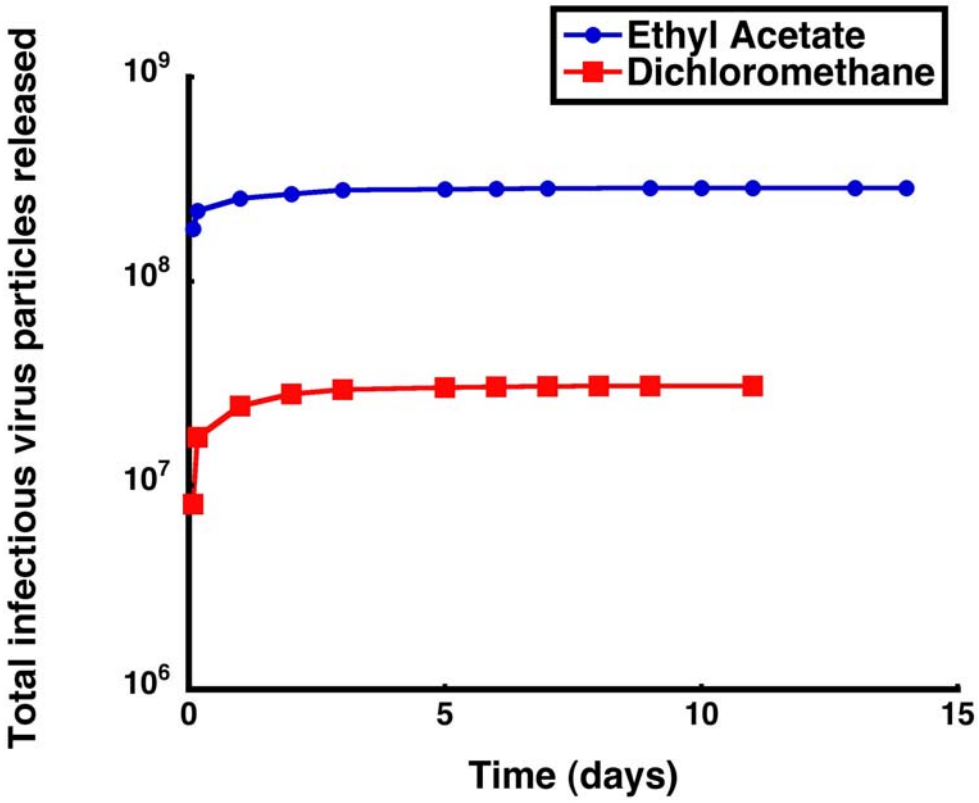

Figure 6: Effect of Organic Solvent on Active Virus Release. The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Microspheres were prepared using general protocol with either dichloromethane or ethyl acetate as the organic solvent, a PLGA polymer concentrations of 100 mg/ml, and lactide:glycolide ratio of 65:35. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant.

7. Comparison of Original and Optimized Formulations

To compare the influence of optimizing all parameters investigated in the previous results, microspheres were prepared using the original and optimized formulation. The original microsphere formulation was prepared following the general protocol utilizing dichloromethane as the solvent to dissolve a 100 mg / ml concentration of PLGA with a 50:50 lactide:glycolide ratio. After taking into consideration the results of changing each parameter investigated, the optimized formulation consisting of ethyl acetate and a 65:35 lactide:glycolide ratio was also formulated. Both the original and optimized formulations were tested with 1 ml of 5×10^{12} vp / ml; however, the infectious virus concentration used for these preparations, 6.1×10^{11} ivp / ml, was larger than any previously used. Total infectious virus particles released by the optimized formulation in the initial 24 hours following resuspension was approximately 50-fold higher (3.76×10^{8} ivp) than the active virus released from the original formulation (7.65×10^{6} ivp). The optimized formulation continued to release detectible amounts of infectious virus particles for 13 days (Figure 7). The total amount released by day 13 was 6.69×10^{8} ivp, over 63 times greater than that released over the course of 7 days by the original formulation (1.06×10^{7} ivp).

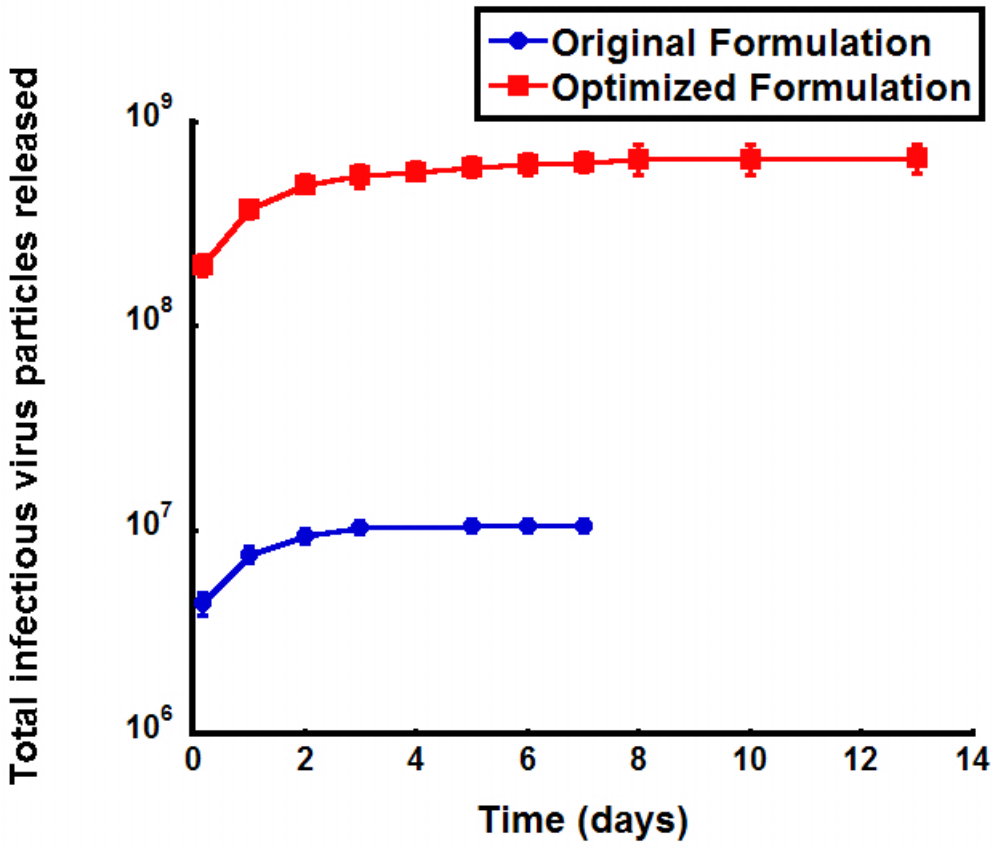

Figure 7: Effect of Optimizing Microsphere Formulation on Active Virus Release. The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Microspheres prepared with the original formulation were formulated according to the general protocol using dichloromethane, a PLGA polymer concentration of 100 mg/ml, and a lactide:glycolide ratio of 50:50. The optimized formulation was prepared with ethyl acetate, a 100 mg / ml polymer concentration, and a polymer ratio of 65:35. 1 ml of virus at a concentration of 6.1 x 10^{11} ivp / ml was incorporated into each preparation. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant.

8. Influence of Sucrose and Sodium Bicarbonate on Formulation

To further optimize the formulation, sucrose (10% w/v) was added to the formulation in the primary aqueous phase and the large secondary aqueous phase to help maintain the osmotic balance during the emulsion process (Srinivason et al., 2005). The sucrose would also help maintain virus activity by displacing some of the virus from the harmful W/O interface. Sodium bicarbonate (2% w/v) was also added to the primary aqueous phase to increase the pH to mildly acidic conditions to help further enhance adenovirus stability during microsphere formation (Rexroad *et al*., 2006). Total infectious virus particles released by the sucrose formulation in the initial 24 hours following resuspension was approximately 3-fold higher (1.05×10^9 ivp) than the active virus released from the optimized formulation (3.76×10^8 ivp). The sucrose formulation continued to release detectible amounts of infectious virus particles for 15 days, which was two days longer than the optimized formulation (Figure 8). The total amount released by day 15 was 2.32×10^9 ivp, over 3.5 times greater than that released over the course of 13 days by the optimized formulation (6.69×10^8 ivp).

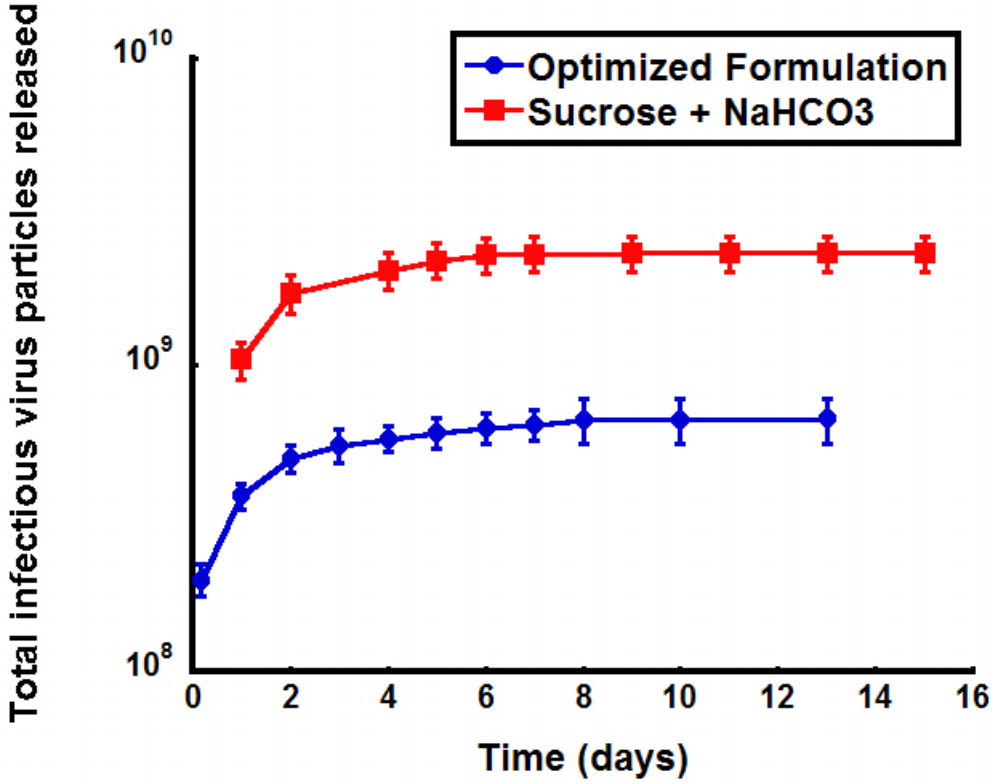

Figure 8: Effect of Sucrose and Sodium Bicarbonate on Active Virus Release. The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Microspheres in both the optimized formulation and the sucrose formulation were prepared according to the general protocol with ethyl acetate, a 100 mg / ml PLGA, a polymer ratio of 65:35, and 1 ml of virus at 6.1 x 10^{11} ivp / ml. Those containing sucrose + $NaHCO_3$ contained 10% sucrose, 2% $NaHCO_3$, and 1% PVA in the primary aqueous phase and 10% sucrose and 0.1% PVA in the large aqueous volume. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant.

9. Representative Particle Size Distribution of Microspheres

The particle size distribution of microspheres was taken at 0, 1, 7, and 14 days after resuspension in release buffer (Table 3). Microspheres were approximately 3800 nm in size. Adenovirus in release buffer was also detected with a particle size of 35 nm. At later sampling times, the free adenovirus becomes aggregated as indicated by the increased particle size.

Table 3: Representative Particle Size Distribution

Time (days)	Particle Size Distribution (nm) ± Standard Deviation	% of Population
0	35.4 3844	5.2 80.6
1	72.11 ± 7.17 3883	8.63 67.7
7	100.2 3887	8.30 88.1
14	50.46 ± 6.92 3586 ± 432.8	3.96 96.04

10. Influence of Simulated Gastric Fluid on Release Profile

To simulated microsphere exposure to the gut, microspheres were exposed to simulated gastric fluid (SGF) for 1 hour at 37°C, washed three times, and resuspended in buffer for release at 37°C. Total virus release was approximately 3.4×10^6 ivp for preparations suspended in SGF for 1 hour or suspended directly in PBS (Figure 9). However, 50% of the virus released from the microspheres incubated in SGF was obtained during incubation in SGF and recovered during the three washing steps (Table 4).

Table 4: Active Virus Released After Exposure to SGF

Sample	Infectious Virus Particles
15 min	**4.2×10^5**
30 min	**1.1×10^6**
60 min	**1.9×10^5**
Wash	**1.2×10^6**
Total From SGF	**2.91×10^6**

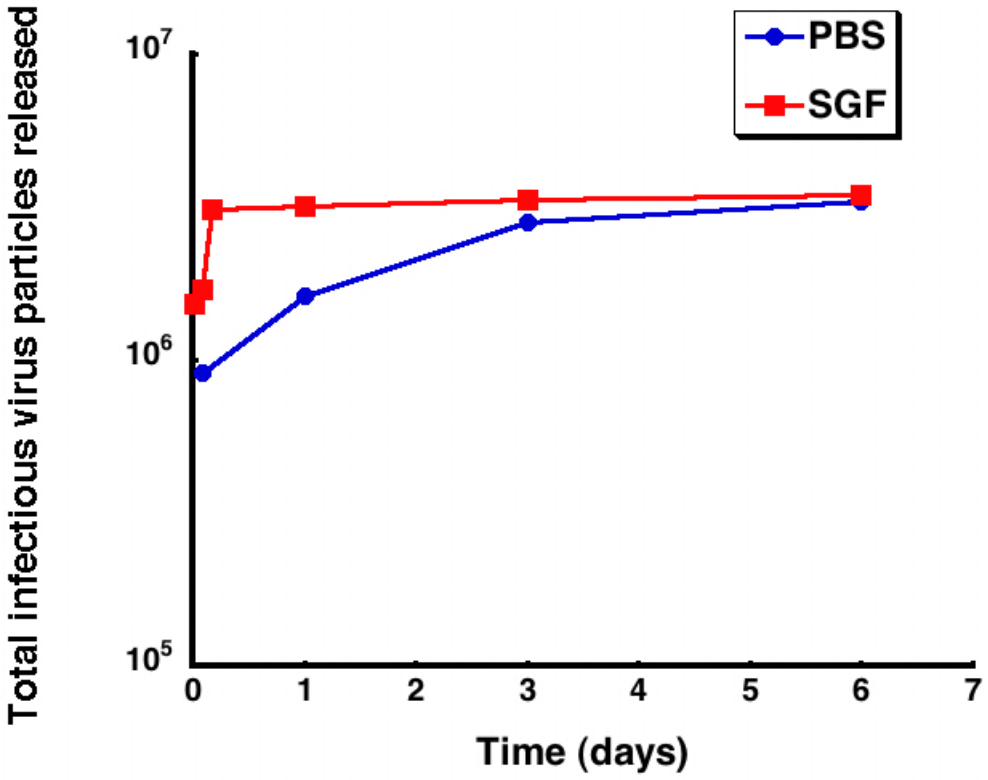

Figure 9: Effect of Simulated Gastric Fluid on Active Virus Release. The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Microspheres were prepared using general protocol with ethyl acetate, 100 mg/ml PLGA, and a ratio of 65:35. Studies denoted by both PBS and SGF consisted of 0.45 g of microspheres taken from the same preparation. The SGF study was exposed to simulated gastric fluid for 1 hour, washed three times, and resuspended in PBS. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant contained in SGF, washes, and PBS.

11. Influence of Air-drying Microspheres on Virus Release

Microsphere preparations were made to determine if the process air-drying had any effect on the release profile of infectious virus particles from the system. The 30 min drying process did not significantly affect the amount of infectious virus particles release ($P > 0.05$, Figure 10). There was a slight difference in the early burst released between the microspheres that were allowed to dry and those immediately resuspended. Within the first 4 hours, the microspheres that were immediately resuspended released 2.2×10^8 ivp, almost twice as much as that released by the dried microspheres (1.2×10^8 ivp). However by 2 days, both the dried and immediately resuspended preparations released approximately 2.6×10^8 ivp. This comparable release profile remained similar for both the immediately resuspended and air-dried preparations releasing approximately 3×10^8 ivp over the course of 15 days.

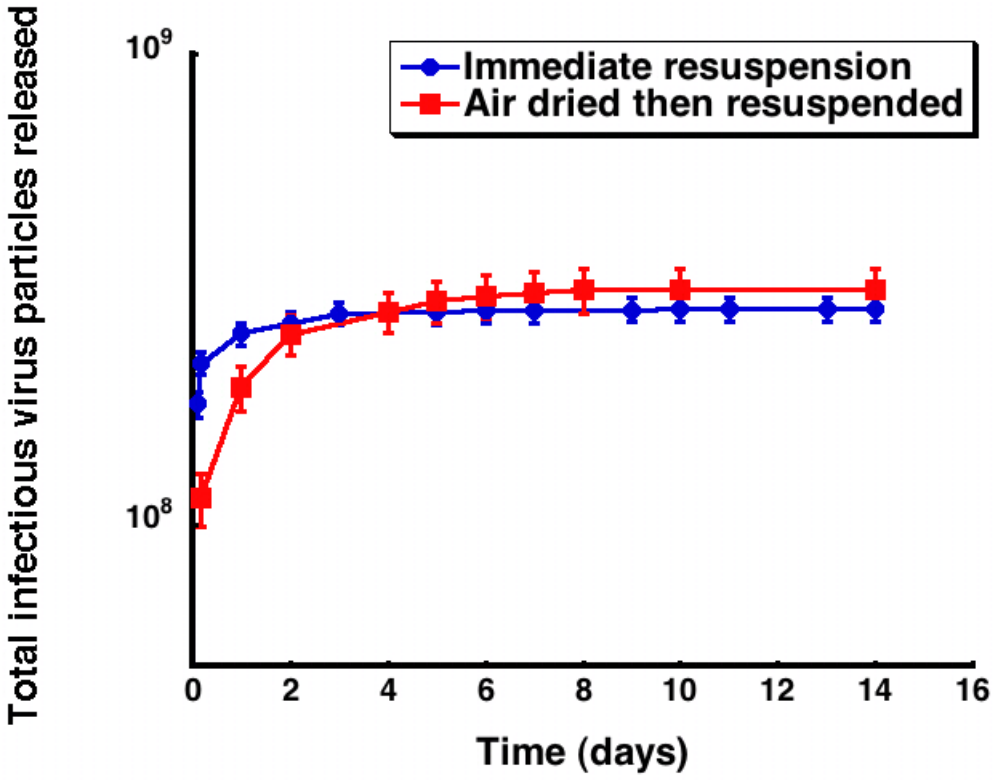

Figure 10: Effect of Drying Microspheres Prior to Resuspension. The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Microspheres were prepared using general protocol with ethyl acetate, 100 mg/ml PLGA, and a ratio of 65:35. After collection, microspheres were either immediately resuspended in PBS for release or air-dried in a laminar flow hood for 30 min prior to resuspension in PBS. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant.

12. Effect of Organic Solvent on Virus Stability

The influence of the choice of organic solvent on the titer of infectious virus particles was investigated by exposing AdlacZ diluted to a concentration of 1×10^{11} vp / ml with either dichloromethane or ethyl acetate. Samples were collected for 60 min and tittered to determine changes from the initial titer of 4.1×10^{9} ivp / ml (Figure 11). AdlacZ exposed to dichloromethane experienced a drop in infectious titer to 1.1×10^{9} ivp / ml after only 5 min in the solvent; whereas the virus exposed to ethyl acetate nearly maintained its initial titer during the same period. By 10 min, dichloromethane reduce the active virus concentration 17-fold (2.3×10^{8} ivp / ml) compared to only a 10% decrease (3.7×10^{9} ivp / ml) observed in virus exposed to ethyl acetate. After 15 min, there was no change in the infectious titer observed throughout the remainder of the 60 min study. Final concentrations of 1.5×10^{9} ivp / ml and 6.5×10^{8} ivp / ml were found at 60 min for the virus exposed to ethyl acetate and dichloromethane, respectively.

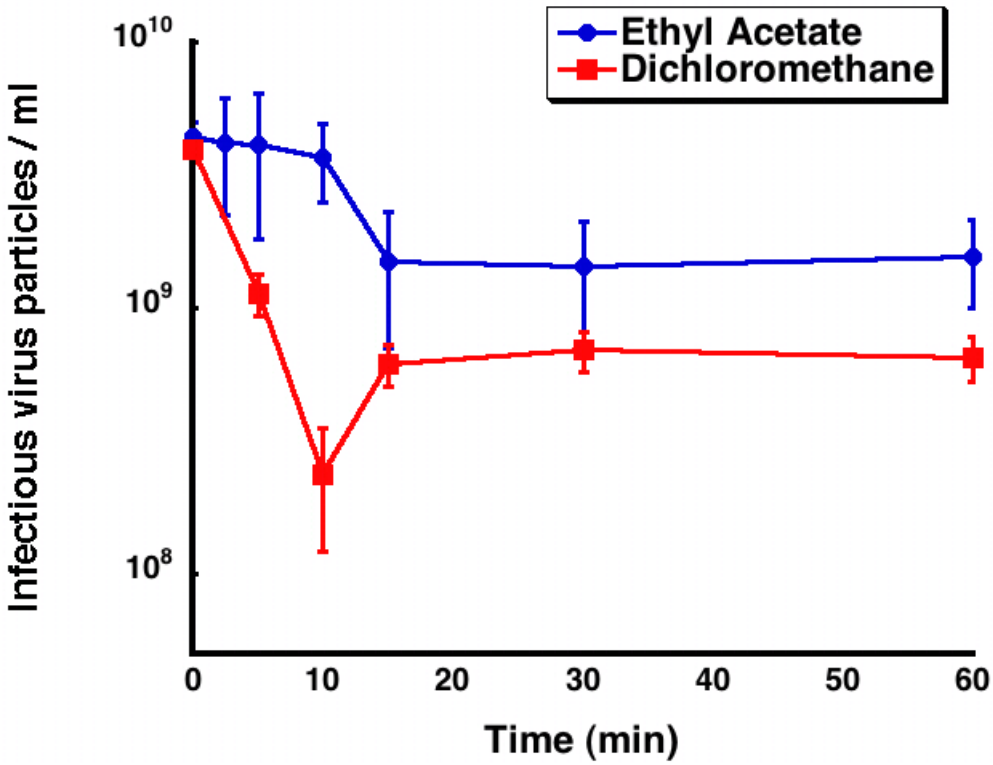

Figure 11: Stability of Adenovirus in Organic Solvent. The data are based on total amount of infectious virus particles determined by X-gal staining of 293T cells infected with samples taken from 1 x 10^{11} vp /ml of AdlacZ diluted in either dichloromethane or ethyl acetate. Initial titer of the dilution used for this study was 4.0 x 10^{9} ivp / ml. All samples were assayed immediately for quantifying the change in the number of ivp / ml. The data represent the mean ± standard error of three separate titers using aliquots from each sample.

13. Effect of Release Buffer on Virus Stability

To determine if the release buffer influenced the stability of AdlacZ released prior to sampling, 1.36 x 10^9 ivp / ml was placed into PBS (pH 7.4) at 37°C for 14 days. Loss of titer in the buffer was experienced with the most significant loss of 54% (7.4 x 10^8 ivp / ml) seen within the first 24 hours (Figure 12). Virus titer continued to decrease at a much slower rate over the remaining 14 days. Over the course of the study, virus titer dropped by 2 logs to 2.6 x 10^7 ivp /ml.

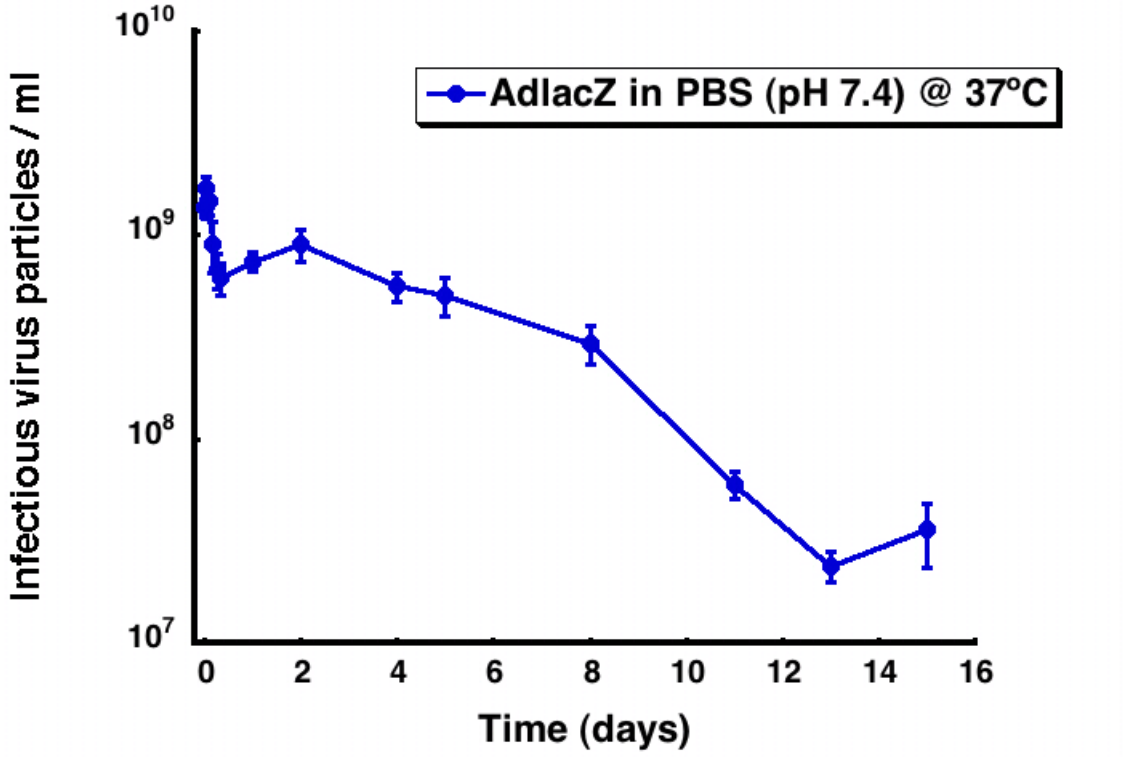

Figure 12: Stability of Adenovirus in Release Buffer. The data are based on total amount of infectious virus particles determined by X-gal staining of 293T cells infected with samples taken from 1 x 10^{11} vp /ml of AdlacZ diluted in PBS (pH 7.4) at 37°C. Initial titer of the dilution used for this study was 1.36 x 10^{9} ivp / ml. All samples were assayed immediately for quantifying the change in the number of ivp / ml. The data represent the mean ± standard error of three separate titers using aliquots from each sample.

14. Effect of Storage Conditions on Microsphere Virus Release

Microsphere preparations were made and either immediately resuspended in PBS for release or stored for 1 day at 24°C, 1 day at 4°C, 1 week at -20°C, 1 week at -80°C, or 1 month at -80°C. Storage at 20°C and -80°C maintained virus stability for 1 week (Figure 13); however preparations stored at 24°C did not release any infectious virus particles after resuspension (Figure not shown). Storage at 4°C for 1 day reduced the number of infectious virus particles released by 73% and also shortened the release time from 12 to 9 days. Adequate stability can be maintained at -80°C for over 1 month (Figure 14).

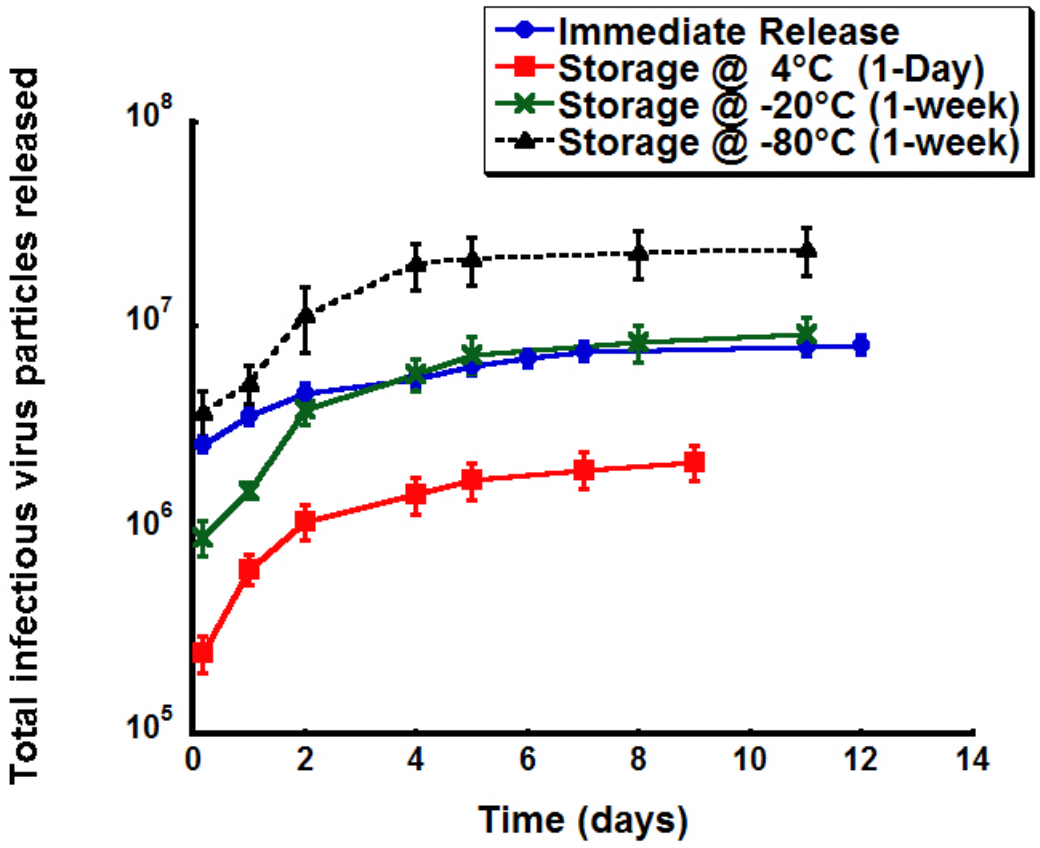

Figure 13: Effect of Short-term Storage on Microsphere Virus Release.
The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Microspheres were prepared using general protocol with ethyl acetate, 100 mg/ml PLGA, and a ratio of 65:35. After collection, 0.4 g of microspheres were either immediately resuspended in PBS for release or stored at 4°C for 1 day, -20°C for 1 week, or -80°C for 1 week prior to resuspension. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant.

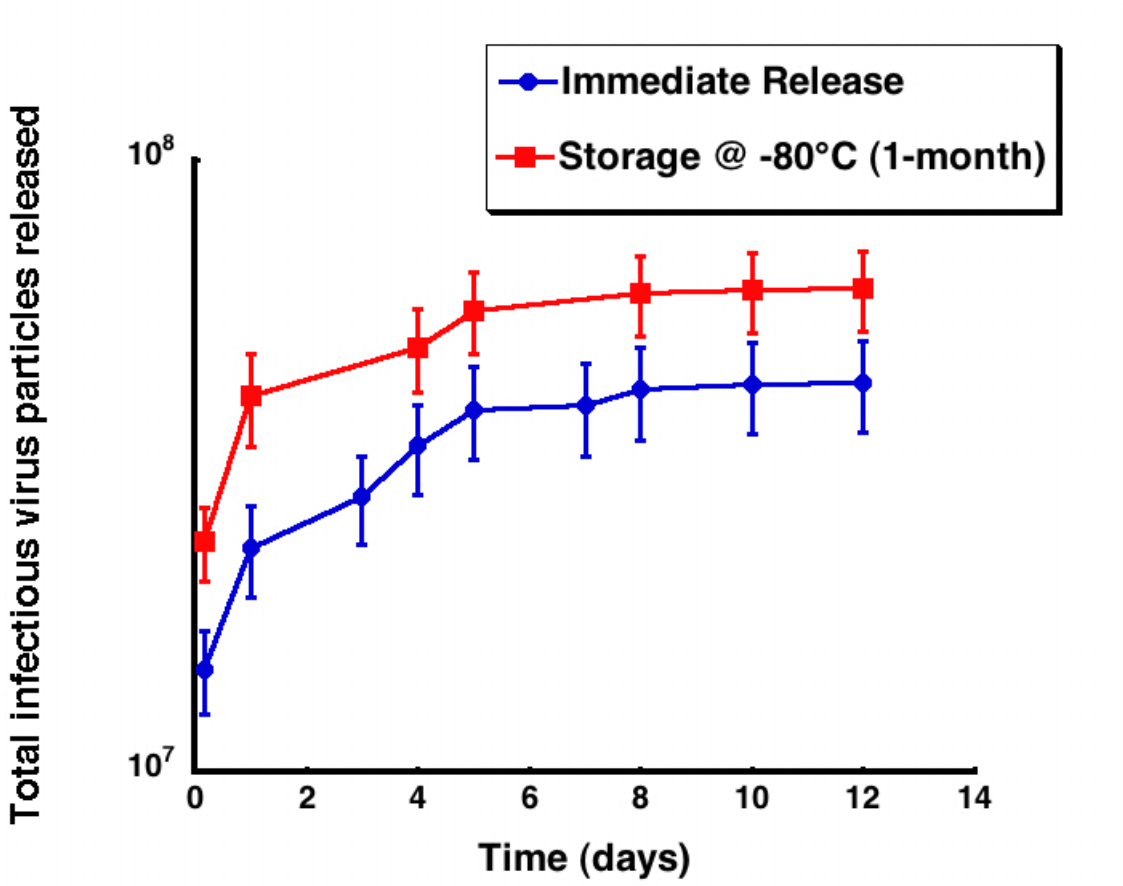

Figure 14: Effect of Long-term Storage on Microsphere Virus Release

The data are based on total release of infectious virus particles determined by X-gal staining of 293T cells infected with supernatant as described in the *Material and Methods* section. Microspheres were prepared using general protocol with ethyl acetate, 100 mg/ml PLGA, and a ratio of 65:35. After collection, 0.65 g of microspheres were either immediately resuspended in PBS for release or stored at -80°C for 1 month prior to resuspension. The data represent the mean ± standard error of three separate titers using aliquots from microsphere supernatant.

Discussion

Objective 1

Our first objective was to increase the total number of infectious virus particles released over time from our original formulation which resulted in only 1.44 x 10^6 ivp released over 10 days. Typical inter-prep variability of *in vitro* release from PLGA microspheres prepared by solvent evaporation or another developed technique call TROMS is typically in the range of 10% to 30% (Barrio *et al*., 2003; Oster *et al*., 2005). The variability in our initial microsphere production process also fell within this range with a RSD of 24.87% for the total release from three preparations. As expected, our initial variability between the three batches at early time points was relatively low with a RSD of only 4.14% at 1 day. Much of the increase in variability over time can be attributed to the variability of 5.68% to 21.84% in our intra-sample titers. Nevertheless, this figure fell within the range of 30% reported by Steven Bauer for this infectious virus particle determination assay (Bauer, 2000).

It is widely known that one of the major factors determining the release kinetics from PLGA microsheres is the lactide to glycolide ratio of the polymer (Kim *et al*., 2005). Du *et al.* demonstrated that a PLGA ratio of 40:60 provided a faster release rate in aqueous medium than other polymer ratios containing higher concentrations of lactide (Du *et al*., 2006). From our study utilizing PLGA lactide to glycolide ratios of 50:50, 65:35, and 75:25, we found that the 65:35 ratio produced the highest initial and total release of infectious virus particles

among the three ratios used. Theoretically, based on the degradation rate of the polymer, the 50:50 PLGA should have released more infectious virus particles within the first 1 day of release than the other two ratios because it has the highest concentration of glycolide units. However, the different polymer ratios can also influence the encapsulation efficiency of the antigen into the polymer matrix (Du *et al*., 2006). We believe that the 65:35 polymer released more infectious virus particles because it provided a higher encapsulation efficiency of the virus that the other two ratios. Another factor that must be considered in this instance is the molecular weight of the polymer. As molecular weight increases, the diffusion of the drug out of the microspheres decreases resulting in a slower release (Samati *et al*., 2006). The molecular weight of both the 50:50 and 65:35 PLGA were 40,000 – 75,000 compared to 66,000 – 107,000 for the 75:25 ratio; therefore the 75:25 could have had the highest encapsulation efficiency, but its decreased rate of release and degradation from the higher molecular weight and lower glycolide unit concentration may mask this.

The polymer concentration utilized to form the primary emulsion also plays a key role in determining the encapsulating efficiency and the average particle size of the microspheres (Singh *et al*., 1998). We determined that a PLGA concentration of 100 mg / ml (2% w / v) released 18 times more infectious virus particles compared to a lower concentration of 50 mg / ml presumably because of increased encapsulation efficiency. The higher concentration slightly increased the size of microsphere particles produced to about 3.8 µm; however, this size is

still small enough to be taken up by Peyer's patches in the gut (Shakweh *et al*., 2005).

Beer *et al.* previously reported that the input volume of adenovirus incorporated into the primary emulsion can influence the percent encapsulation and also the release profile of virus from the microspheres (Beer *et al*., 1998). We increased the volume of virus applied to make the primary emulsion from 1 ml (8.7×10^{10} ivp) to 2 ml (1.74×10^{11} ivp) to determine the effect on the kinetics of infectious virus release. Increasing the volume of adenovirus added to the system actually decreased the amount of infectious virus particles released by 80%. We also observed a decrease in the initial burst release profile in the microspheres produced with the higher concentration of adenovirus. These results correlate with the findings of Samati *et al*. that burst release decreases with increased drug loading (Samati *et al*., 2006).

One of the most critical parameters influencing the formation of polymeric microspheres is the choice of organic solvent because it can affect the rate of solvent removal (altering microsphere hardening), antigen stability, and encapsulation efficiency (Tamber *et al*., 2005; Singh *et al.*, 1998). We compared two of the most popular solvent choices for solvent evaporation, dichloromethane and ethyl acetate, to determine the effect on microsphere formation and the infectious virus release profile. Microspheres formed by ethyl acetate released 10 times more infectious virus particles than those prepared using dichloromethane. Based on these results, we were not able to determine

whether the loss was due to increased encapsulation efficiency using ethyl acetate or the stability of adenovirus in the solvent. Results from a later study discussed in Objective II shed more light on this mater.

After taking all of the results into account, we developed our optimal formulation for improved infectious adenovirus release. The new formulation resulted in an increase in total infectious virus particles 63 times more than the original formulation. A total of 6.69×10^8 ivp was released over the course of 13 days. Our percent efficiency of this formulation was 0.11% compared to the 0.034% reported by another improved encapsulation method (Barrio *et al*., 2004).

We took this optimized formulation one step further to try to minimize the degradation of the virus during the encapsulation process by adding sucrose and sodium bicarbonate to the emulsion. The sucrose was added in equal concentrations of 10% w /v to both the primary and secondary aqueous phases to maintain osmotic balance while forming the emulsion and to minimize the amount of virus coming in contact with the organic solvent at the W / O interface (Srinivason et al., 2005). Sodium bicarbonate (2% w/v) was also added to the primary aqueous phase to increase the pH to mildly acidic conditions to help enhance adenovirus stability during microsphere formation (Rexroad *et al*., 2006). The infectious virus particles released with this formulation was 2.32×10^9 ivp, 3.5 times more than our optimized formulation, improving the percent efficiency of infectious virus particles to 0.38%.

Objective II

The first part of our second objective was to investigate the basis for loss of infectious virus particles released during and after encapsulation. Since our best formulation released only 2.23×10^9 ivp of the 6.1×10^{11} ivp incorporated into the system, we looked at the effect of air-drying microspheres on virus release and the effect of organic solvent and release buffer on adenovirus itself. Air-drying the microspheres for approximately 30 min after collection did not significantly affect the total infectious virus release; however, the initial rate of release within the first day was slower with the air-dried preparations due to the further hardening of the polymer shell during drying.

Since microsphere formulations containing ethyl acetate released on average 10 times more infectious virus particles than those made with dichloromethane, we investigated the how each solvent degraded adenovirus over a one hour period. Titers of adenovirus after 10 min exposure to dichloromethane was 15.5 times less than when exposed to ethyl acetate, indicating that this was the major reason for the decrease observed in the microsphere release profile.

A similar study was conducted to determine how much active virus was lost in between daily sampling times after microsphere suspension in PBS release buffer at 37°C. A 2-log loss in titer was experienced over the course of the 14 day sampling period with the most significant loss (60%) seen within the first 24 hours. Since our sampling interval after the first 4 hours was conducted

on a daily basis, our results underestimate the total number of infectious virus particles released from all of our formulations. We expect more of a loss of infectious virus particles than we estimated with this experiment because as the polymer degrades, the release buffer becomes more and more acidic, causing further degradation of the virus (Rexroad *et al*., 2006).

The second part of this objective was to determine the influence of storage conditions on the release profiles from our microsphere formulation. Storage at -20°C and -80°C maintained virus stability for one week; however, batches stores at 24°C for only one day did not release any detectable infectious virus particles. Storage at 4°C for one day reduced the number of infectious virus particles released by 73% and also shortened the release time from 12 to 9 days. This result is not surprising since adenovirus stored in PBS at 4°C loses 10% of original titer in only 15 min (Croyle *et al*., 2001). Adequate stability can be maintained at -80°C for over one month, the longest time interval we tested.

Conclusions

By optimizing each parameter in the production process, we were able to improve the overall release profile over 218 times that released by our original formulation. This method of production resulted in a formulation over 10 times more efficient than another improved adenovirus encapsulation method and 65 times more efficient than our original formulation (Barrio et al., 2004).

Our original formulation:

- Input: 1.86×10^{11} ivp
- Output: 1.07×10^{7} ivp
- Percent efficiency: 0.0058%
- Release time: 10 days

Our optimized formulation:

- Input: 6.1×10^{11} ivp
- Output: 2.32×10^{9} ivp
- Percent efficiency: 0.38%
- Release time: 14 days

TROMS formulation:

- Input: 1×10^{11} ivp
- Output: 3.4×10^{7} ivp
- Percent efficiency: 0.034%
- Release time: 5 days

Despite our improvements to the encapsulation process, the release was not complete. Losses in virus infectivity were the result of instability in organic solvent, instability during solvent evaporation, and perhaps other parameters

which we did not investigate in these studies herein. Also based on our findings of virus instability in release buffer between sampling times, we acknowledge that the estimation of infectious virus particles reported throughout this paper was below that of the actual titer based upon infectivity assay.

Further characterization of our microsheres is essential before taking this work to the next level. In depth analysis of the particle surface morphology should be ascertained by scanning electron microscopy. Transmission electron microscopy techniques should also be employed to provide evidence for and to determine an estimate of the amount of adenovirus particles remaining at the outer polymer surface after the emulsion process. It is possible that the presence of some virus particles attached to the microshere outer surface could be the reason for the increased virus release in wash steps after exposure to simulated gastric fluid. Testing to determine the total number of virus particles released, including both infectious and inactivated, should also be performed using Real-time PCR methods to detect a specific sequence of the adenovirus genome. The amount of organic solvent remaining in the microsphere product is another crucial variable that needs to be determined because of the toxicity issues associated with ingesting ethyl acetate.

More *in vitro* release profile studies could also be performed using the Caco-2 intestinal cell line to more accurately predict what may happen when implemented *in vivo*. Other future directions could explore the use of mucosal adjuvants in combination with adenovirus and these microspheres to enhance

viral uptake in intestinal cells. These methods could be aimed at disrupting gap junctions allowing for enhanced cellular uptake or provide for specific targeting of the microspheres to particular areas of gastrointestinal tract such as M cells or Peyer's patches.

The implementation of an adenovirus expressing specific antigenic epitopes for vaccination against various pathogens in place of our beta-galactosidase model would be necessary for vaccine applications. The Ebola virus is one possible candidate for our vaccine delivery system. Recently, successful Ebola virus vaccination strategies in non-human primates have employed the use of adenovirus vectors expressing the Ebola glycoprotein. After incorporation into our adenovirus vector and encapsulation into polymeric microspheres, these models could then be tested *in vivo* for efficacy in mice when faced with lethal challenge. These studies should be performed both in the presence and absence of pre-existing immunity to adenovirus to characterize differences in the cellular and humoral immune responses against the Ebola glycoprotein.

Appendix I

Summary of Study Parameters

Figure	Label	Ratio	Polymer (mg/ml)	Solvent	Input (ivp)	Output (ivp)
3	75:25	75:25	100	DCM	2.48×10^{11}	1.02×10^{7}
3	65:35	65:35	100	DCM	2.11×10^{11}	3.07×10^{7}
3	50:50	50:50	100	DCM	2.11×10^{11}	1.42×10^{6}
4	50 mg/ml	65:35	50	DCM	2.11×10^{11}	6.52×10^{5}
4	100 mg/ml	65:35	100	DCM	1.86×10^{11}	1.19×10^{7}
6	Ethyl Acetate	65:35	100	EA	1.86×10^{11}	2.87×10^{8}
6	Dichloromethane	65:35	100	DCM	5.10×10^{11}	3.07×10^{7}
10	Immediate resuspension	65:35	100	EA	1.86×10^{11}	2.86×10^{8}
10	Air dried then resuspended	65:35	100	EA	1.86×10^{11}	3.16×10^{8}
5	1 ml of virus	65:35	100	EA	8.70×10^{10}	1.63×10^{7}
5	2 ml of virus	65:35	100	EA	1.74×10^{11}	2.91×10^{6}
7	Original formulation	50:50	100	DCM	1.86×10^{11}	1.07×10^{7}
7, 8	Optimized formulation	65:35	100	EA	6.10×10^{11}	6.69×10^{8}
8	Sucrose + $NaHCO_3$	65:35	100	EA	6.10×10^{11}	2.32×10^{9}

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